Annales Academiae Medicae Bialostocensis

http://www.roczniki-amb.com

Volume 50
2005

Roczniki Akademii Medycznej w Białymstoku

Pathogenesis and treatment of alcoholic liver disease: progress over the last 50 years
page 7

The gastrointestinal cholecystokinin receptors in health and diseases
page 21

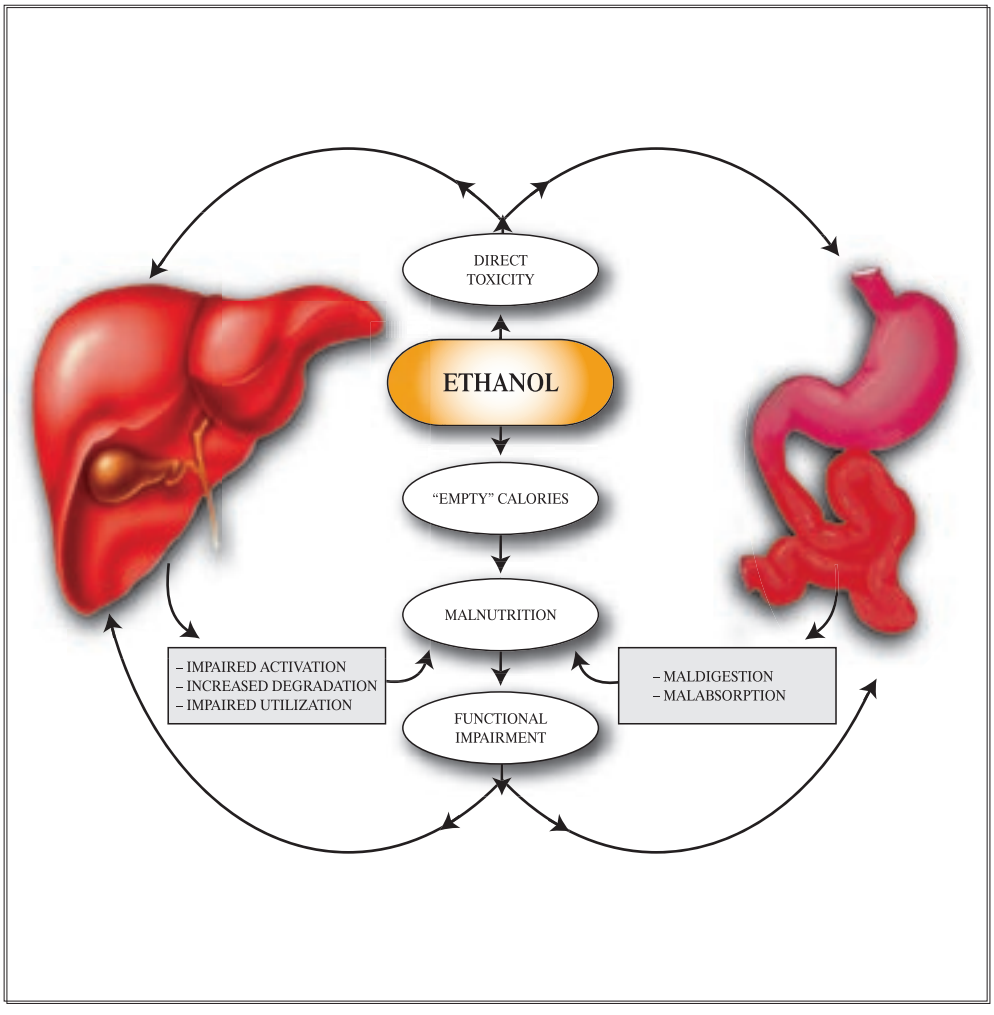
Surgical revascularization and perioperative management in patients with non-ST--elevation acute coronary syndromes
page 37

Surgical treatment of congestive heart failure in coronary artery disease
page 45

Impact of renal dysfunction as a cardiovascular risk factor
page 50

Helicobacter pylori infection and gastric MALT lymphoma
page 54

Somatostatin – receptor mediated diagnosis and treatment in gastrointestinal neuroendocrine tumours (GEP-NET's)
page 62



ISSN 0067-6489

Indexed in: Index Medicus/Medline Chemical Abstracts Index Copernicus

This journal is supported by the State Commitee for Scientific Research, Warszawa, Poland

Published by: Medical University of Białystok, ul. Kilińskiego 1, 15-089 Białystok, Poland, e-mail: editorial_office@amb.edu.pl

Printed on acid-free paper.
Pre-press: "Trans Humana" University Press, Editor-in-chief: E. Kozłowska-Świątkowska, Layout: M. Rabiczko, ul. Świerkowa 20, 15-328 Białystok, Poland
Printed by: "ORTH-DRUK", Białystok, Poland

Contents

7 **Pathogenesis and treatment of alcoholic liver disease: progress over the last 50 years**
Lieber CS

21 **The gastrointestinal cholecystokinin receptors in health and diseases**
Morisset J

37 **Surgical revascularization and perioperative management in patients with non-ST-elevation acute coronary syndromes**
Thielmann M, Jakob H

45 **Surgical treatment of congestive heart failure in coronary artery disease**
Benetis R

50 **Impact of renal dysfunction as a cardiovascular risk factor**
Adamczak M, Ritz E

54 ***Helicobacter pylori*** **infection and gastric MALT lymphoma**
Lehours P, Mégraud F

62 **Somatostatin – receptor mediated diagnosis and treatment in gastrointestinal neuroendocrine tumours (GEP-NET's)**
Öberg K

69 **Important clues to the diagnosis of pancreatic cancer**
Tanaka M

73 **Hereditary pancreatitis and secondary screening for early pancreatic cancer**
Vitone LJ, Greenhalf W, Howes NR, Neoptolemos JP

85 **Extent of lymphadenectomy in the resection of pancreatic cancer. Analysis of the existing evidence**
Pedrazzoli S, Pasquali C, Sperti C

91 **Application of specific cytologic, cytogenetic and molecular-cytogenetic techniques for the characterization of solid tumors**
Schwanitz G, Raff R

97 **Progresses in the medical treatment of advanced colorectal cancer**
Sultan-Amar V, Fabre-Guillevin E, Piedbois P

101 **New strategy for acute necrotizing pancreatitis: Continuous Regional Arterial Infusion (CRAI) therapy**
Mikami Y, Takeda K, Omura N, Abe H, Fukuyama S, Motoi F, Egawa S, Sunamura M, Matsuno S

106 **Anti-cytokine strategies in acute pancreatitis: pathophysiological insights and clinical implications**
Rau BM, Krüger CM, Schilling MK

116 **Antibiotic treatment in acute pancreatitis**
Uomo G

122 **Diabetic nephropathy and cardiovascular diseases**
Czekalski S

126 **Hemostasis in chronic renal failure**
Małyszko J, Małyszko JS, Myśliwiec M, Buczko W

132 **A grand challenge for research: multimodal, multilevel and multiscale systems in medicine and biology**
Coatrieux JL

138 **New approaches to health promotion and informatics education using Internet in the Czech Republic**
Zvárová J

142 Peroxisome proliferator-activated receptor γ ligand prevents the development of chronic pancreatitis through modulating NF-κB-dependent proinflammatory cytokine production and pancreatic stellate cell activation
Hisada S, Shimizu K, Shiratori K, Kobayashi M

148 Single Nucleotide Polymorphisms in exon 3 of the adiponectin gene in subjects with type 2 diabetes mellitus
Krętowski A, Gugała K, Okruszko A, Wawrusiewicz-Kurylonek N, Górska M

151 Interleukin 18 and sICAM-1 serum levels in families with type 1 diabetes mellitus
Mirończuk K, Okruszko A, Wawrusiewicz-Kurylonek N, Krętowski A, Kinalska I, Górska M

155 Long-term outcome of percutaneous transhepatic drainage for benign bile duct stenoses
Eickhoff A, Schilling D, Jakobs R, Weickert U, Hartmann D, Eickhoff JC, Riemann JF

161 Trends in the incidence of the free wall cardiac rupture in acute myocardial infarction. Observational study: experience of a single center
Sobkowicz B, Lenartowska L, Nowak M, Hirnle T, Borys D, Kosicki M, Prajs P, Wrabec K

166 Thrombin activatable fibrinolysis inhibitor (TAFI) in stable angina pectoris patients undergoing coronary artery bypass grafting (CABG)
Lisowski P, Małyszko J, Hirnle T, Lisowska A, Jackowski R, Małyszko JS, Buzun L, Myśliwiec M

173 Concentration of interferon-inducible T cell chemoattractant and monocyte chemotactic protein-1 in serum and cerebrospinal fluid of patients with Lyme borreliosis
Grygorczuk S, Zajkowska J, Świerzbińska R, Pancewicz S, Kondrusik M, Hermanowska-Szpakowicz T

179 HBV-DNA and sFas, sFasL concentrations in serum of healthy HBsAg carriers
Łapiński TW, Kowalczuk O, Prokopowicz D, Chyczewski L, Jaroszewicz J

183 Morpho-functional comparisons in *Helicobacter pylori* – associated chronic atrophic gastritis
Pasechnikov VD, Chukov SZ, Kotelevets SM, Chabannaya TA

188 Gastric juice ammonia and urea concentrations and their relation to gastric mucosa injury in patients maintained on chronic hemodialysis
Blusiewicz K, Rydzewska G, Rydzewski A

193 Laser resection of lung parenchyma – a new technical and clinical approach
Rolle A, Kozłowski M

197 Serum endostatin levels in patients with lung carcinoma
Świdzińska E, Ossolińska M, Naumnik W, Trojan S, Chyczewska E

201 An assessment of telemedicine possibilities in massive casualties situations
Paunksnis A, Barzdžiukas V, Kurapkienė S, Vaičaitienė R, Sereika V

204 Soluble form of TRAIL, Fas and FasL in the serum of patients with B-CLL
Jabłońska E, Kiersnowska-Rogowska B, Rogowski F, Parfieńczyk A, Pużewska W, Bukin M

208 Relationship between insulin-like growth factors (IGF-I and IGF-II), IGF-binding proteins (IGFBP-3, IGFBP-2), leptin and anthropometric parameters (height, body mass index) during antileukaemic treatment in children
Krawczuk-Rybak M, Muszyńska-Rosłan K, Kitszel A, Sawicka-Żukowska M, Wołczyński S

212 Patient acceptance of diagnostic laparoscopy
Weickert U, Bühl A, Jakobs R, Eickhoff A, Löffler W, Riemann JF

216 *Staphylococcus aureus* septicemia in non-neutropenic adult patients hospitalized in internal medicine units
Gallucci F, Amato G, Esposito P, Belli CMC, Russo R, Uomo G

220 The effect of green tea on the activity of aldehyde dehydrogenase (ALDH) in the liver of rats during chronic ethanol consumption
Chrostek L, Tomaszewski W, Szmitkowski M

224 Effects of truncal or highly selective vagotomy on the electron microscopic feature of the rabbit pancreas
Głuszek S, Matykiewicz J

230 The cumulative effect of nuclear factor-κB (NF-κB) inhibition and endothelins in early cerulein-induced acute pancreatitis in rats
Długosz JW, Andrzejewska A, Nowak K, Wróblewski E, Dąbrowski A

237 Human red blood cells′ physiological water exchange with the plasma
Kargol M, Kargol A, Przestalski M, Siedlecki J, Karpińska M, Rogowski M

241 The force-frequency relationship in human heart failure: effect of pyruvate and isoproterenol
Martisiene I, Gendviliene V, Zablockaite D, Gurskaite H

244 Changes of β_2-adrenergic stimulation induced by hyperosmosis in human atrium
Zablockaite D, Gendviliene V, Macianskiene R, Skeberdis VA, Jurevicius J, Kanaporis G, Benetis R

247 Blood oxygen-carrying function during the oxidative stress induced by lipopolysaccharide with a modification of the L-arginine-NO pathway
Glebov A, Zinchuk V

252 Diurnal rhythm of blood pressure, heart rate and adrenergic activity in patients with normotension treated with continuous ambulatory peritoneal dialysis and haemodialysis
Baranowicz-Gąszczyk I, Jóźwiak L, Bednarek-Skublewska A, Książek A

257 Cholecystokinin octapeptide (CCK-8) concentration in plasma is not affected in functional abdominal pain in children
Uścinowicz M, Kaczmarski M

261 Atopy patch test in the diagnosis of food allergy in children with atopic eczema dermatitis syndrome
Cudowska B, Kaczmarski M

268 Immunoblotting in the diagnosis of cross--reactivity in children allergic to birch
Cudowska B, Kaczmarski M, Restani P

274 Hypersensitivity to hydrolyzed cow′s milk protein formula in infants and young children with atopic eczema/dermatitis syndrome with cow′s milk protein allergy
Kaczmarski M, Wasilewska J, Lasota M

279 Acute phase proteins serum concentrations in children are related to urinary iodine excretion
Lialikau SA, Haurylik LL, Sobieska M, Klachko NM, Samborski W

284 Extraction of nerve cells in images with herpetic infections
Nedzved A, Ablameyko S, Oczeretko E

289 Alterations of lymphocyte subpopulations in choroidal melanoma patients undergoing surgery
Manyś-Kubacka K, Kostrzewa A, Sobieska M, Samborski W

293 Phagocytic and bactericidal activity and morphological parameters of blood platelets in patients with *Trichinella spiralis* infection
Matowicka-Karna J, Kemona H, Dymicka-Piekarska V, Butkiewicz A

297 Development and psychokinetic therapy of children suffering from West Syndrome – an overview
Gajewska E, Mojs E, Sobieska M, Samborski W

302 The duration of breastfeeding and attention deficit hyperactivity disorder
Kądziela-Olech H, Piotrowska-Jastrzębska J

307 Chlamydia trachomatis infection in chronically hemodialyzed patients
Bednarek-Skublewska A, Majdan M, Dryglewska M, Książek A

311 Increased serum levels of troponin I and lesions in coronary angiography in hemodialysed patients
Popowska-Drojecka J, Muszytowski M, Sobiło-Jarek L, Majewska A

314 Results of improvement in adequacy of intermittent hemodialysis in uremic patients
Grzegorzewska AE, Banachowicz W, Leander M

319 Patients′ quality assessment of ambulatory obstetric and gynaecological services
Bojar I, Wdowiak L, Ostrowski T

322 Serum isoprostanes levels in patients after abdominal hysterectomy
Szymczyk G, Bełtowski J, Marciniak A, Kotarski J

325 Binding of α_1-antichymotrypsin to the surface of lymphocytes – preliminary study
Sobieska M

329 The analysis of mortality from cardiovascular diseases in Pomeranian province
Bartosińska M, Ejsmont J, Zaborski L, Zagożdżon P

334 Evaluation of secretory mucin concentration of patients with squamous cell carcinoma oral cavity
Dziemiańczyk D, Grabowska SZ, Balicki R

339 Low dose rofecoxib, inflammation and prostacyclin synthesis in acute coronary syndromes
Kuklińska AM, Musiał WJ, Kamiński KA, Kralisz P, Modrzejewski W, Sobkowicz B, Dobrzycki S, Stec S, Wojtkowska I

343 Index of Authors

344 Index of key words

346 Instructions for Authors

Cover Illustration: Interaction of direct toxicity of ethanol on liver and gut with malnutrition secondary to dietary deficiencies, maldigestion, and malabsorption, as well as impaired hepatic activation, inhibition and increased degradation of nutrients (Lieber CS, Roczniki Akademii Medycznej w Białymstoku, 2005; 50, 8).

Pathogenesis and treatment of alcoholic liver disease: progress over the last 50 years

Lieber CS*

Alcohol Research and Treatment Center, Section of Liver Disease and Nutrition, Bronx VA Medical Center, Professor of Medicine and Pathology, Mount Sinai School of Medicine, New York, USA

Abstract

Fifty years ago the dogma prevailed that alcohol was not toxic to the liver and that alcoholic liver disease was exclusively a consequence of nutritional deficiencies. We showed, however, that liver pathology developed even in the absence of malnutrition. This toxicity of alcohol was linked to its metabolism via alcohol dehydrogenase which converts nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide-reduced form (NADH) which contributes to hyperuricemia, hypoglycemia and hepatic steatosis by inhibiting lipid oxidation and promoting lipogenesis. We also discovered a new pathway of ethanol metabolism, the microsomal ethanol oxidizing system (MEOS). The activity of its main enzyme, cytochrome P4502E1 (CYP2E1), and its gene are increased by chronic consumption, resulting in metabolic tolerance to ethanol. CYP2E1 also detoxifies many drugs but occasionally toxic and even carcinogenic metabolites are produced. This activity is also associated with the generation of free radicals with resulting lipid peroxidation and membrane damage as well as depletion of mitochondrial reduced glutathione (GSH) and its ultimate precursor, namely methionine activated to S-adenosylmethionine (SAMe). Its repletion restores liver functions. Administration of polyenylphosphatidylcholine (PPC), a mixture of unsaturated phosphatidylcholines (PC) extracted from soybeans, restores the structure of the membranes and the function of the corresponding enzymes. Ethanol impairs the conversion of β-carotene to vitamin A and depletes hepatic vitamin A and, when it is given together with vitamin A or β-carotene, hepatotoxicity is potentiated. Our present therapeutic approach is to reduce excess alcohol consumption by the Brief Intervention technique found to be very successful. We correct hepatic SAMe depletion and supplementation with PPC has some favorable effects on parameters of liver damage which continue to be evaluated. Similarly dilinoleoylphosphatidylcholine (DLPC), PPC's main component, also partially opposes the increase in CYP2E1 by ethanol. Hence, therapy with SAMe +DLPC is now being considered.

Key words: alcoholic hepatitis, nonalcoholic steatohepatitis (NASH), cytochrome P450 (CYP2E1), S-adenosylmethionine (SAMe), polyenylphosphatidylcholine (PPC).

Introduction

In a prospective survey of 280 subjects with alcoholic liver injury [1] it was found that, within 48 months of follow-up, more than half of those with cirrhosis, and two thirds of those with cirrhosis plus alcoholic hepatitis, had died. This dismal outcome is more severe than that of many cancers, yet it is attracting much less concern, both among the public and the medical profession. This may be due, at least in part, to the general perception that not much can be done about this major public health issue. One purpose of this review is to analyze how concepts about alcoholic liver disease have evolved and how the present state of knowledge allows for a more optimistic outlook in terms of treatment and outcome.

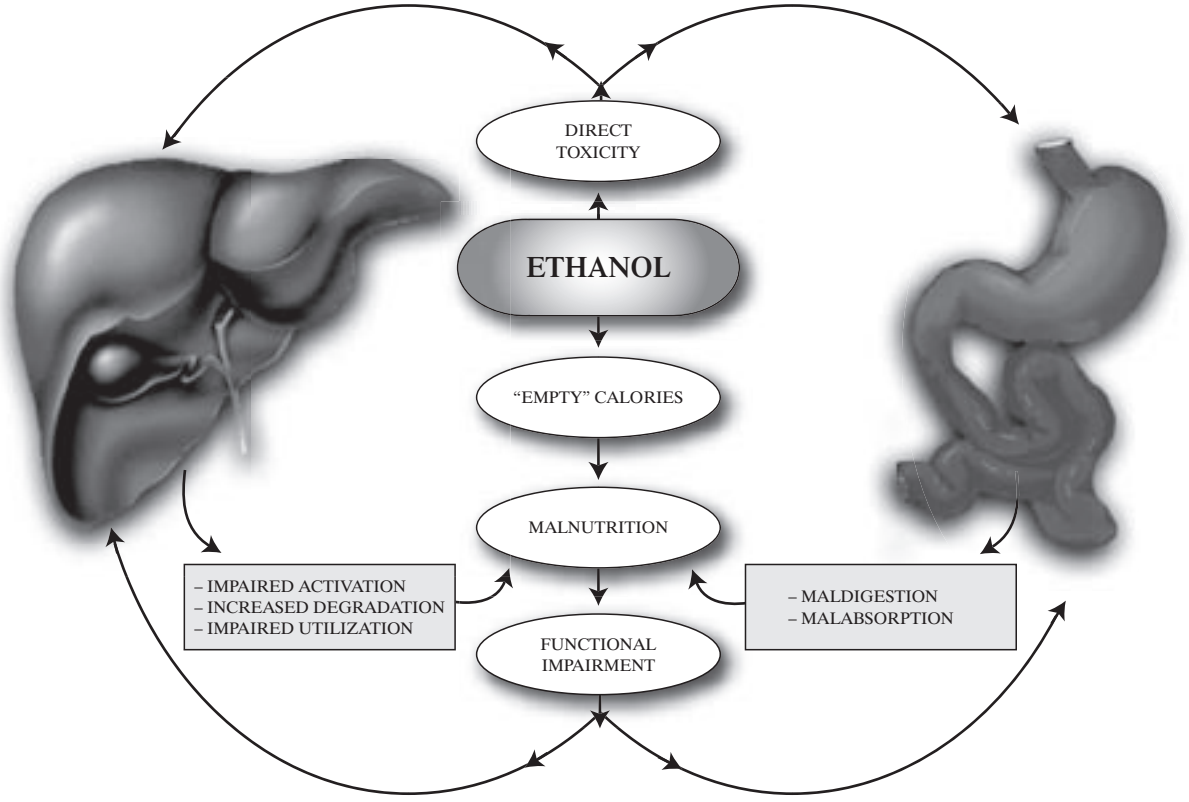
Malnutrition versus toxicity

Because experimentally, nutritional deficiencies cause liver damage, it was postulated that this is also the mechanism

* CORRESPONDING AUTHOR:
VA Medical Center (151-2)
130 West Kingsbridge Road
Bronx, NY 10468, USA
Tel: 718 741 4244; Fax: 718 733 6257
e-mail: liebercs@aol.com (Charles S. Lieber)

Received 30.05.2005 Accepted 13.06.2005

Figure 1. **Interaction of direct toxicity of ethanol on liver and gut with malnutrition secondary to dietary deficiencies, maldigestion, and malabsorption, as well as impaired hepatic activation, inhibition and increased degradation of nutrients (modified from** [176])



whereby alcoholic liver disease develops. Accordingly, fifty years ago the dogma prevailed that alcohol was not toxic to the liver and that alcoholic liver disease was exclusively a consequence of nutritional deficiencies. As a result, the prevailing therapy was nutritional with enriched diets. This view was clearly expressed by Best et al. [2], the co-discoverer of insulin, who stated that "There is no more evidence of a specific toxic effect of pure ethyl alcohol upon liver cells than there is for one due to sugar". This opinion was based on his experiments in rats given alcohol in drinking water. They did not develop any liver damage unless the diet was deficient. However, rats do not develop significant blood levels of alcohol when given in the drinking water. When we overcame the aversion of the rats for alcohol by incorporating 35% of calories as alcohol into nutritionally adequate liquid diets, obvious liver pathology, including fatty liver, developed compared to the control animals pair-fed with an isocaloric diet in which alcohol was replaced by carbohydrates [3]. This was also demonstrated in volunteers given alcohol with enriched diets which did not prevent the development of the first stage of alcoholic liver disease, namely alcoholic fatty liver [3,4] indicating that alcohol exerts some direct toxic effect on the liver independent of malnutrition (*Fig. 1*). This demonstration of the hepatotoxicity of alcohol did, of course, not preclude that one of the mechanisms whereby alcohol affects the liver may also be its interference with nutritional factors.

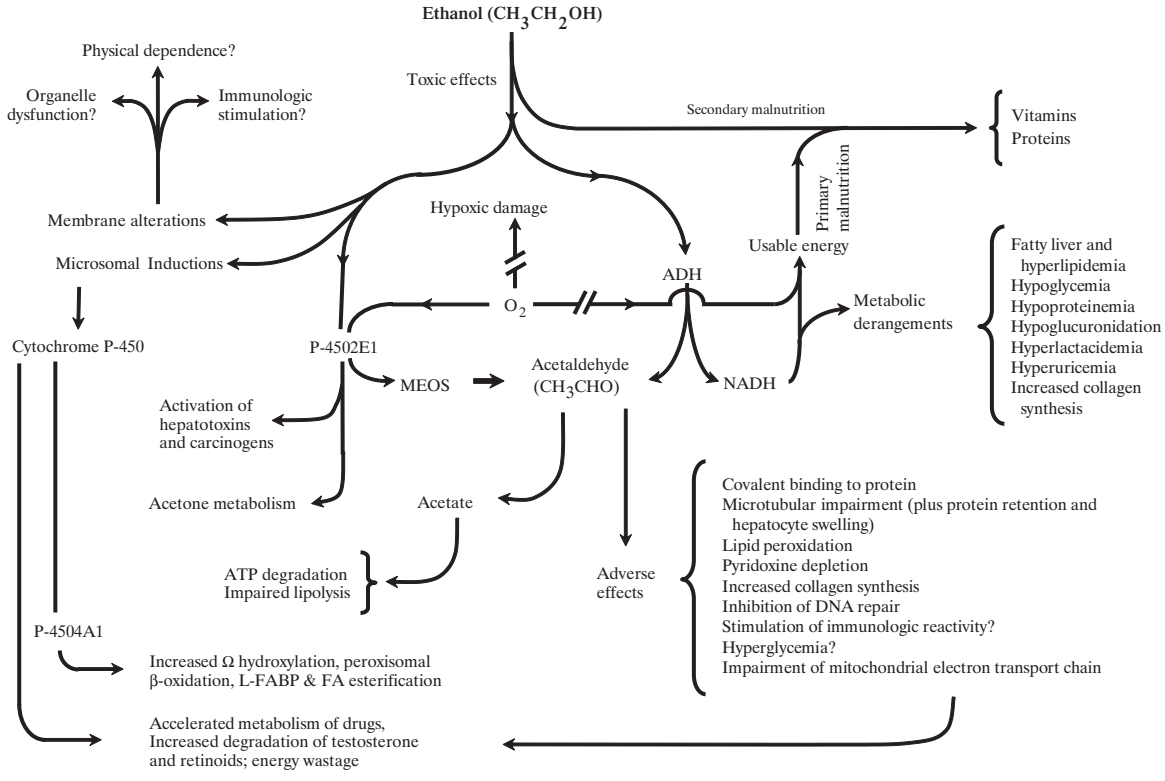
Pathogenesis and treatment of alcoholic and non-alcoholic fatty liver

1. Alcohol and nutrition

Unlike other drugs, ethanol is a substantial source of energy, with 7.1 kcal (29.7 kJ) per gram, a value that exceeds the energy content of carbohydrates or proteins. On average, ethanol accounts for half an alcoholic's caloric intake. It therefore displaces normal nutrients, causing malnutrition (*Fig. 1*), including deficiencies of folate, thiamine, and other vitamins. Secondary malnutrition also occurs through malabsorption due to gastrointestinal complications, such as pancreatic insufficiency and impaired hepatic metabolism of nutrients (*Fig. 1*). In addition, alcohol promotes the degradation of nutrients, as exemplified by its effects on vitamin A [5].

Indeed, in addition to the study of the hepatotoxicity of ethanol, we investigated its associated effect on nutrients with implications for the liver. This included the discovery that liver microsomes harbor previously unrecognized pathways for retinol metabolism [6] which were shown to play a role in the homeostatic control of hepatic vitamin A levels [7]. Using purified cytochrome P-450 isozymes, including the human CYPIIC8 [8], retinol [6,8] and retinoic acid [8,9] metabolizing systems were reconstituted; administration of ethanol or various drugs was shown to result in the induction of the activity of these

Figure 2. **Toxic and metabolic disorders caused by ethanol abuse. Most of the toxic and metabolic disorders that result from long-term ethanol abuse can be explained on the basis of the metabolism of ethanol by alcohol dehydrogenase (ADH) and the associated generation of reduced NADH or by the microsomal ethanol oxidizing system (MEOS), with the induction of cytochrome P-4502E1 and other microsomal enzymes, as well as by the toxic effects of acetaldehyde produced by both pathways. FA denotes fatty acids, and L-FABP denotes liver fatty-acid-binding protein (from** [177])



systems [10,11], a possible mechanism for the striking hepatic vitamin A depletion which was discovered to result from chronic ethanol consumption in rats and non-human primates [12], as well as man [13]. This hepatic vitamin A depletion was found to be associated with significant lysosomal lesions [14].

Depletion of vitamin A called for its replenishment but it was found that vitamin A supplementation in alcohol users is complicated because of the marked exacerbation of vitamin A hepatotoxicity by chronic ethanol consumption. Indeed, amounts of ethanol and vitamin A which, by themselves, do not produce fibrosis, when combined, resulted in necrosis and fibrosis in the liver [15], with development of severe mitochondrial injury [16]. Furthermore, it was discovered in non-human primates that alcohol interferes with the clearance of the retinol precursor β-carotene, possibly by impairing its conversion to vitamin A, resulting in enhanced hepatic and blood levels in baboons [17] and also in man [18], with associated potentiation of their hepatotoxicity [17]. In the aggregate, the above listed studies have led to the recognition of a narrowed therapeutic window for vitamin A and β-carotene in moderate and in heavy drinkers; in turn, this has prompted a redefinition of the optimal conditions for their therapeutic use, in order to avoid adverse interactions with ethanol in terms of hepatotoxicity and carcinogenicity, as reviewed by Leo and Lieber [19].

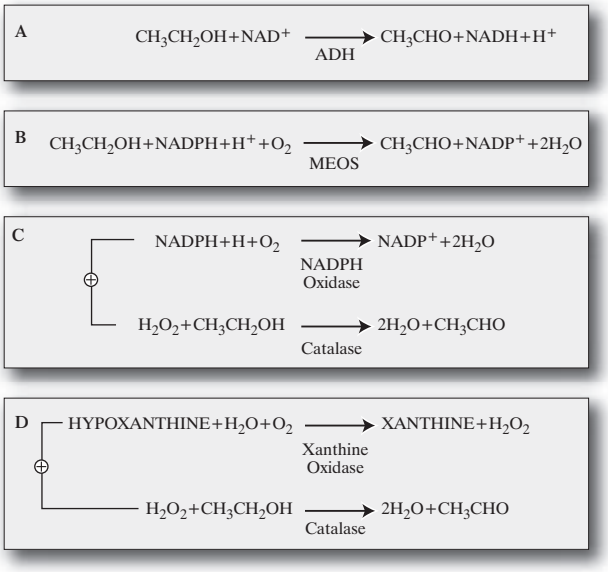
These studies also established the fact that, even in the presence of an adequate diet, alcohol can produce a fatty liver at blood levels which do not necessarily produce intoxication. Increases in dietary long-chain triglycerides enhanced the alcohol-induced steatosis and this effect was attenuated by medium-chain triglycerides [20] as confirmed more recently [21]. Decreasing dietary fat reduced steatosis but, below a level of 10% of calories as dietary fat, there was a reversal of this effect, probably due to the high carbohydrate content of the corresponding diet [22]. These, as well as other effects of alcohol were shown to be due to ethanol's prevailing metabolism in the liver (*Fig. 2*).

2. Toxic effects of hepatic alcohol oxidation

a. Role of Alcohol Dehydrogenase (ADH)

Oxidation of ethanol through the ADH pathway produces acetaldehyde, which is converted to acetate (*Fig. 3*); both reactions reduce nicotinamide adenine dinucleotide (NAD) to nicotinamide adenine dinucleotide-reduced form (NADH). Excess NADH causes a number of metabolic disorders [5], including inhibition of the Krebs cycle and of its fatty acid oxidation. The inhibition of fatty-acid oxidation favors steatosis and hyperlipidemia. The effects of ethanol were reproduced *in vitro* by an alternative NADH-generating system (sorbitol-fructose) and

Figure 3. **Ethanol oxidation by (A) alcohol dehydrogenase (ADH) and nicotinamide adenine dinucleotide (NAD^+); (B) the hepatic microsomal ethanol oxidizing system (MEOS), which involves cytochrome P4502E1 and the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH); (C) a combination of NADPH oxidase and catalase; and (D) xanthine oxidase and catalase (from** [5])



were blocked by a H^+ acceptor (methylene blue) [23,24]. The preventive effect of methylene blue against ethanol-induced fat accumulation was more recently confirmed [25]. Our liquid diet rat model of alcoholic liver disease consistently reproduced a fatty liver but it did not progress to inflammation, fibrosis and cirrhosis. This is probably due to the fact that, even with the liquid diet, the alcohol intake is limited to 35% of total calories whereas alcoholics who develop cirrhosis usually consume at least 50% of their calories as alcohol. Such a high intake of alcohol was then achieved in non-human primates, namely baboons [26]. Under these conditions, not only fatty liver, but also fibrosis and cirrhosis developed despite adequate diets.

It was also known that catalase, located in the peroxisomes, is capable of oxidizing ethanol *in vitro* in the presence of an H_2O_2-generating system [27] (*Fig. 3*), but under physiological conditions, catalase appears to play no major role. In both the rat and baboon models, it was also shown that in addition to the metabolic abnormalities caused by the ADH activity, a new pathway of ethanol metabolism, namely the microsomal ethanol oxidizing system (MEOS), plays a key role in the progression of the disease (vide infra).

b. Role of Acetaldehyde Dehydrogenase

As mentioned, the oxidation of ethanol results in the production of the highly toxic metabolite, namely acetaldehyde which is rapidly further metabolized to acetate, mainly by a mitochondrial low Km aldehyde dehydrogenase (ALDH2), the activity of which is lacking in about 25-50% of Asians. In these individuals, even small amounts of alcohol which have almost no effect on Caucasians can produce a rapid facial flush, frequently associated with tachycardia, headache and nausea [28]. This propensity for flushing is genetically determined and caused by decreased acetaldehyde disposition secondary to the lack of ALDH2 activity [29-32]. In fact, the flushing reaction seen in susceptible Asians mimics to a lesser degree the disulfiram reaction caused by the elevation of acetaldehyde following aldehyde dehydrogenase inhibition. The latter reaction has gained therapeutic use as a reinforcement for abstinence in alcoholism rehabilitation programs. In addition, the aversive cardiovascular effects of acetaldehyde may contribute to the relatively lower incidence of cirrhosis in "flushers" [33]. The flushing phenotype may also confer some resistance to the development of alcoholism [34]. Conversely, however, some Japanese alcoholics with an ALDH2 deficiency and, presumably, higher hepatic acetaldehyde levels during drinking, develop alcoholic liver disease at a lower cumulative intake of ethanol than controls [35]. The ALDH2 activity is also significantly reduced by chronic ethanol consumption [36]. The decreased capacity of mitochondria after chronic alcohol consumption to oxidize acetaldehyde, associated with unaltered or even enhanced rates of ethanol oxidation and hence acetaldehyde generation (e.g. because of MEOS induction, vide infra) results in an imbalance between production and disposition of acetaldehyde. The latter causes the elevated acetaldehyde levels observed after chronic ethanol consumption in man [37] and in baboons [38], with a tremendous increase of acetaldehyde in hepatic venous bloods, reflecting the high tissue level.

Acetaldehyde's toxicity is due, in part, to its capacity to bind to proteins, such as microtobules [39], collagen [40] and microsomal protein [41], including CYP2E1 [42]. By binding to the tubulin of microtubules, acetaldehyde blocks the secretion of proteins. The increases in protein, lipid, water [43] and electrolytes cause hepatocytes to enlarge or "balloon", a hallmark of alcoholic liver disease. Acetaldehyde-protein adducts promote collagen production and may also act as neoantigens, which stimulate an immune response [44,45]. Formation of adducts results in hepatic protein retention, contributing to the hepatomegaly [46], and a score of other toxic manifestations, including decreased GSH and impairment of other antioxidant mechanisms, as reviewed elsewhere [5,47]. The effects include inhibiting the repair of alkylated nucleoproteins [48], decreasing the activity of key enzymes, and markedly reducing oxygen utilization in mitochondria damaged by long-term ethanol consumption [38,49]. Impaired oxygen utilization was also confirmed in baboons *in vivo* [38]. The impaired oxidation capacity of the mitochondria also interferes with the oxidation of acetaldehyde [50], thereby contributing to the vicious circle of progressive acetaldehyde accumulation in turn causing greater mitochondrial injury.

c. Microsomal Ethanol Oxidizing System (MEOS)

1) Discovery of this new pathway

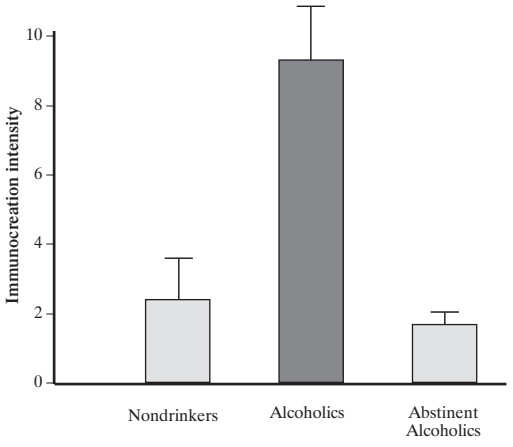
As reviewed elsewhere [51,52], the discovery of this pathway was prompted by the observation that, after chronic ethanol consumption, there is an adaptive increase of ethanol metabolism which could not be explained on the basis of ADH, therefore raising the possibility of the existence of an additional pathway. The first indication of a possible interaction of ethanol with the endoplasmic reticulum of the hepatocyte (also called

microsomal fraction when obtained by ultracentrifugation) was provided by the observation that in rats and humans, ethanol feeding results in a proliferation of the smooth endoplasmic reticulum (SER) [53-55] which was confirmed by an increase in the amount of enzyme activities of the hepatic microsomal membranes [56], including a rise in microsomal glucose-6-phosphatase activity after chronic ethanol administration [57]. This increase in SER resembled that seen after the administration of a wide variety of hepatotoxins [58], barbiturates, and other therapeutic agents [59] and food additives [60]. Because many of the substances that induce a proliferation of the SER are metabolized, at least in part, by the cytochrome P450 enzyme system that is located in the SER, the possibility that ethanol may also be metabolized by a similar process was raised. Indeed, such a system was demonstrated in liver microsomes *in vitro* and found to be inducible by chronic ethanol feeding *in vivo* [22,61]. The proposal that this new pathway (named MEOS) plays a significant role in ethanol metabolism initiated a decade of lively debate: some invoked ADH contaminating the liver microsomes [62], whereas others asserted that this microsomal ethanol oxidation was due to a hydrogen peroxide-dependent reaction promoted by contaminating catalase [63]. Indeed, it had been postulated that the combination of H_2O_2 generation from NADPH oxidase and of catalase could account for microsomal ethanol oxidation [64,65] (*Fig. 3*), especially because a H_2O_2-generating system (glucose-glucose oxidase) can be substituted for NADPH. Actually, the latter is not unexpected, because not only do microsomes contain catalase, but commercial glucose oxidase (used to generate NADPH) is contaminated with catalase. It also had been reported that microsomes from acatalasemic mice fail to oxidize ethanol [66], but this claim was subsequently retracted [67]. In fact, hepatic microsomes of acatalasemic mice subjected to heat inactivation displayed decreased catalase activity, but NADPH-dependent MEOS remained active and unaffected [26,68].

2) Differentiation of the MEOS from ADH and catalase

Eventually, MEOS was solubilized and separated from ADH and catalase activities by diethylaminoethyl cellulose column chromatography [69,70]. Furthermore, whereas catalase reacts peroxidatically primarily with methanol and ethanol, but not with alcohols of longer aliphatic chains [71], the NADPH-dependent MEOS was found capable of metabolizing n-propanol as well as n-butanol. This was shown in hepatic microsomal preparations and in reconstituted systems that contained the microsomal components cytochrome P450, NADPH-cytochrome P450 reductase and phospholipids, and exhibited no ADH or catalase activity [68-70,72]. Such a system was also reconstituted in acatalasemic mice [68]. Furthermore, MEOS activity was inhibited by carbon monoxide and the effect was reversed by lights of wavelengths 430 to 460 nm, which showed the involvement of cytochrome P450 [73]. Finally, successful reconstitution was accomplished with either partially purified or highly-purified microsomal P450 from alcohol [74] or phenobarbital-treated [75] rats. Based on these and various other studies, and regardless of the original claim to the contrary [76], it was finally agreed by the principal contenders involved that catalase cannot account for the microsomal ethanol oxidation [77,78], and that the MEOS is distinct from ADH and catalase and dependent on cytochromes P450, as reviewed elsewhere [51,52].

***Figure 4*. Hepatic cytochrome P4502E1 levels in alcoholics and in nondrinkers. Cytochrome P4502E1 was quantitated by scanning of Western blots of microsomes obtained from percutaneous liver biopsies, using anti-2E1 antibodies. (data from** [79])



Thus, it was established that the key enzyme of the MEOS is an ethanol-inducible cytochrome P450 2E1 (CYP2E1) which was found (*Fig. 4*) to be increased 4- to 10-fold in liver biopsies of recently drinking subjects [79], with a corresponding rise in mRNA [80]. This induction contributes to the metabolic tolerance to ethanol that develops in the alcoholic (in addition to the central nervous tolerance).

3) "Ethanol-specific" cytochrome P450

That chronic ethanol consumption results in the induction of a unique P450 enzyme was shown by Ohnishi and Lieber [74] using a liver microsomal P450 fraction isolated from ethanol-treated rats. An ethanol-inducible form of P450 (LM-3a) was purified from rabbit liver microsomes which catalyzed ethanol oxidation at rates much higher than other P450 isozymes [81,82]. Similar results have been obtained with cytochrome P450j, a major hepatic P450 isozyme purified from ethanol- or isoniazid-treated rats [83,84]. A P450j-like isozyme was also described in humans [85,86]. In a new nomenclature system, it was proposed that the ethanol-inducible form be designated as CYP2El [87].

Despite the discovery of CYP2E1 and its prevailing role in microsomal ethanol oxidation, the term MEOS was maintained because cytochromes P450 other than CYP2E1, as well as hydroxy radicals, can contribute to ethanol metabolism in the microsomes (vide infra). Thus, the term MEOS characterizes total microsomal ethanol oxidation, not only that catalyzed by CYP2E1.

The rat CYP2E1 gene was isolated, characterized, and localized to chromosome 7 [88] and the human gene to chromosome 10 [89]. In rabbits, two genes may be involved [90].

The rat hepatic CYP2E1 gene is transcriptionally activated within 1 day after birth [91]. This activation is accompanied by a demethylation of cytosine residues located within the 5' flanking region of the gene, suggesting that methylation of specific

residues in the 2E1 gene is responsible for the lack of transcription of the 2E1 gene in fetal liver. Cytochrome P4502E1 remains relatively stable during the remainder of the life span, and a tissue-specific relation between the hypomethylation of the human CYP2E1 gene and its hypoexpression was observed [92]. CYP2E1 can be strikingly induced by a variety of substrates.

4) Physiologic role of CYP2E1

As revealed in the studies cited above, the most common cause for CYP2E1 induction is early alcoholic liver injury, such as alcoholic steatosis and alcoholic steatohepatitis (ASH). CYP2E1, however, is also induced in nonalcoholic steatohepatitis (NASH), which can be understood on the basis of the physiologic role of CYP2E1. Indeed, CYP2E1 has a dual role (*Fig. 5*), namely one of detoxification and one of nutritional support. That CYP2E1 contributes to the defense mechanisms of the body against the penetration of toxic xenobiotics is suggested by its location and inducibility at port of entries into the body, and by its broad substrate specificity.

Regarding xenobiotics such as alcohol, CYP2E1 may play a dual detoxification role: through its location in the liver, it may oppose the ethanol resulting from gastrointestinal fermentation to enter the systemic circulation. Moreover, through its marked inducibility at high ethanol concentration, it may prevent alcohol that has entered the circulation from reaching excessive levels. This inducibility was shown not only in experimental animals [93] but also in man [94]. In volunteers, chronic administration of substantial amounts of ethanol resulted in a progressively increased rate of clearance of alcohol from the blood. This accelerated blood alcohol disappearance occurred exclusively at relatively high concentrations of ethanol, the kinetics of which clearly indicate that a system is involved with a Km of approximately 10 mM, which is consistent with that of MEOS (and its CYP2E1) and at least 1-2 orders of magnitude higher than that of ADH. Thus, despite the fact that, at low ethanol concentrations, liver ADH has a much higher capacity for ethanol oxidation than the CYP2E1 system, it is the MEOS, and particularly its CYP2E1, which accounts for the metabolic adaptation to high concentrations of ethanol that develops upon chronic substantial ethanol consumption.

In terms of the nutritional role, CYP2E1 is inducible by fasting in the rat [95]. The increase may be due, at least in part, to ketones. Indeed, in rats [96], rabbits [97], and humans [98], acetone is actively utilized, being metabolized by a microsomal acetone mono-oxygenase identified as CYP2E1 [99, 100]. Acetone is both an inducer and a substrate of CYP2E1 [101,102]. The existence of a gluconeogenic pathway for acetone was shown by the incorporation of [^{14}C] acetone into glucose and aminoacids during fasting and diabetic ketoacidosis [98,103,104]. The conversion of acetone to acetol and then to methylglyoxal, both intermediates in the gluconeogenic pathway, was demonstrated *in vitro* [96,97,105]. The contribution of CYP2E1 to the biotransformation of acetone was shown *in vivo* by the increase in blood acetone after the CYP2E1 inhibitors diallyl sulfide, diallyl sulfoxide, and diallyl sulfone [106]. It is noteworthy, as already pointed out by Reichard et al., [103] that acetone may be a significant gluconeogenic precursor in fasting humans, accounting for 10% of the gluconeogenic demands.

The role of CYP2E1 in fatty acid metabolism also supports the concept of a nutritional role for CYP2E1. Indeed, CYP2E1, in addition to its ethanol oxidizing activity, catalyzes fatty acid ω-1 and ω-2 hydroxylations [107-109]. Ethanol feeding also results in an increased activity of CYP4A1 [110]. The CYP4A subfamily catalyzes ω-hydroxylation at the terminal carbon of fatty acids. Further oxidation of the ω and ω-1 hydroxyacids by alcohol and aldehyde dehydrogenases results in the production of dicarboxylic and oxo-carboxylic acids.

5) Gender difference

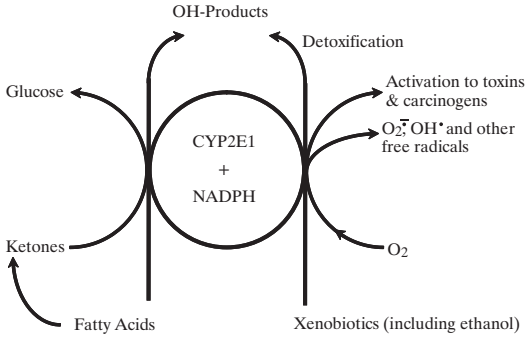
It is of interest that dicarboxylic acids (products of the CYP4A-mediated pathway) play a regulatory role in the hepatic disposition of non-esterified fatty acids by activating a peroxisomal proliferator nuclear receptor alpha (PPNRα) which increases the transcription of key fatty acid disposal pathways, such as the microsomal CYP4A1, the peroxisomal acyl-CoA oxidase and the liver fatty acid-binding protein (L-FABPc) in the cytosol [111]. This, in turn, results in enhanced microsomal ω-oxidation and peroxisomal β-oxidation of fatty acids and in a greater stimulatory effect of L-FABPc on microsomal acylglycerol synthesis [112].

The resulting changes exacerbate the gender difference in the alcohol-induced lipid abnormalities and in the vulnerability to alcoholic liver disease. It was known that gender differences in ethanol distribution [113], bioavailability [114] and hepatic metabolism [115], with increased production of acetaldehyde [116], may contribute to women's vulnerability to alcohol consumption. In addition, gender differences in alcohol-induced derangements of hepatic lipid metabolism, affecting the disposition of potentially toxic fatty acids, have also been reported in animal experiments [117]. Indeed, chronic alcohol administration increased ω-oxidation more effectively in male than in female rats [110], explaining the potentially deleterious accumulation of non-esterified fatty acids in the liver of the alcohol-fed females [117]. This was also associated with a much smaller ethanol-induced increase in cytosolic L-FABPc and microsomal esterification in females than in males, whereas the inhibition of mitochondrial β-oxidation was similar in both genders. Related findings have been observed in humans [118] and contribute to the greater vulnerability of women to the development of alcoholic liver injury.

6) CYP2E1 and oxidative stress

The increase of CYP2E1 has also been shown to play a key role in the pathogenesis of alcoholic liver injury, including ASH, because of the oxidative stress it generates [51]. Actually, CYP2E1 is also invariably elevated in the liver of patients with NASH [119] because fatty acids (which increase in obesity) and ketones (which increase in diabetes) are also substrates for CYP2E1 (vide supra and *Fig. 5*); their excess up-regulates CYP2E1. Although the pathogenesis of NAFLD and NASH has not yet been fully elucidated, a popular mechanism is the "Two Hit" theory [120], the first hit being the accumulation, by several causes (such as obesity), of fatty acids in the liver. The second hit is the peroxidation of these fatty acids because of the oxidative stress produced by different factors, such as CYP2E1 induction [121].

Figure 5. **Physiologic and toxic roles of cytochrome P4502E1 (CYP2E1), the main enzymes of the microsomal ethanol oxidizing system (MEOS). Many endogenous and xenobiotic compounds, including ethanol, ketones, and fatty acids, are substrates for CYP2E1 and induce its activity through various mechanisms, resulting in an array of beneficial as well as harmful effects. NADPH = Reduced form of nicotinamide adenine dinucleotide phosphate (from** [52])



7) Mitochondrial injury

The damage caused by oxidative stress in both ASH and NASH includes mitochondrial injury, which in turn exacerbates the oxidative stress [51]. That mitochondrial damage is a key component of alcoholic liver injury is well established [5], but NASH is also associated with mitochondrial structural defects whereas NAFLD is not. This mitochondrial dysfunction contributes to the oxidative stress in NASH [122].

Like many other useful adaptive systems, when the adaptation becomes excessive, adverse consequences prevail. CYP2E1 leaks oxygen radicals as part of its operation (*Fig. 5*) and when they exceed the cellular defense systems, they result in oxidative stress with its pathologic consequences. This is true when excess alcohol has to be metabolized, as in ASH, or when CYP2E1 is confronted by an excess of ketones and fatty acids associated with diabetes and/or obesity, resulting in NASH.

8) NASH

No therapy for NASH has been proven clearly effective [123-126]. Preliminary data with antioxidants such as vitamin E and C in the treatment of NASH are promising [127], but need to be confirmed. In view of the pathogenic role of CYP2E1 in both ASH and NASH, inhibitors are being considered to oppose the up-regulation of CYP2E1 activity by either ethanol, fatty acids or ketones, which results in increased generation of reactive radicals and toxic metabolites. Indeed, it has been suggested that CYP2E1 inhibitors may eventually provide useful tools for the prevention and treatment of the hepatotoxicity associated with heavy drinking as well as overeating. Indeed, experimentally, a decrease in the inducibility of CYP2E1 was found to be associated with a reduction in associated liver injury [128,129] but when the liver pathology was semiquantitated, it was only partially ameliorated by the CYP2E1 inhibitors used [130,131]. Indeed, the CYP2E1 inhibition was incomplete. Whereas CYP2E1 inhibitors completely blocked lipid peroxidation, they only partially prevented other lipid abnormalities [128]. Thus far, no corresponding human data are available, mainly because of a lack of very effective agents suitable for chronic human use. Several inhibitors [132] have been used or are being developed [133] but there is still a need to obtain CYP2E1 inhibitors which are both effective and also innocuous enough to be used chronically in humans.

It is noteworthy that preliminary results in a rat model of NASH [134] revealed that the combination of SAMe and DLPC, two physiological antioxidants, reduces the oxidative stress resulting from the CYP2E1 induction generated by an excess of lipids, thereby significantly attenuating key changes associated with NASH. Accordingly, since SAMe and DLPC are innocuous, clinical trials with NASH patients can now be considered.

Using the same NASH experimental models, acarbose, a glucosidase inhibitor, was also found to attenuate the NASH [135].

Progression of fatty liver to inflammation and fibrosis

1. Role of products of lipid peroxidation

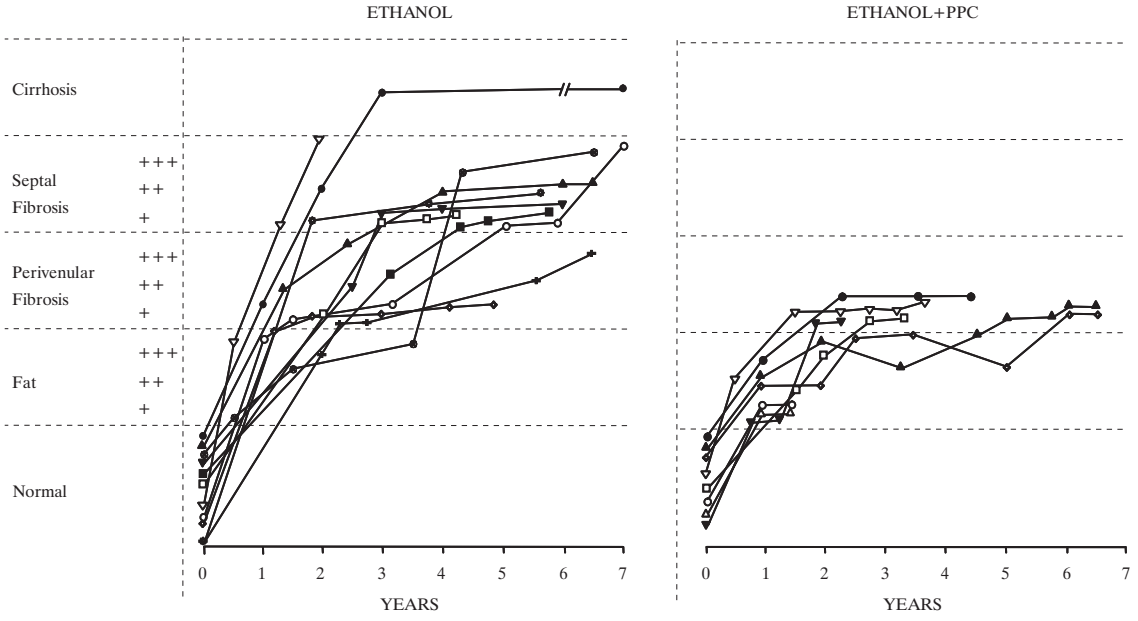
The oxidative stress caused by CYP2E1 induction, acetaldehyde and mitochondrial injury (vide supra) results in lipid peroxidation and membrane damage. In addition, lipid peroxidation products such as 4-hydroxynonenal stimulate fibrogenesis which is also increased through feedback inhibition of collagen synthesis because acetaldehyde forms adducts with the carboxyl-terminal propeptide of procollagen [40].

2. Role of inflammation, cytokines and anticytokine therapy

Oxidative stress promotes inflammation which is aggravated by an increase of the proinflammatory cytokine TNF-α in the Kupffer cells. TNF-α causes metabolic disturbances that are similar to the complications of alcoholic hepatitis, and it is also an important mediator of endotoxic shock and sepsis. Kupffer cells are a major source of cytokines. They also harbor CYP2E1 and its increase after chronic alcohol consumption [136] may act as a major stimulator. Indeed, in both acute and chronic liver diseases, Kupffer cells become activated to produce cytokines and reactive oxygen radicals [137]. Of potential therapeutic interest is the observation that DLPC (the active PC species of PPC) decreases stellate cell activation [138] and that it selectively modulates the LPS-induced activation of Kupffer cells by decreasing the production of the cytotoxic TNF-α, while potentiating the release of the protective IL-1β [139]. This dual action of DLPC on cytokines may provide a potent mechanism against liver injury. In addition to the reduction in TNF-α, the enhanced release of IL-1β could oppose the hepatotoxicity of TNF-α, either directly or indirectly through an IL-1β related increase in the tolerance to TNF-α mediated toxicity.

Recently, it has also been shown that ethanol induces transforming growth factor α (TGF-α) production in hepatocytes, leading to stimulation of collagen synthesis by hepatic stellate cells [140]. These results suggest that TGF-α derived from ethanol-exposed hepatocytes may contribute to the development of hepatic fibrosis in alcoholic liver disease. This effect was spe-

Figure 6. **Sequential development of alcoholic liver injury in baboons fed ethanol with adequate diet (left panel) and prevention of septal fibrosis and cirrhosis by supplementation with polyenylphosphatidylcholine (PPC) (right panel). Liver morphology in animals pair-fed control diets (with or without PPC) remained normal (not shown) (from** [146])



cifically inhibited by anti-TGF-α antibodies. It is noteworthy that experimentally DLPC also decreased TGF-β1-induced collagen mRNA by inhibiting p38 MAPK in hepatic stellate cells [141]. These are activated through induction of CYP2E1, acetaldehyde and by endotoxin. TNF-α is decreased by DLPC, but also in stellate cells not only in Kupffer cells (vide supra) [141]. In addition, collagen accumulation reflects not only enhanced synthesis, but results from an imbalance between collagen degradation and collagen production. Thus, cirrhosis might, in part, represent a relative failure of collagen degradation to keep pace with synthesis. Interestingly, PPC may affect this balance. Indeed, addition of PPC to transformed lipocytes was found to prevent the acetaldehyde-mediated increase in collagen accumulation, possibly by stimulation of collagenase activity [142]. The active ingredient was identified as DLPC [143]. The role of collagenase was also shown indirectly in man by the correlation of the development of alcoholic fibrosis with increased activity of the circulating tissue inhibitor of metalloproteinase (TIMP). Indeed, serum TIMP was significantly increased in alcoholic cirrhosis and may play a role in its pathogenesis through inhibition of collagenase activity. In addition, it can serve as a marker of precirrhotic states, since this test was more sensitive in detecting either perivenular fibrosis or septal fibrosis and offered better discrimination from fatty liver than serum procollagen peptide (PIIIP) [144]. The stimulation of collagenase activity [142] may explain, at least in part, why PPC attenuates the development of fibrosis (including cirrhosis) after chronic alcohol administration [145], an effect confirmed using more purified lecithin extracts, which again pointed to DLPC as the active ingredient of PPC [146] (*Fig. 6*). In the latter studies, the control livers remained normal whereas 10 of 12 baboons fed alcohol without PPC developed septal fibrosis or cirrhosis, with transformation of 81±3% of the hepatic lipocytes to collagen producing transitional cells. By contrast, none of the 8 animals fed alcohol with PPC developed septal fibrosis and cirrhosis, and only 48±9% of their lipocytes were transformed.

Antifibrotic therapy through correction of altered nutrient activation or of the deficiency in selective nutrients

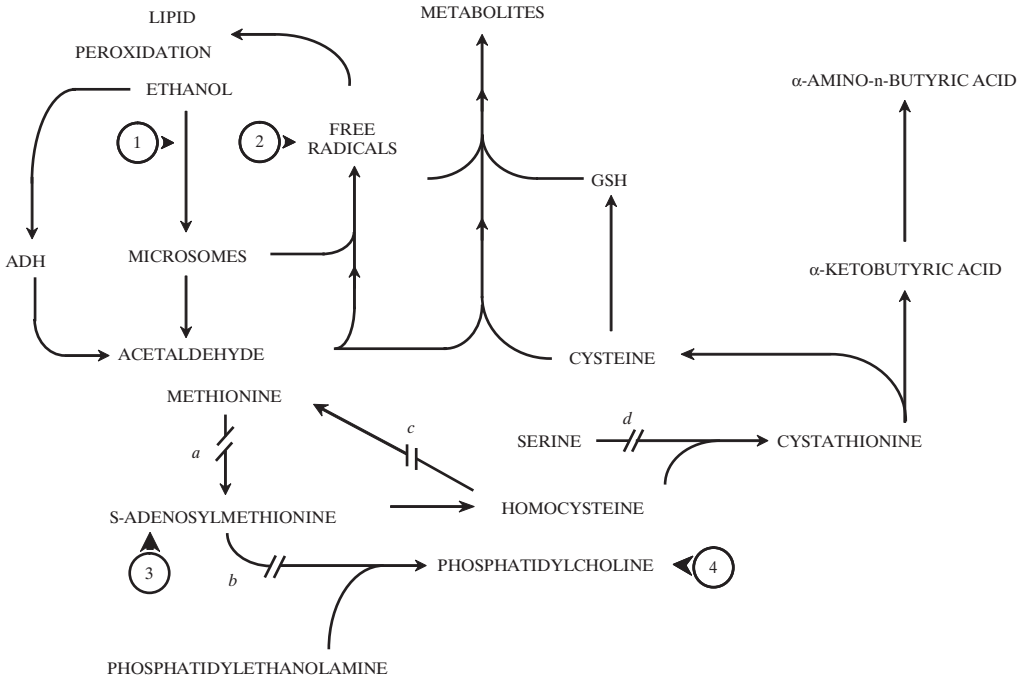
The nutritional approach to liver disease has been improved by recognizing the importance not only of providing a sufficient intake of nutrients but also of correcting the impairment in the activation of key nutrients by alcohol.

1. Methionine and S-adenosylmethionine (SAMe)

a. Pathogenesis of the deficiency and its consequences

Correction of methionine deficiency has been proposed for the treatment of liver diseases, especially the alcoholic variety, but excess methionine was shown to have some adverse effects, as reviewed elsewhere [147]. Whereas in some patients with alcoholic liver disease, circulating methionine levels are normal, in others elevated levels were observed and it was reported that the blood clearance of methionine after an oral load was slowed, suggesting impaired metabolism. Indeed, for most of its functions, methionine must be activated to SAMe and, in cirrhotic livers, Duce et al. [148] reported a decrease in the activity of the enzyme involved, namely SAMe synthetase, also called methionine adenosyltransferase [149] (*Fig. 7*). Furthermore, long-term ethanol consumption in non-human primates was associated

Figure 7. **Link between enhanced lipid peroxidation and accelerated acetaldehyde production as well as increased free radical generation by the induced microsomes, with sites of possible therapeutic interventions. Metabolic blocks caused by liver disease (a, b) or folate (c), B_{12} (c), or B_6 (d) deficiencies are illustrated, with corresponding depletions in S-adenosylmethionine, phosphatidylcholine, and reduced glutathione (GSH). New therapeutic approaches include: ① down-regulation of microsomal enzyme induction, especially of cytochrome P450 2E1 (CYP2E1); ② decrease of free radicals with antioxidants, and replenishment of ③ S-adenosylmethionine as well as "phosphatidylcholine. ADH = Alcohol dehydrogenase. (from** [147])



with a significant depletion of hepatic SAMe [145] which may have a number of adverse effects since SAMe is the principal methylating agent in various transmethylation reactions which are important for nucleic acid and protein synthesis, membrane fluidity and the transport of metabolites and transmission of signals across membranes. Because of impaired methyltransferase activity, SAMe depletion exacerbates the membrane injury induced by alcohol. Furthermore, SAMe plays a key role in the synthesis of polyamines and provides a source of cysteine for glutathione production (*Fig. 7*).

b. Therapeutic applications of SAMe, including clinical trials

In baboons, correction of the ethanol-induced hepatic SAMe depletion with oral SAMe administration resulted in a corresponding attenuation of ethanol-induced liver injury, as shown by a less striking glutathione (GSH) depletion, lesser increases in plasma AST and glutamic dehydrogenase (GDH) activities and fewer megamitochondria [145]. SAMe treatment is also beneficial in man, as shown in a prospective, multicenter, double-blind, placebo-controlled trial [128] performed in 220 inpatients with chronic liver diseases (chronic active hepatitis and cirrhosis, including primary biliary cirrhosis) in whom serum markers of cholestasis and subjective symptoms significantly improved after SAMe. Oral administration of 1 200 mg/day of SAMe for 6 months also resulted in a significant increase of hepatic GSH in patients with alcoholic as well as nonalcoholic liver disease [150]. Noteworthy is a two-year randomized, placebo-controlled, double-blind, multicenter clinical trial of SAMe in patients with alcoholic liver cirrhosis (Child class A and B) in whom SAMe improved survival (29% vs 12%, P=0.025) or delayed liver transplantation [151].

2. Antioxidant therapy

a. Polyenylphosphatidylcholine therapy

This promising therapy involves using polyunsaturated PPC or specifically its main PC species namely DLPC. An early event in the development of ALD is a decrease in hepatic levels of phospholipids, especially PC. This phospholipid deficiency is associated with altered mitochondrial membranes and correlates with morphologic changes and impaired function. Administration of PPC to primates corrected the ethanol-induced decrease in phospholipid levels and prevented progression to alcohol-induced septal fibrosis and cirrhosis [144], with an associated reduction in the number of activated stellate cells. These had been found to be strikingly increased in alcohol-fed baboons, contributing to the progression of fibrosis [152]. A similar transformation of stellate cells was observed in patients with alcoholic liver disease [153], and an attenuation of the activation of stellate cells to myofibroblast-like cells was also observed *in vitro* [138].

A possible explanation for the beneficial effect of PPC, especially DLPC, is the ability of liver cells to directly incorporate the phospholipids into cell membranes. This influ-

ences membrane structure and function. Indeed, the activity of membrane-bound enzymes was normalized: cytochrome oxidase, a key enzyme of the mitochondrial electron-transport chain, which requires a normal phospholipid milieu for optimal activity, and which is severely depressed by chronic ethanol consumption, was restored to normal by addition of PC *in vitro* [154] or by PPC supplementation *in vivo* [155], with improvement of hepatic mitochondrial respiration. It is noteworthy that misoprostol can also attenuate several functional alterations in liver mitochondria during alcohol consumption [156].

PPC promotes collagen breakdown [146] and it also acts as an antioxidant [157] which results in a protection against oxidative stress, as determined by normalization of 4-hydroxynonenal, F2-isoprostanes, and GSH levels [157]. Furthermore, the activity of phosphatidylethanolamine methyltransferase (PEMT), a key enzyme for the regeneration of hepatic PC, and which is depressed in alcoholic liver disease (*Fig. 7*), is also restored with PPC treatment [146].

All these favorable effects of PPC provided a basis for some of the reported therapeutic effects of unsaturated phospholipids in the treatment of liver diseases, including patients with alcoholic hepatitis [158]. Furthermore, PC was shown [146] to interfere with collagen formation from stellate cells which have a predominant role in alcohol-induced fibrogenesis. Subsequently, PPC was found to improve liver tests in patients with hepatitis C [159], and to ameliorate alcoholic liver disease in a pilot study of patients with underlying fibrosis [160]. This was followed by a randomized, prospective, double-blind, placebo-controlled clinical trial conducted in twenty VA medical centers with 789 patients (97% male; mean age 48.8 years) averaging 16 drinks/day (1 drink =14 g alcohol) for nineteen years [161]. A baseline liver biopsy confirmed the presence of perivenular or septal fibrosis or incomplete cirrhosis. Alcohol intake was reduced to approximately two-and-one-half drinks/day as a result of the Brief Intervention approach [162,163]. Accordingly, there was no more progression of the fibrosis and therefore no way to test whether PPC could oppose such a progression except for a subgroup who were still consuming 6 or more drinks a day and who displayed beneficial effects against fibrosis. Ascites, an important secondary clinical measure of liver disease, was also less frequently observed during follow-up of PPC treated patients. Furthermore, improvement in transaminases and bilirubin favoring PPC was seen in the subgroup of HCV^+ drinkers. We found that 25% of all patients with alcoholic liver disease are HCV^+, with an even higher incidence in some U.S. urban areas [164]. In addition to antiviral medications, agents that oppose oxidative stress and fibrosis such as PPC are also being tested for hepatitis C treatment since these two processes contribute much to the pathology and mortality associated with the virus.

b. Silymarin and other antioxidants

Silymarin was found to be effective against various liver injuries in rodents. In patients with alcoholic liver disease, some randomized controlled trials with silymarin showed beneficial effects such as improved survival [165]. However, other studies did not verify such an effect [166]. To clarify this issue, silymarin was studied under controlled conditions in non-human primates and it was found to oppose the alcohol-induced oxidative stress and to retard the development of alcohol-induced hepatic fibrosis [161]. The negative outcome observed in some clinical trials possibly reflects poor compliance. Thus, in view of the innocuity of silymarin, it might be justified to verify its effect in additional clinical studies. Theoretically, other antioxidants, such as alpha-tocopherol, might be useful. Indeed, in patients with cirrhosis, diminished hepatic vitamin E levels have been observed [167]. However, therapeutic results were disappointing [168].

3. Anti-inflammatory therapy

a. Corticosteroids

Because of the potential role of inflammatory factors in the pathogenesis of fibrosis and cirrhosis, anti-inflammatory therapy with corticosteroids has been used. It improved survival rates in encephalopathic patients but not in those with milder illnesses [169]. In patients who had either spontaneous hepatic encephalopathy or a high hepatic discriminant function (based on elevated prothrombin time and bilirubin concentration), prednisolone improved survival by 2 months [170]. However, Christensen and Gluud [171] performed a meta-analysis of 15 treatment trials and concluded that steroids are of no benefit in alcoholic hepatitis. Obviously, multiple factors affect the outcome and present a great challenge to the clinician.

b. Colchicine

Colchicine has also been evaluated as a treatment for alcoholic cirrhosis because of its anti-inflammatory and antifibrotic effect [172]. At the end of this study, patients in the colchicine group had a significantly better 5-year survival. However, these beneficial effects were not confirmed in a recent multicenter trial [173].

Conclusions

The better understanding of the pathogenesis of alcoholic liver disease resulting from extensive research carried out over the last half century has raised prospects for better treatment as well. Our present therapeutic approach is to first reduce excess alcohol consumption by the Brief Intervention technique which, in a recent controlled study, resulted in a significant and striking decrease of alcohol consumption from an average 18 to only 2½ drinks per day sustained for up to 5 years [162]. New tools have also been developed for early recognition and detection of heavy drinkers, based on circulating biologic markers, mainly carbohydrate-deficient transferrin, and preferably in association with γ-glutamyltransferase (GGT) [174]. These biochemical markers, combined with screening for signs of medical complications, help overcome patient denial and facilitate early detection. Craving can be opposed by such pharmacologic agents as naltrexone [175].

Thus, effective prevention and therapy against steatosis and its progression to more severe injury can be achieved by a multifactorial approach: control of alcohol consumption, avoidance of obesity and excess dietary long-chain fatty acids, and replenishment of SAMe and the phosphatidylcholines (using PPC).

Acknowledgement

Original studies were supported, in part, by NIH grants AA11115 and AT001583, the Department of Veterans Affairs, the Kingsbridge Research Foundation and the Christopher D. Smithers Research Foundation. I am grateful to Ms. Y. Rodriguez for her secretarial assistance and Ms. F. DeMara for her editorial assistance.

References

1. Chedid A, Mendenhall CL, Gartside P, French SW, Chen T, Rabin L and the VA Cooperative Group: Prognostic factors in alcoholic liver disease. Am J Gastroenterol, 1991; 82: 210-6.

2. Best CH, Hartroft WS, Lucas CC, Ridout JH. Liver damage produced by feeding alcohol or sugar and its prevention by choline. Brit Med J, 1949; 2:1001-6.

3. Lieber CS, Jones DP, Mendelson J, DeCarli LM. Fatty liver, hyperlipemia and hyperuricemia produced by prolonged alcohol consumption, despite adequate dietary intake. Trans Assoc Am Physicians, 1963; 76: 289-300.

4. Lieber CS, Jones DP, DeCarli LM. Effects of prolonged ethanol intake: Production of fatty liver despite adequate diets. J Clin Invest, 1965; 44: 1009-21.

5. Lieber CS (Ed.) Medical and Nutritional Complications of Alcoholism: Mechanisms and Management. New York: Plenum Press, 1992, p. 579.

6. Leo MA, Lieber CS. New pathway for retinol metabolism in liver microsomes. J Biol Chem, 1985; 260: 5228-31.

7. Leo MA, Kim CI, Lieber CS. Role of vitamin A degradation for the control of hepatic levels in the rat. J Nutr, 1989; 119: 993-1000.

8. Leo MA, Lasker JM, Raucy JL, Kim C, Black M, Lieber CS. Metabolism of retinol and retinoic acid by human liver cytochrome P450IIC8. Arch Biochem Biophys, 1989; 269: 305-12.

9. Leo MA, Iida S, Lieber CS. Retinoic acid metabolism by a system reconstituted with cytochrome P-450. Arch Biochem Biophys, 1984; 234: 305-12.

10. Sato M, Lieber CS. Changes in vitamin A status after acute ethanol administration in the rat. J Nutr, 1982; 112: 1188-96.

11. Leo MA, Lowe N, Lieber CS. Interaction of drugs and retinol. Biochem Pharmacol, 1986; 35: 3949-53.

12. Sato M, Lieber CS. Hepatic vitamin A depletion after chronic ethanol consumption in baboons and rats. J Nutr, 1981; 111: 2015-23.

13. Leo MA, Lieber CS. Hepatic vitamin A depletion in alcoholic liver injury. N Engl J Med, 1982; 307: 597-601.

14. Leo MA, Sato M, Lieber CS. Effect of hepatic vitamin A depletion on the liver in humans and rats. Gastroenterology, 1983; 84: 562-72.

15. Leo MA, Lieber CS. Hepatic fibrosis after long term administration of ethanol and moderate vitamin A supplementation in the rat. Hepatology, 1983; 3: 1-11.

16. Leo MA, Arai M, Sato M, Lieber CS. Hepatotoxicity of vitamin A and ethanol in the rat. Gastroenterology, 1982; 82: 194-205.

17. Leo MA, Kim CI, Lowe N, Lieber CS. Interaction of ethanol with β-carotene: Delayed blood clearance and enhanced hepatotoxicity. Hepatology, 1992; 15: 883-91.

18. Ahmed S, Leo MA, Lieber CS. Interactions between alcohol and beta-carotene in patients with alcoholic liver disease. Am J Clin Nutr, 1994; 60: 430-6.

19. Leo MA, Lieber CS. Alcohol, vitamin A, and beta-carotene: Adverse interactions, including hepatotoxicity and carcinogenicity. Amer J Clin Nutr, 1999; 69: 1071-85.

20. Lieber CS, Lefevre A, Spritz N, Feinman L, DeCarli LM. Difference in hepatic metabolism of long and medium-chain fatty acids: The role of fatty acid chain length in the production of the alcoholic fatty liver. J Clin Invest, 1967; 46: 1451-60.

21. Nanji AA, Yang EK, Fogt F, Hossein Sadrzadeh SM, Dannenberg AJ. Medium chain triglycerides and vitamin E reduce the severity of established experimental alcoholic liver disease. J Pharma Exper Therap, 1996; 277: 1694-700.

22. Lieber CS, DeCarli LM. Quantitative relationship between the amount of dietary fat and the severity of the alcoholic fatty liver. Am J Clin Nutr, 1970; 23: 474-8.

23. Lieber CS, DeCarli, LM, Schmid, R. Effects of ethanol on fatty acid metabolism in liver slices. Biochem Biophys Res Comm, 1959; 1: 302-6.

24. Lieber CS, Schmid R. The effect of ethanol on fatty acid metabolism: Stimulation of hepatic fatty acid synthesis in vitro. J Clin Invest, 1961; 40: 394-9.

25. Galli A, Price D, Crabb D. High-level expression of rat class I alcohol dehydrogenase is sufficient for ethanol-induced fat accumulation in transduced HeLa cells. Hepatology, 1999; 29: 1164-70.

26. Lieber CS, DeCarli LM. An experimental model of alcohol feeding and liver injury in the baboon. J Med Primatol, 1974; 3: 153-63.

27. Keilin D, Hartree EF. Properties of catalase: catalysis of coupled oxidation of alcohols. Biochem J, 1945; 39: 293-301.

28. Wolff PH. Vasomotor sensitivity to alcohol in diverse mongoloid populations. American Journal of Human Genetics, 1973; 25: 193-9.

29. Goedde HW, Harada S, Agarwal DP. Racial differences in alcohol sensitivity: a new hypothesis. Human Genetics, 1979; 51: 331-4.

30. Harada S, Misawa S, Agarwal DP, Goedde HW. Studies on liver alcohol and acetaldehyde dehydrogenase variants in Japanese. Hoppe-Seylers Z Physiol Chem, 1979; 360: 278.

31. Horowitz JH, Rypins EB, Henderson JM, Heymsfield SB, Moffitt SD, Bain RP, Chawla RK, Bleier JC, Rudman D. Evidence for impairment of transsulfuration pathway in cirrhosis. Gastroenterology, 1981; 81: 668-75.

32. Teng YS. Human liver aldehyde dehydrogenase in Chinese and Asiatic Indians: gene deletion and its possible implications in alcohol metabolism. Biochemical Genetics, 1981; 19: 107-4.

33. Yoshihara H, Sato N, Kamada T, Abe H. Low Km ALDH isozyme and alcoholic liver injury. Pharmacol Biochem Behav, 1983; 18(Suppl. 1): 425-8.

34. Harada S, Agarwal DP, Goedde HW, Ishikawa B. Aldehyde dehydrogenase isozyme variation and alcoholism in Japan. Pharmacology, Biochemistry and Behaviour, 1983; 18: 151-3.

35. Enomoto N, Takase S, Takada N, Takada A. Alcoholic liver disease in heterozygotes of mutant and normal aldehyde dehydrogenase-2 genes. Hepatology, 1991; 13: 1071-5.

36. Hasumura Y, Teschke R, Lieber CS. Acetaldehyde oxidation by hepatic mitochondria: Its decrease after chronic ethanol consumption. Science, 1975; 189: 727-9.

37. Di Padova C, Worner TM, Julkunen RJK, Lieber CS. Effects of fasting and chronic alcohol consumption on the first pass metabolism of ethanol. Gastroenterology, 1987; 92: 1169-73.

38. Lieber CS, Baraona E, Hernandez-Munoz R, Kubota S, Sato N, Kawano S, Matsumura T, Inatomi N. Impaired oxygen utilization: A new mechanism for the hepatotoxicity of ethanol in sub-human primates. J Clin Invest, 1989; 83: 1682-90.

39. Baraona E, Leo MA, Borowsky SA, Lieber CS. Pathogenesis of alcohol-induced accumulation of protein in the liver. J Clin Invest, 1977; 60: 546-54.

40. Ma X, Svegliati-Baroni G, Poniachik J, Baraona E, Lieber CS. Collagen synthesis by liver stellate cells is released from its normal feedback regulation by acetaldehyde-induced modification of the carboxyl-terminal propeptide of procollagen. Alcohol Clin Exp Res, 1997; 21; 1204-11.

41. Nomura F, Lieber CS. Binding of acetaldehyde to rat liver microsomes: enhancement after chronic alcohol consumption. Biochem Biophys Res Commun, 1981; 100: 131-7.

42. Behrens UH, Hoerner M, Lasker JM, Lieber CS. Formation of acetaldehyde adducts with ethanol-inducible P450IIE1 in vivo. Biochem Biophys Res Comm, 1988; 154: 584-90.

43. Wondergem R, Davis J. Ethanol increases hepatocyte water volume. Alcohol Clin Exp Res, 1994; 18: 1230-6.

44. Hoerner M, Behrens UJ, Worner T, Lieber CS. Humoral immune response to acetaldehyde adducts in alcoholic patients. Res Commun Chem Pathol Pharmacol, 1986; 54: 3-12.

45. Niemela O, Klajner F, Orrego H, Vidins E, Blendis L, Israel Y. Antibodies against acetaldehyde-modified protein epitopes in human alcoholics. Hepatology, 1987; 7: 1210-4.

46. Baraona E, Lieber CS. Effects of alcohol on hepatic transport of proteins. Ann Rev Med, 1982; 33: 281-92.

47. Lieber CS. Hepatic and other medical disorders. In: Wartenberg AA, Graham AW, (editors). Principles of Addiction Medicine. American Society of Addiction Medicine (ASAM), Washington, DC. 2nd Edition, Ch. 3, pp 755-74, 1998.

48. Espina N, Lima V, Lieber CS, Garro AJ. In vitro and in vivo inhibitory effect of ethanol and acetaldehyde on O6-methylguanine transferase. Carcinogenesis, 1988; 9: 761-6.

49. Baraona E, Matsumura T, Hernandez R, Kubota S, Soong J, Kawano S, Kato S, Inatomi N, Sato S, Lieber CS. Chronic alcohol intake impairs liver oxygenation by decreasing O2 utilization. In: K Kuriyama, A Takada and H Ishii, editors. Biomedical and Social Aspects of Alcohol and Alcoholism. Amsterdam, The Netherlands: Elsevier Science Publishers BV; 1988; p. 387-90.

50. Hasumura Y, Teschke R, Lieber CS. Characteristics of acetaldehyde oxidation in rat liver mitochondria. J Biol Chem, 1976; 251: 4908-13.

51. Lieber CS. Cytochrome P-4502E1: Its physiological and pathological role. Physiol Rev, 1997; 77: 517-44.

52. Lieber CS. Microsomal ethanol-oxidizing system (MEOS), the first 30 years (1968-1998) – a review. Alcohol Clin Exp Res, 1999; 23: 991-1007.

53. Iseri OA, Gottlieb LS, Lieber CS. The ultrastructure if ethanol induced fatty liver. Fed Pro, 1964; 23: 579A.

54. Iseri OA, Lieber CS, Gottlieb LS. The ultrastructure of fatty liver induced by prolonged ethanol ingestion. Am J Path, 1966; 48: 535-55.

55. Lane BP, Lieber CS. Ultrastructural alterations in human hepatocytes following ingestion of ethanol with adequate diets. Am J Path, 1966; 49: 593-603.

56. Ishii H, Joly J-G, Lieber CS. Effect of ethanol on the amount and enzyme activities of hepatic rough and smooth microsomal membranes. Biochim Biophys Acta, 1973; 291: 411-20.

57. Ishii H, Joly J-G, Lieber CS. Increase of microsomal glucose-6-phosphatase activity after chronic ethanol administration. Metabolism, 1973; 22: 799-806.

58. Meldolesi J. On the significance of the hypertrophy of the smooth endoplasmic reticulum in liver cells after administration of drugs. Biochem Pharmacol, 1967; 16: 125-31.

59. Conney AH. Pharmacological implications of microsomal enzyme induction. Pharmacol Rev, 1967; 19: 317-66.

60. Lane BP, Lieber CS. Effects of butylated hydroxytoluene on the ultrastructure of rat hepatocytes. Lab Invest, 1967; 16: 341-8.

61. Lieber CS, DeCarli LM. Ethanol oxidation by hepatic microsomes: Adaptive increase after ethanol feeding. Science, 1968; 162: 917-8.

62. Isselbacher KJ, Carter EA. Ethanol oxidation by liver microsomes: Evidence against a separate and distinct enzyme system. Biochem Biophys Res Commun, 1970; 39: 530-7.

63. Ziegler DM. Discussion in Microsomes and Drug Oxidations (Estabrook RW, Gillette RJ and Lieberman KC eds) p. 458, Williams & Wilkins, Baltimore, MD, 1972.

64. Roach MK, Resse WN Jr., Creaven PJ. Ethanol oxidation in the microsomal fraction of rat liver. Biochem Biophys Res Commun, 1969; 36: 596-602.

65. Tephly TR, Tinelli F, Watkins WD. Alcohol metabolism: Role of microsomal oxidation in vivo. Science, 1969; 166: 627-8.

66. Vatsis KP, Schulman MP. Absence of ethanol metabolism in "acatalatic" hepatic microsomes that oxidize drugs. Biochem Biophys Res Commun, 1973; 52: 588-94.

67. Vatsis KP, Schulman MP. An unidentified constituent of ethanol oxidation in hepatic microsomes (Abstract). Fed Proc, 1974; 33: 554.

68. Teschke R, Hasumura Y, Lieber CS. Hepatic microsomal alcohol-oxidizing system in normal and acatalasemic mice: its dissociation from the peroxidatic activity of catalase-H2O2. Mol Pharmacol, 1975; 11: 841-9.

69. Teschke R, Hasumura Y, Joly JG, Ishii H, Lieber CS. Microsomal ethanol-oxidizing system (MEOS): purification and properties of a rat liver system free of catalase and alcohol dehydrogenase. Biochem Biophys Res Commun, 1972; 49: 1187-93.

70. Teschke R, Hasumura Y, Lieber CS. Hepatic microsomal ethanol oxidizing system: Solubilization, isolation and characterization. Arch Biochem Biophys, 1974; 163: 404-15.

71. Chance B. An intermediate compound in the catalase-hydrogen reaction. Acta Chem Scand, 1947; 1: 236-67.

72. Teschke R, Hasumura Y, Lieber CS. NADPH dependent oxidation of methanol, ethanol, propanol and butanol by hepatic microsomes. Biochem Biophys Res Commun, 1974; 60: 851-7.

73. Fabry TL, Lieber CS. The photochemical action spectrum of the microsomal ethanol oxidizing system. Alcohol Clin Exp Res, 1979; 3: 219-22.

74. Ohnishi K, Lieber CS. Reconstitution of the microsomal ethanol-oxidizing system: Qualitative and quantitative changes of cytochrome P-450 after chronic ethanol consumption. J Biol Chem, 1977; 252: 7124-31.

75. Miwa GT, Levin W, Thomas PE, Lu AYH. The direct oxidation of ethanol by a catalase- and alcohol dehydrogenase-free reconstituted system containing cytochrome P-450. Arch Biochem Biophys, 1978; 187: 464-75.

76. Thurman RG, Ley HG, Scholz R. Hepatic microsomal ethanol oxidation. Hydrogen peroxide formation and the role of catalase. Eur J Biochem, 1972; 25: 420-30.

77. Thurman RG, Brentzel HJ. The role of alcohol dehydrogenase in microsomal ethanol oxidation and the adaptive increase in ethanol metabolism due to chronic treatment with ethanol. Alcohol Clin Exp Res, 1977; 1: 33-8.

78. Teschke R, Matsuzaki S, Ohnishi K, DeCarli LM, Lieber CS. Microsomal ethanol oxidizing system (MEOS): Current status of its characterization and its role. Alcohol Clin Exp Res, 1977; 1: 7-15.

79. Tsutsumi M, Lasker JM, Shimizu M, Rosman AS, Lieber CS. The intralobular distribution of ethanol-inducible P450IIE1 in rat and human liver. Hepatology, 1989; 10: 437-46.

80. Takahashi T, Lasker JM, Rosman AS, Lieber CS. Induction of cytochrome P4502E1 in the human liver by ethanol is caused by a corresponding increase in encoding messenger RNA. Hepatology, 1993; 17: 236-45.

81. Koop DR, Morgan ET, Tarr GE, Coon MJ. Purification and characterization of a unique isozyme of cytochrome P-450 from liver microsomes of ethanol-treated rabbits. J Biol Chem, 1982; 257: 8472-80.

82. Ingelman-Sundberg M, Johansson I. Mechanisms of hydroxyl radical formation and ethanol oxidation by ethanol-inducible and other forms of rabbit liver microsomal cytochromes P-450. J Biol Chem, 1984; 259: 6447-58.

83. Ryan DE, Ramanthan L, Iida S, Thomas PE, Haniu M, Shively JE, Lieber CS, Levin, W. Characterization of a major form of rat hepatic microsomal cytochrome P450 induced by isoniazid. J Biol Chem, 1985; 260: 6385-93.

84. Ryan DE, Koop DR, Thomas PE, Coon MJ, Levin W. Evidence that isoniazid and ethanol induce the same microsomal cytochrome P-450 isozyme 3a. Arch Biochem Biophys, 1986; 246: 633-44.

85. Wrighton SA, Thomas PE, Molowa DT, Haniu M, Shively JE, Maines SL, Watkins PB, Parker G, Mendez-Picon G, Levin W, Guzelian PS. Characterization of ethanol-inducible human liver N-nitrosodimethylamine demethylase. Biochemistry, 1986; 25: 6731-5.

86. Song BJ, Gelboin HV, Park SS, Yang CS, Gonzalez FJ. Complementary DNA and protein sequences of ethanol-inducible rat and human cytochrome P450s: Transcriptional and post-transcriptional regulation of the rat enzyme. J Biol Chem, 1986; 261: 166-89.

87. Nelson DR, Kamataki T, Waxman DJ, Guengerich FP, Estabrook RW, Fetereisen R, Gonzalez FJ, Coon MJ, Gunsalus IC, Gotoh O, Okuda K, Nebert DW. The P450 superfamily: update on new sequences, gene mapping, accession numbers, early trivial names of enzymes, and nomenclature. DNA Cell Biol, 1993; 12: 1-51.

88. Umeno M, Song BJ, Kozak C, Gelboin HV, Gonzalez FJ. The rat p-450IIE1 gene: complete intron and exon sequence, chromosome mapping, and correlation of developmental expression with specific 5' cytosine demethylation. J Biol Chem, 1988; 263: 4956-62.

89. Umeno M, McBride OW, Yang CS, Gelboin HV, Gonzalez FJ. Human ethanol-inducible p-450IIE1: complete gene sequence, promoter characterization, chromosomes mapping, and cDNA directed expression. Biochemistry, 1988; 27: 9006-13.

90. Khani SC, Porter TD, Coon MJ. Isolation and partial characterization of the gene for cytochrome p-450 3a (p-450ALC) and a second closely related gene. Biochem Biophys Res Commun, 1988; 150: 10-7.

91. Ueno T, Gonzalez FJ. Transcriptional control of the rat hepatic CYP2E1 gene. Mol Cell Biol, 1990; 10: 4495-505.

92. Botto G, Serre E, El Khyari S, Cau P, Henric A, DeMeo M, Bergeron P, Barra Y. Hypomethylation and hypoexpression of human CYP2E1 gene in lung tumors. Biochem Biophys Res Commun, 1994; 205: 1086-92.

93. Lieber CS, DeCarli LM. The role of the hepatic microsomal ethanol oxidizing system (MEOS) for ethanol metabolism in vivo. J Pharmacol Exp Ther, 1972; 181: 279-87.

94. Salaspuro MP, Lieber CS. Non-uniformity of blood ethanol elimination: its exaggeration after chronic consumption. Ann Clin Res, 1978; 10: 294-7.

95. Koop DR, Tierney DJ. Multiple mechanisms in the regulation of ethanol-inducible cytochrome P-450IIE1. Bio Essays, 1990; 9: 429-35.

96. Casazza JP, Felver ME, Veech RL. The metabolism of acetone in rat. J Biol Chem, 1984; 259: 231-6.

97. Koop DR, Casazza JP. Identification of ethanol-inducible P450 enzyme 3a as the acetone and acetol monooxygenase of rabbit microsomes. J Biol Chem, 1985; 260: 13607-12.

98. Reichard GA Jr., Skutches CL, Hoeldke RD, Owen OW.

Acetone metabolism in humans during diabetic ketoacidosis. Diabetes, 1986; 35: 668-74.

99. Casazza JP, Veech RL. The production of 1,2 propanediol in ethanol treated rats. Biochem Biophys Res Commun, 1985; 129: 426-30.

100. Johansson IJ, Eliasson E, Norsten C, Ingelman-Sundberg M. Hydroxylation of acetone by ethanol- and acetone-inducible cytochrome P450 in liver microsomes and reconstituted membranes. FEBS Lett, 1986; 196: 59-64.

101. Yang CS, Yoo J-S, Ishizaki H, Hong J. Cytochrome P450IIE1: roles in nitrosamine metabolism and mechanisms of regulation. Drug Metab Rev, 1990; 22: 147-59.

102. Koop DR. Oxidative and reductive metabolism by cytochrome P450 2E1. FASEB J, 1992; 6: 724-30.

103. Reichard GA Jr, Haff AC, Skutches CL, Paul P, Holroyde CP, Owen OE. Plasma acetone metabolism in the fasting human. J Clin Invest, 1979; 63: 619-26.

104. Owen OE, Trapp VE, Skutches CL, Mozzoli MA, Hoieldtke RO, Boden G, Reichard GA Jr. Acetone metabolism during diabetic ketoacidosis. Diabetes, 1982; 31: 242-8.

105. Casazza JP, Fu JL. Measurement of acetol in serum. Anal Biochem, 1985; 148: 344-8.

106. Chen L, Lee M, Hong J-Y, Huang W, Wang E, Yang CS. Relationship between cytochrome P450 2E1 and acetone catabolism in rats as studied with diallyl sulfide as an inhibitor. Biochem Pharmacol, 1994; 48: 2199-205.

107. Laethem RM, Balaxy M, Falck JR, Laethem CL, Koop DR. Formation of 19(S)-, 19(R)-, and 18(R)-Hydroxyeicosatetraenoic acids by alcohol-inducible cytochrome P4502E1. J Biol Chem, 1993; 268: 12912-8.

108. Amet Y, Berthou F, Goasduff T, Salaun JP, Le Breton L, Menez JF. Evidence that cytochrome P450 2E1 is involved in the (ω-1)-hydroxylation of lauric acid in rat liver microsomes. Biochem Biophys Res Comm, 1994; 203: 1168-74.

109. Adas F, Betthou F, Picart D, Lozach P, Beauge F, Amet Y. Involvement of cytochrome P450 2E1 in the (omega-1)-hydroxylation of oleic acid in human and rat liver microsomes. J Lipid Res, 1998; 39: 1210-9.

110. Ma XL, Baraona E, Lieber CS. Alcohol consumption enhances fatty acid ω-oxidation, with a greater increase in male than in female rats. Hepatology, 1993; 18: 1247-53.

111. Kaikaus RM, Chan WK, Ortiz de Montellano PR, Bass NM. Mechanisms of regulation of liver fatty acid-binding protein. Mol Cell Biochem, 1993; 123: 93-100.

112. Burnett DA, Lysenko N, Manning JA, Ockner RK. Utilization of long chain fatty acids by rat liver: Studies of the role of fatty acid binding protein. Gastroenterology, 1979; 77: 241-9.

113. Jones BM, Jones MK. Male and female intoxication levels for three alcohol doses or do women really get higher than men? Alcohol Technical Report, 1976; 5: 544-83.

114. Frezza M, DiPadova C, Pozzato G, Terpin M, Baraona E, Lieber CS. High blood alcohol levels in women: Role of decreased gastric alcohol dehydrogenase activity and first pass metabolism. N Eng J Med, 1990; 322: 95-9.

115. Mishra L, Sharma S, Potter JJ, Mezey E. More rapid elimination of alcohol in women as compared to their male siblings. Alcohol Clin Exp Res, 1989; 13: 752-4.

116. Eriksson CJP, Fukunaga T, Sarkola T, Linholm H, Ahola L. Estrogen-related acetaldehyde elevation in women during alcohol intoxication. Alcohol Clin Exp Res, 1996; 20: 1192-5.

117. Shevchuk O, Baraona E, Ma XL, Pignon JP, Lieber CS. Gender differences in the response of hepatic fatty acids and cytosolic fatty acid-binding capacity to alcohol consumption in rats. Proc Soc Exp Biol Med, 1991; 198: 584-90.

118. Ma X, Baraona E, Goozner BG, Lieber CS. Gender differences in medium chain dicarboxylic aciduria in alcoholics. Am J Med, 1999; 106: 70-5.

119. Weltman MD, Farrell GC, Hall P, lngelman-Sundberg M, Liddle C. Hepatic cytochrome P4502E1 is increased in patients with nonalcoholic steatohepatitis. Hepatology, 1998; 27: 128-33.

120. James FW, Day CP. Non-alcoholic steatohepatitis (NASH): a disease of emerging identity and importance. J Hepatology, 1998; 29: 495-501.

121. Angulo P, Lindor KD. Insulin resistance and mitochondrial abnormalities in NASH: A cool look into a burning issue. Gastroenterology, 2001; 120: 1281-4.

122. Sanyal AJ, Campbell-Sargent C, Mirshahi F, Rizzo WB, Contos MJ, Sterling RK, Luketic VA, Sjiffman ML, Clore JN. Nonalcoholic steatohepatitis: association of insulin resistance and mitochondrial abnormalities. Gastroenterology, 2001; 120: 1183-92.

123. Lee RG, Keeffe EB. Non-alcoholic fatty liver: causes and complications. In: J Bircher, JP Benhamou, M McIntyre, et al., eds. Oxford, Textbook of Clinical Hepatology, 2nd ed. Oxford, UK: Oxford University Press, 1999; 1251-7.

124. Matteoni CA, Younossi ZM, Gramich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver disease: a spectrum of clinical and pathological severity. Gastroenterology, 1999; 116: 1413-9.

125. Kumar KS, Malet PF. Nonalcoholic steatohepatitis. Mayo Clin Proc, 2000; 75: 733-9.

126. Fong DG, Nehra V, Lindor KD, Buchman AL. Metabolic and nutritional considerations in non-alcoholic fatty liver. Hepatology, 2000; 32: 3-10.

127. Harrison SA, Kadakia S, Lang KA, Schenker S. Nonalcoholic steatohepatitis: What we know in the new millennium. Am J Gastroenterol, 2002; 97: 2714-24.

128. French S, Morimoto M, Reitz R, Koop D, Klopfenstein B, Estes K, Clot P, lngelman-Sundberg M, Albano E. Lipid peroxidation, CYP2E1 and arachidonic acid metabolism in alcoholic liver disease in rats. J Nutr, 1997; 127: 907S-11S.

129. Kim ND, Kwak MK, Kim SG. Inhibition of cytochrome P450 2E1 expression by 2-(allylthio) pyrazine, potential chemoprotective agent: hepatoprotective effects. Biochem Pharm, 1997; 53: 261-9.

130. Morimoto M, Hagbjork AL, Nanji AA, lngelman-Sundberg M, Lindros KO, Fu PC, Albano E, French SW. Role of cytochrome P450 2E1 in alcoholic liver disease pathogenesis. Alcohol, 1993; 10: 459-64.

131. Morimoto M, Hagbjork AL, Wan YJY, Fu PC, Clot P, Albano E, lngelman-Sundberg M, French SW. Modulation of experimental alcohol-induced liver disease by cytochrome P450 2E1 inhibitors. Hepatology, 1995; 21: 1610-7.

132. Gebhardt AC, Lucas D, Ménez JF, Seitz HK. Chlormethiazole inhibition of cytochrome P450 2E1 as assessed by chloroxazone hydroxylation in humans. Hepatology, 1997; 26: 957-61.

133. Mathews, JM, Etheridge AS, Raymer JH, Black SR, Pulliam DW, Bucher JR. Selective inhibition of cytochrome P450 2E1 in vivo and in vitro with trans-1,2-dichloroethylene. Chem Res Toxicol, 1998; 11: 778-85.

134. Lieber CS, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, Ponomarenko A, DeCarli LM. Model of non-alcoholic steatohepatitis. Amer J Clin Nutr, 2004; 79: 502-9.

135. Lieber CS, Leo MA, Mak KM, Xu Y, Cao Q, Ren C, Ponomarenko A, DeCarli LM. Acarbose attenuates experimental nonalcoholic steatohepatitis (NASH). Biochem Biophys Res Commun, 2004; 315: 699-703.

136. Koivisto T, Mishin V, Mak KM, Cohen A, Lieber CS. Induction of cytochrome P-4502E1 by ethanol in rat Kupffer cells. Alcohol: Clin Exp Res, 1996; 20: 207-12.

137. Batey R, Cao Q, Madsen G, Pang G, Russell A, Clancy R. Decreased tumor necrosis factor-α and interleukin-1α production from intrahepatic mononuclear cells in chronic ethanol consumption and up regulation by endotoxin. Alcohol Clin Exp Res, 1998; 22: 150-6.

138. Poniachik J, Baraona E, Zhao J, Lieber CS. Dilinoleoylphosphatidylcholine decreases hepatic stellate cell activation. J Lab Clin Med, 1999; 133: 342-8.

139. Oneta CM, Mak KM, Lieber CS. Dilinoleoylphosphatidylcholine selectively modulates lipopolysaccharide-induced Kupffer cell activation. J Lab Clin Med, 1999; 134: 466-70.

140. Kato J, Sato Y, Inui N, Nakano Y, Takimoto R, Takada K, Kobune M, Kuroiwa G, Miyake S, Kohgo Y, Niitsu Y. Ethanol induces transforming growth factor-α expression in hepatocytes, leading to stimulation of collagen synthesis by hepatic stellate cells. Alcohol Clin Exp Res, 2003; 27: 58S-63S.

141. Cao Q, Mak KM, Lieber CS. DLPC decreases TGF-α1-induced collagen mRNA by inhibiting p38 MAPK in hepatic stellate cells. Am J Physiol Gastrointest Liver Physiol, 2002; 283: G1051-61.

142. Li J-J, Kim C-I, Leo MA, Mak KM, Rojkind M, Lieber CS. Polyunsaturated lecithin prevents acetaldehyde-mediated hepatic collagen accumulation by stimulating collagenase activity in cultured lipocytes. Hepatology, 1992; 15: 373-81.

143. Lieber CS, Robins SJ, Leo MA. Hepatic phosphatidylethanolamine methytransferase activity is decreased by ethanol and increased by phosphatidylcholine. Alcohol Clin Exp Res, 1994; 18: 592-5.

144. Li JJ, Rosman AS, Leo MA, Nagai Y, Lieber CS. Tissue inhibitor of metalloproteinase is increased in the serum of precirrhotic and cirrhotic alcoholic patients and can serve as a marker of fibrosis. Hepatology, 1994; 19: 1418-23.

145. Lieber CS, Casini A, DeCarli LM, Kim C, Lowe N, Sasaki R, Leo MA. S-adenosyl-L-methionine attenuates alcohol-induced liver injury in the baboon. Hepatology, 1990; 11: 165-72.

146. Lieber CS, Robins SJ, Li J, Mak KM, Fasulo JM, Leo MA. Phosphatidylcholine protects against fibrosis and cirrhosis in the baboon. Gastroenterology, 1994; 106: 152-9.

147. Lieber CS. Alcoholic liver disease: new insights in pathogenesis lead to new treatments. J Hepatology, 2000; 32: 113-28.

148. Duce AM, Ortiz P, Cabrero C, Mato JM. S-adenosyl-L-methionine synthetase and phospholipid methyltransferase are inhibited in human cirrhosis. Hepatology, 1988; 8: 65-8.

149. Lieber CS. Alcoholic liver injury: pathogenesis and therapy in 2001. Pathol Biol, 2001; 49: 738-52.

150. Vendemiale G, Altomare E, Trizio T, Le Grazie C, Di Padova C, Salerno MT, Carrieri V, Albano O. Effect of oral S-adenosyl-L-methionine on hepatic glutathione in patients with liver disease. Scand J Gastroenterol, 1989; 24: 407-15.

151. Mato JM, Cámara J, Fernández de Paz J, Caballería L, Coll S, Caballero A, Buey-García L, Beltrán J, Benita V, Caballería J, Solá R, Otero-Moreno R, Fèlix B, Duce-Martin A, Correa JA, Parès A, Barro E, Magaz-García I, Puerta JL, Moreno J, Boissard G, Ortiz P, Rodès J. S-Adenosylmethionine in alcoholic liver cirrhosis: A randomized, placebo-controlled, double-blind, multicentre clinical trial. J Hepatology, 1999; 30: 1081-9.

152. Mak KM, Leo MA, Lieber CS. Alcoholic liver injury in baboons: Transformation of lipocytes to transitional cells. Gastroenterology, 1984; 87: 188-200.

153. Mak KM, Lieber CS. Lipocytes and transitional cells in alcoholic liver disease: a morphometric study. Hepatology, 1988; 8: 1027-33.

154. Arai M, Gordon ER, Lieber CS. Decreased cytochrome oxidase activity in hepatic mitochondria after chronic ethanol consumption and the possible role of decreased cytochrome aa3 content and changes in phospholipids. Biochim Biophys Acta, 1984; 797: 320-7.

155. Navder K, Baraona E, Lieber CS. Polyenylphosphatidylcholine attenuates alcohol-induced fatty liver and hyperlipemia in rats. J Nutr, 1997; 127: 1800-6.

156. Dlugosz JW, Korsten MA, Lieber CS. The effect of the prostaglandin analogue – misoprostol on rat liver mitochondria after chronic alcohol feeding. Life Sciences, 1991; 49: 969-78.

157. Lieber CS, Leo MA, Aleynik S, Aleynik M, DeCarli LM. Polyenylphosphatidylcholine decreases alcohol-induced oxidative stress in the baboon. Alcohol Clin Exp Res, 1997; 21: 375-9.

158. Bird GLA, Panos MZ, Polson R, Johnson R, Portman B, Neuberger J, Williams R. Activity of polyunsaturated phosphatidylcholine in HbsAg negative chronic active hepatitis and acute alcoholic hepatitis. Z Gastroenterol, 1991; 29: 21-4.

159. Niederau C, Strohmeyer G, Heinges T, Peter K, Gopfert E, Leich study Group. Polyunsaturated phosphatidyl-choline and interferon alpha for treatment of chronic hepatitis B and C: A multi-center, randomized, double blind, placebo-controlled trial. Hepatogastroenterology, 1998; 45: 797-804.

160. Lieber CS, Leo MA, Aleynik SI, Lowe N, Aleynik MK, Paronetto F. Increasing circulating level of dilinoleoylphosphatidylcholine is associated with protection against alcohol induced oxidative stress and liver fibrosis in man. Hepatology, 2000; 32: 386A.

161. Lieber CS, Leo MA, Cao Q, Ren C, DeCarli LM. Silymarin retards the progression of alcohol-induced hepatic fibrosis in baboons. J Clin Gastroenterol, 2003; 37: 336-9.

162. Lieber CS, Weiss DG, Groszmann R, Paronetto F, Schenker S, for the VA Cooperative Study 391. I. VA Cooperative study of polyenylphosphatidylcholine in alcoholic liver disease: effects on drinking behavior by nurse/physician teams. Alcohol Clin Exp Res, 2003; 27: 1757-64.

163. Lieber CS, Weiss DG, Groszmann R, Paronetto F, Schenker S, for the VA Cooperative Study 391. II. VA Cooperative study of polyenylphosphatidylcholine in alcoholic liver disease. Alcohol Clin Exp Res, 2003; 27: 1765-72.

164. Rosman AS, Waraich A, Galvin K, Casiano J, Paronetto F, Lieber CS. Alcoholism is associated with hepatitis C but not hepatitis B in an urban population. Am J Gastroenterol, 1996; 91: 498-505.

165. Ferenci P, Dragosics B, Dittrich H, Frank H, Benda L, Lochs H, Meryn S, Base W, Schneider B. Randomized controlled trial of silymarin-treatment in patients with cirrhosis of the liver. J Hepatology, 1989; 9: 105-13.

166. Pares A, Planas R, Torres M, Caballeria J, Viver JM, Acero D, Panes J, Rigau J, Santos J, Rodes J. Effects of silymarin in alcoholic patients with cirrhosis of the liver: results of a controlled, double-blind, randomized and multicenter trial. J Hepatology, 1998; 28: 615-21.

167. Leo MA, Rosman A, Lieber CS. Differential depletion of carotenoids and tocopherol in liver diseases. Hepatology, 1993; 17: 977-86.

168. de la Maza MP, Petermann M, Bunout D, Hirsch S. Effects of long-term vitamin E supplementation in alcoholic cirrhotics. J Am Coll Nutr, 1995; 15: 192-6.

169. Carithers RL. Treatment of alcoholic liver disease. Practical Gastroenterology, 1989; 13(6).

170. Ramond MJ, Poynard T, Rueff B, Mathurn P, Teodore C, Chaput JC, Benhamou JP. A randomized trial of prednisolone in patients with severe alcoholic hepatitis. N Engl J Med, 1992; 326: 507-12.

171. Christensen E, Gluud C. Glucocorticoids are effective in alcoholic hepatitis: A meta-analysis adjusting for confounding variables. Gut, 1995; 37: 113-8.

172. Kershenobich D, Vargas F, Garcia-Tsao G, Tamayo RP, Gent M, Rojkind M. Colchicine in the treatment of cirrhosis of the liver. N Engl J Med, 1988; 318: 1709-13.

173. Morgan TR, Weiss DG, Nemchausky B, Schiff ER, Anand B, Simon F, Kidao J, Cecil B, Mendenhall CL, Nelson D, Lieber C, Pedrosa M, Jeffers L, Bloor J, Lumeng L, Marsano L, McClain C, Mishra G, Myers B, Leo M, Ponomarenko Y, Taylor D, Chedid A, French S, Kanel G, Murray N, Pinto P, Fong TL, Sather MR. Colchicine treatment of alcoholic cirrhosis: a randomized, placebo-controlled clinical trial of patient survival. Gastroenterology, 2005; 128: 882-90.

174. Anton RF, Lieber CS, Tabakoff B. Carbohydrate-deficient transferrin and α-glutamyltransferase for the detection and monitoring of alcohol use: results from a multisite study. Alcohol Clin Exp Res, 2002; 26: 1215-22.

175. Volpicelli JR, Alterman AI, Hayashida M, O'Brien CP. Naltrexone in the treatment of alcohol dependence. Arch Gen Psychiatry, 1992; 49: 876-80.

176. Lieber CS. Alcohol and the liver: 1994 Update. Gastroenterology, 1994; 106: 1085-105.

177. Lieber CS. Medical disorders of alcoholism. N Engl J Med, 1995; 333: 1058-65.

The gastrointestinal cholecystokinin receptors in health and diseases

Morisset J*

Service de Gastroentérologie, Université de Sherbrooke, Canada

Key words: cholecystokinin, gastrin, cholecystokinin receptors, pancreas.

Introduction

Over the years, cholecystokinin (CCK) has been accepted as the gastrointestinal hormone mainly responsible for the control of gallbladder contraction, pancreatic enzyme secretion, growth of the pancreatic gland and gut motility. On the contrary, its sister hormone gastrin is recognized to regulate gastric acid secretion and proliferation of the acid secreting portion of the gastric mucosae as well as that of the upper intestine and colon.

These two hormones share the same carboxy-terminal pentapeptide amide sequence but differ in their sulfation sites on the active C-terminal portion of their molecule; indeed, gastrin is sulfated on its sixth tyrosyl residue and CCK on its seventh residue [1]. Because of their structure similarities, both peptides share common biological activities; indeed, CCK possesses weak gastrin activity and gastrin shares slight CCK activity [2].

CCK and gastrin initiate their biological effects through their binding to two subclasses of CCK receptors; the CCK-1 receptor, formerly characterized as the CCK_A receptor (A for alimentary), is highly selective for sulfated CCK [3] whereas the CCK-2 receptor, earlier called CCK_B receptor (B for brain), does not discriminate between sulfated or nonsulfated CCK and gastrin [4].

In this review, we will briefly summarize the different molecular forms and cellular localization of CCK and gastrin. The emphasis will, however, be put on the CCK-1 and CCK-2 receptors describing their biochemical characteristics, their gene in different species, their localization and the results of their specific occupation under normal and pathological states.

* CORRESPONDING AUTHOR:
Service de Gastroentérologie, Département de Médecine
Faculté de Médecine, Université de Sherbrooke
Sherbrooke, Qc, Canada J1H 5N4
Tel: 819 820 6813; Fax: 819 820 6826
e.mail: jean.morisset@USherbrooke.ca (Jean Morisset)

Received 25.04.2005 Accepted 27.04.2005

Cholecystokinin

A. Molecular forms

Shortly after his discovery of CCK-33 in pig intestine [1], Mutt purified the slightly larger form CCK-39 from the same species' intestine [5]. Later on, smaller and larger molecules were isolated from several species' brain and intestine. CCK-58, 8, 5 and 4 were found in porcine brain [6] whereas the molecular forms 58, 39, 33, 25, 18, 8, 7 and 5 were all identified in dog intestine [7,8]. Some of these same peptides were also identified in bovine intestine, 39 and 33, in rat intestine, 58, 22, 8 and in guinea pig intestine, 22 and 8 [9-11]. Early studies indicated that CCK-58 was the major form of CCK in human intestine [12] although it had been previously identified in the blood of dogs [13] and humans [14]. More recently, Reeve demonstrated that the only detectable form of CCK in rat blood was CCK-58 using a new method of extraction to minimize loss and degradation [15]. It was also demonstrated that this CCK-58 peptide exists under sulfated and non-sulfated forms in porcine and dog intestines [16,17]. This CCK-58 molecule was found much less potent than CCK-8 in releasing amylase from dispersed rat pancreatic acini [18]; later on, it was demonstrated that this decreased activity of CCK-58 depended upon its amino terminus shielding the carboxyl terminus responsible for its biological activity [19].

B. Localization

As indicated above, CCK is a peptide that exists in several different molecular forms and all these biologically active molecules contain the same carboxyl terminal phenylalanine amide. CCK is produced and released by the endocrine I cells located within the small intestine mucosae [20]. These I cells are present in larger amounts in the duodenum and proximal jejunum with decreased concentrations further down the gut [21]. CCK was also found in a subpopulation of pituitary cells

[22] and in the brain, its highest concentration was measured in the cerebral cortex [23]; the hormone has been associated in the pathogenesis of schizophrenia [24] and satiety control [25]. CCK was also localized in the celiac plexus and in the vagus nerve in an efferent direction toward the gut [26]. In the nervous system, central and peripheral, CCK is considered as a neurotransmitter. The major question raised at this point regards the form of CCK stored in the intestine and brain; this is being pointed out because of the recent demonstration that CCK-58 appears to be the predominant intestinal form in dogs [8], humans [12] and rats [15] and was found the major molecular form in dog [13] and human [14] plasma following its release. With all these different forms of endocrine CCK circulating in the blood or acting through paracrine and autocrine mechanisms, it is important to characterize these molecular forms because they operate via only two different receptor subtypes, the CCK-1 and CCK-2 receptors, and have different physiological effects on various target tissues.

Gastrin

A. Molecular forms

Although postulated in 1905 by Edkins [27], the existence and nature of gastrin were confirmed by Gregory and Tracy in 1964 when they isolated, sequenced and pharmacologically evaluated the hormone they had purified from hog gastric mucosae [28]. Later on, two heptadecapeptides were isolated with identical amino acid sequences; they were named gastrin I, the non-sulfated form, and gastrin II the sulfated form [29]. Similar forms of gastrin were later found in cat, cow, sheep, dog, goat, rat, guinea pig and rabbit. Few years later, Besson and Yalow identified a 34 amino acid peptide they named "big gastrin" [30] which was later confirmed to exist also in human as an extended form of the G-17 molecule [31]. With the advent of molecular biology and cloning techniques gastrin cDNA was characterized initially from pig antrum in 1982 [32] and a year later it was done for human [33]. From these cloning studies, it was established that from the porcine gastrin precursor cDNA rich of 620 nucleotides, emerged an mRNA of 312 bases coding a 104 amino-acid preprogastrin molecule while the human preprogastrin peptide contained 4 less amino acids [34]. This preprogastrin precursor is further converted to progastrin which through hydrolysis by specific endopeptidases gave glycine-extended Gastrin-34 and then amidated Gastrin-17 [35]. In search for other gastrin molecules, minigastrin (Gastrin-14) was identified [36] along with smaller forms including a c-terminal hexapeptide from porcine antrum [37] and a pentapeptide from canine brain and intestine [38]. Finally, a C-terminal tetrapeptide was purified from the gut [39]. One interesting feature of the gastrin peptide is that in neonatal rat pancreas, the major source of gastrin in the newborn rat, gastrin is totally sulfated; this complete sulfation of gastrin was also observed in the feline pancreas and in the small intestine of human fetus [40]. Later on, the antrum was shown to contain approximately equal proportions of non-sulfated and sulfated gastrin [29].

Human plasma contains the two major forms of gastrin, G-34 and G-17, along with two minor components identified as little gastrin I and II and minigastrin I and II; these forms also circulate as sulfated and non sulfated [41]. Recently, progastrin and glycine-extended gastrins were found to possess biological activities [42,43] and be among the blood circulating forms of gastrin although comprising less than 10% of the circulating hormones [35]. Over the last ten years, Reeve and colleagues claimed that CCK-58 was the major circulating form of CCK in the blood of many species including man and that other forms could be products of degradation happening right after blood collection. Gastrin-34 is also the most abundant form of gastrin into circulation [44] and one may also ask the pertinent question whether the other identified forms can result from degradation.

B. Localization

In the gastrointestinal tract, gastrin is synthesized and released from the endocrine G cells [45] and specific antibodies identified G-34 and G-17 in the same gastric antral cells [46]. During development, at least in the rat, it was observed that patterns of gastrin expression differed in the pancreas, antrum and duodenum [47]. In the pancreas, gastrin is transiently expressed during fetal life before the expression of any other islet hormone; immediately after birth, pancreatic gastrin mRNA expression is fading away to become almost undetectable by day 10 after birth [48]. In the duodenum, gastrin expression remains constant during neonatal development while its expression begins 3 days after birth in the stomach [47].

The highest concentrations of gastrin are present in the antrum of adult mammals whereas the duodenum has the highest concentration in the fetus [49]. Contrary to the duodenum of dog, cat and hog, which are almost depleted of gastrin, that of human is quite rich and contains as much hormone as the antrum [50]. It was also established that around 90% of gastrin found in the human antrum was G-17 while this form found in the duodenum represents between 40 to 50% of its total content [30]. Contrary to human foetal intestinal gastrin which is totally sulfated, only 50% is so in adults [51]. According to Reeve, small amounts of gastrin were found in the pituitary gland [52] with most of the gastrin-like immunoreactivity being CCK-8 or CCK-58 [53].

CCK receptors

The cholecystokinin and gastrin family of peptides share a common carboxy terminal pentapeptide-amide amino sequence and interact with two CCK receptor subtypes, CCK-1 and CCK-2, belonging to the family of seven transmembrane domain receptors.

CCK-1 receptor

A. Gene structure

The genes for the CCK-1 receptor have been cloned from guinea pig gallbladder, pancreas and gastric chief cells [54], human gallbladder [55,56], rabbit gastric [57] and mouse [58] cDNA libraries. This receptor gene consists in all species of five exons and four introns and there is a conserved structural homology in this CCK-1 receptor among different species; as an example, the human gallbladder CCK-1 receptor exhibits 92% identity and 95% similarity with its rat pancreatic counterpart

[59]. The human CCK-1 gene is localized to chromosome 4, that of the mouse has been mapped on chromosome 5 [60] whereas the rat CCK-1 gene was identified on chromosome 14 [61].

B. Biochemical characteristics

Over the years, the CCK-1 receptor structure has been evaluated by at least four different techniques starting with radioligand affinity cross-linking then receptor purification; more recently, deduction of the primary amino acid sequences was done from cDNA structure and the production of more specific receptor antibodies helped in confirming the protein mass. Initial studies on CCK-1 receptor characterization using the cross-linking technique with CCK-33 led to the identification of a major protein of 75 to 95 kDa and minor proteins of 47 and, 140 kDa [62,63]. Using labeled CCK-9 as tracer, CCK receptors proteins of 84 kDa were identified on guinea pig pancreatic acini [64] and some of 78, 45 and 28 kDa were located on dog pancreatic acini [64]. On human gastric smooth muscle membrane, a major band was noticed at 75 kDa and a minor one at 200 kDa [65]. For more details on the CCK-1 receptor biochemical characteristics, consult the review by Silvente-Poirot [66].

From radio labeled CCK agonists binding competition studies, their analysis led to an accepted model that the CCK-1 receptor consists of two binding sites, one of high affinity and low capacity and the other of low affinity and high capacity [67]. Further analysis of competition curves led to a model with three affinity states with high, low and very low affinity states [68]. The two sites model has been the most documented and studies using pancreatic acini from guinea pig and dog [64] have reported high and low affinity states of 0.1 and 35 nM (guinea pig) and 0.5 and 25.0 nM (dog); in rat pancreatic acini, values of 0.06 and 21 nM were reported [3] whereas the receptor from gastric smooth muscle membrane exhibits affinities of 0.07 and 8.4 pM for its high and low affinities, respectively [65]. The concentration of these receptors sites is relatively low with values of 20, 25 and 4 fmol $mgprt^{-1}$ for the high affinity sites on guinea pig, dog [64] and rat [3] pancreatic acini and 500, 600 and 640 fmol $mgprt^{-1}$ for the low affinity sites in these same three species.

C. Localization

Studies on the location of CCK-1 receptors in target cells have been performed using at least four different techniques. Initially, receptors were located from binding studies using labeled specific CCK agonists or antagonists; at the same time, also with labeled hormone, quantitative electron or regular microscope autoradiographs were displayed. More recently, northern blot and in situ hybridization and RT-PCR using species-specific radiolabeled full-length coding sequence cDNA probes or specific primers has been performed using poly $(A)^+$ mRNA from each tissue. Finally, the development of specific CCK receptor antibodies was of great help in the identification of the specific cells containing the receptor; these antibodies were used in association with immunofluorescence and confocal microscopy techniques. As examples, the radiolabeled CCK agonist CCK-8 and antagonist L-364,718 binding studies revealed the presence of the CCK-1 receptor in human gallbladder [55], guinea pig gallbladder and pancreas [54] and rat pancreas [59]. The, 1^{25}I-labeled BH-CCK33 hormone also allowed identification of CCK receptor on mouse [69] and rat [70] pancreatic acini by electron microscopy. Autoradiographs of whole pieces of tissue pictured the CCK-1 receptor in human gallbladder [71], pancreatic nerves [72], in the basal region of the human antral and fundic mucosae as well as in the muscularis propria of the antrum, fundus, and gallbladder [73]. Quantitative RT-PCR experiments indicated that the message levels for the CCK-1 receptors are very low [74] or not expressed at all [75] in human normal pancreas. However, the message was detected in normal gallbladder, intestine, colon, spleen, ovary, cerebellum and frontal lobe [75]. Northern blot and *in situ* hybridization analysis which detect specific mRNAs actually present in a tissue indicate that the CCK-1 mRNA are absent from adult human pancreas [74] whereas detected in rat fundus mucosae and pancreas [76]. From all these binding studies and those utilizing molecular biology techniques, it seems that the northern blot and *in situ* hybridization assays give you a better and more accurate estimation of the true presence of the receptor mRNA in enough amount to expect the presence of the receptor protein. Caution has to be taken with the *in situ* hybridization especially with the pancreas of any species because of the presence of large quantities of RNAse hydrolyzing the probes used.

Once the receptor mRNA has been identified in any given tissue or organ, it is important to establish the presence of the receptor protein for biological function evaluation. This can be done with accuracy and specificity with potent receptor antibodies which have been well characterized and checked by preabsorption of the antiserum with the synthetic peptide used for immunization. These specific antibodies can discriminate any other receptor proteins in any given cell using immunofluorescence or confocal microscopy in co-localization studies with hormones, specific enzymes or proteins. Development of such CCK receptor antibodies is quite recent and already the first published results raised controversies mainly because some of these antibodies were not as specific as they should have been.

The initial studies with such specific CCK-1 receptor antibodies revealed the presence of this receptor on rat myenteric neurons and on fibers in the muscle and mucosae of their stomach, colocalized with VIP or substance P in different neurons [77]. Similar location was also suggested in calf intestine [78]. With another specific antibody, our group described the CCK-1 receptor on rat and mouse pancreatic acinar cells as well as on central cells of rat, mouse and pig pancreatic islets [79], these cells were later confirmed to be the alpha and beta cells in the rat [80]. Although Schweiger [81] failed to visualize the CCK-1 receptor on pig islets' beta cells, he reported its location on the glucagon cells as we previously indicated but not on the acinar cells [79]. We later indicated that their antibody raised in chicken eggs lacked specificity when tested on pig pancreas [80]. Future experiments in this area of research should focus on the development of new specific receptor antibodies that will allow a better mapping of these CCK-1 receptors in target tissues and cells and the use of new sets of "receptor antagonists" for better understanding of their physiological responses.

D. CCK-1 receptor occupation and physiological responses

Occupation of the CCK-1 receptor by CCK and its analogues leads to stimulation of pancreatic enzyme secretion, pancreatic

endocrine hormones release, motility of the gut organs, contraction of the gallbladder, growth of the pancreas and control of satiety and pain in the central and peripheral nervous systems.

1. Secretion of the pancreatic enzymes

One of the first physiological role attributed to CCK was its implication in the control of pancreatic enzyme secretion; this is why one of its initial name was pancreozymin as its action was on the pancreas. Rodents have been the experimental model of choice for such studies and preparation of fresh acini has been the selected technique. Indeed, incubation of pancreatic acini with increasing concentrations of CCK leads to maximal amylase release [82], an effect totally inhibited by the selective and potent CCK-1 receptor antagonist L-364,718 [83]. This CCK-1 receptor antagonist was also very efficient in inhibiting pancreatic enzyme release *in vivo* in rat [83] and dog [84]. Although Cuber showed that CCK-8 increased pancreatic enzyme secretion in pig [85] and Cantor in human [86], it was later demonstrated that in these two species, CCK via its CCK-1 receptor had no direct effect on pancreatic acinar cell secretion [74,87]. In light of these data, we should consider that in large mammals including human, CCK would bind to its CCK-1 receptor present in afferent neurons and activate enzyme secretion by way of a vagal-vagal loop releasing acetylcholine as the final mediator [88]. This is supported by observations in conscious calves that intraluminal administration of CCK-8 resulted in pancreatic enzyme release, an effect atropine-sensitive [89]. It was later confirmed that the calf pancreas does not possess the CCK-1 receptor on its acinar cells [90].

2. Release of pancreatic islet hormones

In the endocrine pancreas, CCK was shown to stimulate insulin release in many species *in vivo* and also from rat isolated islets [91,92]; this insulinotropic effect of CCK is inhibited by the specific CCK-1 receptor antagonist, L-364,718 [93]. Furthermore, it was recently shown that the CCK-1 but not the CCK-2 receptor transcript was detected in rat islets [94], a location later confirmed by immunofluorescence [79]. CCK is also involved in glucagon release [95] and its effect through CCK-1 receptor occupation was confirmed in CCK-1 receptor deficient rats whose islets did not secrete glucagon in response to CCK [96]. This CCK-1 receptor on glucagon cells was also confirmed by immunofluorescence in rat islets [80]. These data disagree with those of Saillan-Barreau [97] who showed that glucagon release from human purified islets in response to CCK resulted from CCK-2 receptor occupation as they showed co-localization of this CCK-2 receptor with glucagon in alpha cells. Our laboratory was unable to confirm such a co-localization and also unable to establish the specificity of their antibody [80].

3. Motility of the gut organs

Stomach: It has been known for many years now that the presence of medium chain fatty acids in the upper gut liberates endogenous CCK which can relax the proximal stomach [98] and delay gastric emptying [99]. Recently, it was demonstrated in humans that endogenous CCK release by fatty acids reduced the tolerated volume of liquid delivered into the stomach via a CCK-1 receptor-mediated delay in gastric emptying by either reducing the proximal gastric tone, antral peristalsis, and/or by increasing pyloric or intestinal tone [100]. These effects on stomach functions were also confirmed in healthy volunteers using GI 181771X, a full specific CCK-1 receptor agonist with no CCK-2 receptor agonist activity [101]. This agonist delayed gastric emptying of solids and increased fasting gastric volumes [102].

Colon: Using receptor autoradiography, it was demonstrated in human colon that the main target of CCK was the myenteric plexus which is rich in CCK-1 receptors. These CCK-1 receptors were also located, at moderate to low density, in the longitudinal muscle [103]. It was then suggested that CCK can affect colonic motility via two different routes involving the neurons of the myenteric plexus and directly on the smooth muscle cells. In the colon, CCK increases colonic transit time [104], thus exerting its inhibitory effect on propulsive motility in the ascending colon [105]. Although physiological serum concentrations of endogenous or exogenous CCK did not affect phasic contractility, tone or transit in healthy subjects, suggesting no CCK physiological implication in the control of interdigestive and postprandial human colonic motility [106], it remains that loxiglumide, a known CCK-1 receptor antagonist, can accelerate colonic transit in normal volunteers [107]. This may suggest that local CCK release can modulate colon motility via paracrine actions involving higher CCK concentrations than those observed in the circulation.

4. Gallbladder contraction

It has to be remembered that CCK was initially discovered for its ability to contract gallbladder. It is now well recognized that CCK is the major hormonal physiological regulator of gallbladder contraction. Indeed, this was established from the demonstration that physiological concentrations of CCK in the blood after a meal were able to cause postprandial gallbladder contraction [108], an effect totally prevented by the administration of a specific CCK-1 receptor antagonist, devazepide [109]. In addition, CCK was postulated to stimulate hepatic bicarbonate secretion into bile [100] and relaxation of the sphincter of Oddi [111]. It thus seems that CCK can physiologically coordinate bile circulation towards its final destination, the duodenum.

5. Growth of gut organs

CCK given to induce enzyme secretion in rats in amount comparable to that in response to a meal, induced growth of the pancreatic gland as indicated by major increases in gland weight, in total enzymes and proteins contents as well as RNA and DNA [112,113]. This trophic action of CCK can be obtained whether CCK was given exogenously [114] or endogenously released either by feeding a high protein diet [115] or in response to pancreatic juice diversion [116]. These growth effects of CCK on the pancreas involve the CCK-1 receptor subtype as its response was totally abolished by the CCK-1 receptor antagonist L-364,718 [115,116]. These trophic effects of CCK on the pancreas have also been observed in the mouse [117] and Syrian hamster [118]. Liver growth however, remained insensitive to the action of either gastrin or CCK at least in the rat [119]. Recently, it was demonstrated that CCK, via its CCK-1 receptor, played a role in

the intrinsic gastric mucosal defense system against injury from luminal irritants, effects which seem to involve the production of nitric oxide from the constitutive form of nitric oxide synthase [120].

6. Control of satiety and pain

Satiety: CCK is now well recognized as an hormonal inhibitor of food intake in many species including humans. It was observed that this suppressive effect of CCK on food intake was enhanced with age in rats, but the observed enhanced sensitivity to the central administration of CCK could not be explained by changes in gene expression of CCK nor of the CCK-1 receptors [121]. The implication of the CCK-1 receptor in satiety control was clearly demonstrated in OLETF (CCK-1 receptor deficient) rats who are hyperphagic during dark and light periods with increased meal size and a concomitant reduction in total number of meals; this decrease in meal number did not compensate for the increased meal size [122]. It was also recently observed that the dorsomedial hypothalamus is one of the few hypothalamic sites in the rat containing CCK-1 receptors and it is exactly there that local CCK injection has the greatest inhibitory effect on food intake [122]. This brain area is also rich in neuropeptide Y (NPY) which was also associated with satiety control. It was then hypothesized that CCK, via its CCK-1 receptor in the dorsomedial hypothalamus, would play a suppressing role on NPY. In the absence of CCK-1 receptor, this inhibition of NPY is absent and therefore we observe failure to compensate for the increased meal size [124].

Gut pain control: Infusion of CCK to patients with IBS (Irritable Bowel Syndrome) caused higher pain scores in patients with functional abdominal pain [125]; higher plasma CCK was also determined in these IBS patients [126] although this finding was not unanimous [127]. Recently, motility patterns were compared between healthy volunteers and IBS patients with abdominal pain and frequent defecation or diarrhea; in the IBS patients, the motility index, the frequency of high-amplitude propagating complexes, and the responses to CCK were all significantly greater than in the control patients. In most patients, the high-amplitude propagating complexes coincided with pain appearance and the effects of CCK were significantly inhibited by a CCK-1 receptor antagonist and also by atropine, suggesting participation of the enteric nervous system [128].

One of the major problems with the use of CCK-1 receptor antagonists in the management of abdominal pain in IBS patients remains their alteration of the normal functions of the gallbladder owing to potential bile stasis and gallstones formation. In a recent study [129] however, it was shown in healthy male volunteers that dexloxiglumide added to a liquid diet partly reversed the increase in colonic transit time caused by the liquid diet without impairing postprandial gallbladder responses [130]. These new data strongly suggest that CCK-1 receptor antagonists can be used to control gut motility and pain without detrimental effects on the gallbladder. This suggestion was later supported in a broader study in which dexloxiglumide, well tolerated by patients, tended to normalize bowel function in a group of female constipation-predominant IBS without promoting gallstone formation [131].

E. CCK-1 receptor occupation under pathological conditions

1. Effects on the stomach

In the early nineties, a Japanese group [132] reported that Otsuka Long Evans Tokushima Fatty (OLETF) rats were spontaneous mutants with little or no expression of the CCK-1 receptor gene [61]. In these CCK-1 receptor deficient rats, TRH which increases vagal efferent activity, indomethacin which decreases mucosal prostaglandin levels, protective molecules for the stomach, HCL and ethanol, known to increase the severity of gastric mucosal damage, all increased the severity of gastric mucosal lesions when compared to control animals. It is known that CCK exhibits anti-ulcer action on the gastric mucosae through its CCK-1 receptor occupation in rats [133]. Recently, CCK-1 and CCK-2 receptors were located in the human stomach [73] and the low degree of variability of CCK receptor density found in human endoscopic biopsy specimens from various individuals supports analysis of their status in pathological states, which has not yet been done to our knowledge.

2. Effects on the exocrine and endocrine pancreas

In CCK-1 receptor deficient rats, pancreatic protein release in response to exogenous CCK-8 or endogenous CCK release by pancreatic juice diversion were significantly impaired [134]; failure to respond to CCK was also observed in isolated acini from these OLETF rats [135].

In CCK deficient mice, the concentration of CCK-1 receptor mRNA remained normal suggesting that agonist binding to its receptor does not regulate receptor gene expression. Moreover, in these CCK-deficient mice, growth of their pancreas and their enzymes adaptation to different diets remained also comparable to control values [136]. These data on CCK-deficient mice agree in some way with findings in rats without CCK-1 receptor [137]. Indeed, in OLETF rats, pancreatic wet weight increases were significantly lower than those in normal LETO rats at all ages examined while total DNA contents in the whole gland and protein concentrations were comparable in both strains. These studies suggest that CCK-1 receptors might not be an absolute requirement for normal pancreatic growth at least in rodents. Although the pancreas develops normally in CCK-1 receptor deficient rats, its regeneration was significantly delayed following 30% pancreatectomy, suggesting its potential need in the gland regeneration process [138].

In the obese Zucker rats, their pancreatic protein secretion in response to increasing doses of CCK-8 was significantly reduced at all doses tested, this impaired release was also associated with significant decreases in CCK-1 receptor high and low affinity sites without any effect on their affinity [139].

Stimulation of the pancreatic gland by supraphysiological doses of caerulein, a CCK analogue, resulted in oedematous pancreatitis with destruction of the pancreas architecture and an important lost of the pancreatic gland, about 40%. Regeneration of the gland can be totally achieved within 5 days following a caerulein treatment at a physiological doses [140], during which the CCK-1 receptor mRNAs were tremendously increased [141]. This regeneration of the pancreatic gland undoubtedly involves the CCK-1 receptors because it can be

prevented by a concomitant treatment with a specific CCK-1 receptor antagonist, L-364,718 [142].

The absence of CCK-1 receptors also impairs functions of the endocrine pancreas; although pancreatic insulin contents were not affected in CCK-1 receptor deficient rats, its release in response to CCK-8 remained at basal level; similarly, CCK-8 failed to induce glucagon secretion while it increased its release in normal rats. In response to a meal, plasma insulin was reduced and associated with transient hyperglycemia in CCK-1 receptor deficient rats. These data clearly demonstrate the importance of the CCK-1 receptor in the control of insulin and glucagon release along with glycemia [96], and agree with the recent co-localization of the CCK-1 receptor with insulin and glucagon in many species by immunofluorescence [80].

3. Effects on the gallbladder

A deletion of a 262-base pair coding region of the human gallbladder CCK-1 receptor led to obesity and cholesterol gallstone disease in a patient with this mutation [143]. After sequencing, the majority of the mRNA produced from this gene was abnormally processed, resulting in deletion of its third exon; this mRNA encoded an inactive receptor unable to recognize CCK agonists. Although not yet proven in substantial number of patients, it is tempting to associate this peculiar mutation with gallstone formation and obesity.

CCK-2 receptor

A. Gene structure

The genes for the CCK-2 receptor have been cloned in human [144], rat [145], mouse [146], dog [147] and rabbit [148]. In human, the nucleotide sequence plus the 3' noncoding region of the human frontal cortex clone is 1969 bp in length [144]. Using Mastomys gastrin receptor cDNAs containing the second and third transmembrane domains and the entire coding region, respectively, five cDNA clones were isolated from a human brain cDNA library with the h CCKB3 clone being the most compatible with the major transcript size of the human brain CCK-2 receptor [149]. From screening a cDNA library constructed from AR42J cells from a rat pancreatic acinar carcinoma cell line, a 2243-base pair clone was isolated from a rat brain cortex cDNA library with identical cDNA sequence to the clone isolated from the AR42J cell cDNA library [145]. The mouse CCK-2 receptor gene was cloned from a 129/SVJλ genomic library using a rat cDNA probe [146]. Restriction mapping and DNA sequencing revealed the gene structure to be comprised of five exons distributed over, 11 kb. The coding region contains 1362 nucleotides. The canine CCK-2 receptor was cloned from a parietal cell cDNA expression library and its cDNA has an open reading frame encoding a 453 amino acid protein [147]. Finally, the rabbit CCK-2 receptor was cloned by screening a rabbit EMBL phage library with a cDNA probe based on the nucleotide sequence of the human gastrin/CCK-2 receptor. The gene contained a 1356-bp open reading frame consisting of five exons interrupted by 4 introns and encoded a protein of 452 amino acids [148]. According to published data, the human CCK-2 receptor has 90% identity to rat and canine receptor. The predicted mouse CCK-2 receptor shares 87% and 92% amino acid identity with the human and rat receptor, respectively. The rabbit protein coding region of the gene exhibits 93 to 97% amino acid similarity with corresponding cDNA identified in human, canine and rat brain or stomach. Fluorescent *in situ* hybridization of human metaphase chromosomal spreads localized the human CCK-2 receptor gene to the distal short arm of chromosome, 11 [150].

B. Biochemical characteristics

The biochemical characterization of the CCK-2 receptor has been done using cell membrane fractions, cells, fixed tissue on slides, group of cells (acini) or transfected cells, usually the COS-7 cells. The binding studies were usually performed using the following radioligands: ^{125}I-CCK-8, ^{125}I-Nleu^{11}hgastrin13, ^{125}I-BH-CCK-8, ^{125}I-BH(Thr.Nle)CCK-9, ^{3}H-L365,260 and ^{125}I-BH(2-17)G17NS.

Pharmacological characterization of the human brain CCK-2 receptor expressed in COS 7 cells indicated agonist affinities consistent with occupation of the CCK-2 receptor. Indeed, calculated IC_{50} values for CCK-8, gastrin 1 and CCK-4 were 0.14, 0.94 and 32 nM, respectively, values comparable to those previously obtained using isolated brain membranes. The CCK-B receptor antagonist L-365,260 bound with approximately 40-fold higher affinity than L-364,718, the CCK-1 receptor antagonist [151]. In another comparable study, L-365,260 was 50-fold more potent than L-364,718 [144]. In tissue sections of the human gastric mucosae expressing the CCK-2 receptor, L-365,260 presented an IC_{50} of 68 nM compared to an IC_{50} greater than 1 μM for L-364-718 [73]. The binding characteristics of ^{3}H-L-365,260 to six different human pancreatic cancer cell homogenates were comparable with K_{DS} in the nM range, 2.0 to 4.3, and receptor concentrations of 125 to 280 fmol/mg protein. Similar binding data were obtained from tumors grown in nude mice [152]. According to binding studies done on pancreatic tissue sections using ^{125}I-BH-CCK-8, it would seem that the human pancreas possesses CCK-2 receptor as the receptor did not discriminate between CCK-8 and gastrin-17-1 binding and bound L-365,260 with higher affinity than lorglumide a known CCK-1 receptor antagonist [71].

In rat adipocytes, CCK-2 receptors were identified on membranes and binding studies were consistent with a single class of high affinity sites with a K_D of 0.2 nM; the absence of CCK-1 receptor was established by RT-PCR in these cells [153]. On dog pancreatic acini, a population of CCK-2 receptor with high affinity sites for G-17 ns and G/CCK-4 was identified which was not associated with amylase release. This population of receptor recognized equally CCK-39, CCK-8 and G-17 ns with IC_{50} of 1 nM [154]. On rabbit isolated gastric mucosal cells, ^{125}I-(Nle^{11})--HG-13 bound specifically to a receptor population with a K_D of 70 pM, binding displaced by gastrin analogues and specific gastrin antagonists [155]. The pig pancreas demonstrated a single class of high affinity sites with as K_D of 0.22 nM established from a saturation analysis of ^{125}I-BH-[Thr.Nle] CCK-9 binding to pancreatic membranes. However, competition binding by specific CCK-1 and CCK-2 agonists and antagonists indicates the presence of both CCK receptor subtypes with CCK-2 receptor being predominant [156]. In general, the CCK-2 receptor cloned from different species exhibits a comparable high affinity for CCK-8

and gastrin-17-1 with IC_{50} in the nM range. On the contrary, they have higher affinity for the CCK-2 receptor antagonist L-365,260 (IC_{50} around 10 nM) than for the CCK-1 receptor antagonist L-364,718 (IC_{50} around 1 μM).

C. Localization

The search for the CCK-2 receptor localization was done using the following techniques: RT-PCR, Northern Blot, immunological staining and autoradiography.

In human

PCR amplification identified a CCK-2 receptor DNA fragment in the human brain, stomach and pancreas but not in the kidney [76]; in a more exhaustive study using the same technique, this CCK-2 receptor was also identified in brain, stomach, pancreas, small intestine, liver, colon, spleen, lung, thymus, ovary, breast, prostate, testes, adrenal and in the kidney [75] contrary to what was observed in the previous study [76]. Still by RT-PCR, in human purified pancreatic acini, messages for the CCK-2 receptor were observed; however, *in situ* hybridization could not confirm this expression probably because of its insufficient level of expression [74]. The CCK-2 receptor mRNA was also identified in human pancreas and in the islets of Langerhans [97] as well as in human gastric mucosae, more precisely in parietal and neuroendocrine cells and in epithelial cells within the neck of the gastric gland [157].

Using Northern blot analysis detecting the mRNA present in a gland or tissue, the CCK-2 receptor was initially detected in human brain, stomach fundus, pancreas and gallbladder [144,151] but not in heart, placenta, lung, liver, skeletal muscle and kidney [149]; some of these organs indicated positive CCK-2 messages by RT-PCR [75].

By autoradiography analysis of ^{125}I-BH-CCK-8 binding, it was shown that the human pancreas predominantly expresses the CCK-2 receptor subtype present all across the gland [71]. Using the same technique but with ^{125}I-[Leu15]-gastrin-1, high concentrations of CCK-2 receptors were detected in the mid glandular region of the human fundic mucosae and circular muscle [73] as well as in pancreatic islets but not in normal acini [72]. Our own study more precisely identified the CCK-2 receptor on human foetal and adult pancreatic islets, specifically on the somatostatin delta cells using a specific CCK-2 receptor antibody; the presence of the receptor was also confirmed by Western blots [158]. These data do not agree with a previous location of this CCK-2 receptor on the human islet's glucagon cells [97] with an antibody we could not evaluate its specificity [80].

In the rat, mouse and guinea pig

In rat and mouse, we encountered the same location problems with the RT-PCR and Northern Blot techniques. By RT-PCR, the CCK-2 receptor was identified in rat total pancreas homogenate, in purified islets [158] and in the antrum mucosae [76] while it could not be detected in two other studies [159,160]. However, by Northern blot analysis, the CCK-2 receptor mRNA remained absent from the rat pancreas and islets [76,145,159,160] and from the rat muscle, kidney, liver and guinea pig gallbladder [76,145]; On the other end, the receptor was detected in the rat brain sub-cortex and cortex and in the fundic mucosae [76,145]. In the adult mouse, the CCK-2-subtype transcripts were detected by Northern Blot analysis in brain and stomach, but not in the pancreas, liver or colon; however, using RT-PCR, a more sensitive technique, transcripts were confirmed in brain and stomach and others were also present in colon, pancreas, kidney and ovary and remained absent in heart, duodenum, small intestine, liver, gallbladder and testis [146]. In the pig [87], the CCK-2 receptor has been identified by Northern Blot in the brain, pancreas and gallbladder.

By immunohistochemistry and electron microscopy, the CCK-2 receptor was localized in guinea pig parietal cells, on chief cells and in endocrine cells of the stomach but not in the lamina propria [161]. By immunocytochemistry, the CCK-2 receptor was shown to be transiently expressed in foetal rat pancreas (E17-E18), expression which disappeared on E20-E22 and after birth. In adult pancreas, the receptor was localized on glucagon islet cells. By immunohistochemistry using fluo-CCK-8, the CCK-2 receptor was identified on gastric ECL cells but surprisingly not on parietal cells; the bioactivity of this agonist was confirmed by its ability to induce histamine release from the ECL cells [162]. In the guinea pig and dog stomach, a specific CCK-2 receptor antibody identified the receptor in a few, small epithelial cells in the bottom part of the corpus mucosae and in the antral mucosae with co-localization with the somatostatin cells [163] as observed in the rat pancreatic islets [158]. Also with a specific CCK-2 receptor antibody, the protein was identified with mucosae neural components of the bovine small intestine [78].

An analysis of all these data on the CCK-2 receptor localization using different techniques suggests that safe localization at the cellular level will come with the use of standardized and specific antibodies ultimately with colocalization with known cellular protein, enzymes or hormones.

D. CCK-2 receptor occupation and physiological responses

The presence of the CCK-2 receptor has been observed in the esophagus, stomach, upper gut, pancreas and on adipose tissue.

1. Growth of the esophagus

CCK-2 receptors have been identified in both lower and mid esophageal mucosae in human [164] and it was previously reported that gastrin can exert trophic effects on esophageal mucosae whether the hormone was infused or endogenously release [165,166].

2. Effects on the stomach

Administration of graded doses of pentagastrin, a gastrin-17 analogue, caused graded increases in both peak acid output and duration of response in rats [167]. With the discovery of the CCK receptor subtypes, explanation of acid release in response to gastrin has moved from a direct effect on the parietal cells to a rather indirect effect through the enterochromaffin-like (ECL) cells. It is now believed that gastrin stimulates acid release primarily through activation of the CCK-2 receptor on the ECL cells via histamine release [168]. The importance of the gastrin-enterochromaffin-like cell axis was strengthened recently by the observation that in gastrin KO mice, histidine

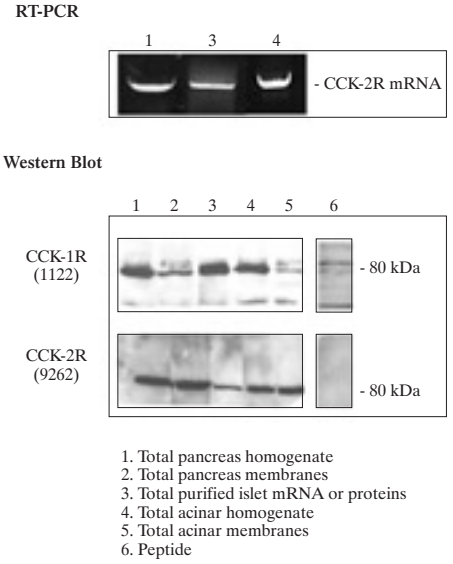
decarboxylase, the enzyme responsible for histamine synthesis (HDC) mRNA was reduced along with a concomitant decrease in HDC activity. In these ECL cells, the number of secretory histamine vesicles was also decreased. Overall, gastric acid output in gastrin KO mice was only 20% of that in the wild type mice [169]. The involvement of the CCK-2 receptor in the control of gastric acid secretion is also supported from data obtained with a new CCK-2 receptor agonists, the diketopiperazine analogues. Indeed, one of these, compound 1, dose-dependently increased gastric acid output in an anesthetized rat, an effect totally blocked by a CCK-2 receptor antagonist, CI-988 [170].

Gastrin is generally considered to be a trophic factor for the oxyntic gland area mucosae of the stomach and may also be involved in regulating mucosal growth in small intestine and colon [171]. However, an extensive study in fed rats using relatively high doses of pentagastrin, 0.25, 1.0 and 4.0 mg kg^{-1} for 5 days did not have any significant effects on thymidine incorporation into oxyntic gland area, duodenum and colon, total organ weight of these three organs as well as their total DNA contents [167]. More recently, it was shown that gastrin elicited increased [^{3}H] thymidine incorporation into ECL cells but failed to do so on parietal cells. Furthermore, gastrin increased tyrosine phosphorylation and activation of MAP kinase and c-fos and c-jun gene expression only in ECL cells. In these respective cells, gastrin dose-dependently increased histamine release and [^{14}C]-aminopyrine uptake [172]. These data clearly indicate that gastrin can activate intracellular pathways related to cell growth in the ECL cells but not in the parietal cells and suggest that gastrin may act to promote commitment or differentiation of precursor cells to parietal cells [173].

3. Effects on the pancreas

Although previous pharmacological data in humans support the involvement of CCK-1 receptor in the regulation of pancreatic exocrine secretion based on the potent response to CCK and the limited one to gastrin [86,174], it was later suggested that the CCK-1 receptors were present on neurons, rather than on pancreatic acinar cells, and that the final mediator would be acetylcholine [175]. This hypothesis was recently supported by the observation that the human pancreatic acinar cells lack functional responses to cholecystokinin and gastrin; in that study, however, it was clearly demonstrated that the CCK-2 receptor has the potential to induce enzyme secretion when transiently transfected into human acini with a secretory response to CCK-8 comparable to that induced by carbachol [74]. This potential of the CCK-2 receptor coupled to pancreatic enzyme secretion was also demonstrated in transgenic mice expressing the human CCK-2 receptor in their acini as they secreted amylase in response to CCK and gastrin in a dose-dependent manner in the presence of a CCK-1 receptor antagonist; the pharmacological characteristics of these secretory responses to both stimuli reflect occupation of the CCK-1 rather than that of the CCK-2 receptor with EC_{50} in the pM range [176]. In the rat, our own data [177] (*Fig. 1*) indicate that their pancreas expresses both CCK receptors subtypes with secretion in response to gastrin being the result of CCK-1 receptor occupation; indeed, it is inhibited by the CCK-1 receptor antagonist, L-364,718 but not by the CCK-2 receptor antagonist, L-365,260. In pig acini, amylase

Figure 1. **Amylase release in response to pentagastrin (PG) and PG plus the CCK-1 (L-364,718) and the CCK-2 (L-365,260) receptor antagonists**
Freshly prepared acini were incubated 30 min at 37°C with increasing concentrations of pentagastrin, L-364,718 and L-365,260



release was stimulated by caerulein concentrations above 1 nM, remained insensitive to the high affinity CCK-1 receptor agonist JMV-180 and caerulein-induced amylase secretion was inhibited only by the CCK-1 receptor antagonist MK-329 at concentrations above 100 nM [87]. These data are difficult to reconcile with the absence of either CCK receptor subtype on the pig acinar cells [79,80]; one possibility remains that the acini preparation contained functional nerve ending sensitive to CCK as part of the short reflex system described by Konturek [175]. In response to CCK and gastrin in the presence of SR27,897, a CCK-1 receptor antagonist, glucagon secretion from purified human pancreatic islets reached its maximal release at 13 and 8 pM, respectively, a response inhibited by RPR-101048, a specific CCK-2 receptor antagonist [97]. Again, maximal secretory responses to CCK in the pM range pleads for CCK-1 receptor occupation [3] and such high affinity of gastrin for its CCK-2 receptor are unusual [177].

The implication of the CCK-2 receptor in the control of pancreas growth via its agonist gastrin is far from being established. Indeed, in the rat, under conditions which increased serum gastrin levels, gastrin infusion or chronic injections, omeprazole treatment, and fundectomy, all were without effect on the weight and DNA content of the pancreatic gland [167,178]. Earlier studies, however, claimed that gastrin exerts trophic effects in the pancreas [179,180].

In normal rats, levels of circulating leptin observed 2 and 6 h after feeding were significantly reduced by treatment with YM022, a specific CCK-2 receptor antagonist. By reducing plasma leptin, the antagonist simultaneously increased epididymal fat tissue leptin content after refeeding. Concomitantly, the antagonist also dose-dependently inhibited leptin mRNA recovery observed after refeeding. This study also indicated that

the CCK-2 receptor was present in all the adipose tissues tested: mesenteric, epididymal and perirenal [153].

E. CCK-2 receptor occupation under pathological conditions

1. The esophagus

Over recent years, there has been an enormous increase in the incidence of Barrett's metaplasia and esophageal adenocarcinoma. Since gastrin plays an important role in the regulation of gastrointestinal organs proliferation and differentiation, it became important to evaluate its implication in the regulation of Barrett's metaplasia and a search for its CCK-2 receptor.

In SEG-1 cells, a human esophageal adenocarcinoma cell line, the presence of the CCK-2 receptor has been established by RT-PCR and gastrin shown to cause a dose-dependent increase in their proliferation when compared to controls; this proliferative effect of gastrin was abolished in the presence of the specific CCK-2 receptor antagonist L-365,260 [181]. In analyzing human tissue samples, the relative expression levels of gastrin and the CCK-2 receptor were significantly increased by 29 and 8 fold, respectively in the Barrett's samples when compared to their paired normal; in esophageal cells, high basal levels of activated PKB/AkT were associated to endogenous gastrin expression and reduced in the presence of YMO22, a specific CCK-2 receptor antagonist. It is then suggested that gastrin acting in an autocrine manner may aid progression of Barrett's metaplasia through amplification of antiapoptotic pathways [182]. In another study, CCK-2 receptors were detected in 30% of normal patients, in 80% of patients with esophagitis, in 100% of patients with Barrett's metaplasia and in 70% of patients with esophageal adenocarcinomas. In the Barrett's group, all patients expressed CCK-2 receptors twice as much as in controls. Furthermore, in Barrett's mucosal biopsies, gastrin significantly increased DNA synthesis, an effect blocked by the CCK-2 receptor antagonist L-740,093. It thus seems that overexpression of the CCK-2 receptor in Barrett's metaplasia may have implications in the management of patients in whom gastrin is elevated by acid suppression therapy [164]. However, an earlier study indicated that in esophageal cancers, only one sample exhibited a low level of CCK-2 receptor mRNA; in that study, the CCK-1 receptor was overexpressed in esophageal cancers [183]. These contradictory results definitely plead for new studies to clarify which CCK receptor subtypes are overexpressed in Barrett metaplasia and cancer of the esophagus.

2. The stomach

In the human gastric adenocarcinoma cell line, amidated gastrin caused a dose-dependent increase in DNA synthesis, an effect attenuated by the CCK-2 receptor antagonist L-365,260; this growth-promoting effect of gastrin was associated with increased levels of cyclin D1 transcripts, protein and promoter activity [184].

In rat, sustained hypergastrinaemia resulted in ECL cell hyperplasia and later on lead to ECL cell carcinoid; in human, after 5 years of proton pump treatment, ECL cell hyperplasia rather than carcinoids was observed [185]. In the African rodent Mastomys, induced hypergastrinemia by histamine-2 receptor blockade, resulted in ECL cells carcinoids associated with slight elevation of the CCK-2 receptor mRNA but an 8 fold increase in histidine decarboxylase (HDC) mRNA not influenced by CCK-2 receptor inhibition. These data suggest that HDC mRNA expression in neoplastic ECL cells is not under the influence of the CCK-2 receptor [186].

Many studies indicate that gastrin is an important factor in the progression to gastric cancer; however, the presence of gastrin and its CCK-2 receptor in gastric adenocarcinomas remains a controversial issue. Indeed, gastrin and its receptor have been detected in human gastric adenocarcinomas by immunohistochemistry [187]. However, the CCK-2 receptor expression was detected in only 7% of gastric cancer samples by RT-PCR [188], or by receptor autoradiography [189]. In another study using RT-PCR, transcripts of CCK-2 receptors were present in 7 out of 8 specimens of gastric adenocarcinoma [183]. Again, the expression of the CCK-2 receptor in gastric cancer cells and tissues has to be reevaluated with standardized techniques.

3. The pancreas

In the rat with caerulein-induced pancreatitis, CCK-2 receptor mRNA expression was tremendously increased above control values, and its overexpression was maintained only in animals treated with growth-promoting doses of caerulein used to induce pancreas regeneration [141].

Transfection of the human CCK-2 receptor in mouse pancreatic acinar cells, a location where it does not normally belong [80], led to larger pancreas at age 50 days. When these mice were crossed with gastrin-mice expressing gastrin in their pancreatic β cells, some of these animals (3 out of 20 homozygous), developed malignant transformations through an acinar-ductal carcinoma sequence [190]. On the contrary, transfection of the CCK-2 receptor in human pancreatic cells, the MiaPaCa-2 and Panc-1, led to inhibited anchorage-dependent growth upon CCK stimulation along with decreased DNA synthesis [191]. These data suggest that occupation of overexpressed CCK-2 receptors on at least two pancreatic tumor cell lines can result in cell growth inhibition. However, similar MIA-PaCa-2 cells were shown to possess specific CCK-2 receptors whose occupation by CCK resulted in their proliferation [192]. This inhibitory effect of CCK is also totally opposite to growth stimulation of the hormone on normal acinar cells in culture [193]. These opposite responses are not yet quite understood; the ductal origin of the pancreatic cancer cells could be the reason; however, this possibility is not supported by the observation that ductal complexes induced in duct-ligated rat pancreas are stimulated to grow in response to gastrin; these cells have a higher expression of the CCK-2 receptor absent on normal ductal cells [194]. Furthermore, CCK-2 receptors have been identified by binding assays with the CCK-2 receptor antagonist L-365,260, on six different human pancreatic cancer cells of ductal origin [152].

Controversy also exists in the distribution of the CCK receptor subtypes in human tumors, mostly those of the pancreas. According to Reubi using his autoradiography technique for identification, 22% of the gastroenteropancreatic tumors express the CCK-2 receptor with 38% expressing the CCK-1 subtype [195]. In another study [72], the same authors indicated that ductal pancreatic carcinoma rarely expressed CCK receptors; the CCK-2 receptor mRNA and protein were identified in

Figure 2. **RT-PCR of CCK2-R mRNA and Western blots of the CCK-1 and CCK-2 receptor proteins from different pancreatic preparations using the specific CCK-1 (1122) and CCK-2 (9262) receptor antibodies. Conditions of both experiments are described in** [158]

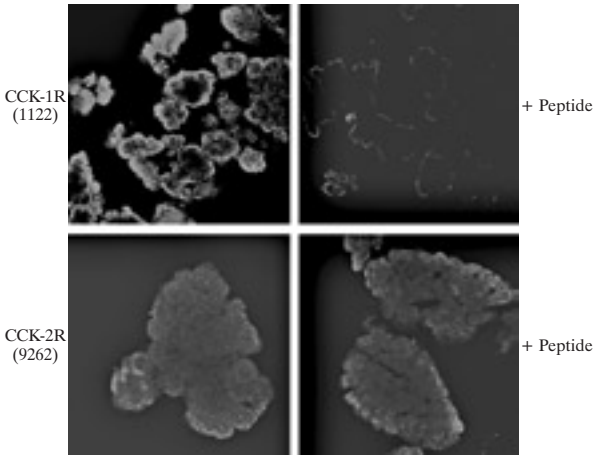
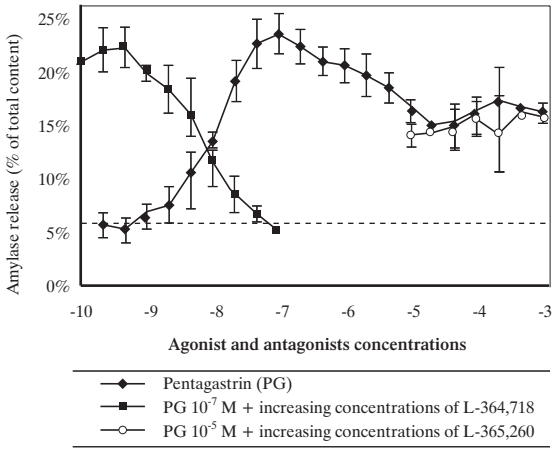


Figure 3. **Localization of the CCK-1 and CCK-2 receptors on purified rat pancreatic acini by confocal microscopy. Conditions of the experiment and the use of the specific antibodies are described in** [158]



a few tumors characterized by neuroendocrine differentiation. The major source of CCK-2 receptors in pancreatic tumor was found in islets [72].

Using RT-PCR, expression of the CCK-2 receptors was detected in all normal pancreatic tissue samples and adenocarcinomas whereas the CCK-1 receptors were observed in gallbladder, intestine, brain, ovary, spleen, thymus and in all pancreatic adenocarcinomas but not in any normal pancreas specimen [75]. Using the same technique, CCK was undetectable in all tumors and normal pancreas; the CCK-2 receptor mRNA was detected in all pancreatic tumors, all resection tissue, and all normal pancreatic tissue. Furthermore, the CCK-1 receptor mRNAs were detected in 12 of 18 tumors, in 5 of 10 resection margins and in all normal tissue samples [196], this last result being different from those of the previous study [75]. In order to solve the dilemma of CCK receptors presence in normal and tumoral pancreatic tissues and identify the specific cells expressing these receptors, an immunohistochemical study with specific CCK receptor subtype antibodies has to be done.

4. The colon

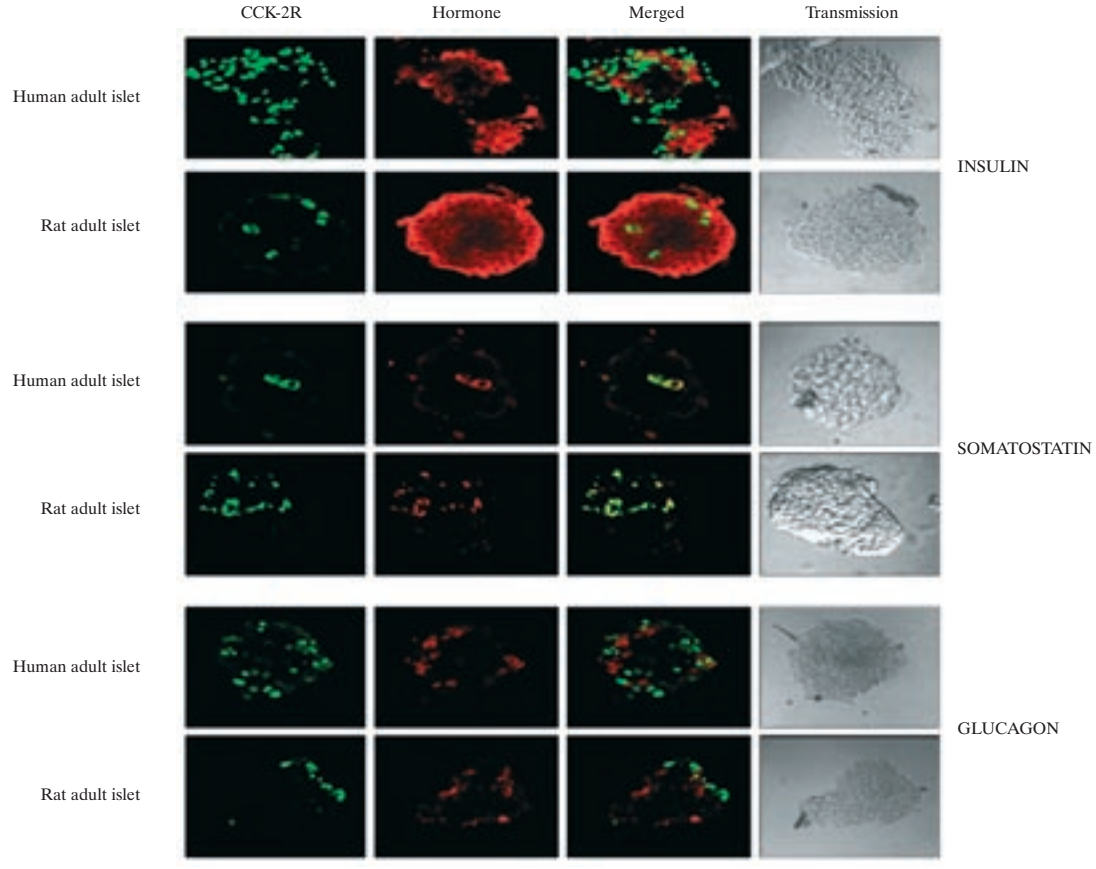
Until recently, the predominant thought was that the CCK-2 receptor could play a role in the proliferation of colon cancers; this view was deduced mostly from studies performed on cancer cell lines. However, the presence of the CCK-2 receptors in human colon cancer still remains controversial. In one study using receptor autoradiography, the CCK-2 receptors could not be detected in 22 specimens of colorectal carcinomas [195]. In another study using RT-PCR, a low level of CCK-2 receptor mRNA was found in only 2 out of 12 colon adenocarcinoma samples [183]. From binding studies, very low CCK-2 receptor binding sites were detected in some primary human colon tumors [197] whereas others failed to detect specific binding in the majority of colon cancers [198]. The present overall view is that gastrin and its CCK-2 receptor do not play a major role in the growth of the majority of colorectal carcinomas. It is also evident that future research will have to be focused on the receptor protein cellular localization using specific potent antibodies.

5. Cellular co-localization of the CCK-1 and CCK-2 receptors

Characterization of the CCK-1 and CCK-2 receptors by binding studies on pancreatic acinar cells led to the discovery that those acini may possess both receptor subtypes. An initial study indicated that dog pancreatic acini expressed high affinity sites for G17 and G/CCK-4 and that their occupation was not related to amylase release [154]. Similarly, gastrin receptors were also characterized by binding studies on guinea pig acini and shown to be distinct from the previously described CCK receptors [199]. In purified human acini, using quantitative RT-PCR, it was shown that the CCK-1 receptor was expressed at less than one copy/acinar cell and approximately at five copies/acinar cell for the CCK-2 receptor. These acini did not release amylase in response to CCK-8 and gastrin and this failure was assumed to result from insufficient level of receptor expression [74].

As shown in *Fig. 2*, our own data indicate that the rat pancreas expressed the CCK-2 receptor mRNA in the whole gland (band 1), in purified islets (band 3) and in hand-picked acini (band 4). This message corresponds to the CCK-2 receptor expressed as an 80 kDa protein present in all fractions; acinar cells and islets have the CCK-1 receptor also present as an 80 kDa protein. Specificity of both antibodies is demonstrated in band 6 with preincubation of each antibody with its specific peptide used for its development. The presence of both CCK receptor proteins on purified acini is also confirmed by confocal microscopy with the same antibodies used for the Western blots. As shown in *Fig. 3*, it is evident that the CCK-1 receptor proteins are more abundant on purified rat acinar cells than the CCK-2 receptors which also seem to be present on all cells. Amounts of this CCK-2 receptor are evidently more important in islets delta cells as observed in *Fig. 4*.

***Figure 4*. Colocalization of the pancreatic CCK-2 receptor with three islets hormones. Islets purification from human and rat pancreas was performed as described in** [158]



The major question raised following the demonstration that individual cells contain both subtypes of the CCK receptor, remains its physiological relevancy. The phenomenon is not unique to the CCK receptors as oligomerization [200] and heterodimerization [201] of the somatostatin receptor subtypes have been previously observed. One result of such heterodimerization of the SST3 and SST2A receptors is the resistance of the SST2A to agonist-induced desensitization [201]. Heterodimerization of the CCK-1 and CCK-2 receptors was observed following their stable transfection in CHO cells and such complexes exhibited enhanced agonist-stimulated cellular signaling, delayed agonist-induced receptor internalization and increased cell growth [202]. We previously demonstrated [177] (*Fig. 1*) that the presence of the CCK-2 receptor on rat pancreatic acini was not associated with enzyme release confirming what was obtained in dog acini [154].

Conclusions

The CCK receptor subtypes remain one of the key elements to all the unanswered questions and controversial responses to CCK and gastrin related to their effects in normal cell physiology and cancer development. It is therefore mandatory to establish their respective cellular location in normal and cancerous organs for potential therapies with selective and appropriate hormones and receptor antagonists. In the near future, we should look forward for a better understanding of CCK receptors' homo- and heterodimerization and their consequences on potential integrated physiological responses and/or association to cancer development.

Acknowledgement

We thank Mrs Christiane Gauvin for secretarial assistance, Drs M. Biernat and S. Julien and J. Lainé for their technical assistance. This research was supported by grant GP6369 from the Natural Sciences and Engineering Research Council of Canada.

References

1. Mutt V, Jorpes JE. Structure of porcine cholecystokinin-pancreozymin. Cleavage with thrombin and with trypsin. Eur J Biochem, 1968; 6: 156-62.

2. Cantor P, Petronijevic L, Pedersen JF, Worning H. Chole-

cystokinetic and pancreozymic effect of O-sulfated gastrin compared with nonsulfated gastrin and cholecystokinin. Gastroenterology, 1986; 91: 1154-63.

3. Sankaran H, Goldfine ID, Deveney CW, Wong KY, Williams JA. Binding of cholecystokinin to high affinity receptors on isolated pancreatic acini. J Biol Chem, 1980; 255: 1849-53.

4. Saito A, Sankaran H, Goldfine ID, Williams JA. Characterization of receptors for cholecystokinin and related peptides in mouse cerebral cortex. J Neurochem, 1981; 37: 483-90.

5. Mutt V. Further investigations on intestinal hormonal polypeptides. Clin Endocrinol, 1976; 5: 175S-83S.

6. Rehfeld JF, Hansen HF. Characterization of preprocholecystokinin products in the porcine cerebral cortex: evidence of different neuronal processing pathways. J Biol Chem, 1986; 261: 5832-40.

7. Eysselein VE, Reeve JR Jr, Shively JE, Hawke D, Walsh JH. Partial structure of a large canine cholecystokinin (CCK-58): amino acid sequence. Peptides, 1982; 3: 687-91.

8. Reeve JR Jr, Eysselein V, Walsh JH, Ben-Avram CM, Shively JE. New molecular forms of cholecystokinin-microsequence analysis of forms previously characterized by chromatographic methods. J Biol Chem, 1986; 261: 16392-7.

9. Carlquist M, Mutt V, Jornvall H. Characterization of two novel forms of cholecystokinin isolated from bovine upper intestine. Regul Pept, 1985; 11: 27-34.

10. Eng J, Du B, Pan YE, Chang M, Hulmes JD, Yalow RS. Purification and sequencing of a rat intestinal 22 amino acid C-terminal CCK fragment. Peptides, 1984; 5: 1203-6.

11. Zhou ZZ, Eng J, Pan YE, Chang M, Hulmes JD, Raufman JP, Yalow RS. Unique cholecystokinin peptides isolated from guinea pig intestine. Peptides, 1985; 6: 337-41.

12. Eysselein VE, Eberlein GA, Schaeffer M, Grandt D, Goebell H, Niebel W, Rosenquist GL, Meyer HE, Reeve JR Jr. Characterization of the major form of cholecystokinin in human intestine: CCK-58. Am J Physiol, 1990; 258: G253-60.

13. Eysselein VE, Eberlein GA, Hesse WH, Singer MV, Goebell H, Reeve JR Jr. Cholecystokinin-58 is the major circulating form of cholecystokinin in canine blood. J Biol Chem, 1987; 262: 214-7.

14. Eberlein GA, Eysselein VE, Hesse WH, Goebell H, Schaefer M, Reeve JR Jr. Detection of cholecystokinin-58 in human blood by inhibition of degradation. Am J Physiol, 1987; 253: G477-82.

15. Reeve JR Jr, Green GM, Chew P, Eysselein VE, Keire DA. CCK-58 is the only detectable endocrine form of cholecystokinin in rat. Am J Physiol 2003; 285: G255-65.

16. Bonetto V, Jornvall H, Andersson M, Reulund S, Mutt V, Sillard R. Isolation and characterization of sulphated and nonsulphated forms of cholecystokinin-58 and their action on gallbladder contraction. Eur J Biochem, 1999; 264: 336-40.

17. Reeve JR Jr, Liddle RA, McVey DC, Vigna SR, Solomon TE, Keire DA, Rosenquist G, Shively JE, Lee TD, Chew P, Green GM, Coskun T. Identification of nonsulfated cholecystokinin-58 in canine intestinal extracts and its biological properties. Am J Physiol, 2004; 287: G326-33.

18. Reeve JR Jr, Eysselein V, Eberlein GA, Chew P, Ho FJ, Huebner VD, Shively JE, Lee TD, Liddle RA. Characterization of canine intestinal cholecystokinin-58 lacking its carboxyl-terminal nonapeptide. Evidence for similar post-translational processing in brain and gut. J Biol Chem, 1991; 266: 13770-6.

19. Reeve JR Jr, Eysselein VE, Rosenquist G, Zeeh J, Regner V, Ho FJ, Chew P, Davis MT, Lee TD, Shively JE, Brazer SR, Liddle RA. Evidence that CCK-58 has structure that influences its biological activity. Am J Physiol, 1996; 270: G860-8.

20. Buffa R, Solcia E, Go VLW. Immunohistochemical identification of the cholecystokinin cell in the intestinal mucosa. Gastroenterology, 1976; 70: 528-32.

21. Larsson LI, Rehfeld JF. Distribution of gastrin and CCK cells in the rat gastrointestinal tract. Histochemistry, 1978; 58: 23-31.

22. Rehfeld JF, Hansen HF, Larsson LI, Stengaard-Pedersen K, Thorn NA. Gastrin and cholecystokinin in pituitary neurons. Proc Natl Acad Sci USA, 1984; 81: 1902-5.

23. Fallon JH, Seroogy KB. The distribution and some connections of cholecystokinin neurons in the rat brain. Ann NY Acad Sci, 1985; 448: 121-32.

24. Stevens J. An anatomy of schizophrenia. Arch Gen Psych, 1973; 29: 177-89.

25. Crawley JN, Kiss JZ. Paraventricular nucleus lesions abolish the inhibition of feeding induced by systemic cholecystokinin. Peptides, 1985; 6: 927-35.

26. Dockray GJ, Gregory RA, Tracy HJ, Zhou WY. Transport of cholecystokinin octapeptide-like immunoreactivity toward the gut in afferent vagal fibers in cat and dog. J Physiol, 1981; 314: 501-11.

27. Edkins JS. On the chemical mechanism of gastric secretion. Proc R Soc Lond (Biol), 1905; 76: 376.

28. Gregory RA, Tracy HJ. The constitution and properties of two gastrins extracted from hog antral mucosa. Gut, 1964; 5: 103-117.

29. Gregory RA, Tracy HJ, Grosmann MI. Isolation of two gastrins from human antral mucosa. Nature, 1966; 209: 583.

30. Berson SA, Yalow RS. Nature of immunoreactive gastrin extracted from tissues of gastrointestinal tract. Gastroenterology, 1971; 60: 215-22.

31. Gregory RA. A review of some recent developments in the chemistry of the gastrins. Bioorg Chem, 1979; 8: 497-511.

32. Yoo OJ, Powell CT, Agarwal KL. Molecular cloning and nucleotide sequence of full-length cDNA coding for porcine gastrin. Proc Natl Acad Sci USA, 1982; 79: 1049-53.

33. Kato K, Himeno S, Takahashi Y, Wakabayaski T, Tarui S, Matsubara K. Molecular cloning of human gastrin precursor cDNA. Gene, 1983; 26: 53-7.

34. Baldwin GS, Shulkes A. Gastrin, gastrin receptors and colorectal cancer. Gut, 1998; 42: 581-84.

35. Aly A, Shulkes A, Baldwin GS. Gastrins, cholecystokinins and gastrointestinal cancer. Biochim Biophys Acta, 2004; 1704: 1-10.

36. Gregory RA, Tracy HJ, Harris JI, Runswick MJ, Moore S, Kenner GW, Ramage R. Minigastrin: corrected structure and synthesis. Hoppe Seylers Z Physiol Chem, 1979; 360: 73-80.

37. Gregory RA, Dockray GJ, Reeve JR Jr, Shively SE, Miller C. Isolation from porcine antral mucosa of a hexapeptide corresponding to the C-terminal sequence of gastrin. Peptides, 1983; 4: 319-23.

38. Shively J, Reeve JR Jr, Eysselein VE, Ben-Avram CM, Vigna SR, Walsh JH. CCK-5: sequence analysis of a small cholecystokinin from canine brain and intestine. Am J Physiol, 1987; 252: G272-5.

39. Rehfeld JF, Larsson LI. The predominating molecular form of gastrin and cholecystokinin in the gut is a small peptide corresponding to their COOH-terminal tetrapeptide amide. Acta Physiol Scand, 1979; 105: 117-9.

40. Brand SJ, Andersen BN, Rehfeld JF. Complete tyrosine-O-sulphation of gastrin in neonatal rat pancreas. Nature, 1984; 309: 456-8.

41. Rehfeld JF, Stadel F, Vikelsoe J. Immunoreactive gastrin components in human serum. Gut, 1974; 15: 102-11.

42. Seva C, Dickinson C, Yamada T. Growth-promoting effects of glycine-extended progastrin. Science, 1994; 265: 410-2.

43. Singh P, Lu XB, Cobb SL, Miller BT, Tarasova N, Varro A, Owlia A. The prohormone, progastrin, 1-80, stimulates growth of intestinal epithelial cells in vitro via high affinity binding sites. Am J Physiol, 2003, 284: G328-39.

44. Lamers CB, Walsh JH, Jansen JB, Harrison AR, Ippolili AF, Van Tongeren JH. Evidence that gastrin 34 is preferentially released from the human duodenum. Gastroenterology, 1982; 83: 233-9.

45. McGuigan JI. Gastrin mucosal intracellular localization of gastrin by immunofluorescence. Gastroenterology, 1968; 55: 315-27.

46. Vaillant C, Dockray G, Hopkins CR. Cellular origins of different forms of gastrin. The specific immunocytochemical localization of related peptides. J Histochem Cytochem, 1979; 27: 932-5.

47. Brand SJ, Fuller PJ. Differential gastrin gene expression in rat gastrointestinal tract and pancreas during neonatal development. J Biol Chem, 1988; 263: 5341-7.

48. Morisset J, Wong H, Walsh JH, Lainé J, Bourassa J. Pancreatic CCK_B receptors: their potential roles in somatostatin release and δ-cell proliferation. Am J Physiol, 2000; 279: G148-56.

49. Larsson LI, Rehfeld JF, Goltermann N. Gastrin in the human fetus. Distribution and molecular forms of gastrin in the antro-pyloric gland area, duodenum and pancreas. Scan J Gastroenterol, 1977; 12: 869-72.

50. Nilsson G, Yalow RS, Berson SA. Distribution of gastrin in the gastrointestinal tract in human, dog, cat and hog. In: Frontiers in Gastrointestinal Hormone Research. Proceedings of the, 16th Nobel Symposium. Ed. Andersson S, 1973; 95-101.

51. Anderson BN, Abramovich D, Brand JJ, Petersen B, Rehfeld JF. Complete sulfation of jejunal gastrin in human fetus. Regul Peptides, 1985; 10: 329-38.

52. Reeve JR Jr, Walsh JH, Tompkins RK, Hawke D, Shively, JE. Amino terminal fragments of human progastrin from gastrinoma. Biochem Biophys Res Commun, 1984; 123: 404-9.

53. Eysselein VE, Reeve JR Jr Shively JE, Miller C, Walsh JH. Isolation of a large cholecystokinin precursor from canine brain. Proc Natl Acad Sci USA, 1984; 81: 6565-8.

54. DeWeerth A, Pisegna JR, Wank SA. Guinea pig gallbladder and pancreas possess identical CCK-A receptor subtypes: receptor cloning and expression. Am J Physiol, 1993; 265: G1116-21.

55. DeWeerth A, Pisegna JR, Huppi K, Wank SA. Molecular cloning, functional expression and chromosomal localization of the human cholecystokinin type A receptor. Biochem Biophys Res Commun, 1993; 194: 811-8.

56. Ulrich CD, Ferber I, Holicky E, Hadac E, Buell G, Miller LJ. Molecular cloning and functional expression of the human gallbladder cholecystokinin A receptor. Biochem Biophys Res Commun, 1993; 193: 204-11.

57. Reuben M, Rising L, Prinz C, Hersey S, Sachs G. Cloning and expression of the rabbit gastric CCK-A receptor. Biochim Biophys Acta, 1994; 1219: 321-7.

58. Lacourse KA, Lay JM, Swanberg LJ, Jenkins C, Samuelson LC. Molecular structure of the mouse CCK-A receptor gene. Biochem Biophys Res Commun, 1997; 236: 630-5.

59. Wank SA, Harkins RT, Jensen RT, Shapira H, deWeerth A, Slattery T. Purification, molecular cloning and functional expression of the cholecystokinin receptor from rat pancreas. Proc Natl Acad Sci USA, 1992; 89: 3125-9.

60. Huppi K, Siwarski D, Pisegna JR, Wank S. Chromosomal localization of the gastric and brain receptors for cholecystokinin (CCK-AR and CCK-BR) in human and mouse. Genomics, 1995; 25: 727-9.

61. Takiguchi S, Takata Y, Funakoshi A, Miyasaka K, Kataoka K, Fujimura Y, Goto T, Kono A. Disrupted cholecystokinin type-A receptor (CCKAR) gene in OLETF rats. Gene, 1997; 197: 169-75.

62. Pearson RK, Miller LJ. Affinity labeling of novel cholecystokinin-binding protein in rat pancreatic plasmalemna using new short probes for the receptor. J Biol Chem, 1987; 262: 869-76.

63. Sakamoto C, Goldfine ID, Williams JA. Characterization of cholecystokinin receptor subunits on pancreatic plasma membranes. J Biol Chem, 1983; 258: 12707-11.

64. Fourmy D, Zahidi A, Fabre R, Guidet M, Pradayrol L, Ribet A. Receptors for cholecystokinin and gastrin peptides display specific binding properties and are structurally different in guinea pig and dog pancreas. Eur J Biochem, 1987; 165: 683-92.

65. Miller LJ. Characterization of cholecystokinin receptors on human gastric smooth muscle tumors. Am J Physiol, 1984; 247: G402-10.

66. Silvente-Poirot S, Dufresne M, Vaysse N, Fourmy D. The peripheral cholecystokinin receptors. Eur J Biochem, 1993; 215: 513-29.

67. Sankaran H, Goldfine ID, Bailey H, Licko V, Williams JA. Relationship of cholecystokinin receptor binding to regulation of biological functions in pancreatic acini. Am J Physiol, 1982; 242: G250-7.

68. Talkad DV, Fortune KP, Pollo DP, Shah GN, Wank SA, Gardner JD. Direct demonstration of three different states of the pancreatic cholecystokinin receptor. Proc Natl Acad Sci USA, 1994; 91: 1868-72.

69. Goldfine ID, Williams JA. Receptors for insulin and CCK in the acinar pancreas: relationship to hormone action. Int Rev Cytol, 1983; 85: 1-38.

70. Rosenzweig SA, Miller LJ, Jamieson JD. Identification and localization of cholecystokinin binding sites on rat pancreatic plasma membranes and acinar cells: a biochemical and autoradiography study. J Cell Biol, 1983; 96: 1288-97.

71. Tang C, Biemond I, Lamers CBHW. Cholecystokinin receptors in human pancreas and gallbladder muscle; a comparative study. Gastroenterology, 1996; 111:1621-6.

72. Reubi JC, Waser B, Gugger M, Friess H, Kleeff J, Kayed H, Buchler MW, Laissue JA. Distribution of CCK1 and CCK2 receptors in normal and diseased human pancreatic tissue. Gastroenterology, 2003; 125: 98-106.

73. Reubi JC, Waser B, Laderach U, Stettler C, Friess H, Halter F, Schmassmann A. Localization of cholecystokinin A and cholecystokinin B-gastrin receptors in the human stomach. Gastroenterology, 1997; 112: 1197-205.

74. Ji B, Bi Y, Simeone D, Mortensen RM, Logsdon CD. Human pancreatic acinar cells lack functional responses to cholecystokinin and gastrin. Gastroenterology, 2001; 121: 1380-90.

75. Weinberg DS, Ruggeri B, Barber MT, Biswas S, Miknyocki S, Waldman SA. Cholecystokinin A and B receptors are differentially expressed in normal pancreas and pancreatic adenocarcinoma. J Clin Invest, 1997; 100: 597-603.

76. Monstein HJ, Nylander AG, Saleki A, Chen D, Lundquist I, Hakanson R. Cholecystokinin-A and cholecystokinin-B/gastrin receptor mRNA expression in the gastrointestinal tract and pancreas of the rat and man. Scand J Gastroenterol, 1996; 31: 383-90.

77. Sternini C, Wong H, Pham T, DeGiorgio R, Miller LJ, Kuntz SM, Reeve JR Jr, Walsh JH, Raybould HE. Expression of cholecystokinin A receptors in neurons innervating the rat stomach and intestine. Gastroenterology, 1999; 117: 1136-46.

78. Zabielsky R, Morisset J, Podgurniak P, Romé V, Biernat M, Bernard C, Chayvialle JA, Guilloteau P. Bovine pancreatic secretion in the first week of life: potential involvement of intestinal CCK receptors. Regul Peptides, 2002; 103: 93-104.

79. Bourassa J, Lainé J, Kruse ML, Gagnon MC, Calvo E, Morisset J. Ontogeny and species differences in the pancreatic expression and localization of the CCKA receptor. Biochem Biophys Res Commun, 1999; 260: 820-8.

80. Morisset J, Julien S, Lainé J. Localization of the cholecystokinin receptor subtypes in the endocrine pancreas. J Histochem Cytochem, 2003; 51: 1501-13.

81. Schweiger M, Erhard MH, Amselgruber WM. Cell-specific localization of the cholecystokinin A receptor in the porcine pancreas. Anat Histol Embryol, 2000; 29: 357-61.

82. Jensen RT, Wank SA, Rowley WH, Sato S, Gardner JD. Interaction of CCK with pancreatic acinar cells. Trends Pharmacol Sci, 1989; 10: 418-23.

83. Louie DS, Liang JP, Owyang C. Characterization of a new CCK antagonist, L364,718: in vitro and in vivo studies. Am J Physiol, 1988; 255: G261-6.

84. Hosotani R, Chowdhury P, McKay D, Rayford PL. L364718, a new CCK antagonist, inhibits biological actions of CCK in conscious dogs. Peptides, 1987; 8: 1061-4.

85. Cuber JC, Corring T, Levenez F, Bernard C, Chayvialle JA. Effects of cholecystokinin octapeptide on the pancreatic exocrine secretion in the pig. Can J Physiol Pharmacol, 1989; 67: 1391-7.

86. Cantor P, Petronijevic L, Pedersen JF, Worning H. Cholecystokinetic and pancreozymic effect of 0-sulfated gastrin compared with nonsulfated gastrin and cholecystokinin. Gastroenterology, 1986; 91: 1154-63.

87. Morisset J, Levenez F, Corring T, Benrezzak O, Pelletier G, Calvo E. Pig pancreatic acinar cells possess predominantly the CCK-B receptor type. Am J Physiol, 1996; 271: E397-402.

88. Owyang C. Physiological mechanisms of cholecystokinin action on pancreatic secretion. Am J Physiol, 1996; 271: G1-7.

89. Zabielski R, Onaga R, Mineo H, Kato S, Pierzynowski SG. Intraduodenal cholecystokinin octapeoptide (CCK-8) can stimulate pancreatic secretion in the calf. Int J Pancreatol, 1995; 17: 271-8.

90. Morisset J, Lainé J, Bourassa J, Lessard M. Romé V, Guilloteau P. Presence and localization of CCK receptor subtypes in calf pancreas. Regul Peptides, 2003; 111: 103-9.

91. Karlsson S, Ahren B. Cholecystokinin and the regulation of insulin secretion. Scand J Gastroenterol, 1992; 27: 161-5.

92. Verspohl EJ, Ammon HPT, Williams JA, Goldfine ID. Evidence that cholecystokinin interacts with specific receptors and regulates insulin release in isolated rat islets of Langerhans. Diabetes, 1986; 35: 38-43.

93. Zawalich WS, Diaz VA, Zawalich KC. Stimulatory effects of cholecystokinin in isolated perfused islets inhibited by potent and specific antagonist L364718. Diabetes, 1988; 37: 1432-7.

94. Karlsson S, Sundler F, Ahren B. CCK receptor subtype in insulin-producing cells: a combined functional and in situ hybridization study in rat islets and a rat insulinoma cell line. Regul Peptides, 1998; 78: 95-103.

95. Hermansen K. Effects of cholecystokinin (CCK)-4, nonsulfated CCK-8, and sulfated CCK-8 on pancreatic somatostatin, insulin, and glucagon secretion in the dog: studies in vitro. Endocrinology, 1984; 114: 1170-5.

96. Funakoski A, Miyasaka K, Kanai S, Masuda M, Yasunami Y, Nagai T, Ikeda S, Jimi A, Kawanami T, Kono A. Pancreatic endocrine dysfunction in rats not expressing the cholecystokinin-A receptor. Pancreas, 1996; 12: 230-6.

97. Saillan-Barreau C, Dufresne M, Clerc, P, Sanchez D, Coromainola H, Moriscot C, Guy-Crotte O, Escieut C, Vaysse N, Gomis R, Tarasova N, Fourmy D. Evidence for a functional role of the cholecystokinin-B/gastrin receptor in the human foetal and adult pancreas. Diabetes, 1999; 48: 2015-21.

98. Mesquita MA, Thompson DG, Troncon LE, D'Amato M, Rovati LC, Barlow J. Effect of cholecystokinin-A receptor blockade on lipid-induced gastric relaxation in humans. Am J Physiol, 1997; 273: G118-23.

99. Moran TH, McHugh PR. Cholecystokinin suppresses food intake by inhibiting gastric emptying. Am J Physiol, 1982; 242: R491-7.

100. Lal S, McLaughlin J, Barlow J, D'Amato M, Giacovelli G, Varro A, Dockray GJ, Thompson DG. Cholecystokinin pathways modulate sen-

sations induced by gastric distention in humans. Am J Physiol, 2004; 287: G72-9.

101. Adamkiewicz B. Clinical Investigator's Brochure. Research Triangle Park, NC: Glaxo Smith Kline, 2000.

102. Castillo EJ, Delgado-Aros S, Camilleri M, Burton D, Stephens D, O'Connor-Semmes R, Walker A, Shachoy-Clark A, Zinsmeinster AR. Effect of oral CCK-1 agonist GI, 181771X on fasting and postprandial gastric functions in healthy volunteers. Am J Physiol, 2004; 287: G363-9.

103. Rettenbacher M, Reubi JC. Localization and characterization of neuropeptide receptors in human colon. Naunyn-Schmiedeberg's Arch Pharmacol, 2001; 364: 291-304.

104. Scarpignato C, Pelosini I. Management of irritable bowel syndrome: novel approaches to the pharmacology of gut motility. Can J Gastroenterol, 1999; 13: 50A-65A.

105. Fosatti-Marchal S, Coffin B, Flourie B, Lemann MCF, Jian R, Rambaud JC. Effects of cholecystokinin octapeptide (CCK-OP) on the tonic and phasic motor activity of the human colon. Gastroenterology, 1994; 106: A499.

106. Niederau C, Faber S, Karaus M. Cholecystokinin's role in regulation of colonic mobility in health and in irritable bowel syndrome. Gastroenterology, 1992; 102: 1889-98.

107. Meyer BM, Werth BA, Beglinger C, Hildebrand P, Jansen JB, Zach D, Rovati LC, Stalder GA. Role of cholecystokinin in regulation of gastrointestinal motor functions. Lancet, 1989; 2: 12-5.

108. Liddle RA, Goldfine ID, Rosen MS, Taplitz RA, Williams JA. Cholecystokinin bioactivity in human plasma: molecular forms, responses to feeding, and relationship to gallbladder contraction. J Clin Invest, 1985; 75: 1144-52.

109. Liddle RA, Gertz BJ, Kanayama S, Beccaria L, Coher LD, Turnbull TA, Morita ET. Effects of a novel cholecystokinin (CCK) receptor antagonist, MK-329, on gallbladder contraction and gastric emptying in humans: implications for the physiology of CCK. J Clin Invest, 1989; 84: 1220-5.

110. Shaw RA, Jones RS. The choleretic action of cholecystokinin and cholecystokinin octapeptide in dogs. Surgery, 1978; 84: 622-5.

111. Sarles JC, Bidart JM, Devaux MA, Echinard C, Castagnini A. Action of cholecystokinin and caerulein on the rabbit sphincter of Oddi. Digestion, 1976; 14: 415-28.

112. Solomon TE, Petersen H, Elashoff J, Grossman MI. Interaction of caerulein and secretin on pancreatic size and composition in rat. Am J Physiol, 1978; 235: E714-9.

113. Solomon TE, Vanier M, Morisset J. Cell site and time course of DNA synthesis in pancreas after caerulein and secretin. Am J Physiol, 1983; 245: G99-105.

114. Rivard N, Rydzewska G, Lods JS, Martinez J, Morsisset J. Pancreas growth, tyrosine kinase, PTlIns3-kinase, and PLD involve high-affinity CCK-receptor occupation. Am J Physiol, 1994; 266: G62-70.

115. Morisset J, Guan D, Jurkowska G, Rivard N, Green GM. Endogenous cholecystokinin, the major factor responsible for dietary protein-induced pancreatic growth. Pancreas, 1992; 7: 522-9.

116. Rivard N, Guan D, Maouyo D, Grondin G, Bérubé FL, Morisset J. Endogenous cholecystokinin release responsible for pancreatic growth observed after pancreatic juice diversion. Endocrinology, 1991; 129: 2867-74.

117. Niederau C, Liddle RA, Williams JA, Grendell JH. Pancreatic growth: interaction of exogenous cholecystokinin, a protease inhibitor, and a cholecystokinin receptor antagonist in mice. Gut, 1987; 28: 63-9.

118. Pfeiffer CJ, Chernenko GA, Kohli Y, Barrowman JA. Trophic effects of cholecystokinin octapeptide on the pancreas of the Syrian hamster. Can J Physiol Pharmacol, 1982; 60: 358-62.

119. Chen D, Ding XQ, Rehfeld JF, Hakanson R. Endogenous gastrin and cholecystokinin do not promote growth of rat liver. Scan J Gastroenterol, 1994; 29: 688-92.

120. West SD, Helmer KS, Chang LK, Cui Y, Greeley GH, Mercer DW. Cholecystokinin secretagogue-induced gastroprotection: role of nitric oxide and blood flow. Am J Physiol, 2003; 284: G399-410.

121. Miyasaka K, Kanai S, Masuda M, Ohta M, Kawanami T, Matsumoto M, Funakoski A. Gene expressions of cholecystokinin (CCK) and CCK receptors, and its satiety effect in young and old male rats. Arch Gerontol Geriatrics, 1995; 21: 147-55.

122. Blevins JE, Stanley BG, Reidelberger RD. Brain regions where cholecystokinin suppresses feeding in rats. Brain Res, 2000; 860: 1-10.

123. Bi S, Ladenheim EE, Schwartz GJ, Moran TH. A role for NPY overexpression in the dorsomedial hypothalamus in hyperphagia and obesity of OLETF rats. Am J Physiol, 2001; 281: R254-60.

124. Moran TH, Kinzig KP. Gastrointestinal satiety signals II. Cholecystokinin. Am J Physiol, 2004; 286: G183-8.

125. Roberts-Thomson IC, Fettman MJ, Jonsson JR, Frewin DB. Responses to cholecystokinin octapeptide in patients with functional abdominal pain syndromes. J Gastroenterol Hepatol, 1992; 7: 293-7.

126. Sjolund K, Ehman R, Lindgren S, Rehfeld JF. Disturbed motilin and cholecystokinin release in the irritable bowel syndrome. Scand J Gastroenterol, 1996; 31: 1110-4.

127. Simren M, Abrahamsson H, Bjornsson ES. An exaggerated sensory component of the gastrocolonic response in patients with irritable bowel syndrome. Gut, 2001; 48: 20-7.

128. Chey WY, Jin HO, Lee MH, Sun SW, Lee KY. Colonic motility abnormality in patients with irritable bowel syndrome exhibiting abnormal pain and diarrhea. Am J Gastroenterol, 2001; 96: 1499-506.

129. Meier R, D'Amato M, Pullwitt A, Schneider H, Rovati LC, Beglinger C. Effect of a CCK-A receptor antagonist in an experimental model of delayed colonic transit time in man. Gastroenterology, 1994; 106: A538.

130. Meier R, Beglinger C, Giacovelli G, D'Amato M. Effect of a CCK-A receptor antagonist dexloxiglumide on post-prandial gallbladder emptying and colonic transit time in healthy volunteers. Gastroenterology, 1997; 112: A788.

131. D'Amato M, Whorwell PJ, Thompson DC, Spiller RC, Giacovelli G, Griffin PH. The CCK-1 receptor antagonist dexloxiglumide is effective and safe in female patients with constipation predominant irritable bowel syndrome. Am J Gastroenterol, 2001; 96: S31.

132. Kawano K, Hirashima T, Mari S, Saitoh Y, Kurosumi M. Spontaneous long-term hyperglycemic rat with diabetic complications. Diabetes, 1992; 41: 1422-8.

133. Ohumura T, Shoji E, Onodera S, Kohgo Y. Susceptibility to the development of gastric mucosal damage in OLETF rat not expressing the CCK-A receptor. Biochem Biophys Res Commun, 1996; 224: 728-33.

134. Funakoshi A, Miyasaka K, Jimi A, Kawanai T, Takata Y, Kono A. Little or no expression of the cholecystokinin-A receptor gene in the pancreas of diabetic rats (Otsuka Long-Evans Tokushima Fatty = OLETF rats). Biochem Biophys Res Commun, 1994; 199: 482-8.

135. Funakoshi A, Miyasaka K, Shinozaki H, Masuda M, Kawanami T, Takata Y, Kono A. An animal model of congenital defect of gene expression of cholecystokinin (CCK)-A receptor. Biochem Biophys Res Commun, 1995; 210: 787-96.

136. Lacourse KA, Swanberg LJ, Gillespie PJ, Rehfeld JF, Saunders TL, Samuelson LC. Pancreatic function in CCK-deficient mice: adaptation to dietary protein does not require CCK. Am J Physiol, 1999; 276: G1302-9.

137. Miyasaka K, Ohta M, Kanai S, Sato Y, Masuda M, Funakoshi A. Role of cholecystokinin (CCK)-A receptor for pancreatic growth after weaning: a study in a new rat model without gene expression of CCK_A receptor. Pancreas, 1996; 12: 351-6.

138. Miyasaka K, Ohta M, Masuda M, Funakoshi A. Retardation of pancreatic regeneration after partial pancreatectomy in a strain of rats without CCK-A receptor gene expression. Pancreas, 1997; 14: 391-9.

139. Praissman M, Izzo RS. A reduced pancreatic protein secretion in response to cholecystokinin (CCK) in the obese Zucker rat correlates with a reduced receptor capacity for CCK. Endocrinology, 1986; 119: 546-53.

140. Jurkowska G, Grondin G, Massé S, Morisset J. Soybean trypsin inhibitor and caerulein accelerate recovery of caerulein-induced pancreatitis in rats. Gastroenterology, 1992; 102: 550-62.

141. Morisset J, Calvo E. Trophicity of the pancreas from rodents to man. J Physiol Pharmacol, 1998; 49(Suppl 2): 25-37.

142. Jurkowska G, Grondin G, Morisset J. Involvement of endogenous cholecystokinin in pancreatic regeneration after caerulein-induced pancreatitis. Pancreas, 1992; 7: 295-304.

143. Miller LJ, Holicky EL, Ulrich CD, Wieben ED. Abnormal processing of the human cholecystokinin receptor gene in association with gallstones and obesity. Gastroenterology, 1995; 109: 1375-80.

144. Pisegna JR, deWeerth A, Huppi K, Wank SA. Molecular cloning of the human brain and gastric cholecystokinin receptor: structure, functional expression and chromosomal localization. Biochem Biophys Res Commun, 1992; 189: 296-303.

145. Wank SA, Pisegna JR, deWeerth A. Brain and gastrointestinal cholecystokinin receptor family: structure and functional expression. Proc Natl Acad Sci USA, 1992; 89: 8691-5.

146. Lay JM, Jenkins C, Friis-Hansen L, Samuelson LC. Structure and developmental expression of the mouse CCK-B receptor gene. Biochem Biophys Res Commun, 2000; 272: 837-42.

147. Kopin AS, Lee YM, McBride EW, Miller LJ, Lu M, Lin HY, Kolakowski LF, Beinborn M. Expression cloning and characterization of the canine parietal cell gastrin receptor. Proc Natl Acad Sci USA, 1992; 89: 3605-9.

148. Blandizzi C, Song I, Yamada T. Molecular cloning and structural analysis of the rabbit gastrin/CCK_B receptor gene. Biochem Biophys Res Commun, 1994; 202: 947-53.

149. Ito M, Matsui T, Taniguchi T, Tsukamoto T, Murayama T, Arima N, Nakata H, Chiba T, Chihara K. Functional characterization of a human brain cholecystokinin-B receptor atrophic effect of cholecystokinin and gastrin. J Biol Chem, 1993; 268: 18300-5.

150. Zimonjic DB, Popescu NC, Matsui T, Ito M, Chihara K. Localization of the human cholecystokinin-B/gastrin receptor gene (CCK-BR) to chromosome, 11p15.5 → p15.4 by fluorescence *in situ* hybridization. Cytogenet Cell Genet, 1994; 65: 184-5.

151. Lee YM, Beinborn M, McBride EW, Lu M, Kolakowski LF, Kopin AS. The human brain cholecystokinin-B/gastrin receptor: cloning and characterization. J Biol Chem, 1993; 268: 8164-9.

152. Palmer Smith J, Liu G, Soundararajan V, McLaughlin PJ, Zagon IS. Identification and characterization of CCK-B/gastrin receptors in human pancreatic cancer cell lines. Am J Physiol, 1994; 266: R277-83.

153. Attoub S, Levasseur S, Buyse M, Goiot H, Laigneau JP, Moizo L, Hervatin F, LeMarchand-Brustel Y, Lewin JMM, Bado A. Physiological role of cholecystokinin B/gastrin receptor in leptin secretion. Endocrinology, 1999; 140: 4406-10.

154. Fourmy D, Zahidi A, Pradayrol L, Vayssette J, Ribet A. Relationship of CCK/gastrin receptor binding to amylase release in dog pancreatic acini. Regul Peptides, 1984; 10: 57-68.

155. Magous R, Bali JP. High-affinity binding sites for gastrin on isolated rabbit gastric mucosal cells. Eur J Pharmacol, 1982; 82: 47-54.

156. Philippe C, Lhoste EF, Dufresne M, Moroder L, Corring T, Fourmy D. Pharmacological and biochemical evidence for the simultaneous expression of CCK_B/gastrin and CCK_A receptors in the pig pancreas. Brt J Pharmacol, 1997; 120: 447-54.

157. Schmitz F, Goke MN, Otte JM, Schrader H, Reimann B, Kruse ML, Siegel EG, Peters J, Herzig KH, Folsch UR, Schmidt WE. Cellular expression of CCK-A and CCK-B/gastrin receptors in human gastric mucosa. Regul Peptides, 2001; 102: 101-10.

158. Julien S, Lainé J, Morisset J. The rat pancreatic islets: a reliable tool to study islet responses to cholecystokinin receptor occupation. Regul Peptides, 2004; 121: 73-81.

159. Zhou W, Povaski SP, Bell RH. Overexpression of messenger RNA for cholecystokinin-A receptor and novel expression of messenger RNA for gastrin (cholecystokinin-B) receptor in azaserine-induced rat pancreatic carcinoma. Carcinogenesis, 1993; 14: 2189-92.

160. Zhou W, Povaski SP, Bell RH. Characterization of cholecystokinin receptors and messenger RNA expression in rat pancreas: evidence for expression of cholecystokinin-A receptors but not cholecystokinin-B (gastrin) receptors. J Surg Res, 1995; 58: 281-9.

161. Tarasova NI, Romanov VI, DaSilva PP, Michejda CJ. Numerous cell targets for gastrin in the guinea pig stomach revealed by gastrin/CCK_B receptor localization. Cell Tissue Res, 1996; 283: 1-6.

162. Bakke I, Qvigstad G, Sandvik AK, Waldum HL. The CCK-2 receptor is located on the ECL cell, but not on the parietal cell. Scand J Gastroenterol, 2001; 36: 1128-33.

163. Helander HF, Wong H, Poorkhalkali N, Walsh JH. Immunohistochemical localization of gastrin/CCK-B receptors in the dog and guinea pig stomach. Acta Physiol Scand, 1997; 159: 313-20.

164. Haigh CR, Attwood SEA, Thompson DG, Jankowski JA, Kirton CM, Pritchard DM, Varro A, Dimaline R. Gastrin induces proliferation in Barrett's metaplasia through activation of the CCK2 receptor. Gastroenterology, 2003; 124: 615-25.

165. VanNieuwenhove Y, DeBacker T, Chen D, Hakanson R, Willems G. Gastrin stimulates epithelial cell proliferation in the esophagus of rats. Virchows Arch, 1998; 432: 371-5.

166. VanNieuwenhove Y, Chen D, Willems G. Postprandial cell proliferation in the esophageal epithelium of rats. Regul Peptides, 2001; 97: 131-7.

167. Solomon TE, Morisset J, Wood JG, Bussjaeger LJ. Additive interaction of pentagastrin and secretin on pancreatic growth in rats. Gastroenterology, 1987; 92: 429-35.

168. Schmidt WE, Schmitz F. Genetic dissection of the secretory machinery in the stomach. Gastroenterology, 2004; 126: 606-9.

169. Chen D, Zhao CM, Hakanson R, Samuelson LC, Rehfeld JF, Friis-Hansen L. Altered control of gastric acid secretion in gastrin-cholecystokinin double mutant mice. Gastroenterology, 2004; 126: 476-87.

170. Weng JH, Bado A, Garbay C, Roques BP. Novel CCK-B receptor agonists: diketopiperazine analogues derived from CCK_4 bioactive conformation. Regul Peptides, 1996; 65: 3-9.

171. Johnson LR. Regulation of gastrointestinal growth. In: Johnson LR, ed. Physiology of the gastrointestinal tract. New-York: Raven Press, 1981: 169-96.

172. Kinoshita Y, Nakata H, Kishi K, Kawanami C, Sawada M, Chiba T. Comparison of the signal transduction pathways activated by gastrin in enterochromaffin-like and parietal cells. Gastroenterology, 1998; 115: 93-100.

173. Koh TJ, Goldenring JR, Ito S, Mashimo H, Kopin AS, Varro A, Dockray GJ, Wang TC. Gastrin deficiency results in altered gastric differentiation and decreased colonic proliferation in mice. Gastroenterology, 1997; 113: 1015-25.

174. Valenzuela JE, Walsh JH, Isenberg JI. Effect of gastrin on pancreatic enzyme secretion and gallbladder emptying in man. Gastroenterology, 1976; 71: 409-11.

175. Konturek SJ, Zabielski R, Konturek JW, Czarnecki J. Neuroendocrinology of the pancreas; role of brain-gut axis in pancreatic secretion. Eur J Pharmacol, 2003; 481: 1-14.

176. Saillan-Barreau C, Clerc P, Adato M, Escrieut C, Vaysse IV, Fourmy D, Dufresne M. Transgenic CCK-B/gastrin receptor mediates murine exocrine pancreatic secretion. Gastroenterology, 1998; 115: 988-96.

177. Morisset J, Lainé J, Biernat M, Julien S. What are the pancreatic target cells for gastrin and its CCKB receptor? Is this a couple for cancerous cells? Med Sci Monit, 2004; 10: RA242-6.

178. Chen D, Nylander AG, Norlen P, Hakanson R. Gastrin does not stimulate growth of the rat pancreas. Scan J Gastroenterol, 1996; 31: 404-10.

179. Mayston PD, Barrowman JA. The influence of chronic administration of pentagastrin on the rat pancreas. Q J Exp Physiol, 1971; 56: 113-22.

180. Majumdar APN, Goltermann N. Chronic administration of pentagastrin. Effects on pancreatic protein and nucleic acid contents and protein synthesis in rats. Digestion, 1979; 19: 144-7.

181. Carlton Moore T, Jepeal LT, Boylan MO, Singh SK, Boyd N, Beer DG, Chang AJ, Wolfe MM. Gastrin stimulates receptor-mediated proliferation of human esophageal adenocarcinoma cells. Regul Peptides, 2004; 120: 195-203.

182. Harris JC, Clarke PA, Awan A, Jankowski J, Watson SA. An antiapoptotic role for gastrin and the gastrin/CCK-2 receptor in Barrett's esophagus. Cancer Res, 2004; 64: 1915-9.

183. Clerc P, Dufresne M, Saillan C, Chastre E, André T, Escrieut C, Kennedy K, Vaysse N, Gespach C, Fourmy D. Differential expression of the CCK-A and CCK-B/gastrin receptor genes in human cancers of the esophagus, stomach and colon. Int J Cancer, 1997; 72: 931-6.

184. Song DH, Rana B, Wolfe JR, Crimmins G, Choi C, Albanese C, Wang TC, Pestell RG, Wolfe MM. Gastrin-induced gastric adenocarcinoma growth is mediated through cyclin D1. Am J Physiol, 2003; 285: G217-22.

185. Rindi G, Bordi C, Rappel S, LaRosa S, Slolte M, Solcia E. Gastric carcinoids and neuroendocrine carcinomas: pathogenesis, pathology, and behavior. World J Surg, 1996; 20: 168-72.

186. Kolby L, Wangberg B, Ahlman H, Modlin IM, Theodorsson E, Nilsson O. Altered influence of CCK-B/gastrin receptors on HDC expression in ECL cells after neoplastic transformation. Regul Peptides, 1999; 85: 115-23.

187. Henwood M, Clarke PA, Smith AM, Watson SA. Expression of gastrin in developing gastric adenocarcinoma. Br J Surg, 2001; 88: 564-8.

188. Okada N, Kubota A, Imamura T, Suwa H, Kawaguchi Y, Ohshio G. Evaluation of cholecystokinin, gastrin, CCK-1 receptor, and CCK-2/gastrin receptor gene expressions in gastric cancer. Cancer Lett, 1996; 106: 257-62.

189. Reubi JC, Waser B, Schmassmann A, Laissue JA. Receptor autoradiographic evaluation of cholecystokinin, neurotensin, somatostatin and vasoactive intestinal peptide receptors in gastro-intestinal adenocarcinoma samples: where are they really located? Int J Cancer, 1999; 81: 376-86.

190. Clerc P, Leung-Theung-Long S, Wang TC, Dockray GJ, Bouisson M, Delisle MB, Vaysse N, Pradayrol L, Fourmy D, Dufresne M. Expression of CCK2 receptors in the murine pancreas: proliferation, transdifferentiation of acinar cells, and neoplasia. Gastroenterology, 2002; 122: 428-37.

191. Detjen K, Fenrich MC, Logsdon CD. Transfected cholecystokinin receptors mediate growth inhibitory effects on human pancreatic cancer cell lines. Gastroenterology, 1997; 112: 952-9.

192. Kaufmann R, Schalberg H, Rudroff C, Henklein P, Nowak G. Cholecystokinin B-type receptor signaling is involved in human pancreatic cancer cell growth. Neuropeptides, 1997; 31: 573-83.

193. Logsdon CD. Stimulation of pancreatic acinar cell growth by CCK, epidermal growth factor, and insulin in vitro. Am J Physiol, 1986; 251: G487-94.

194. Rooman I, Lardon J, Flamez D, Schmit F, Bouwens L. Mitogenic effect of gastrin and expression of gastrin receptors in duct-like cells of rat pancreas. Gastroenterology, 2001; 121: 940-9.

195. Reubi JC, Schaer JC, Waser B. Cholecystokinin (CCK)-A and CCK-B/gastrin receptors in human tumors. Cancer Res, 1997; 57: 1377-86.

196. Goetze JP, Nielsen FC, Burcharth F, Rehfeld JF. Closing the gastrin loop in pancreatic carcinoma. Coexpression of gastrin and its receptor in solid human pancreatic adenocarcinoma. Cancer, 2000; 88: 2487-94.

197. Upp RH, Singh P, Townsend HC, Thompson JC. Clinical significance of gastrin receptors in human colon cancer. Cancer Res, 1989; 49: 488-92.

198. Imdahl A, Mantamadiotis T, Eggstein S, Farthmann EH, Baldwin GS. Expression of gastrin, gastrin/CCK-B and gastrin CCK-C receptors in human colorectal carcinomas. J Cancer Res Clin Oncol, 1995; 121: 661-6.

199. Yu DH, Noguchi M, Zhou ZC, Villanueva ML, Gardner JD, Jensen RT. Characterization of gastrin receptors on guinea pig pancreatic acini. Am J Physiol, 1987; 253: G793-801.

200. Patel RC, Kumar U, Lamb DC, Eid JS, Rocheville M, Grant M, Rani A, Hazlett T, Patel SC, Gratton E, Patel YC. Ligand binding to somatostatin receptors induces receptor-specific oligomer formation in live cells. Proc Natl Acad Sci USA, 2002; 99: 3294-9.

201. Pfeiffer M, Koch T, Schroder H, Klutzny M, Kirscht S, Kreienkamp HJ, Hollt V, Schulz S. Homo- and heterodimerization of somatostatin receptor subtypes. Inactivation of SST3 receptor function by heterodimerization with SST2A. J Biol Chem, 2001; 276: 14027-36.

202. Cheng ZJ, Harikumar KG, Holicky EL, Miller LJ. Heterodimerization of type A and B cholecystokinin receptors enhance signaling and promote cell growth. J Biol Chem, 2003; 278: 52972-9.

Surgical revascularization and perioperative management in patients with non-ST-elevation acute coronary syndromes

Thielmann M*, Jakob H

Department of Thoracic and Cardiovascular Surgery, West-German Heart Center Essen, University Clinic of Essen, Germany

Abstract

Purpose: **The management and surgical revascularization treatment of patients with acute coronary syndromes (ACS) have undergone great evolution over the past decade. The objective of the present study was therefore to analyze the outcome and predictors of survival in patients unresponsive to maximal non-surgical treatment referred to emergency coronary artery bypass grafting (CABG) with ACS.**

Material and methods: **Between October 1999 and September 2004, a total of 3571 CABG patients underwent primary isolated CABG at our institution. Out of these, non-ACS (N-ACS) was present in 3124 patients (group 1), 386 patients (group 2) had non-ST-elevation ACS (NSTE-ACS), whereas 61 patients (group 3) had ST-elevation ACS (STE-ACS). Clinical data, in-hospital morbidity and mortality were prospectively recorded and studied retrospectively in the groups.**

Results: **Left main stem stenosis was observed in 25%, 32%, and 41%, respectively (P<0.02). Previous myocardial infarction was found in 33%, 43%, and 73% (P<0.001). Overall in-hospital mortality was 1.5% in group 1, 4.2% in group 2, and 13.0% in group 3 (P<0.001). Logistic regression and receiver operating characteristic analyses identified cTnI as the strongest preoperative predictor significantly related to in-hospital mortality. A preoperative cTnI level above 1.5 ng/ml was the best single predictor for in-hospital mortality amongst patients with ACS.**

Conclusions: **The present study clearly demonstrates a significant difference of in-hospital morbidity and mortality between patients with ACS undergoing CABG. A more precise patient's risk stratification on admission and improvements in the perioperative management with adjunctive pharmacological therapies and the use of intraaortic balloon counter pulsation may improve patients' outcome.**

Key words: coronary artery bypass grafting, acute coronary syndromes, perioperative management, outcomes.

Introduction

To date, patients undergoing coronary artery bypass grafting (CABG) with acute coronary syndromes (ACS) ranging from unstable angina or acute myocardial infarction (AMI) without ST-segment elevations up to evolving AMI with persistent ST--segment elevations, offer a challenge from the standpoint of diagnosis, treatment, and prognosis, as the clinical manifestations vary considerably. The differences between the entities of ACS are related to the symptoms severity, the initial electrocardiographic pattern, and the degree of acute myocardial cellular necrosis, as expressed by cardiac troponins elevation as a biochemical marker of irreversible myocardial necrosis [1-3]. Patients with ST-elevation acute myocardial infarction have been clearly identified to have an increased risk of suffering death in hospital and, moreover, to have an increased adverse prognosis in the setting of percutaneous coronary intervention [4,5] as well as in patients undergoing emergency CABG [6,7]. However, the reasons for higher adverse prognosis and increased mortality rates in patients with non-ST-elevation ACS (NSTE-ACS) associated with 'minor myocardial damage' resulting in minor elevations of cardiac troponins are not fully understood. Nonetheless, the surgical revascularization in

* CORRESPONDING AUTHOR:
Department of Thoracic and Cardiovascular Surgery
West-German Heart Center, University Clinic of Essen
Hufelandstraße 55
45122 Essen, Germany
Tel: +49 201 7234928; Fax: +49 201 7235451
e-mail: matthias.thielmann@uni-essen.de (Matthias Thielmann)

Received 17.01.2005 Accepted 25.01.2005

patients with ACS has, so far, an important indication based on the obstructive coronary artery lesion; all of them considered of high risk according to the ACC/AHA guidelines [8-10].

The outcomes of patients with ACS undergoing CABG surgery during their hospitalization are expected to be worse than of non-ACS (N-ACS) patients [11,12]. Although there is a plethora of data regarding the outcomes of CABG in general, there are less of contemporary data regarding the frequency and outcomes of CABG among a wide spectrum of patients refferd to CABG due to ACS, although surgical revascularization treatment and management of ACS patients have undergone a great evolution over the past decade [9,12].

The objective of the present study was therefore to analyze the outcome and predictors of survival in patients unresponsive to maximal non-surgical treatment referred to emergency CABG due to ACS at our institution.

Material and methods

Patient population and data collection

From 1999 to 2004, a total of 3510 consecutive patients who underwent primary isolated CABG at the West-German Heart Center Essen were prospectively studied. Of these, 3124 patients had N-ACS (Group 1), whereas a NSTE-ACS (Group 2) was preoperatively identified in 386 CABG patients. NSTE-ACS was supposed to be present if patients had symptoms indicative of an ACS within the preceding 24 hours like new onset of chest pain or accelerating chest pain within the previous 24 hours occurring at rest or with minimal exertion, alleviated by nitroglycerin and/or rest in the absence of ST-segment elevation of >1 mm on the electrocardiogram or with an elevated serum level of cardiac troponin I (cTnI) or creatine kinase (CK) on admission. Informed consent was obtained for all patients and the study protocols were approved by the Institutional Review Board of the West-German Heart Center Essen. Patients were excluded from the study, if any of the following criteria were present: (1) preoperative myocardial infarction with ST-segment elevation on the electrocardiogram (STEMI); (2) new onset left bundle branch block; (3) reoperations; (4) any concomitant heart surgery besides CABG. All clinical data were prospectively recorded and documented with more than 1800 variables per case using a database tool according to the "Heidelberger Verein zur Multizentrischen Datenanalyse e.V." (HVMD) [13].

The primary study endpoint for comparison between the two groups were in-hospital mortality, defined as all cause of death within 30 days after surgery or during the same time period of hospitalization. Secondary study endpoints were postoperative major adverse events (MAE) during the period of hospitalization including: (1) perioperative myocardial infarction (PMI); (2) low cardiac output syndrome (LOS) with high-dose inotropic support with or without requiring the use of an intraaortic balloon counterpulsation; (3) stroke, and minor adverse events like; (4) new-onset ventricular arrhythmias; (5) major bleeding; (6) necessity for rethoracotomy and (7) postoperative renal failure requiring temporary hemodialysis. Perioperative myocardial infarction was considered to have occurred, if one of the following diagnostic criteria were present: (1) a postoperaive cTnI serum level above 10.5 ng/ml within the first 24 hours after CABG, as previously described [14]; (2) the appearance of ST-segment deviations at the J point in two or more contiguous leads with cut-off points ≥0.2 mV in leads V1, V2, or V3 and ≥0.1 mV in other leads or T-wave abnormalities in two or more contiguous leads or the development of new Q-waves [2]. LOS was present, if high-dose inotropic support was necessary in the postoperative course during hospital stay with or without the need of an intraaortic balloon couterpulsation (IABP).

Preoperative risk stratification

In patients with an established diagnosis of ACS, the management strategy to be selected in a particular patient depends on the perceived risk of progression to myocardial infarction or death. All patients presenting with NSTE-ACS were stratified to their individual risk, using the established independent predictors like: (A) markers of thrombotic risk, such as recurrence of chest pain, ECG-changes, or elevated levels of cardiac markers for myocardial damage and severity of coronary artery disease, and (B) risk-factors and comorbidities, such as age, history of previous MI, left ventricular function, renal dysfunction, etc [15-17].

Preoperative management

Patients presenting NSTE-ACS were treated preoperatively on the evidence of clinical trials or meta-analyses with: (1) hemodynamic monitoring; (2) adjunctive pharmacological therapeutic measures, using beta-blockers, nitrates, low-molecular-weight or intravenous heparin, and finally ADP receptor antagonists, such as ticlopidine and clopidogrel resulting in inhibition of platelet aggregation in patients with NSTE-ACS; (3) the optimal timing of CABG surgery, which depends on the preoperative dynamics of acute myocardial injury and (4) the evaluation of the prophylactic use of preoperative IABP treatment, especially in high-risk patients due to (a) impaired left ventricular ejection fraction, (b) preoperative hemodynamic instability with necessity of inotropic support, (c) unstable angina at the time of operation despite intravenous nitroglycerin and heparin, and (d) filiform left mainstem disease or left main equivalent or severe three-vessel disease.

Surgical management

Standard anesthetic and monitoring techniques were used in all patients. Internal thoracic artery, radial artery, and saphenous vein grafts were used as graft conduits. Heparin was administered in order to achieve an activated coagulation time above 400 s. Standard cardiopulmonary bypass (CPB) technique was used with ascending aortic and two-stage venous cannulation. During CPB, moderate hemodilution with a hematocrit level between 20% and 25% using mild systemic hypothermia (>32°C) was maintained. A maximum of myocardial protection was achieved using simultaneous antegrade and retrograde cristalloid cardioplegic arrest (Bretschneider) with additional topical cooling, and single aortic cross clamping for all distal anastomosis. In addition, cardioplegia was administered through the distal grafts until aortic unclamping if necessary. Depending on the severity of coronary artery disease and the

resultant estimated extent of acute myocardial injury, the C-1-esterase inhibitor (Berinert) was administered intravenously 5 minutes before reperfusion. Reperfusion was performed using a modified protocol with aortic systolic blood pressure <50 mmHg at aortic unclamping for the first 3 minutes of reperfusion. Proximal graft anastomoses to the aorta were performed with partial occlusion of the ascending aorta. IABP support was applied intra- and postoperatively according to the morphology of coronary arteries, necessity of inotropic support and/or hemodynamic difficulties during CPB weaning time or in the early postoperative course.

Postoperative management

Postoperative management for high-risk CABG patients was standardized. Patients were monitored with respect to arterial pressure, pulmonary pressure, central venous pressure. A 12-lead ECG as well as the serum biomarkers for myocardial damage, such as cardiac troponin I, myoglobin, and creatin kinase were determined immediately after arrival on the intensive care unit and at 6, 12 and 24 hours postoperatively and once a day thereafter. A medication of 500 mg acetylsalicylic acid was administered intravenously within the first 6 hours after surgery in the absence of significant bleeding.

Statistical analysis

Data are reported as mean ±SD and categorical variables by their percentage. For all categorical variables the odds ratios (OR) and 95% confidence intervals (CI) were calculated. Comparisons of categorical variables between the groups were performed by Pearson's Chi-square test, since expected frequencies <5 occurred all P values were calculated exactly. Comparisons of continuous variables between groups were analyzed by students t-test. Univariate and multivariate logistic regression analyses were performed to identify preoperative independent predictors for in-hospital mortality. All preoperative predictor variables that were identified as significant at a two-tailed nominal P value of less than 0.10 in univariate regression analysis were then entered into a multivariate logistic regression analysis model. Receiver operating curve (ROC) analyses were applied to determine optimal cut-off values of cTnI and to evaluate the predictive power for in-hospital mortality. A P value less than 0.05 was considered to indicate statistical significance. All statistical analyses between groups were performed using the SPSS software package (SPSS Inc., Chicago, IL, USA).

Results

The demographics and baseline data of the two groups are summarized in *Tab. 1*. Preoperative baseline characteristics and demographics of the patients were comparable with the contemporary coronary surgery patient profile. A preoperative significant difference between the groups could be observed in terms of age, smoking history, previous myocardial infarction, symptoms of angina, left mainstem disease, left ventricular ejection fraction as well as the preoperative cTnI serum level (*Fig. 2A*) and CK activity. The preoperative cTnI serum level was likewise significantly different according to the survival staus of the entire study cohort (*Fig. 2B*). As demonstrated in *Tab. 2* the intraoperative data did not differ between the groups, except the amount of cardioplegia to be used, which was slightly but significantly more in group 2 compared to group 1 (P=0.01). The aortic cross clamping time and the cardiopulmonary bypass time tended to be longer in group 2. The number of graft conduits per patient did not differ between the groups and the percentage of internal mammary artery grafts to be used was also not different. The intraoperative mean graft flow, as measured by Doppler flowmetry, did also not differ between the groups. According to the postoperative data, as shown in *Tab. 2* and *Fig. 1*, a significant difference in the necessity for intraoperative and postoperative intraaortic balloon pump (IABP) support between the groups (P<0.001) accompanied by a significantly prolonged postoperative ventilation time (P<0.001) and a longer ICU stay (P<0.001) could be observed. Among the 3124 patients with N-ACS of group 1, 47 (1.5%) died

Table 1. **Baseline characteristics**

	Group 1 N-ACS (n=3124)	Group 2 NSTE-ACS (n=386)	P value
Demographics			
Age, y	66±9	67±9	0.01
Gender, female	635 (20)	95 (25)	0.11
Body weight, kg	81±14	82±16	0.44
Cardiovascular risk factors			
Diabetes mellitus	941 (30)	122 (30)	0.72
Hypertension	2588 (83)	333 (86)	0.15
Hyperlipidemia	2525 (81)	295 (76)	0.09
Family history	1409 (45)	178 (45)	0.82
Smoking history	1902 (61)	199 (62)	0.004
Comorbidities			
History of stroke	221 (7)	22 (6)	0.77
COPD	497 (16)	70 (16)	0.18
PVD	472 (15)	54 (15)	0.62
Renal disease*	436 (14)	51 (13)	0.92
Dialysis	65 (2)	7 (2)	0.59
Cardiac history			
Previous MI**	1028 (33)	189 (43)	<0.0001
Previous PCI	625 (20)	66 (17)	0.18
CCS III-IV	1839 (59)	268 (66)	0.0001
Extent of CAD			
Left-mainstem disease	782 (25)	123 (32)	0.02
One-vessel disease	90 (3)	11 (4)	0.06
Two-vessel disease	472 (15)	57 (14)	0.44
Three-vessel disease	2595 (83)	318 (82)	0.97
LV function			
LV-EF, %	60±15	56±15	<0.0001
Preoperative serum marker			
cTnI, ng/mL	0.03±0.04	2.4±7.6	<0.0001
CK, IU/L	55±63	85±120	<0.0001

Data are presented as mean ±SD or number (%); COPD – Chronic obstructive pulmonary disease; PVD – Peripheral vascular disease; MI – Myocardial infarction; PCI – Percutaneous coronary intervention; CCS – Canadian Cardiovascular Society; CAD – Coronary artery disease; LV – Left ventricle; EF – Ejection fraction; * – Serum Creatinine >0.2 μmol/l; ** – >7 days

Figure 1. **Preoperative, intraoperative and postoperative use of intraaortic ballon counterpulsation support in CABG patients with NACS versus NSTE-ACS (P<0.001)**

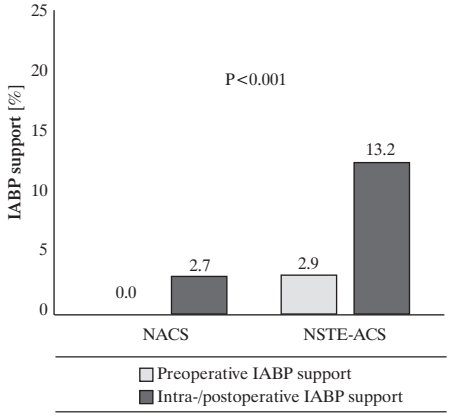
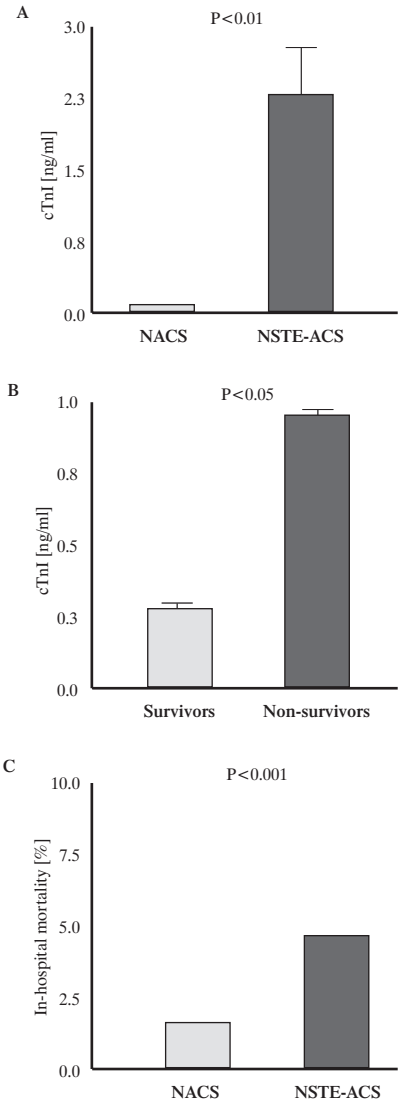


Table 2. **Intra- and postoperative characteristics**

	Group 1 N-ACS (n=3124)	Group 2 NSTE-ACS (n=386)	OR (95% CI)	P value
Intraoperative data				
ACC time, min	68±23	72±23	–	0.19
CPB time, min	106±39	110±33	–	0.21
Cardioplegia, mL	1516±473	1579±445	–	0.01
Reperfusion time, min	31±16	34±14	–	0.11
Grafts per patient, n	3.0±0.8	3.0±0.8	–	0.30
Postoperative data				
Ventilation time, h	8 (7-11)	8 (7-13)	–	<0.001
IABP support	84 (2.7)	51 (13)	5.5(3.8-8.1)	<0.001
ICU stay, d	1 (1-2)	1 (1-4)	–	<0.001
Hospital stay, d	8 (6-12)	8 (6-12)	–	0.21
Major adverse events				
Death in hospital	47 (1.5)	16 (4.2)	2.8(1.5-5.2)	<0.001
LCOS	48 (1.5)	22 (5.7)	3.9(2.2-6.7)	<0.001
PMI	171 (5.5)	44 (11.5)	2.2(1.5-3.2)	<0.001
Stroke	62 (2.0)	6 (1.6)	0.8(0.3-1.9)	0.56
Other complications				
Major bleeding	110 (3.5)	11 (2.8)	0.8(0.4-1.6)	0.50
Rethoracotomy	88 (2.8)	16 (4.1)	1.5(0.8-2.6)	0.15
Arrhythmia	410 (13)	78 (20)	1.7(1.3-2.1)	<0.001
Renal failure (dialysis)	172 (5.5)	62 (16)	3.3(2.4-4.5)	<0.001

Data are presented as mean ±SD, median (25% – 75% Interquartile) or number (%); ACC – Aortic cross-clamp; CPB – Cardiopulmonary bypass; IABP – Intraaortic ballon counterpulsation; ICU – Intensive care unit; LOS – Low cardiac output syndrome; PMI – Perioperative myocardial infarction; OR – odds ratio and 95% confidence interval between group 1 and 2

Figure 2. **(A) Preoperative serum cTnI levels in patients with NACS versus NSTE-ACS (P<0.01). (B) Prognostic value of pre-CABG cTnI serum levels: survival status of the entire study cohort according to pre-CABG cTnI levels (P<0.05). (C) In-hospital mortality in CABG patients with NACS versus NSTE-ACS (P<0.001)**



in the postoperative course within 30 days or within the same time of hospital stay, whereas 16 (4.2%) deaths occurred among 386 patients with NSTE-ACS in group 2 (P<0.001; *Fig. 2C*). The difference of in-hospital mortality was accompanied by a significant difference in the appearence of postoperative low cardiac output syndrome (P<0.001) and the incidence of perioperative myocardial infarction (P<0.001). The incidence of postoperative stroke was not significantly different. Other postoperative complications and adverse events like major bleeding (>200 ml/h first 6 h) and all causes of rethoracotomy were not different between the groups. The occurrence of new-onset arrhythmia (P<0.001) and the incidence of postoperative renal failure requiring temporary veno-venous hemofiltration or hemodialysis was significantly different between the two groups (P<0.001).

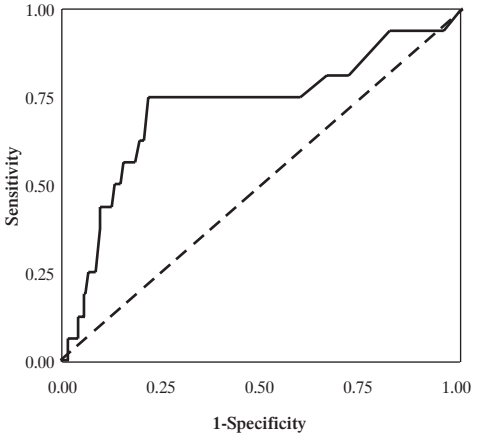
Univariate and multivariate logistic regression analyses identified a number of risk factors and preoperative variables like age, peripheral vascular disease, previous PCI, angina class and preoperative cTnI serum level to be related with in-hospital mortality. These independent predictors of death are detailed in *Tab. 4*. In this regard, the preoperative cTnI serum level

***Table 4*. Univariate and multivariate logistic regression analysis of variables between groups 1 and 2**

	Univariate Regression Analysis		Multivariate Regeression Analysis	
	Odds Ratio [95% CI]	P Value	Odds Ratio [95% CI]	P Value
Age	1.1 (1.0-1.1)	<0.001	1.1 (1.0-1.1)	0.02
Gender	2.1 (1.1-4.1)	0.02	1.3 (0.6-2.8)	0.46
Obesity	0.9 (0.5-1.7)	0.80	—	—
Smoking	0.4 (0.2-0.8)	0.01	0.6 (0.3-1.2)	0.17
Diabetes mellitus	1.9 (1.0-3.5)	0.05	1.3 (0.7-2.7)	0.44
Hypertension	3.8 (0.9-15.9)	0.07	2.1 (0.5-8.9)	0.33
Hyperlipidemia	1.8 (0.7-4.5)	0.24	—	—
Renal disease	1.0 (0.3-2.8)	0.96	—	—
COPD	1.5 (0.7-3.2)	0.29	—	—
PVD	2.6 (1.3-5.2)	0.01	2.5 (1.2-5.2)	0.01
Previous MI	1.5 (0.8-2.9)	0.19	—	—
Previous PCI	2.4 (1.2-4.7)	0.01	2.7 (1.3-5.6)	0.01
Angina Class III-IV	5.5 (2.0-15.5)	0.01	3.5 (1.2-10.2)	0.02
Left-mainstem disease	1.0 (0.5-2.0)	0.91	—	—
LV-EF (%)	0.98 (0.96-1.0)	0.12	—	—
CK (IU/L)	1.0 (0.99-1.0)	0.62	—	—
cTnI (ng/mL)	1.1 (1.0-1.1)	<0.0001	1.1 (1.0-1.1)	<0.001

* – All cause of death; CI – Confidence interval

***Figure 3*. ROC curve analysis demonstrating the discriminatory power of preoperative cTnI levels for in-hospital mortality. The optimal per-CABG cTnI serum level was found with 1.5 ng/ml with an area under curve of 0.72±0.08, a sensitivitiy of 75.0% and a specificity of 78.5%**



was found to be the strongest independent risk factor for death (P<0.0001). The discriminative power of the preoperative cTnI serum level for in-hospital mortality by using a receiver operating characteristic (ROC) curve analysis revealed an optimal cTnI cut-off value of 1.5 ng/ml, with an area under curve of 0.72±0.08, a sensitivitiy of 75.0% and a specificity of 78.5% (*Fig. 3*).

Discussion

Our findings in the present study suggest that in a surgical population of patients undergoing CABG, the existence of preoperative non-ST-elevation ACS is associated with a significantly higher mortality within 30 days and a higher incidence of major adverse cardiac events, such as perioperative myocardial infarction or low cardiac output syndrome. Moreover, the present study could clearly demonstrate, that increased mortality rates after CABG due to NSTE-ACS are significantly associated with several independent preoperative predictors, most notably, with the degree of preoperative cTnI serum elevation. Furthermore, the present study suggest that the surgical revascularization with the concomitant perioperative management strategies is safe and effective in the clinical course of patients presenting NSTE-ACS. At our institution, the average mortality rate was 4.2% during hospital stay or within 30 days for CABG patients with NSTE-ASC, which was significantly higher compared to CABG patients without ACS during the same interval. This overall hospital mortality rate is well in the range of actual data of the current literature [11].

Multiple studies have shown that patients undergoing coronary artery bypass grafting who present acute myocardial infarction with ST-segment elevations on the electrocardiogram have an clearly increased risk of suffering death in hospital. It has been clearly shown that emergency revasculratzation of patients with an acute transmural myocardial infarction have an increased adverse prognosis in the setting of percutaneous coronary intervention [5,18] as well as in patients following emergency CABG [6,7]. However, the occurences and reasons for higher adverse prognosis and increased mortality rates in patients with non-ST-elevation ACS (NSTE-ACS) associated with 'minor myocardial damage' resulting in minor elevations of cardiac troponins are not fully understood. In the setting of ACS, elevations of cardiac troponins were found to be associated with multivessel disease, complex coronary lesions with unstable and ruptured plaques, distal coronary microembolization of platelet microaggregates and plaque debris [19,20] as

well as abnormal microvascular myocardial perfusion [5], which might be an alternative or contributory cause of elevations of cardiac troponins. In several recent studies, it has been shown that patients with unstable CAD and elevated cardiac troponins had more widespread CAD than those without elevated cardiac troponins and had more often complex coronary lesions and visible thrombus in the culprit vessel [21,22]. It has also been demonstrated, that minor elevations of cardiac troponins and thus, a 'minor myocardial damage', is present in approximately 30% of patients with rest angina and negative CK/CK-MB values [23].

According to risk stratification among patients with NSTE-ACS, there is an increased risk of death within 6 weeks in those with elevated serum levels of cardiac troponins and the risk of death continues to increase as the cardiac troponin serum level increases [8,10]. Reversible ST-segment depression is associated with an increase by a factor of 3-6 in the likelihood of death, myocardial infarction, ischemia at rest, or provocable ischemia during a test to stratify risk. Although the conditions of the majority of patients with unstable angina will stabilize with effective antiischemic medications, approximately 50-60% of such patients will require revascularization treatment because of the "unresponsiveness" of medical therapy [12]. High-risk patients are those who have had angina at rest, prolonged angina, or persistent angina with positive serum levels of cardiac troponins or dynamic ST-segment changes or hemodynamic instability, and these patients urgently require invasive diagnostic evaluation and subsequent revascularization treatment. In accordance to the present guidelines of the ACC/AHA, patients with NSTE-ACS require, first of all, an aggressive medical treatment in order to stabilize and control the symptoms if possible. Medical therapy should be adjusted rapidly to relieve manifestations of ischemia and should include antiplatelet therapy (aspirin, or ticlopidine or clopidogrel if aspirin is contraindicated), antithrombotic therapy (unfractionated heparin or low-molecular-weight heparin), beta-blockers, nitrates, and possibly calcium-channel blockers. Early administration of glycoprotein IIb/IIIa inhibitors may be particularly important, especially in high-risk patients with positive troponin tests or those in whom implantation of coronary stents is anticipated [8-10]. However, even in patients with NSTE-ACS were surgical revascularization is indicated, a recently published randomized trial have demonstrated that the benefit (freedom from cardiovascular death, MI, stroke) of administering clopidogrel early on admission appear to outweight the risk of life-threatening bleeding in patients with NSTE-ACS following CABG [24].

Surgical myocardial revascularization with CABG for patients with unstable angina and left main stem stenosis or equivalent with evidence of myocardial ischemia, or with triple vessel disease and impaired left ventricular function, improves prognosis and has, so far, a clear indication based on the obstructive coronary artery lesion; all of them considered of high-risk according to the ACC/AHA guidelines [8-10]. The perioperative treatment strategies of NSTE-ACS patients undergoing CABG are basically based on numerous suggestions and recommendations that have been made to reduce the risk for CABG surgery in patients with AMI 'in general', including better selection and optimal timing of surgery [7], adjunctive pharmacological therapy [25], and accurately timed IABP support [26].

The benefical effects of preoperative IABP treatment on outcome in high-risk patients have been clearly demonstrated in several non-randomized and randomized trials. Even in patients undergoing CABG with preoperative evidence of myocardial ischemia and/or preoperative hemodynamic instability, there is strong evidence of the benefical effect for preoperative and intraoperative use of the IABP [26-28].

The optimal timing of surgical revascularization in patients with AMI has been described in several previous studies. It has been shown that hospital mortality decreases with increasing time interval between CABG and AMI [7,29]. However, no data are currently available according to the optimal timing of CABG in patients with NSTE-ACS. At our institution, the timing for surgery of patients with NSTE-ACS was depending on the severity of symptoms and the level and/or kinetic of preoperative cTnI value indicating ongoing myocardial ischemia despite maximal non-surgical therapy on the one hand, and the complexity and severity of the coronary artery lesions on the other hand.

In terms of using the optimal myocardial protection during CABG, the type of cardioplegia (blood versus cristalloid, warm versus cold) has been the subject of numerous experimental and clinical studies, but this issue still remains controversial [30-32]. The benefical effect with optimal delivery of the cardioplegic solution by the use of simultaneous antegrade/retrograde cardioplegia and additional administration through the distal grafts, however, have been clearly demonstrated [33-35].

The adjunctive pharmacological therapy and thus, the optimal cardiac protection during AMI and/or myocardial injury due to ischemia/reperfusion is still a challenging field of cardiovascular research since numerous treatment options have been investigated so far to reduce myocardial infact size. Intravenous beta-blockers administered in the early hours of infarction were clearly shown to be of benefit [36]. Intravenous adenosine appeared promising for AMIs and myocardial protection during CABG, as did C1-esterase inhibitors and cariporide in some studies [37-40]. However, the majority of other medications were studied with negative or marginal results. Moreover, no data are currently available according to the optimal adjunctive pharmacological therapy of patients undergoing CABG with NSTE-ACS.

Conclusions

The present study demonstrates a significantly higher risk for patients with NSTE-ACS undergoing surgical revascularization compared to patients with N-ACS with significantly increased in-hospital morbidity and mortality rates. Multivariate logistic regression analysis revealed the preoperative cardiac troponin I level as the most strongest independent predictor of death in-hospital. Therefore, a more precise patient's risk assessment and a tailored perioperative management strategy may improve patients outcome.

Acknowledgement

The results of the study were presented at the Euroregional Workshop on Cardiology – standards and new trends in the treatment of the acute coronary syndromes, Medical University of Bialystok, Poland, 19–20 November, 2004

References

1. Adams III JE, Bodor GS, Dávila-Román VG, Delmez JA, Apple FS, Ladenson JH, Jaffe AS. Cardiac troponin I: A marker with high specificity for cardiac injury. Circulation, 1993; 88: 101-6.

2. Alpert JS, Thygesen K, Antman E, Bassand JP. Myocardial infarction redefined – a consensus document of The Joint European Society of Cardiology/American College of Cardiology Committee for the redefinition of myocardial infarction. JACC, 2000; 36: 959-69.

3. Antman EM, Tanasijevic MJ, Thompson B, Schactman M, McCabe CH, Cannon CP, Fischer GA, Fung AY, Thompson C, Wybenga D, Braunwald E. Cardiac-specific troponin i levels to predict the risk of mortality in patients with acute coronary syndromes. The New England Journal of Medicine, 1996; 335: 1342-9.

4. Matetzky S, Sharir T, Domingo M, Noc M, Chyu K-Y, Kaul S, Eigler N, Shah PK, Cercek B. Elevated troponin I level on admission is associated with adverse outcome of primary angioplasty in acute myocardial infartion. Circulation, 2000; 102: 1611-6.

5. Giannitsis E, Muller-Bardorff M, Lehrke S, Wiegand U, Tolg R, Weidtmann B, Hartmann F, Richardt G, Katus HA. Admission troponin T level predicts clinical outcomes, TIMI flow, and myocardial tissue perfusion after primary percutaneous intervention for acute ST-segment elevation myocardial infarction. Circulation, 2001; 104: 630-5.

6. Albes JM, Gross M, Franke U, Wippermann J, Cohnert TU, Vollandt R, Wahlers T. Revascularization during acute myocardial infarction: risks and benefits revisited. The Annals of Thoracic Surgery, 2002; 74: 102-8.

7. Lee DC, Oz MC, Weinberg AD, Ting W. Appropriate timing of surgical intervention after transmural acute myocardial infarction. The Journal of Thoracic and Cardiovascular Surgery, 2003; 125: 115-20.

8. Bertrand ME, Simoons ML, Fox KAA, Wallentin LC, Hamm CW, McFadden E, De Feyter PJ, Specchia G, Ruzyllo W. Management of acute coronary syndromes in patients presenting without persistent ST-segment elevation. European Heart Journal, 2002; 23: 1809-40.

9. Eagle KA, Guyton RA, Davidoff R, Edwards FH, Ewy GA, Gardner TJ, Hart JC, Herrmann HC, Hillis LD, Hutter AM Jr., Lytle BW, Marlow RA, Nugent WC, Orszulak TA, Antman EM, Smith SC Jr., Alpert JS, Anderson JL, Faxon DP, Fuster V, Gibbons RJ, Gregoratos G, Halperin JL, Hiratzka LF, Hunt SA, Jacobs AK, Ornato JP. ACC/AHA 2004 Guideline Update for Coronary Artery Bypass Graft Surgery: Summary Article: A Report of the American College of Cardiology/American Heart Association Task Force on Practice Guidelines (Committee to Update the 1999 Guidelines for Coronary Artery Bypass Graft Surgery). Circulation, 2004; 110: 1168-76.

10. Hamm C. Guidelines: acute coronary syndrome (ACS). 1: ACS without persistent ST segment elevations. Z Kardiol, 2004; 93: 72-90.

11. Louagie YAG, Jamart J, Buche M, Eucher PM, Schoevaerdts D, Collard E, Gonzalez M, Marchandise B, Schoevaerdts JC. Operation for unstable angina pectoris: factors influencing adverse in-hospital outcome. The Annals of Thoracic Surgery, 1995; 59: 1141-9.

12. Solodky A, Behar S, Boyko V, Battler A, Hasdai D. The outcome of coronary artery bypass grafting surgery among patients hospitalized with acute coronary syndrome: the Euro Heart Survey of acute coronary syndrome experience. Cardiology, 2005; 103: 44-7.

13. Vahl CF, Tochtermann U, Gams E, Hagl S. Efficiency of a computer network in the administrative and medical field of cardiac surgery. Concept of and experience with a departmental system. European Journal of Cardio-Thoracic Surgery, 1990; 4: 632-8.

14. Thielmann M, Massoudy P, Marggraf G, Kamler K, Herold U, Piotrowski J, Schmermund A, Erbel R, Jakob H. Diagnostic discrimination between early graft failure and non-graft related perioperative myocardial infarction with cardiac troponin I following coronary artery bypass surgery. Circulation, 2003; 108 (Suppl. S): 391-2.

15. Parsonnet V, Dean D, Bernstein AD. A method of uniform stratification of risk for evaluating the results of surgery in acquired adult heart disease. Circulation, 1989; 79 (Suppl. I): 3-12.

16. Higgins TL, Estafanous FG, Loop FD, Beck GJ, Blum JM, Paranandi L. Stratification of morbidity and mortality outcome by preoperative risk factors in coronary artery bypass patients. A clinical severity score. JAMA, 1992; 267: 2344-8.

17. Nashef SAM, Roques F, Michel P, Gauducheau E, Lemeshow S, Salamon R. European system for cardiac operative risk evaluation (EuroSCORE). European Journal of Cardio-Thoracic Surgery, 1999; 16: 9-13.

18. Opfermann UT, Peivandi AA, Dahm M, Hilgenstock H, Hafner G, Loos A, Oelert H. Postoperative patterns and kinetics of cTnI, cTnT, CK-MB-activity and CK-activity after aortic valve replacement. Swiss Med Wkly, 2001; 131: 550-5.

19. Topol EJ, Yadav JS. Recognition of the Importance of embolization in atherosclerotic vascular disease. Circulation, 2000; 101: 570-80.

20. Heusch G, Schulz R, Haude M, Erbel R. Coronary microembolization. Journal of Molecular and Cellular Cardiology, 2004; 37: 23-31.

21. Jurlander B, Farhi ER, John J, Keany CM, Balu D, Grande P, Ellis AK. Coronary angiographic findings and troponin t in patients with unstable angina pectoris*1. The American Journal of Cardiology, 2000; 85: 810-4.

22. Lindahl B, Diderholm E, Lagerqvist B, Venge P, Wallentin L. Mechanisms behind the prognostic value of troponin T in unstable coronary artery disease: a FRISC II substudy. Journal of the American College of Cardiology, 2001; 38: 979-86.

23. Katus HA, Remppis A, Neumann FJ, Scheffold T, Diederich KW, Vinar G, Noe A, Matern G, Kuebler W. Diagnostic efficiency of troponin T measurements in acute myocardial infarction. Circulation, 1991; 83: 902-12.

24. Fox KAA, Mehta SR, Peters R, Zhao F, Lakkis N, Gersh BJ, Yusuf S. Benefits and risks of the combination of clopidogrel and aspirin in patients undergoing surgical revascularization for non-st-elevation acute coronary syndrome: the clopidogrel in unstable angina to prevent recurrent ischemic events (CURE) trial. Circulation, 2004; 110: 1202-8.

25. Kloner RA, Rezkalla SH. Cardiac protection during acute myocardial infarction: Where do we stand in 2004? Journal of the American College of Cardiology, 2004; 44: 276-86.

26. Christenson JT, Simonet F, Badel P, Schmuziger M. Optimal timing of preoperative intraaortic balloon pump support in high-risk coronary patients. The Annals of Thoracic Surgery, 1999; 68: 934-9.

27. Cohen M, Urban P, Christenson JT, Joseph DL, Freedman RJ Jr., Miller MF, Ohman EM, Reddy RC, Stone GW, Ferguson JJ, III, on behalf of the Benchmark Registry collaborators. Intra-aortic balloon counterpulsation in US and non-US centres: results of the Benchmark(R) Registry. European Heart Journal, 2003; 24: 1763-70.

28. Stone GW, Ohman EM, Miller MF, Joseph DL, Christenson JT, Cohen M, Urban PM, Reddy RC, Freedman RJ, Staman KL. Contemporary utilization and outcomes of intra-aortic balloon counterpulsation in acute myocardial infarction*1: The Benchmark Registry. Journal of the American College of Cardiology, 2003; 41: 1940-5.

29. Lee DC, Oz MC, Weinberg AD, Lin SX, Ting W. Optimal timing of revascularization: transmural versus nontransmural acute myocardial infarction. The Annals of Thoracic Surgery, 2001; 71: 1198-204.

30. Elvenes OP, Korvald C, Myklebust R, Sorlie D. Warm retrograde blood cardioplegia saves more ischemic myocardium but may cause a functional impairment compared to cold crystalloid. European Journal of Cardio-Thoracic Surgery, 2002; 22: 402-9.

31. Kamlot A, Bellows SD, Simkhovich BZ, Hale SL, Aoki A, Kloner RA, Kay GL. Is Warm retrograde blood cardioplegia better than cold for myocardial protection? The Annals of Thoracic Surgery, 1997; 63: 98-104.

32. Pichon H, Chocron S, Alwan K, Toubin G, Kaili D, Falcoz P, Latini L, Clement F, Viel J, Etievent JP. Crystalloid versus cold blood cardioplegia and cardiac troponin I release. Circulation, 1997; 96: 316-20.

33. Tian G, Shen J, Sun J, Xiang B, Oriaku GI, Zhezong L, Scarth G, Somorjai R, Saunders JK, Salerno TA, Deslauriers R. Does simultaneous antegrade/retrograde cardioplegia improve myocardial perfusion in the areas at risk? A magnetic resonance perfusion imaging study in isolated pig hearts. The Journal of Thoracic and Cardiovascular Surgery, 1998; 115: 913-24.

34. Tian G, Dai G, Xiang B, Sun J, Lindsay WG, Deslauriers R. Effect on myocardial perfusion of simultaneous delivery of cardioplegic solution through a single coronary artery and the coronary sinus*1. Journal of Thoracic and Cardiovascular Surgery, 2001; 122: 1004-10.

35. Gundry SR, Razzouk AJ, Vigesaa RE, Wang N, Bailey LL. Optimal delivery of cardioplegic solution for "redo" operations. The Journal of Thoracic and Cardiovascular Surgery, 1992; 103: 896-901.

36. Beta-Blocker Heart Attack Research Group. A randomized trial of propanolol in patients with acute myocardial infarction. I. Mortality results. JAMA: The Journal of the American Medical Association, 1982; 247: 1707-14.

37. Horstick G, Berg O, Heimann A, Gotze O, Loos M, Hafner G, Bierbach B, Petersen S, Bhakdi S, Darius H, Horstick M, Meyer J, Kempski O. Application of C1-esterase inhibitor during reperfusion of ischemic myocardium: dose-related beneficial versus detrimental effects. Circulation, 2001; 104: 3125-31.

38. Horstick G. C1-esterase inhibitor in ischemia and reperfusion. Immunobiology, 2002; 205: 552-62.

39. Bauernschmitt R, Bohrer H, Hagl S. Rescue therapy with C1-esterase inhibitor concentrate after emergency coronary surgery for failed PTCA. Intensive Care Med, 1998; 24: 635-8.

40. Boyce SW, Bartels C, Bolli R, Chaitman B, Chen JC, Chi E, Jessel A, Kereiakes D, Knight J, Thulin L. Impact of sodium-hydrogen exchange inhibition by cariporide on death or myocardial infarction in high-risk CABG surgery patients: results of the CABG surgery cohort of the GUARDIAN study*1. Journal of Thoracic and Cardiovascular Surgery, 2003; 126: 420-7.

Surgical treatment of congestive heart failure in coronary artery disease

Benetis R*

Clinic of Cardiac Surgery, Kaunas Heart Center, Kaunas Medical University, Lithuania

Abstract

Heart failure (HF) is a patophysiological condition, when the heart can not provide adequate blood flow to the body organs. The main cause of HF is now ischemic heart disease (IHD), and the number of patients with HF in aging society is growing. HF is becoming the leading cause of death. Medical therapy does not provide satisfactory results in respect of symptoms and survival (5 year survival 28-40%). Therefore there is a trend towards early invasive methods of IHD treatment: percutaneous or surgical revascularisation and surgical reconstruction of myocardial damage.

Most common surgical procedure in IHD is coronary artery bypass grafting (CABG). This treatment is safe and effective in patients with normal ventricular function (operative mortality 0.5%, 5 year survival >92%). Results in patients with impaired left ventricular (LV) function are better than conservative therapy, but still not satisfactory (operative mortality 8.4%, 5 year survival 65%). The modern surgical concept for improvement of ventricular function is left ventricular (LV) shape and volume restoration (SVR) accompanied by CABG. In cases of severe damage of myocardium resulting in left ventricular aneurysm or akinesia, SVR improves LV function and prevents further LV remodeling. At present it is under investigation whether SVR is of benefit for moderate-sized ventricles and NYHA class II symptoms. In case of ischemic mitral insufficiency mitral valve repair is a method of choice. The results of combined procedures in Heart Failure group (CABG + MV reconstruction or SVR) are better than CABG alone. Other surgical alternatives for HF treatment are: heart transplantation, ventricular assist devices (VAD), dynamic cardiomyoplasty, constrictive devices and cellular transplantation therapy. Heart transplantation is reserved for younger patients with less comorbidities. Shortage of donor organs and poor long-term results remains a main problem of such a treatment. VAD at present is still very expensive, and serves particularly as a "bridge to heart transplantation" or "bridge to recovery" rather then destination therapy. Despite of all achievements in medical or invasive HF treatment further basic and clinical works as well as new organization systems are necessary to find optimal strategies to reduce cost of care, improve quality of life and survival.

Key words: congestive heart failure, coronary artery, surgical treatment.

Definition of heart failure (HF) currently describes pathophysiological status when heart muscle as pump can not provide adequate blood flow to the body organs to meet metabolic needs of above tissue. Following last years, incidence of heart failure has been increasing. Reaching annual prevalence of 14-16 patients per 1 000 health care system patients [1]. However, more recent data showed kind of plateau regarding mortality from heart failure in 5 years adjusted for age. The same have been followed analyzing changes in relative risk of death after heart failure onset within a different age cohorts. Relative risk of death in younger group of patients (mean age of 60 years) showing signs of heart failure, have been reduced from 0.84 during 1985-1990 to 0.63 in a year 1991-1995 and to 0.48 during 1996-2000. However, within a group of more elderly patients (mean age 80 years) relative risk of death from heart failure at the same time frames have been reduced only slightly: 0.85-0.88-0.72. The same trend has been followed in both sex groups [2]. Also it is well recognized that ischemic heart disease (IHD) together with cerebrovascular disease defined as atherothrombosis remains leading cause of death and heart failure [3].

* CORRESPONDING AUTHOR:
Department of Cardiac Surgery
Kaunas Medical University
Eiveniu 2 LT-50009 Kaunas, Lithuania
Tel: +37 37733412; Fax: +37 37733412
e-mail: rimantas.benetis@kmuk.lt (Rimantas Benetis)

Received 05.01.2005 Accepted 16.04.2005

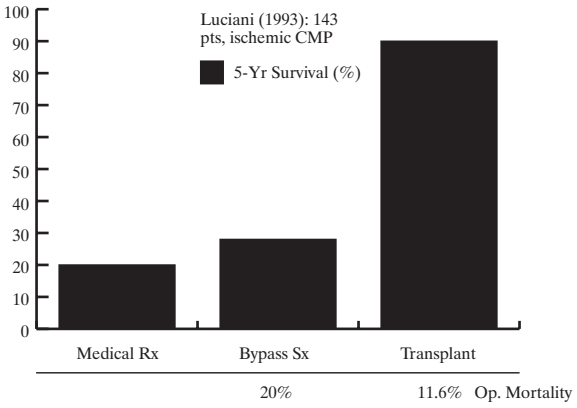
Table 1. **Arts II Arterial Revascularization Therapies Study Part II: Sirolimus-Eluting Bx Velocity Stent for the Treatment of Multivessel de Novo Coronary Artery Lesions (Luis Gruberg MD) Presenter: Patric W. Serruys, on Behalf of the ARTS II Investigators**

MACCE: 1 – Month follow-up

Characteristic	ARTS II (n=606)	ARTS I	
		CABG (n=605)	PCI (n=600)
Death (%)	0	0.5	1.5
CVA (%)	0.2	1.0	0.5
MI (%)	0.3	2.3	2.5
Reapeat CABG (%)	1.4	0.2	2.0
Reapeat PCI (%)	1	0.2	1.7
Total MACCE (%)	2.9	4.2	8.2

CABG = coronary artery bypass graft; CVA = cerebrovascular accident; MACCE = major adverse cardiac and cerebrovascular events; PCI = percutaneous coronary intervention

Figure 1. **Myocardial Revascularization**



After myocardial infarction (MI) there is a direct correlation between the quantity of HF biomarkers like Troponin I (Tn I), C-reactive protein (CRP) for B type natriuretic peptide (BNP) and incidence of onset of HF or death from it. Applying multimarkers approach to determine 30 day mortality risk in acute coronary ischemia cases in an OPUS-TIMI (16) and TACTICS-TIMI (18) studies, all 3 biomarkers increase correlated well with the increased risk of 30 days mortality rate from progressing heart failure after MI [4]. Those, whom sustain from MI, develop minor or major symptoms of HF because of left ventricular (LV) remodeling. HF is diagnosed in approximately 1% of population at the age of more then 65 years and remains as a main cause at hospitalization for the patients of the same age. More then 30% of them being rehospitalized within 90 days because of progression of HF symptoms, inadequate medical treatment and inadequate follow-up of patients: discrepancies between planned treatment and treatment after discharge.

Medical treatment of HF remains as a treatment core and includes: diuretics, digoxine, aldosterone antagonists, B blokers, ACE inhibitors and currently angiotensine II receptors inhibitors. However, they do not substantially improve symptoms of HF either prolong survival of HF patients [5].

Speaking about treatment of IHD as from the main cause of HF, there is well accepted trend towards early and aggressive mechanical revascularization of stenotic or acutely occluded coronary vessel.

PCI and stenting studies within international cardiological society, however, still showed rather high rate of death, MI and major adverse cardiac event rate in a large group of studies: TAXUS VI, ENDEAVOR I, ARTS II, TROPICAL, SVELTE, SICTO, PISCES, ISAR, IMPRESS-2 MVD, etc. [6] (*Tab. 1*).

Large numbers of invasive coronary procedures seems did not reduce, rather increased financial burden to cardiosurgical and intensive care unit budgets, because of higher numbers of more elderly and more sick patients. For instance in Denmark number of coronary artery bypass grafting (CABG) operations have decreased approximately by 20% since 2002, at the same time treatment costs within ICU have increased approximately 20% (personal communication with Danish CABG registry).

Surgical treatment of IHD complicated by HF creates number of socioeconomical, ethical and medical problems. Though, currently there is little doubt and controversy regarding indications for surgery for high risk myocardial revascularization or ischemic mitral valve repair. Indications for surgery of LV volume and shape restoration operations remain unclear.

CABG operations can provide rather low (less then 0.5%) mortality and good survival 5 years after surgery (>92%) in a group of patients with good LV function (ejection fraction /EF/>50%). However, decreased EF correlates with increased operative mortality: 6.2% 5 year survival 65%, respectively EF<0.30 – operative mortality – 8.4% (CASS registry) (*Fig. 1*)

High risk myocardial revascularization without concomitant valve or LV size and shape correction may carry high operative mortality – up to 20% comparing to 11.6% mortality rate in a heart transplant group. However, late 5 year survival is better (80%) in CABG group (80%) and heart transplantation group (85%) compared to only medical group, where 5 year survival is at best from 28% to 40% of patients [5].

We have analyzed our low EF <30% CAGB patient group. Operative mortality being 3.9% and when this group of patients was divided in to a group with restrictive diastolic dysfunction, operative mortality reached 8.7%. Analysis of HF symptoms or functional capacity after 1 year following surgery within a group of patients with low LVEF <30% showed general shift of patients from NYHA class III (66.5%) before surgery to NYHA class II postoperatively (70.4%), however 28% of pts remaining in NYHA class III. While analyzing group of pts with restrictive diastolic dysfunction after 1 year following CABG, there was a positive trend towards improvement of a postoperative NYHA class comparing to preoperative. Being 78.3% of pts in a NYHA class III and 17.4% in a NYHA class IV preoperatively and postoperatively NYHA class III – 42.9% but NYHA class IV – 28.6% (*Fig. 2*).

Currently it is still unclear whether the patients will sustain high risk CABG surgery and will benefit from it regarding reduction of HF symptom. We do continue operating on them irrespectively with or without clear evidence of myocardical viability, tests, providing optimal myocardial preservation

Figure 2. **NYHA changes in a PTS group with restrictive LV diastolic dysfunction**

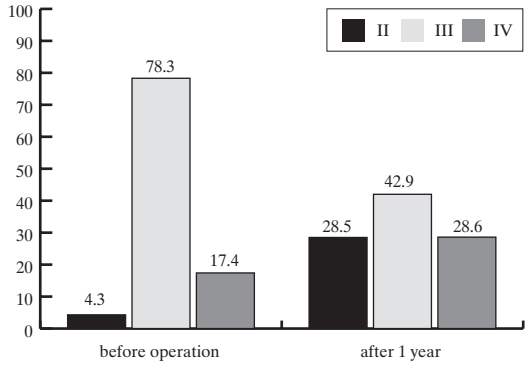


Figure 3. **Mechanisms of IMR**

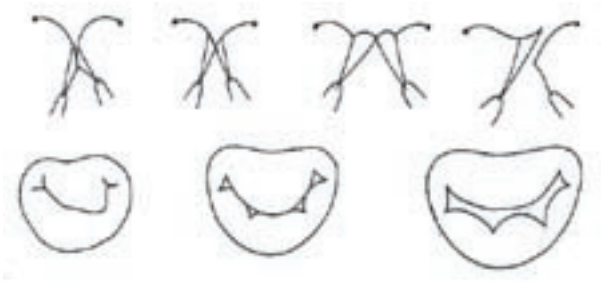


Figure 4. **Papillary muscle shortening (total) or partial with or without papillotomy including translocation**

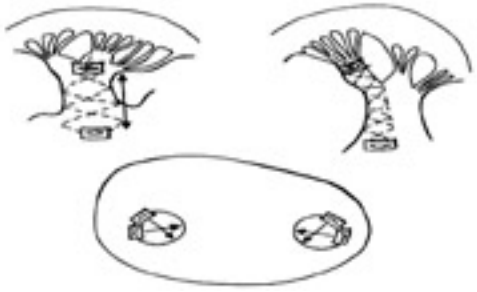


Figure 5. **Interpapillary muscle distance shortening**

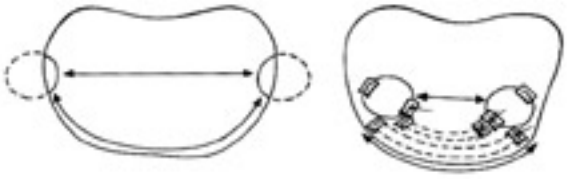


Figure 6. **Papillary muscle base – AV ring distance shortening**

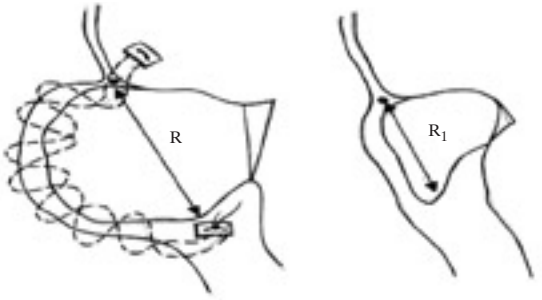
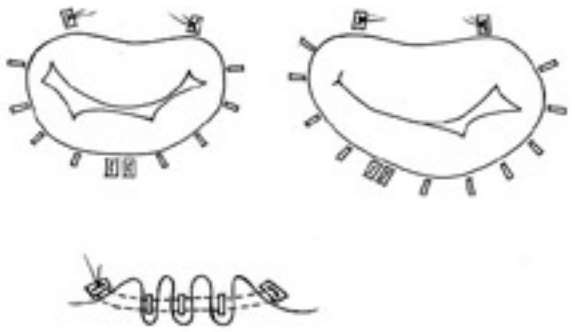


Figure 7. **Annuloplasty (symmetric or asymmetric)**



and revascularization techniques in order to facilitate optimal postoperative medical treatment, and for some young patients as a first step cardiac operation before advocating heart transplantation.

Generally the same is applied for the patients with ischemic mitral regurgitation (IMR). The mechanism of IMR depends on a changes localy and remote from MI area: post infarction remodeling of LV, which in general leads to general LV enlargement, different local LV deformities, depending on a localization of MI, leading to distortion of all MV apparatus: MV annulus dilatation, restriction or prolapse of leaflets, elongation or dysfunction of papillary muscles. Complex changes of all the LV and MV apparatus geometry would finally lead to IMR: central, paracommissural or both (*Fig. 3*).

Undersized annuloplasty for IMR and dilative CMD proposed by Steven F. Bolling remains as a good alternative to treat IMR along with CABG. Providing low operative mortality and better postoperative results comparing to mitral valve replacement. However, up to 8-12% of ischemic mitral valve annuloplasty repairs will fail within 1-3 years because of further LV remodeling and progressive LV failure. Some of the surgeons at this stage would advocate mitral valve replacement by valve substitute, for instance A. Calafiore: when valve leaflet coaptation point is beyond atrioventricular ring line more then 1 cm. Having more then 550 cases of mitral valve repair including 470 patients with IMR repair, we would advocate "future oriented ischemic mitral valve repair". Those would include: correction of the whole mitral valve apparatus: papillary muscle elongation, partial or total shortening (*Fig. 4*) or translocation techniques (*Fig. 5*) along with LV basal segment, mitral valve annulus and papillary muscle base or interpapillary muscle distance correction (*Fig. 7*). All procedure is selected after careful transesophageal echo investigation before surgery. Effectiveness of mitral valve repair is controlled in operating room, also when the heart starts beating and generating physiological haemodynamic parameters. Second attempt of mitral

Figure 8. **Results OR IMV REPAIR II**

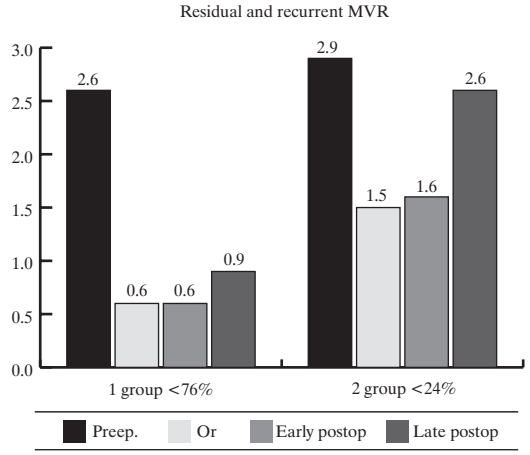
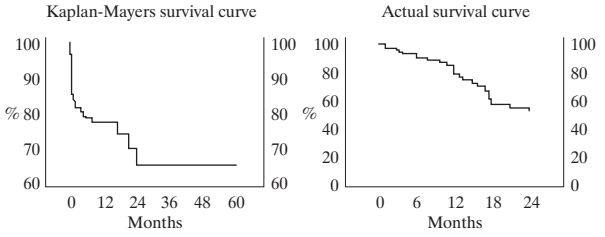


Figure 9. **Results of IMV III**



valve repair is undertaken if residual MR is more or equal to grade two. Whenever effective mitral valve repair can not be achieved, mitral valve is replaced, in our experience – 4 cases (less then 1%). Currently all annuloplasties now are performed using two double 2/0 Ethibond semi pursue, Teflon pledget reinforced sutures (*Fig. 7*). Carefully following suture annuloplasty procedure, both symmetric and asymmetric, suture tear through tissue can be completely avoided, providing long-term effective stabilization of MV annulus and subsequently clinical improvement of HF symptoms postoperatively. In our IMR repair group 86% of pts showed minor or negligible residual and recurrent MR, no more then grade 1 (mean 0.9). 14% of pts in whom TEE intra and early postoperatively revealed MR grade 1 and more, but no more then grade 2, (mean intraoperative MR 1.5; early postoperatively – 1.6) in a late postoperative period (more then 12 month) had recurrent MR with mean MR grade 2.6 (*Fig. 8*). However, only 10% (7 pts) required redo surgery on mitral valve in a late postoperative period. All valves have been replaced using mechanical (2 pts) and biological (5 pts) valve substitutes with 0% mortality. Most of them have been operated on using fibrillating heart technique. This restrospective single center analysis would confirm the indications to correct even moderate IMR, whenever left ventricular end diastolic diameter reaches or exceeds 50 mm. Nevertheless it is worth to mention that actuarial survival of our pts is 65% in 2 years after surgery (*Fig. 9*).

As LV remodeling certainly have an effect onto IMR it does have impact onto LV function impairment also. Segmental MI leads to increased LV end diastolic pressure and volume subsequently to increased wall tension and stress, and finally congestion. At present only paramount experience of Vincent Dor (Centre Cardiothoracique de Monaco) led surgeons to turn onto LV reconstruction – aneurysmectomies. At present RESTORE (Reconstructive Endoventricular Surgery Returning Torsion Original Radius Elliptical Shape to the Left Ventricle) registry demonstrated five-year findings in 1198 pts operated on between 1998-2003. Surgical ventricular restoration (SVR) using patch in 80% and without it in 20% was accompanied by CABG in 95%, MV repair in 22% and MV replacement in 1%. SVR can be performed relatively safe with overall 30 day mortality – 5.3%: 8.7% with mitral valve repair, vs 4.0% without repair. LVEF improvement is significant, from mean preoperative EF of 29.6 % to 39.5% postoperatively. Preoperative LV end systolic volume index decreased from a mean of 80.4 ml/m^2 to 56.6 ml/m^2 postoperatively. NYHA class being 3-4 in 67% of pts, in postoperative period 85% of pts were in NYHA class 1 or 2. Over all 5 year survival was 68.6% and survival was better in the group of pts who had dyskinetic as compared with akinetic LV (80% vs 65%). Risk factors for death were EF<30%, LVESVI>8 ml/m^2, NYHA class 3-4 and advanced age >75% [7].

At present STICH; Surgical Treatment for Ischemic Heart Failure trial may help to answer the question of wether SVR is of benefit to those with more moderate-sized ventricles and NYHA class 2 HF symptoms. STICH is randomizing pts with ischemic cardiomyopathy to medical therapy, CABG alone, or CABG with SVR [8]. Currently we have introduced aneurismal compression plication technique for small aneurysmus or diffuse hipo-akinetic areas (*Fig. 10*).

Other alternatives in treating heart failure of ischemic origin are less effective and some of them have more or less experimental or historical importance. That would be dynamic cardiomyoplasty (A. Carpentier), cellular transplantation, constrictive devices, cardiac resynchronization therapy along with ICD implantation and least but not last heart transplantation and ventricular assist devices (VAD).

However, perhaps only heart transplantation and VAD therapy are under closer investigation. Although, heart transplantation techniques are well established and results quite well understood and accepted, this method of treatment remains reserved for younger patients with les comorbidities. Heart transplantation results because of dilative cardiomyopathy are certainly better comparing to those of ischemic. Also shortage of donor organs does not warrant to become heart transplantation as a method of choice for those with four advanced ischemic heart failure.

VAD therapy may become another, yet still expensive alternative for those with IHF. From existing registries it is well known, even FDA have approved the whole line of assist devices

Figure 10. **LV wall compression – reinforcement**

in treating terminal heart failure, 20-30% on VAD therapy will die before heart transplantation. Infection at the site of cables and cannulas or device endocarditis will reach to 70% in 2 years period. Thromboembolic complications also would account up to 25%. Only in USA 4000 pts with congestive heart disease awaits for heart transplantation, and only 2200 will get this type of surgery. For the VAD (biVAD's) only in USA there is a need of 100 systems per year.

Though it can be achieved rather good survival in all three treatment modalities: "bridge to transplantation", "bridge to recovery" and "destination therapy", vast majority of pts will die within 2 to 3 years, however, quality of life during those years may be improved dramatically.

Despite of all achievements in medical or invasive coronary artery disease therapy surgical approach to treat ischemic heart failure remains in majority of cases main method of treatment.

Finally establishment of new organization systems and models like multidisciplinary approach to IHF pts may reduce hospitalization rate more then 80%, more then by 85% can be reduced visits to emergency units, reduced cost of care, improved quality of life and survival.

References

1. McCullough PA, Philbin EF, Spertus JA, Kaatz S, Sandberg KR, Weaver WD. Confirmation of a heart failure epidemic: findings from the Resource Utilization Among Congestive Heart Failure (REACH) study. J Am Coll Cardiol, 2002; 39: 60-9.
2. Roger VL, Weston SA, Redfield MM, Hellermann-Homan JP, Killian J, Yawn BP. Trends in heart failure incidence and survival in a community-based population. JAMA, 2004; 292: 344-50.
3. The World Health Report 2001. Geneva, WHO, 2001.
4. Sabatine MS, Aretz HT, Fang LS, Dec GW. Images in cardiovascular medicine. Fibrillary/immunotactoid glomerulopathy with cardiac involvement. Circulation, 2002; 105: 120-1.
5. Young JB. Management of chronic heart failure: what do recent clinical trials teach us? Rev Cardiovasc Med, 2004; 5(Suppl. 1): S3-9.
6. Euro PCR 2004, the Paris Course on Revascularisation, May 25-28, 2004, Paris, France.
7. Athanasuleas CL, Buckberg GD, Stanley AW, Siler W, Dor V, Di Donato M. Surgical ventricular restoration in the treatment of congestive heart failure due to post-infarction ventricular dilation. J Am Coll Cardiol, 2004; 44: 1439-45.
8. Owen A. NICE try but a long way to go in heart faiture. Br J Cardiol, 2004; 95: 339-41.

Impact of renal dysfunction as a cardiovascular risk factor

Adamczak M[1,2], Ritz E[1]*

[1] Department Internal Medicine, Ruperto-Carola-University, Heidelberg, Germany,
[2] Department of Nephrology, Endocrinology and Metabolic Diseases, Silesian University School of Medicine, Katowice, Poland

Abstract

This review summarizes the available evidence concerning the relationship between renal dysfunction and cardiovascular risk in non-diabetic patients. Based on numerous studies, there remains no more doubt today, that even minor renal dysfunction, as reflected by microalbuminuria and/or decreased estimated glomerular filtration rate, causes a dramatic increase in cardiovascular risk. Renal dysfunction as a novel risk indicator should be incorporated into currently used algorithms to assess risk factor profile, not to the least because evaluation of renal function helps to select the most appropriate strategy to reduce the cardiovascular risk.

Key words: cardiovascular risk, microalbuminuria, reduced glomerular filtration rate.

Introduction

It has recently been recognized that both microalbuminuria and a reduced glomerular filtration rate impact heavily on the cardiovascular risk in non-diabetic patients. This had been well known for a long time in diabetic patients but the information in non-diabetic patients is novel [1].

* CORRESPONDING AUTHOR:
Nierenzentrum
im Neuenheimer Feld 162
D 68120 Heidelberg, Germany
Tel: +49 6221 601705
e-mail: Prof.E.Ritz@t-online.de (Eberhard Ritz)

Received 08.02.2005 Accepted 05.05.2005

Epidemiological information

Following several early observations the best currently available information comes from the so-called PREVEND-Study covering 40,856 inhabitants of the Dutch city of Groningen [2]. A normal urinary albumin concentration in one spot urine sample was seen in 73%, a high normal concentration between 10-20 mg/l in 19% and frank microalbuminuria in 8%. Those patients had mostly no comorbidity but in approximately 25% of the patients hypertension, diabetes or a combination of both was noted. It is of interest to look at the correlates, which were associated with increased albumin excretion. In men a highly significant relation between fasting plasma glucose concentration and urinary albumin excretion rate was found; this was less pronounced in women. It is of particular note that this was true not only for plasma glucose concentrations in the hyperglycaemic range but was seen even in the high normal range of plasma glucose concentration. This finding is not without interest because impaired plasma glucose concentration is a feature of the metabolic syndrome, which is associated with a particularly high cardiovascular risk. Another facet of the metabolic syndrome, i.e. an increased body-mass-index (BMI), was also correlated to the urinary albumin excretion rate – again more pronounced in men compared to women. This was not only true for morbid obesity. The relation extended even into the range of low normal BMI values. This finding is of interest in view of the observation of Mykkanen [3]: in a large study covering 982 non-diabetic individuals he had noted that insulin sensitivity, when measured with the intravenous glucose tolerance test, was significantly reduced in patients who exhibited microalbuminuria. In those patients elevated concentrations of immune reactive insulin were also noted.

Fliser et al. [4] examined patients with primary renal disease using the intravenous glucose tolerance test. Even when the inulin clearance was only not below 80 ml/min. patients on average had significantly impaired insulin sensitivity. Conversely Chen et al. [5] found in the general population that insulin resistance (measured as the HOMA-index) increased the risk

of a future development of renal dysfunction. Finally in renal patients, similarly as in non-renal patients, insulin resistance is an independent predictor of total mortality and cardiovascular mortality [6]. So the two phenomena of insulin resistance and renal dysfunction are tightly related and may be linked by common pathomechanisms.

Microalbuminuria and reduced glomerular filtration rate (GFR) are two different aspects of renal dysfunction. The question arises whether the two are somehow interrelated. Unfortunately, there are no studies available where renal function had been measured with exact clearance techniques. Nevertheless, Pinto Sietsma [7] noted in the PREVEND-Study in Groningen that in individuals with a moderate increase in urinary albumin excretion the average creatinine clearance, was significantly higher. Only in the highest quantiles of albuminuria was a reduction of creatinine clearance found. This is reminiscent of the finding of Mogensen [8] in patients with type 2 diabetes in whom he found increased GFR in the early stage of microalbuminuria. It was only when proteinuria progressed markedly that the GFR decreased.

A further question is whether such minor albuminuria is relevant for all-cause mortality and cardiovascular mortality respectively? Again the Groningen study [9] noted a slight but significant increase of mortality which started in the range of high normal urinary albumin concentrations and rose progressively in the microalbuminuric range (20-200 mg/l). The HOPE study had also shown that the risk rises progressively with increasing albuminuria, similar to the relation between cardiovascular events and plasma cholesterol concentration [10]. This was true even in the range of normal urinary albumin concentrations. It follows that the definition of "microalbuminuria" is somewhat arbitrary and that urinary albumin excretion rate should be treated as a continuous value.

This continuous relationship corresponds to what Rachmani [11] had noted in patients with diabetes. Compared to the range of low normal albuminuria, albuminuria in the range 10-20 mg/day increased the risk to progress to frank microalbuminuria by a factor of 2.34 and the risk to experience a cardiovascular endpoint by factor of 1.9. In the range of frank microalbuminuria the risk was significantly higher by a factor of 12.4 and 9.8 respectively.

The high cardiovascular risk associated with reduced renal function

In parallel with the interest in microalbuminuria, the issue of the relation between moderately impaired renal function and cardiovascular risk has recently also attracted considerable interest.

The first observation in this direction was made in the Hypertension Detection and Follow up Programme. Shulman [12] reported on a 96-months follow up study in which he found a highly significant correlation between serum creatinine concentration and cumulative mortality. An increase was seen even between serum creatinine concentrations of 1.20 and 1.49 mg/dl. This finding has considerably epidemiological importance. *Tab. 1* summarizes the epidemiological analysis of Levey [13] based upon the data of NHANES (National Health and Nutrition Survey) in the USA. The number of patients with chronic kidney disease (CKD) stage 5 is very low, i.e. 300 000. In contrast in USA 5.3 million inhabitants have CKD stage 2 and 7.6 million inhabitants CKD stage 3.

Table 1. **Frequency of impaired renal function in the general population of the USA (NHANES data)**

Chronic kidney disease (CKD)	Estimated glomerular filtration (eGFR)	Percent population
stage	ml/min	%
5	<15	0.1
4	15-29	0.2
3	30-59	4.3
2	60-89	3.0
1	>90	3.3

A relation between impaired renal function and increased cardiovascular risk was found in several distinct cohorts, i.e. in the general population, in hypertensive patients, in patients at high cardiovascular risk and patients with impaired cardiac function. Furthermore, a similar relation has also been noted in patients with an acute ischemic cardiac event.

In the Dutch city of Hoorn Henry observed elderly patients for up to 10 years [14]. He noted that the risk to die from cardiovascular causes increased by 26% per 5 ml decrease in GFR. In other words, if the filtrate decreases by 20 ml/min the risk doubles.

In hypertensive individuals Luis Ruilope noted in the HOT (Hypertension Optimal Treatment) study that – independent of the diastolic target blood pressure to which the patient was randomised – the frequency of cardiovascular event was significantly higher by a factor of 2, if the estimated creatinine clearance was less than 60 ml/min compared to hypertensive patients with a higher GFR [15]. Similarly in the HOPE study Mann [16] noted that with increasing concentrations of serum creatinine the rate of cardiac events was significantly higher if patients had a serum creatinine concentration higher than 1.4 mg/dl. The risk was then increased by 40% compared to an increase of 60% when patients had microalbuminuria. The risk was increased by 108% when the patients had elevated serum creatinine plus microalbuminuria.

A particularly impressive increase in the risk of death in individuals with impaired renal function is seen in patients suffering from congestive heart failure. In a randomised prospective study Hillege [17] examined the relation between estimated GFR, severity of congestive heart failure (New York Heart Association category) and use of ACE inhibitors. In the highest compared to the lowest quartile the relative risk was increased by a factor of 2.85. An inverse relation was noted between GFR and atrial natriuretic peptid (ANP) plasma concentration. This finding may point to an important role of hypervolemia. The adverse effect of an elevated serum creatinine concentration was not completely explained by the severity of heart failure suggesting that there was an independent pathogenetic relation between renal dysfunction and cardiovascular risk.

Figure 1. **Cockroft-Gault formula for creatinine clearance (C_{crea}) estimation**

$$C_{crea} = \frac{(140 - age) \times body\ weight\ [kg]}{S\text{-}creatinine\ [mg/dl] \times 72} \quad (x0.85)_{woman}$$

C_{crea}[ml/min.]

It is also known that patients with impaired renal function, who have an ischemic cardiac event, have higher in-hospital mortality as well as postdischarge mortality than patients with normal renal function. In a national sample of elderly Medicare-patients who had an acute ischemic event Shlipak [18] found a clear relation between survival after discharge from the hospital and serum creatinine concentration. In a monocentric study assessing 3000 patients with an acute cardiac ischemic event, Wright noted that hospital mortality as well as postdischarge mortality was dramatically increased when the estimated GFR was decreased. Disappointingly the patients with impaired renal function, despite their extreme cardiovascular risk, had also received less aspirin, less betablockers, and less invasive procedures such as thrombolysis or percutaneous transluminal coronary angioplasty (PTCA). But this iatrogenic factor does not fully explain the extremely high cardiac risk. Even when individuals with a creatinine clearance of 100 ml/min were compared to patients with a creatinine clearance of 90 ml/min, the risk was almost double in the latter. Reinicke [19] examined the cumulative mortality of patients who had been discharged after PTCA. Even when comparing a serum creatinine of 1 mg/dl with a concentration of 1.2 mg/dl the mortality increased from 5 to 8.5%.

What practical conclusions can we draw from these observations?

It is unfortunate, that the cardiovascular risk increases in a concentration range of serum creatinine where this analyte is very insensitive to changes in the glomerular filtration rate. It is therefore recommended that the chemical laboratory reports not only the serum creatinine concentration, but estimates the GFR based on information concerning age, body weight and gender. Several formulas such as the Cockroft-Gault formula (see *Fig. 1*) or the MDRD formula are currently available. The risk in a patient with moderate impaired renal function is comparable in magnitude to that of a patient with diabetes mellitus. The National Institute of Health (NIH) emphasized that, in analogy to what is done in diabetes mellitus, cardiovascular prophylaxis in the patients with renal dysfunction should be considered secondary and no longer primary prophylaxis.

Underlying pathomechanisms

Although the exact pathomechanisms linking renal dysfunction to cardiac mortality have not been completely clarified some very interesting recent observations suggest that a whole array of factors is involved.

Kielstein [20] examined a cohort of patients with primary renal disease and found elevated concentrations of asymmetric dimethyl-L-arginin (ADMA), an inhibitor of endothelial nitric oxide synthase (eNOS). It is remarkable that this was true even in patients in whom normal clearance values had been measured. It is of course clear that normal whole kidney clearance is perfectly compatible with a major reduction in renal parenchymal mass, because the remaining glomeruli compensate to a large extent by increasing single nephron GFR. Why should increased ADMA be linked to cardiovascular risk. ADMA interferes with the production of the endogenous vasodilator and endothelial protective factor nitric oxide (NO) and inhibits several diverse endothelial cell functions.

ADMA is excreted via the kidney but this is not the only explanation. There are very good arguments that a catabolic enzyme in endothelial cells, dimethylarginine dimethylaminohydrolase, is diminished as well.

As another potential pathogenetic factor Kronenberg [21] found marked abnormalities of apolipoproteins, particularly an increase in apolipoprotein-A-IV (which of course is cardioprotective but the finding illustrates that the regulation of apo-lipoprotein metabolism is abnormal). Kronenberg also found very early in renal disease an increase of the concentration of the cardiovascular risk factor Lp(a) [22].

In hypertensive patients with autosomal dominal polycystic kidney disease Klein [23] measured increased sympathetic nerve activity even when the glomerular filtration rate was still normal.

Furthermore, Shlipak [24] found that patients having only a minor increase of serum creatinine had elevated serum concentrations of biomarkers indicating a state of microinflammation, e.g. increased CRP, fibrinogen, interleukin 6 as well as evidence of a prothrombotic state (elevated factor VIII, D-dimers).

Finally, even when inulin clearance was still normal, Stefański [25] found that the night-time decrease of blood pressure was attenuated and left ventricular wall thickness was increased as evidence of concentric remodelling.

All the above factors could interact to increase the cardiovascular risk.

Finally, since the early days of dialysis it had been postulated that atherogenesis is accelerated in uremia [26]. Recently there have been several studies in a genetic model of spontaneous atherogenesis the apo-e -/- mouse. In these animals resection of renal parenchyma increases the rate of growth of atherosclerotic plaques. Of note, this was even seen when only one kidney was removed [27].

Conclusion

It is obvious from the above that minor renal dysfunction, as reflected by microalbuminuria or decreased estimated GFR, has a major impact on cardiovascular risk. Renal dysfunction is thus a novel risk factor, which must be incorporated into currently used algorithms to assess risk factor profile. Evaluation

of renal function is important in order to select the appropriate strategy to reduce the cardiovascular risk (www.eshonline.org/documents/2003_guidelines and www.escardio.org/scinfo/guidelines.htm).

References

1. Ritz E. Minor renal dysfunction: an emerging independent cardiovascular risk factor. Heart, 2003; 89: 963-4.
2. Hillege HL, Janssen WM, Bak AA, Diercks GF, Grobbee DE, Crijns HJ, Van Gilst WH, De Zeeuw D, De Jong PE. Microalbuminuria is common, also in a nondiabetic, nonhypertensive population, and an independent indicator of cardiovascular risk factors and cardiovascular morbidity. J Intern Med, 2001; 249: 519-26.
3. Mykkanen L, Zaccaro DJ, Wagenknecht LE, Robbins DC, Gabriel M, Haffner SM. Microalbuminuria is associated with insulin resistance in nondiabetic subjects: the insulin resistance atherosclerosis study. Diabetes, 1998; 47: 793-800.
4. Fliser D, Pacini G, Engelleiter R, Kautzky-Willer A, Prager R, Franek E, Ritz E. Insulin resistance and hyperinsulinemia are already present in patients with incipient renal disease. Kidney Int, 1998; 53: 1343-7.
5. Chen J, Muntner P, Hamm LL, Fonseca V, Batuman V, Whelton PK, He J. Insulin resistance and risk of chronic kidney disease in nondiabetic US adults. J Am Soc Nephrol, 2003; 14: 469-77.
6. Shinohara K, Shoji T, Emoto M, Tahara H, Koyama H, Ishimura E, Miki T, Tabata T, Nishizawa Y. Insulin resistance as an independent predictor of cardiovascular mortality in patients with end-stage renal disease. J Am Soc Nephrol, 2002; 13: 1894-1900.
7. Pinto-Sietsma SJ, Janssen WM, Hillege HL, Navis G, De Zeeuw D, De Jong PE. Urinary albumin excretion is associated with renal functional abnormalities in a nondiabetic population. J Am Soc Nephrol, 2000; 11: 1882-8.
8. Mogensen CE. Prediction of clinical diabetic nephropathy in IDDM patients. Alternatives to microalbuminuria? Diabetes, 1990; 39: 761-7.
9. Hillege HL, Fidler V, Diercks GF, van Gilst WH, de Zeeuw D, van Veldhuisen DJ, Gans RO, Janssen WM, Grobbee DE, de Jong PE. Urinary albumin excretion predicts cardiovascular and noncardiovascular mortality in general population. Circulation, 2002; 106: 1777-82.
10. Gerstein HC, Mann JF, Yi Q, Zinman B, Dinneen SF, Hoogwerf B, Halle JP, Young J, Rashkow A, Joyce C, Nawaz S, Yusuf S. Albuminuria and risk of cardiovascular events, death, and heart failure in diabetic and nondiabetic individuals. JAMA, 2001; 286: 421-6.
11. Rachmani R, Levi Z, Lidar M, Slavachevski I, Half-Onn E, Ravid M. Considerations about the threshold value of microalbuminuria in patients with diabetes mellitus: lessons from an 8-year follow-up study of 599 patients. Diabetes Res Clin Pract, 2000; 49:187-94.
12. Shulman NB, Ford CE, Hall WD, Blaufox MD, Simon D, Langford HG, Schneider KA. Prognostic value of serum creatinine and effect of treatment of hypertension on renal function. Results from the hypertension detection and follow-up program. The Hypertension Detection and Follow-up Program Cooperative Group. Hypertension, 1989; 13: I80-93.
13. Levey AS, Coresh J. Should the K/DOQI definition of chronic kidney disease be changed? Am J Kidney Dis, 2003; 42: 626-30.
14. Henry RM, Kostense PJ, Bos G, Dekker JM, Nijpels G, Heine RJ, Bouter LM, Stehouwer CD. Mild renal insufficiency is associated with increased cardiovascular mortality: The Hoorn Study. Kidney Int, 2002; 62: 1402-7.
15. Ruilope LM, Salvetti A, Jamerson K, Hansson L, Warnold I, Wedel H, Zanchetti A. Renal function and intensive lowering of blood pressure in hypertensive participants of the hypertension optimal treatment (HOT) study. J Am Soc Nephrol, 2001; 12: 218-25.
16. Mann JF, Gerstein HC, Pogue J, Bosch J, Yusuf S. Renal insufficiency as a predictor of cardiovascular outcomes and the impact of ramipril: the HOPE randomized trial. Ann Intern Med, 2001; 134: 629-36.
17. Hillege HL, Girbes AR, de Kam PJ, Boomsma F, de Zeeuw D, Charlesworth A, Hampton JR, van Veldhuisen DJ: Renal function, neurohormonal activation, and survival in patients with chronic heart failure. Circulation, 2000; 102: 203-10.
18. Shlipak MG, Heidenreich PA, Noguchi H, Chertow GM, Browner WS, McClellan MB. Association of renal insufficiency with treatment and outcomes after myocardial infarction in elderly patients. Ann Intern Med, 2002; 137: 555-62.
19. Reinecke H, Trey T, Matzkies F, Fobker M, Breithardt G, Schaefer RM. Grade of chronic renal failure, and acute and long-term outcome after percutaneous coronary interventions. Kidney Int, 2003; 63: 696-701.
20. Kielstein JT, Boger RH, Bode-Boger SM, Frolich JC, Haller H, Ritz E, Fliser D. Marked increase of asymmetric dimethylarginine in patients with incipient primary chronic renal disease. J Am Soc Nephrol, 2002; 13: 170-6.
21. Kronenberg F, Kuen E, Ritz E, Konig P, Kraatz G, Lhotta K, Mann JF, Muller GA, Neyer U, Riegel W, Riegler P, Schwenger V, von Eckardstein A. Apolipoprotein A-IV serum concentrations are elevated in patients with mild and moderate renal failure. J Am Soc Nephrol, 2002; 13: 461-9.
22. Kronenberg F, Kuen E, Ritz E, Junker R, Konig P, Kraatz G, Lhotta K, Mann JF, Muller GA, Neyer U, Riegel W, Reigler P, Schwenger V, Von Eckardstein A. Lipoprotein(a) serum concentrations and apolipoprotein(a) phenotypes in mild and moderate renal failure. J Am Soc Nephrol, 2000; 11: 105-15.
23. Klein IH, Ligtenberg G, Oey PL, Koomans HA, Blankestijn PJ. Sympathetic activity is increased in polycystic kidney disease and is associated with hypertension. J Am Soc Nephrol, 2001; 12: 2427-33.
24. Shlipak MG, Fried LF, Crump C, Bleyer AJ, Manolio TA, Tracy RP, Furberg CD, Psaty BM. Elevations of inflammatory and procoagulant biomarkers in elderly persons with renal insufficiency. Circulation, 2003; 107: 87-92.
25. Stefanski A, Schmidt KG, Waldherr R, Ritz E. Early increase in blood pressure and diastolic left ventricular malfunction in patients with glomerulonephritis. Kidney Int, 1996; 50: 1321-6.
26. Lindner A, Charra B, Sherrard DJ, Scribner BH. Accelerated atherosclerosis in prolonged maintenance hemodialysis. N Engl J Med, 1974; 290:697-701.
27. Buzello M, Tornig J, Faulhaber J, Ehmke H, Ritz E, Amann K. The apolipoprotein e knockout mouse: a model documenting accelerated atherogenesis in uremia. J Am Soc Nephrol, 2003; 14: 311-6.

Helicobacter pylori infection and gastric MALT lymphoma

Lehours P*, Mégraud F

INSERM–ERI 10, Centre National de Référence des Campylobacters et Hélicobacters
Laboratoire de Bactériologie, CHU Pellegrin, Bordeaux cedex, France

Abstract

***Helicobacter pylori* infection is implicated in the development of two different gastric cancers: gastric adenocarcinoma and gastric MALT lymphoma. The association with the gastric MALT lymphoma is strong and causal. It is currently the only cancer which can be treated by a simple antibiotic treatment. However, the evolution of an *H. pylori* infection towards lymphoma is exceptional. Host susceptibility factors and environmental factors predisposing a patient to lymphoma have not yet been determined. The bacterial factors are currently being identified.**

Key words: lymphoma, MALT, *H. pylori*.

Abbreviations: JHP – *Helicobacter pylori* strain J99 open reading frame region; IPSID – Immuno proliferative small intestinal disease; NHL – Non-Hodgkin lymphoma; PGIL – Primitive gastrointestinal lymphoma; MALT – Mucosa associated lymphoid tissue; ORF – Open reading frame region; PCR – Polymerase chain reaction

Introduction

Twenty-five years have gone by since two Australian researchers were able to culture *Helicobacter pylori (H. pylori)* for the first time [1,2]. This bacterium which is strictly adapted to humans has unique properties. First of all, approximately half of the world population is said to be infected. Furthermore, the bacterium's adaptation to the gastric mucosa, thanks to its large production of urease which neutralizes the gastric acidity, insures its survival. Only about 10% of infected subjects develop gastroduodenal diseases, all within a very heterogeneous spectrum: gastritis, ulcers, gastric adenocarcinoma or mucosa associated lymphoid tissue (MALT)-type gastric lymphoma. *H. pylori* infection is therefore a potentially carcinogenic infection and, as such, has been recognized as a type I carcinogen (maximum level) by the International Agency In Research against Cancer [3]. The discovery of the bacterium has revolutionized one of the most important domains of gastroenterology. In fact, despite initial skepticism, ulcer treatment is now comprised of an antibiotic therapy aimed at eradicating *H. pylori*. The beneficial effect of this intervention was subsequentially applied to gastric MALT lymphoma as it is indeed possible to cure this particular cancer on a long term basis following *H. pylori* eradication [4].

For a given subject, individual factors, environmental factors and factors linked to the bacterium itself, contribute to the evolution towards a chronic infection and leads to the appearance of a lymphoma.

This article is comprised of three successive aspects: a general review of gastrointestinal lymphomas, a part dedicated especially to gastric MALT lymphoma, and finally an up-to-date on the most recent genetic data concerning *H. pylori* strains which are associated with gastric MALT lymphoma.

Primitive gastrointestinal lymphomas

The term of "lymphoma" evokes primarily a lymph node pathology. However, lymphoid tissue is also present in certain organs as the digestive tract. Indeed, 25% of all lymphomas are found elsewhere than in the lymph nodes and, amongst them, digestive lymphomas are the most frequent [5]. Work done by Isaacson and Wright led to regrouping most of the extra-gan-

* CORRESPONDING AUTHOR:
INSERM–ERI 10
Centre National de Référence des Campylobacters et Hélicobacters
Laboratoire de Bactériologie, CHU Pellegrin
Place Amélie Raba Léon
33076 Bordeaux cedex, France
Tel: (33) 5 57 57 12 86; Fax: (33) 5 56 51 41 82
e-mail: philippe.lehours@labhel.u-bordeaux2.fr (Philippe Lehours)

Received 08.07.2005 Accepted 08.07.2005

Table 1. **Histopathological classification of gastrointestinal lymphomas**

Phenotype B	
Low grade MALT B lymphoma – Western type (focalized) – Mediterranean type (extensive): IPSID (essentially alpha chain disease)	From the marginal zone of MALT
High grade MALT B lymphoma, with or without a component of weak malignancy including: – centroblast – immunoblast – large anaplasic cells	Diffuse large B cells
Centrocytic lymphoma = digestive lymphomatic polyposis	From the mantle zone
Burkitt's lymphoma or Burkitt type lymphoma	From Burkitt
Other types (equivalent to ganglionnary lymphomas)	Follicular
Phenotype T	
T lymphomas associated (EATL) with an enteropathy	T intestinal type
T lymphomas not associated with an enteropathy	

MALT – Mucosa Associated Lymphoid Tissue
IPSID – Immuno Proliferative Small Intestinal Disease
EATL – Enteropathy-Associated T Lymphoma

glionary lymphomas into one entity, the MALT lymphomas [6]. The diagnosis of gastrointestinal lymphomas should now be made following the recent classification of the World Health Organization (*Tab. 1*) [7,8].

I. Epidemiology of and predisposing factors for gastrointestinal lymphomas

Digestive lymphoma localizations represent 12.5% of all non-Hodgkin lymphomas (NHL) and are the most frequently found extraganglionary form (36%), gastric localization being the most frequent [9].

There is a male predominance in primitive gastrointestinal lymphomas (PGIL) (ratio 2:1). The average age is 57 years, but the age is much lower in Burkitt's lymphoma cases which concern young patients [10,11].

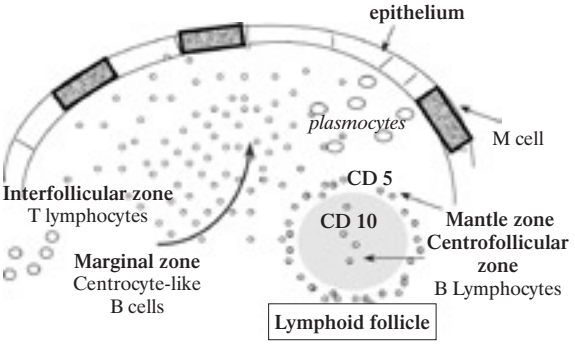
The etiology of PGIL is most often unknown. Acquired immune deficiency syndromes (AIDS) or genetic problems (chromosome X-linked deficiencies) have been associated with different kinds of lymphoma like Burkitt's lymphoma or lymphomatic polyposis syndrome. The infection hypothesis has also been considered, for example, in the case of certain T intestinal lymphomas which are associated with human T-cell leukemia retrovirus type I (HTLV-I). Recently *Campylobacter jejuni* has been suspected to play a role in the genesis of certain cases of immuno proliferative small intestinal disease (IPSID), but this hypothesis has not received unanimous approval [12,13]. *H. pylori* infection therefore represents the infectious cause of lymphoma which has been studied and characterized the most thoroughly.

II. Comparison of morphological characteristics of MALT lymphomas and Peyer patches

MALT lymphomas share morphologic characteristics which bring them close to Peyer patches and allow them to be differentiated from lymph node lymphomas, as follows:

– the constant presence of lymphoid follicles with a clear center;
– lymphoepithelial lesions formed by invasion of individuals glands by aggregates of lymphoma cells.

Figure 1. **Schematic representation of lymphoid tissue from the digestive tract (MALT)** [60]



Peyer patches are indeed comprised of lymphoid follicles with a clear center which, when activated, are surrounded by a mantle zone and a marginal zone. The T lymphocytes are arranged along the venules and histocytes. The follicles bulge towards the intestinal lumen and establish a zone called "the dome", found between the follicle and the epithelium and comprised of B lymphocytes from the marginal zone (*Fig. 1*). Immunohistochemical studies show that only at the dome level are there intraepithelial B lymphocytes (CD20+, CD79a+) in formations resembling miniature lymphoepithelial lesions.

Gastric MALT lymphoma

Gastric lymphoma is considered to be the classic lymphoma of MALT-type of the digestive tract. It is a B cell lymphoma with a very unusual pathogenesis and evolution which slowly progresses and stays localized in the stomach for a long time. The development of the lymphoma is directly linked to the *H. pylori* infection although it is not known why this evolution is present in only a very small number of infected subjects.

I. Epidemiology of gastric MALT lymphoma

Epidemiological data on gastric MALT lymphoma are very heterogeneous. An epidemiological study carried out in Germany reported on the detection of 94 cases in a total population of 3.5 million inhabitants over a 3 year period, and estimated the incidence to be 0.7-0.8 per 100000, with an average age of 62.1 years and a sex ratio slightly in favor of the male gender [9]. This incidence seems to be comparable to that of other European countries with the exception of England where the incidence is lower (0.2 per 100000). Amongst the North African countries, in Tunisia, the incidence is estimated at 6.3 per 100000 for men and 3.8 per 100000 for women [14].

II. Relationship between *H. pylori* infection and gastric MALT lymphoma

In order to analyze the relationship between *H. pylori* infection and gastric MALT lymphoma, it is interesting to review the Bradford Hill criteria which were first used to show the causal link between lung cancer and tobacco smoking [15]. These criteria include: 1) an association and a temporal relationship between the two situations, 2) the biological plausability, i.e. the pathophysiological mechanisms underlying this association, and 3) the efficacity of an intervention. The existence of an animal model is another argument in favour of a causal relationship.

2-1. Association and temporal relationship

Numerous epidemiological arguments back the fact that an association exists between gastric MALT lymphoma and *H. pylori* infection.

First, the prevalence of *H. pylori* infection in patients suffering with gastric MALT lymphoma, based on several studies, is between 80 and 90%, whereas the prevalence in the adult population in France for example, ranges from 25 to 30% [16]. Parsonnet et al. [17] offered a major epidemiological argument implicating *H. pylori* infection in MALT lymphoma. These authors showed in a case-control study nested in two large cohorts that the relative risk of developing this type of lymphoma was six times higher in the presence of an *H. pylori* infection. Furthermore, this increase was noted only for the gastric lymphomas; there was no increase in the relative risk for nodal lymphomas. They found that 88% of the patients had anti-*H. pylori* antibodies in blood samples collected fifteen years before the diagnosis of lymphoma, which clearly indicates that the *H. pylori* infection preceded the appearance of the gastric MALT lymphoma.

For certain cases, another species of the *Helicobacter* genus has been incriminated: *Helicobacter heilmannii* [18]. However, these cases are very rare, especially since the prevalence of *H. heilmannii* infection in humans in very low: 0.5% compared to an average of 20 to 25% in France for *H. pylori* [19].

2-2. Pathophysiological mechanism

The stomach normally is lacking in lymphoid tissue. After an *H. pylori* infection, a lymphoid infiltrate appears, which constitute a chronic gastritis. In certain cases the lympoid tissue can be organized as lymphoid follicles. MALT lymphoma emerges from these lymphoid structures [20]. Therefore, these lymphoid follicles appear after an antigenic stimulation by *H. pylori*. This hypothesis was validated *in vitro* by showing that T lymphocytes sensitized for *H. pylori* produce cytokines which stimulate B lymphoid proliferation [21-23]. The remaining question is whether the activation of the B cells requires the presence of a continuous antigenic stimulation by *H. pylori* or whether it is the consequence of an autoimmune mechanism [24]. In fact, the neoplastic B cells frequently produce antibodies directed toward autoantigens. Furthermore, the cells need to be in contact with the intratumoral T cells in order to proliferate, a CD40 and CD40 ligand interaction occurs [25]. This would explain the tendency for lymphomas with a weak degree of malignancy to remain localized and to regress following an *H. pylori* eradication. The existence of a clonal lymphocytic proliferation over several years would favour the occurrence of genetic alterations and the lymphoma would progressively proliferate independently of *H. pylori* [26]. Even if the mechanism for the evolution of a *H. pylori* infection to the gastritis stage and then to MALT lymphoma remains unknown, the role played by the bacterium seems to be likely.

Table 2. **Summary of the main studies performed from 1993 à 2002 evaluating the impact of *H. pylori* eradication on the regression of low grade gastric MALT lymphoma**

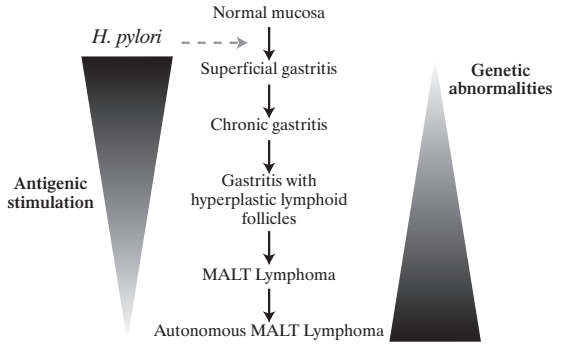
Author	Reference	Year	Number of patients	% remission
Wotherspoon et al.	[27]	1993	6	83
Bayerdörffer et al.	[61]	1995	33	69
Savio et al.	[62]	1996	12	84
Roggero et al.	[63]	1995	25	60
Fischbach et al.	[64]	1996	15	93
Montalban et al.	[65]	1997	9	88
Pinotti et al.	[66]	1997	45	68
Neubauer et al.	[67]	1997	50	80
Nobre-Leitao et al.	[68]	1998	17	100
Steinbach et al.	[69]	1999	28	50
Thiede et al.	[70]	2000	84	81
Fischbach et al.	[71]	2000	36	89
Ruskone-Fourmestraux et al.	[72]	2001	34	56
De Jong et al.	[73]	2001	23	56
Matsushima et al.	[74]	2002	14	71
Diz-Lois Palomares et al.	[75]	2002	14	71
Levy et al.	[30]	2002	48	69
Liu et al.	[44]	2002	111	43
Accumulated data	**1993-2002**		**604**	**72.8**

2-3. Effect of an intervention

The possibility of obtaining a regression of the lymphoma by taking an *H. pylori* eradication treatment constitutes the definitive proof of the causal role of the infection.

Wotherspoon et al. [27] showed in a pilot study including six patients that it was possible to obtain a regression of a low grade gastric MALT lymphoma in five of these patients, 22 months after the eradication of *H. pylori*. Several studies have determined that the tumor regression is 70 to 80%, with extremes of 50 to 100%, within a minimum delay of 4 to 6 months and after a maximum of 18 months, and after a post-treatment lapse greater than 6 years [4,28]. The variety of results obtained in the different studies stems mainly from the heterogeneity of the patients studied and the differences related to the locoregional extension of the lymphoma. Indeed, if only patients at stage EI were included in the different studies, the regression rate would be close to 80%. Furthermore, the time interval chosen between the eradication treatment and the lymphoma control may have a slight effect on the disparity of the results obtained (*Tab. 2*).

Figure 2. **Hypothetical cascade from the appearance of a MALT lymphoma at the gastric mucus level after infection with *H. pylori***



In France, the most commonly used eradication treatment combines amoxicillin (1 g bid) and clarithromycin (500 mg bid) with a double dose of proton pump inhibitor (PPI), compared to the usual dose, for 7 to 14 days. A control, based on *H. pylori* culture of gastric biopsies, should be carried out at least one month after the end of the treatment to verify *H. pylori* eradication and eventually modify the treatment depending on the antibiotic susceptibility results. The slow evolution of the disease allows a delay of at least 6 months before checking the histological and endoscopic evolution during the first two years [29,30]. In the case of a lymphoma with a high degree of malignancy, the chances of healing due to an *H. pylori* eradication are smaller [31]. Although it is now well accepted that a *H. pylori* infection contributes to the appearance of lymphoid tissue in the stomach and to its evolution towards a malignant proliferation, the *H. pylori*-dependent characteristics of the lymphoma disappears as genetic abnormalities accumulate (*Fig. 2*).

Regarding lymphomas linked to *H. heilmannii*, a tumoral regression has also been obtained after an eradication treatment identical to that of *H. pylori* [18].

The *H. pylori* eradication treatment seems to have a positive effect on extragastric MALT lymphomas (salivary gland, duodenum, colon, bladder, lung) even though it is often impossible to detect *H. pylori* in these extra-abdominal sites [32,33].

Lastly, treatment of *H. pylori* positive lymphomas which do not respond to eradication treatment, i.e. most of those of high grade of malignancy or, certain of those of low grade malignancy, as well as treatment of *H. pylori* negative gastric MALT lymphomas, is based on more classic therapeutic approaches for lymphomas. Gastric resection is the oldest treatment still being used. This approach can be helpful in removing big tumors but an average survival rate of 63% at most after 5 years can be expected for lymphomas at the EI2 stage [11]. Radiotherapy is currently preferred to surgery [34]. Finally chemotherapy using alkylant agents like chlorambucil or cyclophosphamide can be used successfully on patients diagnosed at an advanced stage of lymphoma or after a treatment failure [34].

2-4. Existence of an animal model

A prolonged gastric *H. pylori* infection in the BALB/c mouse constitutes a lymphogenesis model [35,36]. In these experimental lymphomas, one finds the characteristics of centrocyte-like cells, lymphoepithelial lesions, glandular destruction and consequently an aspect which is quite similar to that of human lymphomas. However, in the BALB/c model, lymphoepithelial lesions which evoke a gastric MALT lymphoma appear only in certain animals infected orally with *H. pylori* (an average of 40%) and approximately 20 months post-infection [36]. In some cases lymphoma can also evolve toward high grade gastric MALT lymphoma. One should note that in this model an eradication treatment induces the regression of the lymphoma.

III. Associated molecular abnormalities

Several translocations have been identified in the tumoral cells of gastric MALT lymphomas. Their significance is becoming more and more clear; in fact, they cause an antiapoptotic effect which is strictly related to a malignant expansion [37].

3-1. The translocation t(1;14)(p22;q32)

The translocation t(1;14) is found in approximately 5% of gastric MALT lymphomas. It leads to the overexpression of Bcl-10 protein whose gene is put under the control of the promotor gene of the immunoglobulin heavy chains. Bcl-10 possesses a CARD amino-terminal domain ("caspase recruitment domain") and can activate the NF-κB transcription factor [38]. In the lymphomatous cells, the Bcl-10 gene is overexpressed but it is also mutated, provoking the synthesis of a truncated protein capable of activating NF-κB and cell proliferation, but its overexpression does not induce apoptosis [39]. The t(1;14) translocation is also frequently associated with other supernumerary genetic abnormalities like those at the 3, 8 and 12 chromosome level. Trisomy 3 is found in 30% of gastric MALT lymphomas even though its role in the progression of the disease has not been well established [40].

3-2. The t(11;18) translocation

The t(11;18) translocation has been implicated in 21 to 60% of gastric MALT lymphomas. This translocation is also found in the marginal zone of other mucus sites in lymphomas [41]. It involves two genes: a human para-capsase, MLT1 (MALT lymphoma associated translocation-1); and c-IAP2 or API, a function of which is to inhibit the capsases by interacting directly with them. The translocation produces an IAP2-MLT1 fusion which has the capacity of activating the NF-κB pathway and therefore the cell protection against apoptosis [42]. It is associated with the most advanced stages, in particular with tumors having started to invade the submucosa, and also with the absence of regression in EI and EII stage lymphomas undergoing *H. pylori* eradication treatment [43-45]. The translocation should therefore be identified because its detection is predictive of the efficiency of an eradication treatment.

3-3. The t(14;18)(q32;q21) translocation

A third translocation was reported, t(14;18)(q32;q21), initially associated with a MALT type lymphoma of the skin and the liver, and then found in different localizations like the salivary glands [46]. Its identification implies a third pathway of carcinogenesis. However, for the moment this translocation does not appear to be associated with gastric lymphomas.

Studies on *H. pylori* strains associated with gastric MALT lymphoma

H. pylori was the first bacterium to be classified as a type I carcinogen (maximum level) by the International Agency of Research against Cancer [3]. Since its discovery, many research projects have focused on virulence factors or genetic markers but few studies have included *H. pylori* strains associated with gastric MALT lymphoma.

I. Study of the major virulence factors associated with *H. pylori*

H. pylori is perfectly adapted to the human stomach thanks to factors which allow it to: 1) resist against gastric acidity, 2) move around in the gastric mucus, and 3) escape from the immune response of the host. The major virulence factors found in *H. pylori* are those stimulating inflammation and the cell damage which results thereof, and in particular the products of the cag pathogenicity island and other proinflammatory proteins. Eight virulence factors were recently evaluated by studying a large collection of French strains issued from patients with gastric MALT lymphoma or gastritis [47]. Four factors involved in gastric inflammation and tissue lesions (CagA, CagE, OipA and IceA) as well as the vacuolizing cytotoxin VacA were tested. CagA is the virulence factor which has been most thoroughly studied along with VacA, and it has been associated in particular with duodenal ulcer and gastric adenocarcinoma. Four external membrane proteins were also studied: BabA, SabA, HopZ and HopQ. BabA, SabA and HopZ are adherence factors, BabA and SabA recognize in particular Lewis type antigens [48-50]. None of these factors tested individually could be significantly associated with strains from low grade gastric MALT lymphoma whereas three of them (IceA1, SabA and HopZ) showed a tendency to be associated. These strains have probably not a proinflammatory potential which distinguishes them from strains associated with ulcers or gastric adenocarcinoma [47].

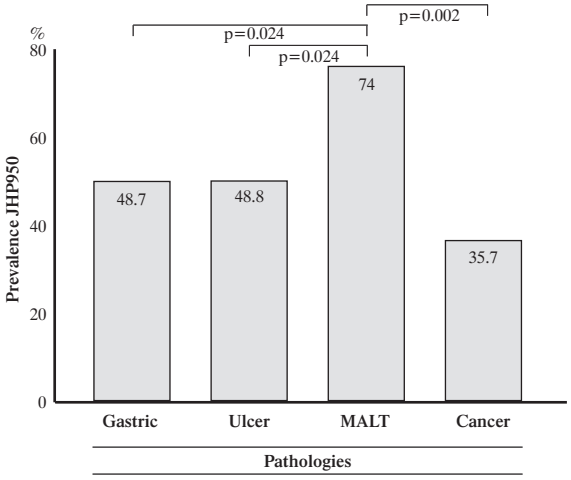
II. Study of the genetic characteristics of *H. pylori* strains associated with gastric MALT lymphoma

The development of a low grade gastric MALT lymphoma is most likely the consequence of an infection with an *H. pylori* strain, which harbours a particular set of genes or a particular gene expression, in a host with a particular genetic susceptibility. Within *H. pylori* species a considerable genomic as well as phenotypic diversity do exist. It is therefore possible that some strains have evolved towards a capacity to induce disease. Since known pathogenicity factors have not be implicated, the genetic material from these strains must be analyzed in order to determine either specific genetic markers or new virulence factors.

The new method for studying the genetic material in bacteria is comprised of DNA chips which detect the presence of ORFs in a set of strains compared to the genome of the strains for which the whole genomic sequence is known [51]. As no *H. pylori* strain associated with MALT lymphoma has ever been sequenced, a subtractive hybridization technique was used [52]. This technique has the advantage of extracting from the genome of a particular strain what is specific to it when compared to a control strain. Indeed, it is within the variable part of the genome of strains associated with gastric MALT lymphoma that new virulence factors are expected to be found. A specific marker for low grade gastric MALT lymphoma strains, the ORF JHP950, has reinforced the hypothesis that these MALT strains share a common genetic profile. Indeed, the prevalence of the JHP950 ORF in gastric MALT lymphoma strains was significantly higher than in strains isolated from duodenal ulcer and gastric adenocarcinoma (*Fig. 3*) [53]. This ORF belongs to the so-called plasticity zone of *H. pylori*. This zone is not considered to be a pathogenicity island per se but more likely a large sized genomic island [52]. However, ORF JHP950 is a part of the predicted operon containing ORF JHP947 which has been associated with strains isolated from patients with gastric adenocarcinoma [54]. ORF JHP950 is associated with gastric MALT lymphoma on the same level as the cagA gene is associated with ulcer strains [55].

Furthermore, by comparing the data obtained with this marker to those in the study on *H. pylori* major virulence factors, a significant association between this ORF and the genes, IceA1 and SabA, was shown in gastric MALT lymphoma strains [53]. Studies on the genetic diversity of *H. pylori*, such as the one performed by Salama et al. [51], showed a cluster of genes, in particular cag pathogenicity island ORFs associated to certain genes like *babA* or *hopQ* which were consequently identified as virulence factors [56,57]. The gene cluster identified in MALT strains could be a result of the phylogenetic evolution of lymphoma strains; i.e., they would have been selected during evolution because they offer an advantage, which remains to be determined, for the strains that contain them. ORF JHP950 codes for a protein with an undetermined function like 33% of the ORFs in the *H. pylori* genome, and therefore its role cannot be integrated into the pathophysiology of lymphoma [58]. Only

Figure 3. **Prevalence of ORF JHP950 in *H. pylori* strains associated with low grade gastric MALT lymphoma (MALT) (n=43) in comparison to strains associated with chronic gastritis (Gastritis) (n=39), duodenal ulcer (Ulcer) (n=41) or gastric adenocarcinoma (Cancer) (n=28)** [53]



p= Fisher exact test

complementary approaches such as reverse genetics, proteomics or others will allow us to answer this question. This study therefore allowed us to show the presence of genetic markers in certain *H. pylori* strains associated with gastric MALT lymphoma which could be used in the screening of strains with a high risk of causing a lymphoma.

II. Recent data obtained by comparative genomics

H. pylori strains associated with gastric MALT lymphoma therefore apparently share common genes. This hypothesis was recently confirmed by results obtained by comparative genomic studies. DNA from the collection of 43 lymphoma strains previously mentioned were hybridized on high density membranes containing a selection of 248 non-ubiquitous genes (the variable part of the *H. pylori* genome) and 50 ubiquitous genes (the stable part of the *H. pylori* genome). A statistical analysis carried out using the normalized values of these hybridizations revealed that 80% of the strains associated with lymphoma could be grouped together in the same cluster, distinguishing them from strains associated with other pathologies linked to *H. pylori* (gastritis, ulcer and intestinal metaplasia) [59].

These recent data do not allow us yet to understand the pathophysiological mechanisms of these particular strains but incite us strongly to look at the genetic material of these strains from every angle. It is from this perspective that a complete genome sequencing of a strain originating from a low grade gastric MALT lymphoma was conducted, in order to be able to compare it to the genomes of other *H. pylori* strains issued from other diseases.

Conclusions

The implication of *H. pylori* in the genesis of gastric MALT lymphoma has been clearly established. This discovery has revolutionized the treatment of this lymphoma because a *H. pylori* eradication treatment is capable of curing the lymphoma in most instances. This infection portrays a magnificent model of carcinogenesis induced by a chronic bacterial infection. The pathophysiological mechanisms leading to the occurrence of the lymphoma are beginning to be understood. The most recent molecular data indicates that strains associated with this cancer share common genetic characteristics. The identification of a first genetic marker for these strains and the results which are expected from the complete sequencing of a MALT lymphoma strain should lead to a screening method at the precancerous stage of strains with a high risk of causing lymphoma, and therefore, to propose a targeted antibiotic treatment. However, all of the elements involved in lymphogenesis are probably not limited only to bacteria and therefore a joint research effort involving individual susceptibility factors should be carried out.

References

1. Warren JR, Marshall B. Unientified curved bacilli on gastric epithelium in active chronic gastritis. Lancet, 1983; 1: 1273-5.
2. Marshall BJ, Warren JR. Unidentified curved bacilli in the stomach of patients with gastritis and peptic ulceration. Lancet, 1984; 1: 1311-5.
3. IARC. Schistosomes, liver flukes and *Helicobacter pylori*. in "Monographs on the evaluation of carcinogenic risks to humans" IARC Working Group on the Evaluation of Carcinogenic Risks to Humans. Lyon, 7-14 June 1994; 61: 1-241.
4. Stolte M, Bayerdörffer E, Morgner A, Alpen B, Wundisch T, Thiede C, Neubauer A. *Helicobacter* and gastric MALT lymphoma. Gut, 2002; 50 (Suppl 3): 19-24.
5. Freeman C, Berg JW, Cutler SJ. Occurrence and prognosis of extranodal lymphomas. Cancer, 1972; 29: 252-60.
6. Isaacson PG, Wright DH. Malignant lymphoma of mucosa-associated lymphoid tissue. A distinctive type of B-cell lymphoma. Cancer, 1983; 52: 1410-6.
7. Isaacson PG, Spencer J, Wright DH. Classifying primary gut lymphomas. Lancet, 1988; 2: 1148-9.
8. Harris NL, Jaffe ES, Diebold J, Flandrin G, Muller-Hermelink HK, Vardiman J, Lister TA, Bloomfield CD. World Health Organization classification of neoplastic diseases of the hematopoietic and lymphoid tissues: report of the Clinical Advisory Committee meeting-Airlie House, Virginia, November 1997. J Clin Oncol, 1999; 17: 3835-49.
9. Ullrich A, Fischbach W, Blettner M. Incidence of gastric B-cell lymphomas: a population-based study in Germany. Ann Oncol, 2002; 13: 1120-7.
10. Fischbach W, Kestel W, Kirchner T, Mossner J, Wilms K. Malignant lymphomas of the upper gastrointestinal tract. Results of a prospective study in 103 patients. Cancer, 1992; 70: 1075-80.
11. Radaszkiewicz T, Dragosics B, Bauer P. Gastrointestinal malignant lymphomas of the mucosa-associated lymphoid tissue: factors relevant to prognosis. Gastroenterology, 1992; 102: 1628-38.
12. Peterson MC. Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. N Engl J Med, 2004; 350: 1685-6.
13. Lecuit M, Abachin E, Martin A, Poyart C, Pochart P, Suarez F, Bengoufa D, Feuillard J, Lavergne A, Gordon JI, Berche P, Guillevin L, Lortholary O. Immunoproliferative small intestinal disease associated with *Campylobacter jejuni*. N Engl J Med, 2004; 350: 239-48.
14. Ben-Khelifa H. Gastric lymphoma: is the worldwide incidence rising? Gastrointest Endosc, 2002; 56: 955.
15. Hill AB. The environment and disease: association or causation? Proc R Soc Med, 1965; 295-300.
16. Eck M, Greiner A, Schmausser B, Eck H, Kolve M, Fischbach W, Strecker P, Muller-Hermelink HK. Evaluation of *Helicobacter pylori* in gastric MALT-type lymphoma: Differences between histologic and serologic diagnosis. Mod Pathol, 1999; 12: 1148-51.
17. Parsonnet J, Hansen S, Rodriguez L, Gelb AB, Warnke RA, Jellum E, Orentreich N, Vogelman JH, Friedman GD. *Helicobacter pylori* infection and gastric lymphoma. N Engl J Med, 1994; 330: 1267-71.
18. Morgner A, Lehn N, Andersen LP, Thiede C, Bennedsen M, Trebesius K, Neubauer B, Neubauer A, Stolte M, Bayerdorffer E. *Helicobacter heilmannii*-associated primary gastric low-grade MALT lymphoma: Complete remission after curing the infection. Gastroenterology, 2000; 118: 821-8.
19. Broutet N, Mégraud F. Epidemiology of *Helicobacter pylori* infection. 1996 Epidemiology of *Helicobacter pylori* infection. *Helicobacter pylori* (Mégraud F, and Lamouliatte H, Eds.), 1 Epidémiologie, Pathogénie, Diagnostic, Paris.
20. Miyamoto M, Haruma K, Hiyama T, et al. High incidence of B-cell monoclonality in follicular gastritis: a possible association between follicular gastritis and MALT lymphoma. Virchows Arch, 2002; 440: 376-80.
21. Hussell T, Issacson PG, Crabtree JE, Spencer J. The response of cells from low-grade B-cell gastric lymphomas of mucosa-associated lymphoid tissue to *Helicobacter pylori*. Lancet, 1993; 342: 571-4.
22. Hussell T, Isaacson PG, Crabtree JE, Spencer J. *Helicobacter pylori*-specific tumour-infiltrating T cells provide contact dependent help for the growth of malignant B cells in low-grade gastric lymphoma of mucosa-associated lymphoid tissue. J Pathol, 1996; 178: 122-7.
23. D'Elios MM, Amedei A, Manghetti M. Impaired T-cell regulation of B-cell growth in *Helicobacter pylori*-related gastric low-grade MALT lymphoma. Gastroenterology, 1999; 117: 1105-12.
24. Kawahara Y, Yokota K, Mizuno M, Yunoki N, Uesu T, Okada H, Kobayashi K, Hirai Y, Oguma K, Tsuji T. Antibodies to human gastric epithelial cells and heat shock protein 60 in *Helicobacter pylori* positive mucosa associated lymphoid tissue lymphoma. Gut, 1999; 45: 20-3.
25. Guindi M. Role of activated host T cells in the promotion of MALT lymphoma growth. Semin Cancer Biol, 2000; 10: 341-4.
26. Dierlamm J, Baens M, Wlodarska I, Stefanova-Ouzounova M, Hernandez JM, Hossfeld DK, De Wolf-Peeters C, Hagemeijer A, Van den Berghe H, Marynen P. The apoptosis inhibitor gene API2 and a novel

18q gene, MLT, are recurrently rearranged in the t(11;18)(q21;q21) associated with mucosa-associated lymphoid tissue lymphomas. Blood, 1999; 93: 3601-9.

27. Wotherspoon AC, Doglioni C, Diss TC, Pan L, Moschini A, de Boni M, Isaacson PG. Regression of primary low-grade B-cell gastric lymphoma of mucosa-associated lymphoid tissue type after eradication of *Helicobacter pylori*. Lancet, 1993; 342: 575-7.

28. Savio A, Zamboni G, Capelli P, Negrini R, Santandrea G, Scarpa A, Fuini A, Pasini F, Ambrosetti A, Paterlini A, Buffoli F, Angelini GP, Cesari P, Rolfi F, Graffeo M, Pascarella A, Valli M, Mombello A, Ederle A, Franzin G. Relapse of low-grade gastric MALT lymphoma after *Helicobacter pylori* eradication: true relapse or persistence? Long-term post-treatment follow-up of a multicenter trial in the north-east of Italy and evaluation of the diagnostic protocol's adequacy. Recent Results Cancer Res, 2000; 156: 116-24.

29. Sandmeier D, Benhattar J, Bouzourene H. The natural history of a gastric low grade B cell MALT lymphoma followed during 11 years without treatment. J Clin Pathol, 2002; 55: 548-50.

30. Levy M, Copie Bergman C, Traulle C. Conservative treatment of primary gastric low-grade B-cell lymphoma of mucosa-associated lymphoid tissue: Predictive factors of response and outcome. Am J Gastroenterol, 2002; 97: 292-7.

31. Ng WW, Lam CP, Chau WK, Fen-Yau Li A, Huang CC, Chang FY, Lee SD. Regression of high-grade gastric mucosa-associated lymphoid tissue lymphoma with *Helicobacter pylori* after triple antibiotic therapy. Gastrointest Endosc, 2000; 51: 93-6.

32. Raderer M, Pfeffel F, Pohl G, Mannhalter C, Valencak J, Chott A. Regression of colonic low grade B cell lymphoma of the mucosa associated lymphoid tissue type after eradication of *Helicobacter pylori*. Gut, 2000; 46: 133-5.

33. van den Bosch J, Kropman RF, Blok P. Disappearance of a mucosa-associated lymphoid tissue (MALT) lymphoma of the urinary bladder after treatment for *Helicobacter pylori*. Eur J Haematol, 2002; 68: 187-8.

34. Schechter NR, Portlock CS, Yahalom J. Treatment of mucosa-associated lymphoid tissue lymphoma of the stomach with radiation alone. J Clin Oncol, 1998; 16: 1916-21.

35. Lee A, O'Rourke J, Enno A. Gastric mucosa-associated lymphoid tissue lymphoma: implications of animal models on pathogenic and therapeutic considerations-mouse models of gastric lymphoma. Recent Results Cancer Res, 2000; 156: 42-51.

36. Mueller A, O'Rourke J, Grimm J. Distinct gene expression profiles characterize the histopathological stages of disease in *Helicobacter*-induced mucosa-associated lymphoid tissue lymphoma. Proc Natl Acad Sci USA, 2003; 100: 1292-7.

37. Isaacson PG, Du MQ. MALT lymphoma: from morphology to molecules. Nat Rev Cancer, 2004; 4: 644-53.

38. Zhang Q, Siebert R, Yan M, Hinzmann B, Cui X, Xue L, Rakestraw KM, Naeve CW, Beckmann G, Weisenburger DD, Sanger WG, Nowotny H, Vesely M, Callet-Bauchu E, Salles G, Dixit VM, Rosenthal A, Schlegelberger B, Morris SW. Inactivating mutations and overexpression of BCL10, a caspase recruitment domain-containing gene, in MALT lymphoma with t(1;14)(p22;q32). Nat Genet, 1999; 22: 63-8.

39. Ruland J, Duncan GS, Elia A, del Barco Barrantes I, Nguyen L, Plyte S, Millar DG, Bouchard D, Wakeham A, Ohashi PS, Mak TW. Bcl10 is a positive regulator of antigen receptor-induced activation of NF-kappaB and neural tube closure. Cell, 2001; 104: 33-42.

40. Wotherspoon AC, Finn TM, Isaacson PG. Trisomy 3 in low-grade B-cell lymphomas of mucosa-associated lymphoid tissue. Blood, 1995; 85: 2000-4.

41. Baens M, Maes B, Steyls A. The product of the t(11;18), an API2-MLT fusion, marks nearly half of gastric MALT type lymphomas without large cell proliferation. Am J Pathol, 2000; 156: 1433-9.

42. Uren AG, O'Rourke K, Aravind LA, Pisabarro MT, Seshagiri S, Koonin EV, Dixit VM. Identification of paracaspases and metacaspases: two ancient families of caspase-like proteins, one of which plays a key role in MALT lymphoma. Mol Cell, 2000; 6: 961-7.

43. Liu HX, Ruskone-Fourmestraux A, LavergneSlove A. Resistance of t(11;18) positive gastric mucosa-associated lymphoid tissue lymphoma to *Helicobacter pylori* eradication therapy. Lancet, 2001; 357: 39-40.

44. Liu HoX, Ye HT, Ruskone Fourmestraux A, De Jong D, Pileri S, Thiede C, Lavergne A, Boot H, Caletti G, Wundisch T, Molina T, Taal BG, Elena S, Thomas T, Zinzani PL, Neubauer A, Stolte M, Hamoudi RA, Dogan A, Isaacson PG, Du MQ. T(11;18) is a marker for all stage gastric MALT lymphomas that will not respond to *H. pylori* eradication. Gastroenterology, 2002; 122: 1286-94.

45. Nakamura S, Matsumoto T, Jo Y, Fujisawa K, Suekane H, Yao T, Tsuneyoshi M, Iida M. Chromosomal translocation t(11;18)(q21;q21) in gastrointestinal mucosa associated lymphoid tissue lymphoma. J Clin Pathol, 2003; 56: 36-42.

46. Streubel B, Lamprecht A, Dierlamm J, Cerroni L, Stolte M, Ott G, Raderer M, Chott A. T(14;18)(q32;q21) involving IGH and MALT1 is a frequent chromosomal aberration in MALT lymphoma. Blood, 2003; 101: 2335-9.

47. Lehours P, Ménard A, Dupouy S, Bergey B, Richy F, Zerbib F, Ruskone-Fourmestraux A, Delchier JC, Megraud F. Evaluation of the association of nine *Helicobacter pylori* virulence factors with strains involved in low grade gastric MALT lymphoma. Infect Immun, 2004; 74: 880-8.

48. Ilver D, Arnqvist A, Ogren J, Frick IM, Kersulyte D, Incecik ET, Berg DE, Covacci A, Engstrand L, Boren T. *Helicobacter pylori* adhesin binding fucosylated histo-blood group antigens revealed by retagging. Science, 1998; 279: 373-7.

49. Mahdavi J, Sonden B, Hurtig M, Olfat FO, Forsberg L, Roche N, Angstrom J, Larsson T, Teneberg S, Karlsson KA, Altraja S, Wadstrom T, Kersulyte D, Berg DE, Dubois A, Petersson C, Magnusson KE, Norberg T, Lindh F, Lundskog BB, Arnqvist A, Hammarstrom L, Boren T. *Helicobacter pylori* SabA adhesin in persistent infection and chronic inflammation. Science, 2002; 297: 573-8.

50. Peck B, Ortkamp M, Diehl KD, Hundt E, Knapp B. Conservation, localization and expression of HopZ, a protein involved in adhesion of *Helicobacter pylori*. Nucl Acids Research, 1999; 27: 3325-33.

51. Salama N, Guillemin K, McDaniel TK, Sherlock G, Tompkins L, Falkow S. A whole-genome microarray reveals genetic diversity among *Helicobacter pylori* strains. Proc Natl Acad Sci USA, 2000; 97: 14668-73.

52. Occhialini A, Marais A, Alm R, Garcia F, Sierra R, Megraud F. Distribution of open reading frames of plasticity region of strain J99 in *Helicobacter pylori* strains isolated from gastric carcinoma and gastritis patients in Costa Rica. Infect Immun, 2000; 68: 6240-9.

53. Lehours P, Dupouy S, Bergey B, Ruskone-Foumestraux A, Delchier JC, Rad R, Richy F, Tankovic J, Zerbib F, Megraud F, Menard A. Identification of a genetic marker of *Helicobacter pylori* strains involved in gastric extranodal marginal zone B cell lymphoma of the MALT-type. Gut, 2004; 53(7): 931-7.

54. Santos A, Queiroz DM, Ménard A, Marais A, Rocha GA, Oliveira CA, Nogueira AM, Uzeda M, Megraud F. New pathogenicity marker found in the plasticity region of the *Helicobacter pylori* genome. J Clin Microbiol, 2003; 41: 1651-5.

55. Labigne A, Lamouliatte H, Birac C. Distribution of the *cagA* gene among *Helicobacter pylori* strains associated with peptic ulcer. Am J Gastroenterol, 1994; 89: 1326.

56. Gerhard M, Lehn N, Neumayer N, Boren T, Rad R, Schepp W, Miehlke S, Classen M, Prinz C. Clinical relevance of the *Helicobacter pylori* gene for blood-group antigen-binding adhesin. Proc Natl Acad Sci USA, 1999; 96: 12778-83.

57. Cao P, Cover TL. Two different families of *hopQ* alleles in *Helicobacter pylori*. J Clin Microbiol, 2002; 40: 4504-11.

58. Boneca IG, de Reuse H, Epinat JC, Pupin M, Labigne A, Moszer I. A revised annotation and comparative analysis of *Helicobacter pylori* genomes. Nucleic Acids Res, 2003; 31: 1704-14.

59. Thiberge JM, Dillies MA, Coppée JY. Analyse comparative du génome de 120 souches de *H. pylori* associées à différentes pathologies. 2004. Abstract. Journées Francophones de Pathologie Digestive 2004.

60. Ruskone-Fourmestraux A. MALT gastric lymphomas. Rev Med Interne, 2004; 25: 573-81.

61. Bayerdorffer E, Neubauer A, Rudolph B, Thiede C, Lehn N, Eidt S, Stolte M. Regression of primary gastric lymphoma of mucosa-associated lymphoid tissue type after cure of *Helicobacter pylori* infection. MALT Lymphoma Study Group. Lancet, 1995; 345: 1591-4.

62. Savio A, Franzin G, Wotherspoon AC, Zamboni G, Negrini R, Buffoli F, Diss TC, Pan L, Isaacson PG. Diagnosis and posttreatment follow-up of *Helicobacter pylori*-positive gastric lymphoma of mucosa-associated lymphoid tissue: Histology, polymerase chain reaction, or both? Blood, 1996; 87: 1255-60.

63. Roggero E, Zucca E, Pinotti G. Eradication of *Helicobacter pylori* infection in primary low-grade gastric lymphoma of mucosa-associated lymphoid tissue. Ann Intern Med, 1995; 122: 767-9.

64. Fischbach W, Kolve M, Engemann R. Unexpected success of *Helicobacter pylori* eradication in low-grade lymphoma. Gastroenterology, 1996; 110.

65. Montalban C, Manzanal A, Boixeda D, Redondo C, Alvarez I, Calleja JL, Bellas C. *Helicobacter pylori* eradication for the treatment of low-grade gastric MALT lymphoma: Follow-up together with sequential molecular studies. Ann Oncol, 1997; 8: 37-9.

66. Pinotti G, Zucca E, Roggero E, Pascarella A, Bertoni F, Savio A, Savio E, Capella C, Pedrinis E, Saletti P, Morandi E, Santandrea G, Cavalli F. Clinical features, treatment and outcome in a series of 93 patients with low-grade gastric MALT lymphoma. Leuk Lymphoma, 1997; 26: 527-37.

67. Neubauer A, Thiede C, Morgner N, Alpen B, Ritter M, Neubauer B, Wundisch T, Ehninger G, Stolte M, Bayerdorffer E. Cure of *Helicobacter pylori* infection and duration of remission of low-grade gastric mucosa-associated lymphoid tissue lymphoma. J Natl Cancer Inst, 1997; 89: 1350-5.

68. Nobre-Leitao C, Lage P, Cravo M, Cabecadas J, Chaves P, Alberto-Santos A, Correia J, Soares J, Costa-Mira F. Treatment of gastric MALT lymphoma by *Helicobacter pylori* eradication: a study controlled by endoscopic ultrasonography. Am J Gastroenterol, 1998; 93: 732-6.

69. Steinbach G, Ford R, Glober G, Sample D, Hagemeister FB, Lynch PM, McLaughlin PW, Rodriguez MA, Romaguera JE, Sarris AH, Younes A, Luthra R, Manning JT, Johnson CM, Lahoti S, Shen Y, Lee JE, Winn RJ, Genta RM, Graham DY, Cabanillas FF. Antibiotic treatment of gastric lymphoma of mucosa-associated lymphoid tissue. An uncontrolled trial. Ann Intern Med, 1999; 131: 88-95.

70. Thiede C, Wundisch T, Neubauer B, Alpen B, Morgner A, Ritter M, Ehninger G, Stolte M, Bayerdorffer E, Neubauer A. Eradication of Helicobacter pylori and stability of remissions in low-grade gastric B-cell lymphomas of the mucosa-associated lymphoid tissue: results of an ongoing multicenter trial. Recent Results Cancer Res, 2000; 156: 125-33.

71. Fischbach W, Dragosics B, KolveGoebeler ME. Primary gastric B-cell lymphoma: Results of a prospective multicenter study. Gastroenterology, 2000; 119: 1191-202.

72. Ruskoné Fourmestraux A, Lavergne A, Aegerter PH, Megraud F, Palazzo L, de Mascarel A, Molina T, Rambaud JL. Predictive factors for regression of gastric MALT lymphoma after anti-*Helicobacter pylori* treatment. Gut, 2001; 48: 297-303.

73. de Jong D, Vyth Dreese F, Dellemijn T, Verra N, Ruskone-Fourmestraux A, Lavergne-Slove A, Hart G, Boot H. Histological and immunological parameters to predict treatment outcome of *Helicobacter pylori* eradication in low-grade gastric MALT lymphoma. J Pathol, 2001; 193: 318-24.

74. Matsushima Y, Kinoshita Y, Fukui H, Maekawa T, Yazumi S, Okada A, Nakase H, Kawanami C, Iwano M, Hashimoto K, Takeda Z, Okazaki K, Chiba T. Immunological and molecular analysis of B lymphocytes in low-grade MALT lymphoma of the stomach. Are there any useful markers for predicting outcome after *Helicobacter pylori* eradication? J Gastroenterol, 2002; 37: 428-33.

75. Diz-Lois Palomares MT, Souto Ruzo J, Yanez Lopez JA, Alvarez Garcia A, Arnal Monreal F, Vazquez-Iglesias JL. Early-stage gastric MALT lymphomas: eradication of *H. pylori* and outcome. Rev Esp Enferm Di, 2002; 94: 669-78.

Somatostatin – receptor mediated diagnosis and treatment in gastrointestinal neuroendocrine tumours (GEP-NET's)

Öberg K*

Department of Medical Sciences, Endocrine Oncology, University Hospital, Uppsala, Sweden

Key words: somatostatin, gastrointestional neuroendoctrine tumours (GEP-NET's).

Introduction

Neuroendocrine tumours constitute a group of tumours that originate from neuroendocrine cells throughout the body. That includes endocrine tumours of the thymus, lung, pancreas and gastrointestinal tract. Neuroendocrine gastrointestinal tumours have classically been divided into carcinoid tumours and endocrine pancreatic tumours. Many of these tumours produce hormones that can induce clinical symptoms in the patient [1]. Since these hormones are stored in secretory granules containing chromogranin A, a common feature for patients with neuroendocrine tumours is elevated levels of chromogranin A in plasma. Measurement of this hormone is a very sensitive marker for neuroendocrine tumours [2].

Patients with endocrine pancreatic tumours (EPT) can develop different syndromes according to the hormone produced. These syndromes include the Zollinger-Ellison syndrome for patients producing gastrin, the Verner-Morrison syndrome for patients with vasoactive intestinal peptide (VIP) production, the insulinoma syndrome due to excess of insulin/ /proinsulin and the glucagonoma syndrome for patients with high glucagon production respectively. A subgroup of endocrine pancreatic tumours (30-40%) does not produce any hormone that give rise to clinical symptoms and these tumours are called non-functioning endocrine pancreatic tumours.

Patients with gastrointestinal endocrine tumours have traditionally been divided into foregut (gastric, duodenal), midgut (ileal, jejunal, appendicial) and hindgut carcinoids (colonic and rectal). For patients with midgut carcinoid tumours the carcinoid syndrome becomes overt when the patient develops liver metastases. The carcinoid syndrome consists of flushes, diarrhea, the carcinoid heart disease and sometimes bronchial constriction. These tumours produce serotonin and tachykinins and the most frequently measured tumour marker is the urinary 5-HIAA (5-hydroxyindoleacetic acid) which is a degradation product of serotonin. Patients with foregut carcinoid tumours may produce several different hormones including gastrin, ACTH (adrenocorticotropin releasing hormone), ghrelin and somatostatin, and therefore the symptoms may vary considerably in these patients.

Recently a new classification has been proposed by WHO. The tumours are divided into well differentiated neuroendocrine tumours, well differentiated neuroendocrine carcinoma, tumours with uncertain behavior and low differentiated carcinoma. Proliferation index (Ki-67, MIB-1), angioinvasion and mitoses are important factors in the classification [3]. This classification is now being introduced into the clinic.

Somatostatin and somatostatin analogs

Somatostatin is a peptide hormone that was first isolated in 1973 [4]. The primary function attributed to this hormone was the inhibition of growth hormone release. Subsequently several other functions of somatostatin have been identified such as a general inhibitory effect on hormone release from endocrine cells, inhibition of secretion from the exocrine pancreas, reduction of gastrointestinal motility and a neurotransmitter function and immunoregulatory [5]. Already in 1978 the first report discussing the use of natural somatostatin for the treatment of a patient with a carcinoid tumour in order to reduce hormone related symptoms was published [6]. However, the use of natural somatostatin for long-term treatment is difficult since the half-life of this hormone is only 90 seconds necessitating continuous intravenous infusion. In the early 1980s the first long-acting

* CORRESPONDING AUTHOR:
Department of Medical Sciences
Endocrine Oncology, University Hospital
SE-751 85 Uppsala, Sweden
Tel: +46-18-611 49 17; Fax: +46-18-50 72 68
e-mail: kjell.oberg@medsci.uu.se (Kjell Öberg)

Received 30.05.2005 Accepted 13.06.2005

somatostatin analog, octreotide, was introduced for clinical use. Octreotide is an octapeptide with a half-life in the circulation of about 115 minutes after subcutaneous injection [7]. The initial clinical applications were acromegaly and hormone producing tumours of the gastrointestinal tract [8]. Beside octreotide, another somatostatin analog is now available, lanreotide [9]. Both these somatostatin analogs have the same receptor binding profile with a high affinity for somatostatin receptor subtypes 2 and 5.

The primary effect of the somatostatin analog treatment is to inhibit the secretion of excessive hormones produced by the tumour cells and thereby significantly reduce hormone related symptoms. In patients with carcinoid crises or the VIPoma syndrome, treatment with a somatostatin analog may be life-saving. This relief of symptoms is usually concomitant with reduced hormone levels detected in plasma and urine. In different studies, significantly reduced hormone levels are found in 50-70% of patients treated with somatostatin analogs. In most patients an improvement of quality-of-life can be seen following treatment with somatostatin analogs. Today long acting formulations of both octreotide (Sandostatin LAR®) and lanreotide exist (Somatuline Autogel®) [10].

In *in vitro* experiments somatostatin analogs have been shown to inhibit proliferation of many different tumour cell lines. It has been demonstrated a stabilization of tumour growth in 30-50% of patients possibly by reduction of growth promoting factors and inhibition of angiogenesis [11]. However, only very few patients (2-5%) respond by reduction in tumour size (50%) during treatment with somatostatin analogs [11].

New somatostatin analogs with different receptor subtype binding profiles and biological actions have been reported. It has been argued that analogs with specificity for new subsets of receptors or single somatostatin receptor subtypes may prove valuable for treatment of both malignant and non-malignant diseases. However, until now, only three analogs with very similar binding and biological profiles have been tested in clinical trials (octreotide, lanreotide and vapreotide). A new somatostatin analog is being introduced with a more universal binding profile than the previously available analogs. This new analog, SOM230, binds with high affinity to somatostatin receptors 1, 2, 3 and 5 [12] and effectively inhibits secretion of growth hormone from primary cultures of rat pituitary cells. The *in vivo* effect of growth hormone suppression in rats is also very high. In long-term studies, the reduction in IGF-1 levels in plasma was 75% in rats treated with SOM230 and 28% in animals treated with octreotide. The inhibitory effect on growth hormone secretion seems to be rather specific since insulin and glucagon levels were reduced only at high doses of SOM230. These results could also be confirmed in primates [13]. SOM230 is now applied in phase II trials in midgut carcinoids. In an early trial with patients with midgut carcinoid refractory to octreotide LAR, 30% responded to SOM230 at doses of 900 μg bid [14].

Somatostatin receptors

Somatostatin acts through specific receptors expressed on the plasma membrane. Five different subtypes have been cloned and they belong to the seven-transmembrane receptor superfamily [15-17]. The receptors are G protein-coupled but several other second messenger systems are also used for intracellular signal transduction. The somatostatin receptors are expressed in a tissue specific manner [18]. All somatostatin receptor subtypes can be found in the brain and in the endocrine cells of the pancreas. In the endocrine pancreatic cells the receptor expression shows considerable variability [19]. In the endocrine cells of the pancreatic islets somatostatin receptor subtypes 1, 3 and 4 are almost always expressed in all four cell types, while somatostatin receptor subtype 2 is found in most alpha cells and beta cells, but only in about half of the delta and very few PP (pancreatic polypeptide) cells. Somatostatin receptor subtype 5 is found in all beta cells and delta cells but almost never in alpha cells or PP cells. This expression pattern differs from the expression found in endocrine cells scattered throughout the exocrine pancreas and also from endocrine cells found in the ductal epithelium. In these locations fewer receptor subtypes can be identified although all receptor subtypes are present in some cells.

Somatostatin receptor subtype 2 is found in the gastrointestinal tract together with subtype 1 and 5. The receptor subtype most rarely expressed is subtype 4 that can be found in the lung, however, very little research has been directed towards this receptor and future studies may show other locations for this receptor as well. It has been shown that somatostatin receptor subtype 2 is the subtype mainly responsible for the inhibitory effect on hormone release from endocrine cells [18]. This action involves inhibition of cAMP formation and also a reduction in intracellular calcium levels leading to inhibition of hormone secretion. Patel et al. showed that somatostatin receptor subtype 3 might be involved in apoptosis [20]. In a recent paper another group has been able to couple induction of apoptosis to somatostatin receptor subtype 2 as well [21]. The increase of apoptosis was shown in a cell line, LH-60, that has a deletion of the p53 gene. Thus, in these cells the induction of apoptosis has to be independent of p53 accumulation.

Receptor subtypes 1, 2 and 5 may mediate a growth-inhibiting signal at least *in vitro* [22,23]. It has been shown that SHP-1 protein plays an important role in somatostatin mediated cell growth arrest signaled through receptor subtype 2 [24]. The activity of SHP-1 is stimulated by somatostatin receptor subtype 2 and the activated SHP-1 inhibits the cell proliferation by accumulation of hypophosphorylated retinoblastoma protein leading to growth arrest.

The possibility of G protein-coupled receptor to form dimers has been proposed previously. Somatostatin receptors have been shown to form dimers, both between different somatostatin receptor subtypes [25] and also with dopamine receptors [26]. The exact physiological function of this dimerization is not clear, but in experimental systems it has been shown that somatostatin receptor subtype 5 can form dimers with receptor subtype 1 but not with somatostatin receptor subtype 4. Thus, there seem to be some restriction as to the dimers that can be formed. This phenomenon was studied in transfected CHO-cells and it was shown that somatostatin receptor subtype 5 could be found as a monomer which was considered to be inactive and as a homodimer or a heterodimer with somatostatin receptor subtype 1 after stimulation of a ligand binding to the receptor

Table 1. **Expression of somatostatin receptor subtypes in neuroendocrine tumours examined by immunohistochemistry and RT-PCR**

	Sst1	Sst2	Sst3	Sst4	Sst5
Endocrine pancreatic tumours					
Papotti et al. [28]	30/33*	37/48	30/48	8/33*	29/48
Kulaksiz et al. [29]	21/69	54/69	54/69	ND	53/69
Oda et al. [31]	6/7	6/7	6/7	ND	ND
Midgut carcinoid tumour					
Papotti et al. [28]	12/13*	21/26	17/26	3/13*	21/26
Kulaksiz et al. [29]	13/35	30/35	26/35	ND	29/35

ND: not done; * indicates use of RT-PCR method

[25]. It was also shown that somatostatin receptor subtype 1 was internalized only as a heterodimer, and this might explain why normal endocrine cells adapt to somatostatin analogue treatment and maintain a normal responsiveness in cells that express all five receptor subtypes.

Somatostatin receptor expression in neuroendocrine tumours

The expression of somatostatin receptors in neuroendocrine tumours was first shown by Reubi in 1987 by autoradiography using somatostatin labeled with radioactive iodine [27]. After the somatostatin receptor became cloned, in situ hybridization and RT-PCR took the place of autoradiography. During the past few years polyclonal antibodies specific for the five different somatostatin receptor subtypes have been developed and there are now some reports of the somatostatin receptor expression in neuroendocrine tumours based on immunohistochemical staining. See *Tab. 1* for a summary of results concerning somatostatin receptor expression in different neuroendocrine tumours.

There are some papers reporting on the expression of somatostatin receptors in endocrine pancreatic tumours. Papotti et al. [28] have investigated the expression of somatostatin receptor subtypes by RT-PCR (all subtypes) and immunohistochemistry (subtypes 2, 3 and 5). They found that most tumours expressed somatostatin receptors subtype 1, 2, 3 and 5. However, only a minority of tumours expressed receptor subtype 4. They reported that all patients included with gastrinomas and glucagonomas expressed somatostatin receptor subtype 2 while all somatostatinomas expressed receptor subtype 5. The expression in insulinomas was, however, variable.

Another group has also reported on the expression of somatostatin receptors 1, 2, 3 and 5 in endocrine pancreatic tumours [29]. They found a high expression of receptor subtypes 2, 3 and 5 while the expression of subtype 1 was intermediate. They did not investigate the somatostatin receptor subtype 4 expression. For details see *Tab. 1*.

In carcinoid tumours, the expression of somatostatin receptor subtype 2 has been correlated to responsiveness to treatment with somatostatin analogs [30]. In a recent publication the predictive value of somatostatin receptor expression was investigated in patients with endocrine pancreatic tumours [31]. Tumour specimens from seven patients with endocrine pancreatic tumours were stained for somatostatin receptor subtypes 1, 2 and 3. The only patient responding to octreotide injection was a patient with an insulinoma who had a very strong expression of somatostatin receptor subtype 2. In this patient octreotide could reduce the hypoglycemias. In this report most patients expressed all three subtypes that were examined, but the expression was often rather weak.

Both Papotti and Kulaksiz have reported on the expression of somatostatin receptors in carcinoid tumours as well [28,29]. The results are similar to the results for endocrine pancreatic tumours with a high expression for subtypes 2, 3 and 5 reported by both authors. Papotti reported a high expression also for receptor subtype 1 while this was low in the paper by Kulaksiz. Somatostatin receptor subtype 4 was only investigated by RT-PCR in Papottis' work and showed a low expression. A considerable variation in the receptor expression was not only seen between patients, but also within the same patient's tumour tissue sample.

Imaging

Somatostatin analogs can be labeled with radioactive isotopes, injected intravenously and the distribution of tracer can subsequently be detected with a gamma camera. Octreoscan is the most frequently used method, and today this investigation is included in the basic work-up of patients with neuroendocrine tumours [32]. The investigation will give information both about the receptor status of the tumour and also of the tumour spread. This information is used when decisions are made about the treatment of the patients. Patients with somatostatin receptor expressing tumours usually respond to treatment with somatostatin analogs, while those that lack such expression usually fail to respond with a decrease in hormone levels [32].

The use of somatostatin receptor scintigraphy for radio nuclear imaging of neuroendocrine tumours has been compared with other diagnostic methods. A comparison was made between ^{123}I-meta-iodobenzylguanidine (^{123}I-MIBG) and ^{111}In-pentetreotide in 54 patients with different neuroendocrine tumours [34]. The difference in sensitivity in detecting metastases seen on computerized tomography or magnetic resonance tomography was investigated. It was shown that ^{111}In-pentetreotide was more sensitive than ^{123}I-MIBG with a detection rate of 67% vs 50% for carcinoid tumours and 91% vs 9% for endocrine pancreatic tumours. These differences may in part be explained by the fact that the two different methods reflect different biological features and in some patients they might be complementary to each other. For patients with midgut carcinoid tumours and endocrine pancreatic tumours both spread of disease and somatostatin receptor status might be of importance and therefore an ^{111}In-pentetreotide scintigraphy should be performed.

In another investigation, fluorodeoxyglucose (FDG) positron emission tomography (PET) and somatostatin receptor scintigraphy were compared in patients most of whom had carcinoid tumours [35]. In this study FDG PET was performed

in 17 patients and somatostatin receptor scintigraphy in 16 patients. Most patients had typical carcinoid tumours with a low proliferation rate. FDG PET correctly confirmed 4/7 primary tumours and 8/11 metastatic lesions, while somatostatin receptor scintigraphy identified 6/7 primary tumours and 10/11 metastases. There was no correlation between the tracer uptake in tumour lesions and histological features such as proliferation rate measured by Ki-67 or p53 expression. It was concluded that somatostatin receptor scintigraphy should be performed in most patients and that FDG PET should be used in patients that are negative at somatostatin receptor scintigraphy. In a most recent study PET with C^{11}-5HTP had significantly higher sensitivity than Octreoscan® in patients with neuroendocrine GEP-NET's [36].

Treatment with somatostatin analogs

Midgut carcinoid tumours

A large number of clinical trials with octreotide in midgut carcinoid tumours have been published [37-39]. The reported subjective and histochemical response rate has been between 40% and 60% when the tumor responses have been less than 5%. In patients with midgut carcinoid tumours the use of somatostatin analogs has been considered to be first line treatment in the presence of a carcinoid syndrome. In some studies the somatostatin analog is combined with alpha-interferon [40]. Aparicio et al. recently discussed the use of somatostatin analogs in neuroendocrine tumours [41]. They used somatostatin analogs (octreotide 100 μg thrice daily and/or lanreotide 30 mg every 14 days) in 35 patients with progressive disease, 12 midgut carcinoid patients, 13 patients with endocrine pancreatic tumours, 5 with primary tumours in the lung and 5 patients with other locations of the primary tumour. A partial reduction in tumour size was observed in one patient, while the tumour growth was stabilized in 20 other patients. The authors also divided the patients into those with tumours with a high proliferation rate and those with a low proliferation rate. They found a significantly lower response rate in patients with rapidly progressing tumours (4/12) as compared to patients with slowly growing tumours (13/17), $p<0.02$. The median duration of treatment in this study was 7 months. The dose of somatostatin analog might be critical. Ultra high doses of somatostatin analogs have demonstrated significant clinical benefit in patients resistant to standard doses of octreotide or lanreotide [42,43]. Guidelines for the use of octreotide in clinical practice have recently been published [44].

Endocrine pancreatic tumours

The use of somatostatin analogs in the treatment of endocrine pancreatic tumours is well established. Single treatment with somatostatin analogs produces good symptomatic and biochemical responses, but the effect on tumour size is disappointing with only about 5% objective responses. The use of a combination of somatostatin analogs and alpha-interferon has been proposed as a possible strategy to control both hormone symptoms and tumour growth. Such a combination was reported on by Frank et al. [45]. In their study 21 patients with progressing tumours were included. One patient had a decrease in tumour size while 60% of the patients remained stable for a median of 12 months. However, their material was a mixed material with several different tumour groups and they used response criteria that are not commonly accepted, designating a reduction in tumour size by >30% as an objective response. Therefore, results from this study are difficult to compare with results from other groups.

Another study investigating the potential of this combination to control both symptoms and tumour growth has been presented [46]. Only patients with malignant endocrine pancreatic tumours were included. A total of 16 patients were treated with a combination of a somatostatin analog (octreotide or somatuline) and alpha-interferon (interferon-alpha2b, lymphoblastoid interferon or human leukocyte interferon). During treatment with this combination 3 patients (19%) showed a reduction in tumour size by >50% for about 2 years (19-25 months). Eleven patients showed stabilization of tumour size for 13 months (4-32 months) while 2 patients continued to progress. In 62.5% (10 of 16) of the patients a biochemical response was detected for a median of 22 months (range 10-32 months). Five patients remained stable for 9 months (4-20 months) and only one patient progressed. From this study it seems that the combination can be used with good results in patients with endocrine pancreatic tumours and can be considered as an alternative for patients who do not want to receive chemotherapy as first line treatment.

In another study 15 patients with malignant gastrinomas were treated with octreotide [47]. All patients had liver metastases and were in a progressive state. The patients were treated with octreotide 200 μg twice daily and were eventually switched over to long-acting release octreotide 20-30 mg every month. After 3 months of treatment 7 patients (47%) had stabilization of their previously progressive disease and one patient had a reduction in tumour size (6%). The mean duration of response was 25 months (range 5.5-54.1 months) and six of the eight responders were still responding at the time of last follow-up. This response could not be correlated to pre-study clinical parameters such as tumour extent, gastrin levels or acid secretory rates. Patients with slow-growing tumours tended to have a higher response rate. During follow-up only 25% of patients responding to somatostatin analog treatment died as compared to 71% of the non-responders. The authors claimed that octreotide is an effective antitumour treatment and might be considered early in the treatment of patients with endocrine pancreatic tumours.

Tumour targeting

During the last few years the same substances that are used for somatostatin receptor scintigraphy have been used for high-dose radioactive tumour targeting therapy. There have been some reports on small clinical trials with good results both on hormone levels and on tumour size [48,49]. The toxicity is mainly limited to impairment of kidney function and bone marrow suppression with a decrease in platelets and white blood cells.

In a phase II study, 41 patients with neuroendocrine gastroenteropancreatic and bronchial carcinoid tumours were included [50]. Thirty-four patients had progressive disease.

They were treated with four courses of ^{90}Y-DOTATOC up to a total dose of 6000 MBq/m^2. Complete remission was found in 1 patient while 9 of 41 showed a partial response. A minor response was observed in 5 patients and 6 patients progressed. The rest of the patients remained stable. The median duration of response was not reached after 26 months of follow-up and the two-year survival was 76%. A reduction in morphine dependent tumour-associated pain was observed and 83% of patients with a carcinoid syndrome had a reduction in symptoms. Side-effects included grade III pancytopenia in 5% and vomiting in 23% of patients.

In another study the same group reported on a similar study including 39 patients with neuroendocrine tumours, treated with 7.4 GBq/m^2 of ^{90}Y-DOTATOC [51]. The response rates were within the same range with only 3 patients progressing and 2 patients showing a complete remission. Bone marrow suppression was seen in the same range as in the previous study. However, in this study one patient developed grade 2 renal insufficiency.

^{111}In-pentetreotide can also be used to treat patients with neuroendocrine tumours. In a small study, 27 patients with advanced gastroenteropancreatic tumours who had failed all forms of conventional therapy were treated with at least 2 monthly injections of 180 mCi ^{111}In-pentetreotide [52]. A total of 16 patients were considered to have clinical benefit from the treatment. A radiological response was found in 2 patients and tumour necrosis in 7 patients. Tumour markers decreased by >50% in 81% of the patients. An inclusion criterion for entering this study was that patients should have less than 6 months expected survival. The median survival after treatment was 18 months (range 3-54 months) and the treatment was well tolerated. Thus, the authors argued that treatment with ^{111}In-pentetreotide might prolong the survival in these severely ill patients.

The adverse reactions to tumour targeting treatment with radioactive somatostatin analogues mainly affects bone marrow and kidney function. In a report concerning a patient with midgut carcinoid tumours treated with ^{90}Y-DOTATOC, a severe deterioration of kidney function occurred 15 months after treatment was discontinued [53]. The patient had received 4 doses of ^{90}Y-DOTATOC reaching a cumulative dose of 9.62 MBq. Injections were administrated every 6th week. In an attempt to prevent renal toxicity, the patient received an amino acid solution, Hartmann-Hepa 8%, together with the fourth treatment cycle. Before and during treatment the patient had normal levels of serum creatinine and urea nitrogen. After 15 months a progressive deterioration of renal function was observed leading to end-stage renal disease. The patient was treated with intermittent haemodialysis when creatinine clearance declined to less than 10 ml/min. A contributing factor to this renal failure might be that the patient did not receive treatment with amino acids until the last treatment period.

In nuclear medicine, cationic amino acids are used in order to prevent renal damage from high doses of radioactive isotopes. The hypothesis is that positively charged amino acids bind to the negatively charged sites in the renal tubular cells and hence decrease the tubular reabsorption and further degradation of the injected conjugate. It has been shown that infusion of arginine and lysine can reduce the kidney uptake of ^{111}In-pentetreotide and radiolabeled Fab-fragments both in experimental models and in patients [54,55].

In another report, 5 patients treated with ^{90}Y-DOTATOC developed renal failure [56]. In three of these patients a kidney biopsy could be performed showing a thrombotic microangiopathy. This pathological-anatomical diagnosis is the same as the picture seen in patients receiving external radiotherapy. In these patients the renal failure became overt only 3 months after the last injection of ^{90}Y-DOTATOC. This severe adverse reaction indicates that further studies are needed to understand how kidney protection should be administrated and the level and frequency of doses of ^{90}Y-DOTATOC that should be used.

New isotopes will be tested in the future in order to treat patients with somatostatin receptor expressing tumours. One such new isotope is 177-lutetium (^{177}Lu), a beta- and gamma-emitting radionuclide. An advantage of this radionuclide as compared to 90-yttrium is that it has a shorter penetration in tissue, making it more suitable for treatment of small tumours. The somatostatin analog DOTA-0-Tyr3-octreotate binds with a very high affinity to somatostatin receptor subtype 2 and can be labeled with ^{177}Lu. In animal experiments with a rat model, ^{177}Lu-octreotate had a favorable impact on survival. In a study comparing the uptake of the two different radioactive compounds, ^{90}Y-DOTATOC and ^{177}Lu-octreotate, performed in six patients with somatostatin receptor expressing tumours, the uptake in spleen, liver and kidney was equal, while the tumour uptake was three to fourfold higher in four of five patients from ^{177}Lu-octreotate [57]. Also in this study, infusion of amino acids reduced the kidney radiation dose by almost 50%. Thus, a higher absorbed dose can be obtained in most tumours without increase in doses absorbed by potentially dose-limiting organs. In a recent paper by Kwekkeboom and co-workers, the results of treatment of 131 patients with neuroendocrine tumours, treated with cumulative doses up to 600-800 m Ci of ^{177}Lu-Octreotate. Complete remission was obtained in 2%, partial remission in 26%, minor responses in 19%, stable disease in 35% and progression in 18%. Hematology toxicity occurred in less than 2% and renal insufficiency in one patient and hepatorenal syndrome in another [58].

Future aspects

SOM230 is a new somatostatin analog with high affinity for somatostatin receptor subtype 1, 2, 3 and 5 that has entered early clinical studies and results will soon be available. Much effort is also being placed into development of methods to investigate the expression of the different somatostatin receptors in normal and malignant tissue. Several groups have developed receptor subtype specific antibodies that can be used in immunohistochemistry and hopefully these will soon be included in the diagnostic work-up. The use of different radioactively labeled somatostatin analogs is also a subject which draws much attention at the moment and new trials with new analogs and new isotopes will be initiated very soon. In the future mixtures of isotopes might be used in order to treat both very small and

medium sized metastases at the same time. Radio sensitizers that may improve the effect of radioactive targeting therapy are also being developed.

References

1. Öberg K. Neuroendocrine gastrointestinal tumors – a condensed overview of diagnosis and treatment. Ann Oncol, 1999; 10: S3-8.
2. Stridsberg M, Eriksson B, Öberg K, Janson ET. A comparison between three commercial kits for chromogranin A measurements. J Endocrinol, 2003; 177: 337-41.
3. Rindi G, Capella C, Solcia E. Cell biology, clinicopathological profile, and classification of gastro-enteropancreatic endocrine tumors. J Mol Med, 1998; 76: 413-20.
4. Brazeau P, Vale W, Burgus R, Ling N, Butcher M, Rivier J, Guillemin R. Hypothalamic polypeptide that inhibits the secretion of immunoreactive pituitary growth hormone. Science, 1973; 179: 77-9.
5. Reichlin S. Somatostatin I and II. N Engl J Med, 1983; 309: 1495-501, 1556-63.
6. Thulin L, Samnegård H, Tydén G, Long DH, Efendic S. Efficacy of somatostatin in a patient with a carcinoid syndrome. Lancet, 1978; 2: 43.
7. Bauer W, Briner U, Doepfner W, Haller R, Huquenin R, Marbach P, Petcher TJ, Pless M. SMS 201-995: a very potent and selective octapeptide analogue of somatostatin with prolonged action. Life Sciences, 1982; 31: 1133-40.
8. Lamberts SWJ, van der Lely AJ, de Herder WW, Hofland LJ. Octreotide. N Engl J Med, 1996; 334: 246-54.
9. Heron I, Thomas F, Dero M, Gancel A, Ruiz JM, Schatz B, Kuhn JM. Pharmacokinetics and efficacy of a long-acting formulation of the new somatostatin analogue BIM 23014 in patients with acromegaly. J Clin Endocrinol Metab, 1993; 76: 721-7.
10. Lancranjan I, Bruns C, Grass P. Sandostatin LAR: Pharmacokinetics, pharmacodynamics, efficacy, and tolerability in acromegalic patients. Metabolism, 1995; 44(Suppl. 1): 18-26.
11. Patel YC. Somatostatin and its receptor family. Front Neuroendocrinol, 1999; 20: 157-98.
12. Bruns C, Lewis I, Briner U, Meno-Tetang G, Weckbecker G. SOM230: a novel somatostatin peptidometic with broad somatotropin release inhibiting factor (SRIF) receptor binding and a unique antisecretory profile. Eur J Endocrinol, 2002; 146: 707-16.
13. Weckbecker G, Briner U, Lewis I, Bruns C. SOM230: a new peptidomimetic with potent inhibitory effects on the growth hormone/ /insulin-like growth factor-1 axis in rats, primates and dogs. Endocrinol, 2002; 143: 4123-30.
14. Kvols L, Öberg K, de Herder W. Early data on the efficacy and safety of the novel multi-ligand somatostatin analog, SOM230 in patients with metastatic carcinoid tumours refractory or resistant to octreotide LAR. ASCO, 2005; abstr. 8024.
15. Yamada Y, Post SR, Wang K, Tager HS, Bell GI, Seino S. Cloning and functional characterization of a family of human and mouse somatostatin receptors expressed in brain, gastrointestinal tract, and kidney. Proc Natl Acad Sci, 1992; 89: 251-5.
16. Yamada Y, Reisine T, Law SF. Somatostatin receptors, an expanding gene family: Cloning and functional characterization of human SSTR3, a protein coupled to adenylyl cyclase. Mol Endocrinol, 1992; 6: 2136-42.
17. Yamada Y, Kagimoto S, Kubota A, Yasuda K, Masuda K, Someya Y, Ihara Y, Li Q, Imura H, Seino S. Cloning, functional expression and pharmacological characterization of a fourth (hSSTR4) and a fifth (hSSTR5) human somatostatin receptor subtype. Biochem Biophys Res Commun, 1993; 195: 844-52.
18. Patel YC. Molecular pharmacology of somatostatin receptor subtypes. J Endocrinol Invest, 1997; 20: 348-67.
19. Portela-Gomes GM, Stridsberg M, Grimelius L, Öberg K, Janson ET. Expression of the five different somatostatin receptor subtypes in endocrine cells of the pancreas. App Immunohist Mol Morphol, 2000; 8: 126-32.
20. Sharma K, Patel YC, Srikant CB. Subtype-elective induction of wild-type p53 and apoptosis, but not cell cycle arrest, by human somatostatin receptor 3. Mol Endocrinol, 1996; 10: 1688-96.
21. Teijeiro R, Ríos R, Costoya JA, Castro R, Bello JL, Devesa J, Arce VM. Activation of human somatostatin receptor 2 promotes apoptosis through a mechanism that is independent from induction of p53. Cell Physiol Biochem, 2002; 12: 31-8.
22. Buscail L, Delesque N, Estève JP, Saint-Laurent N, Prats H, Clerc P, Robberecht P, Bell GI, Liebow C, Schally AV. Stimulation of tyrosine phosphatase and inhibition of cell proliferation by somatostatin analogues: Mediation by human somatostatin receptor subtypes SSTR1 and SSTR2. Proc Natl Acad Sci, 1994; 91: 2315-9.
23. Buscail L, Estève JP, Saint-Laurent N, Bertrand V, Reisine T, O'Carroll AM, Bell GI, Schally AV, Vaysse N, Susini C. Inhibition of cell proliferation by the somatostatin analogue RC-160 is mediated by somatostatin receptor subtypes SSRT2 and SSTR5 through different mechanisms. Proc Natl Acad Sci, 1995; 92: 1580-4.
24. Pages P, Benali N, Saint-Laurent N, Estève JP, Schally AV, Tkaczuk J, Vaysse N, Susini C, Buscail L. Sst2 somatostatin receptor mediates cell cycle arrest and induction of p27 (Kip1). Evidence for the role of SHP-1. J Biol Chem, 1999; 274: 15186-93.
25. Rocheville M, Lange DC, Kumar U, Sasi R, Patel RC, Patel YC. Subtypes of the somatostatin receptor assemble as functional homo- and heterodimers. J Biol Chem, 2000; 275: 7862-9.
26. Rocheville M, Lange DC, Kumar U, Patel Z, Patel RC, Patel YC. Receptors for dopamine and somatostatin: formation of hetero-oligomers with enhanced functional activity. Science, 2000; 288: 154-7.
27. Reubi JC, Kvols LK, Waser B, Nagorney DM, Heitz PU, Charboneau JW, Reading CC, Moertel C. Detection of somatostatin receptors in surgical and percutaneous needle biopsy samples of carcinoids and islet cell carcinomas. Cancer Res, 1990; 50: 5969-77.
28. Papotti M, Bongiovanni M, Volante M, Allia E, Landolfi S, Helhoe L, Schindler M, Cole SL, Bussolati G. Expression of somatostatin receptor types 1-5 in 81 cases of gastrointestinal and pancreatic endocrine tumors. A correlative immunohistochemical and reverse-transcriptase polymerase chain reaction analysis. Virchows Archiv, 2002; 440: 461-75.
29. Kulaksiz H, Eissele R, Rössler D, Schulz S, Hollt V, Cetin Y, Arnold R. Identification of somatostatin receptor subtypes 1, 2A, 3 and 5 in neuroendocrine tumours with subtype specific antibodies. Gut, 2002; 50: 52-60.
30. Janson ET, Stridsberg M, Gobl A, Westlin JE, Öberg K. Determination of somatostatin receptor subtype 2 in carcinoid tumors by immunohistochemical investigation with somatostatin receptor subtype 2 antibodies. Cancer Res, 1998; 58: 2375-8.
31. Oda Y, Tanaka Y, Naruse T, Sasanabe R, Tsubamoto M, Funahashi H. Expression of somatostatin receptor and effects of somatostatin analog on pancreatic endocrine tumors. Surg Today, 2002; 32: 690-4.
32. Krenning EP, Kooij PP, Pauwels S, Breeman WA, Postema PT, De Harder WW, Valkema R, Kwekkeboom D. Somatostatin receptor: Scintigraphy and radionuclide therapy. Digestion, 1996; 57(S1): 57-61.
33. Janson ET, Westlin JE, Eriksson B, Ahlström H, Nilsson S, Öberg K. [111In-DTPA-D-Phe]-octreotide scintigraphy in patients with carcinoid tumours: the predictive value for somatostatin analogue treatment. Eur J of Endocrinol, 1994; 131: 577-81.
34. Kaltsas G, Korbonits M, Heintz E, Mukherjee JJ, Jenkins PJ, Chew SL, Reznek R, Monson JP, Besser GM, Foley R, Britton KE, Grossman AB. Comparison of somatostatin analog and meta-iodobenzylguanidine radionuclides in the diagnosis and localization of advanced neuroendocrine tumors. J Clin Endocrinol Metab, 2001; 86: 895-902.
35. Belhocine T, Foidart J, Rigo P, Najjar F, Thiry A, Quatresooz P, Hustinx R. Fluorodeoxyglucose positron emission tomography and somatostatin receptor scintigraphy for diagnosing and staging carcinoid tumours: correlations with the pathological indexes p53 and Ki-67. Nucl Med Comm, 2002; 23: 727-34.
36. Orlefors H, Sundin A, Ahlstrom H, Bjurling P, Bergstrom M, Lilja A, Langstrom B, Öberg K, Eriksson B. Positron emission tomography with 5-hydroxytryprophan in neuroendocrine tumors. J Clin Oncol, 1998; 16: 2534-41.
37. Öberg K. Chemotherapy and biotherapy in neuroendocrine tumors. Curr Opin Oncol, 1993; 5: 110-20.
38. Lamberts SW, van der Lely AJ, de Herder WW, Hofland LJ. Octreotide. N Engl J Med, 1996; 334: 246-54.
39. Gorden P, Comi RJ, Maton PN, Go VL. NIH conference. Somatostatin and somatostatin analogue (SMS 201-995) in treatment of hormone-secreting tumors of the pituitary and gastrointestinal tract and non-neoplastic diseases of the gut. Ann Intern Med, 1989; 110: 35-50.
40. Janson ET, Ahlström H, Andersson T, Öberg K. Octreotide and interferon-α: A new combination for the treatment of malignant carcinoid tumors. Eur J Cancer, 1992; 28A: 1647-50.
41. Aparicio T, Ducreux M, Baudin E, Sabourin JC, De Beare T, Mitry E, Schlumberger M, Rougier P. Antitumor activity of somatostatin analogues in progressive metastatic neuroendocrine tumours. Eur J Cancer, 2001; 37: 1014-9.
42. Welin SV, Janson ET, Sundin A, Stridsberg M, Lavenius E,

Granberg D, Skogseid B, Öberg KE, Eriksson BK. High-dose treatment with a long-acting somatostatin analogue in patients with advanced midgut carcinoid tumours. Eur J Endocrinol, 2004; 151: 107-12.

43. Faiss S, Wiedenmann B. Dose-dependent and antiproliferative effects of somatostatin. J Endocrinol Invest, 1997; 20: 68-70.

44. Öberg K, Kvols L, Caplin M, Delle Fave G, de Herder W, Rindi G, Ruszniewski P, Woltering EA, Wiedenmann B. Consensus report on the use of somatostatin analogs for the management of neuroendocrine tumors of the gastroenteropancreatic system. Ann Oncol, 2004; 15: 966-73.

45. Frank M, Klose KJ, Wied M, Ishaque N, Schade-Brittinger C, Arnold R. Combination therapy with octreotide and alpha-interferon: Effect on tumor growth in metastatic endocrine gastroenteropancreatic tumors. Am J Gastroenterol, 1999; 94: 1381-7.

46. Fjällskog ML, Sundin A, Westlin JE, Öberg K, Janson ET, Eriksson B. Treatment of malignant endocrine pancreatic tumors with a combination of alpha-interferon and somatostatin analogs. Med Oncol, 2002; 19: 35-42.

47. Shojamanesh H, Gibril F, Louie F, Ojeaburu JV, Bashir S, Abou-Saif A, Jensen RT. Prospective study of the antitumor efficacy of long-term octreotide treatment in patients with metastatic gastrinoma. Cancer, 2002; 94: 331-43.

48. McCarthy KE, Woltering EA, Espenan GD, Cronin M, Maloney TJ, Anthony LB. In situ radiotherapy with 111In-Pentetreotide: Initial Observations and future directions. Cancer J Sci Am, 1998; 4: 94-102.

49. Janson ET, Eriksson B, Öberg K, Skogseid B, Ohrvall V, Nilsson S, Westlin JE. Treatment with high dose Octreoscan in patients with neuroendocrine tumors-evaluation of therapeutic and toxic effects. Acta Oncol, 1999; 38: 373-7.

50. Waldherr C, Pless M, Maecke HR, Haldemann A, Mueller-Brand J. The clinical value of 90Y-DOTA-D-Phe1-Tyr3-octreotide, 90Y-DOTATOC, in the treatment of neuroendocrine tumours: A clinical phase II study. Ann Oncol, 2001; 12: 941-5.

51. Waldherr C, Plesss M, Maecke HR, Schumacher T, Crazzolara A, Nitzsche EU, Haldemann A, Mueller-Brand J. Tumor response and clinical benefit in neuroendocrine tumors after 7.4 GBq ^{90}Y-DOTATOC. J Nucl Med, 2002; 43: 610-6.

52. Anthony LB, Woltering EA, Espenan GD, Cronin MD, Maloney TJ, McCarthy KE. Indium-111-pentetreotide prolongs survival in gastroenteropancreatic malignancies. Sem Nucl Med, 2002; 32: 123-32.

53. Cybulla M, Weiner SM, Otte A. End-stage renal disease after treatment with ^{90}Y-DOTATOC. Eur J Nucl Med, 2001; 28: 1552-4.

54. Bernard BF, Krenning EP, Breeman WAP, Rolleman EJ, Bakker WH, Visser TJ, Macke H, de Jong M. D-lysine reduction of Indium-111 octreotide and Yttrium-90 octreotide renal uptake. J Nucl Med, 1997; 38: 1929-33.

55. Behr TM, Sharkey RM, Sgouros G, Blumenthal RD, Dunn RM, Kolbert K, Griffiths GL, Siegel JA, Becker WS, Goldenberg DM. Overcoming the nephrotoxicity of radiometal-labeled immunoconjugates – improved cancer therapy administered to a nude mice model in relation to the internal radioation dosimetry. Cancer, 1997; 80: 2591-610.

56. Moll S, Nickeleit V, Mueller-Brand J, Brunner FP, Maecke HR, Mihatsch MJ. A new cause of renal trombotic microangiopathy: yttrium 90-DOTATOC internal radiopathy. Am J Kidney Dis, 2001; 37: 847-51.

57. Kwekkebom DJ, Bakker WH, Kooij PP, Konijnenberg MW, Srinivasan A, Erion JL, Schmidt MA, Bugaj JL, De Jong M, Krening EP. 117lu-DOTAOtyr3-octreotate: comparison with 111In-DTPAo-octreotide in patients. Eur J Nucl Med, 2001; 28: 1319-25.

58. Kwekkebom DJ, Teunissen JJ, Bakker WH, Kooij PP, de Herder WW, Feelders RA, van Eijck CH, Esser JP, Kam BL, Krenning EP. Radiolabeled somatostatin analog [177Lu-DOTA0,Tyr3]octreotate in patients with endocrine gastroenteropancreatic tumors. J Clin Oncol, 2005; 23: 2754-62.

Important clues to the diagnosis of pancreatic cancer

Tanaka M*

Department of Surgery and Oncology, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan

Abstract

The incidence of pancreatic carcinoma is recently increasing but the prognosis remains extremely poor. Widespread awareness of important clues to the diagnosis is particularly important to improve the prognosis. Dilatation of the main pancreatic duct on ultrasonograms and/or CT scans, hyperamylasemia incidentally found during routine blood examinations, and recent onset diabetes mellitus must lead to thorough imaging studies of the pancreas. Death from pancreatic carcinoma occurs in 0.2-1.9% of all diabetic patients, being more than 300 times frequent compared to general population. Diabetes may be the only clinical sign of pancreatic carcinoma in some patients. In our recent study, of 163 diabetic patients selected by several criteria who underwent ERCP screening, 12 patients (7.4%) proved to have pancreatic carcinoma. The prevalence of pancreatic carcinoma was more frequent in those with a recent onset (<3 years) of diabetes (13.7% (8/58)) than in those with a longer history (>3 years, 3.8% (4/105)). Furthermore, intraductal papillary mucinous neoplasm (IPMN) is reported to be associated with pancreatic carcinoma. Concomitant carcinoma was found in 9 of our series of 94 patients (9.5%) who underwent surgical resection of branch duct IPMN. Of particular interest is the fact that two of the 9 patients had carcinoma in situ that could be diagnosed only by cytology of the pancreatic juice. IPMN may be the only clue to the early diagnosis of pancreatic carcinoma presenting with no clinical symptoms or abnormalities on imaging studies.

* CORRESPONDING AUTHOR:
Department of Surgery and Oncology
Graduate School of Medical Sciences
Kyushu University, Maidashi 3-1-1
Fukuoka 812-8582, Japan
Fax: +81-92-642-5457
e-mail: masaotan@med.kyushu-u.ac.jp (Masao Tanaka)

Received 04.05.2005 Accepted 06.05.2005

Key words: pancreatic cancer, early diagnosis, diabetes, intraductal papillary mucinous neoplasm.

Introduction

The incidence of pancreatic carcinoma is increasing probably due to aging of the general population and/or some other reasons, but the early diagnosis remains difficult. There are a few fortunate patients who are incidentally diagnosed as having small pancreatic carcinoma due to the advent of a variety of imaging studies, but most of the patients with pancreatic carcinoma are diagnosed in a far-advanced stage because they do not present with specific symptoms and usual work-up imaging studies do not include the pancreas. The extremely high malignant potential of pancreatic carcinoma and its early extrapancreatic invasion due to the retroperitoneal location readily make it unresectable, keeping the prognosis dismal. A high index of suspicion and awareness of a variety of diagnostic clues are essential to make early diagnosis of pancreatic carcinoma. This is an overview of various important clues to the diagnosis of this disease entity, i.e. 1) dilation of the pancreatic duct and/or hyperamylasemia, 2) diabetes mellitus, and 3) intraductal papillary mucinous neoplasms (IPMN) of the pancreas. In particular, the importance of IPMN as a new hint for the diagnosis is emphasized.

1. Dilation of the pancreatic duct and/or hyperamylasemia

The most frequent key to the diagnosis of pancreatic carcinoma is dilation of the main pancreatic duct detected by US and/or CT. If this dilation is erroneously considered as chronic pancreatitis and further examinations are not conducted, the diagnosis of pancreatic carcinoma would be missed. Diffuse main pancreatic duct dilation tends to be regarded as chronic pancreatitis; however, thorough examinations to negate the

presence of periampullary carcinoma must be performed before any treatment or follow-up as chronic pancreatitis is begun. Hyperamylasemia and/or hyperamylasuria have similar significance and can be the first clue to the diagnosis of pancreatic carcinoma, because these findings can be found by routine blood and urine chemistry. Since the amylase level may vary from day to day, an attitude toward a thorough examination even of one time detection of hyperamylasemia or hyperamylasuria is important.

On the other hand, there has been much controversy as to carcinogenesis in chronic pancreatitis. Chronic inflammation is reported to lead to carcinogenesis in patients with hereditary and nonhereditary chronic pancreatitis. A genetic injury and cell overgrowth caused by chronic persistent inflammation may lead to carcinogenesis by increased cell cycles due to production of inflammatory mediators such as cytokines, superoxide, and NF-κB and cyclooxygenase [1-3]. On the other hand, there were some reports that none of patients with chronic pancreatitis developed pancreatic cancer at long term. Gambill [4] found no single case of pancreatic cancer in their series of 56 patients with chronic pancreatitis followed-up for >20 years. Oguchi et al. [5] also described that none of 53 patients with pancreatolithiasis had pancreatic cancer during observation up to 16 years. A few groups of authors reported that pancreatic cancer occurred in only 1-2% of patients with chronic pancreatitis [6-8]. Therefore, whether persistent inflammation of chronic pancreatitis leads to carcinogenesis or not remains controversial. However, it can be said that diffuse dilation of the main pancreatic duct seemingly due to chronic pancreatitis should prompt thorough examinations of the pancreas to negate the presence of pancreatic cancer.

2. Diabetes mellitus

DiMagno [9] listed recent onset diabetes mellitus as well as chronic pancreatitis, intraductal papillary mucinous neoplasm (IPMN), hereditary pancreatic cancer, hereditary chronic pancreatitis, familial adenomatous polyposis of the colon and hereditary dysplastic nevus syndrome as a high-risk group of pancreatic cancer. He emphasized the importance of recognition of the high-risk population for early diagnosis of pancreatic cancer by reviewing the fact that 15% of all patients with pancreatic cancer had sought medical advice more than 6 months before the diagnosis of cancer but spent that useless period, undergoing various examinations under suspicion of other diseases.

The rate of death from pancreatic cancer reaches 0.2-1.9% in diabetic patients and obviously higher than 0.004-0.008% in ordinary population [10]. Diabetes results from endocrine impairment due to upstream pancreatitis caused by obstruction of the main pancreatic duct, extensive replacement of pancreatic parenchyma by cancer, and/or effects of amylin to raise blood sugar or islet amyloid polypeptide (IAPP), that increases resistance to insulin, produced by pancreatic cancer cells [11,12].

In our previous report, we determined the prevalence of pancreatic cancer in patients with diabetes [13]. We selected a high-risk group in diabetic patients by identifying our original criteria, including: 1) an onset of diabetes after 55 years of age, 2) deterioration of diabetes or body weight loss despite strict medical control, 3) elevation of serum amylase and/or CA19-9 levels, and 4) pancreatobiliary abnormalities such as pancreatic duct dilation, enlargement, and hypoechoic mass on routine ultrasonography, and conducted ERCP screening of pancreatic cancer. We found 6 patients (7.0%) with pancreatic cancer in a consecutive series of 86 such patients with diabetes. When confined to 36 patients with recent-onset diabetes within 3 years, 5 patients (13.9%) were diagnosed as having pancreatic cancer. These prevalence rates remain unchanged even after the number of patients studied has increased to 163, being 7.4% in the whole series and 13.7% in the recent-onset. Pancreatic cancer could be demonstrated by noninvasive imaging modalities such as ultrasonography, computed tomography, and magnetic resonance cholangiopancreatography in all these patients. We should be well aware of the fact that diabetes may be a sign of pancreatic cancer.

3. Intraductal papillary mucinous neoplasm (IPMN)

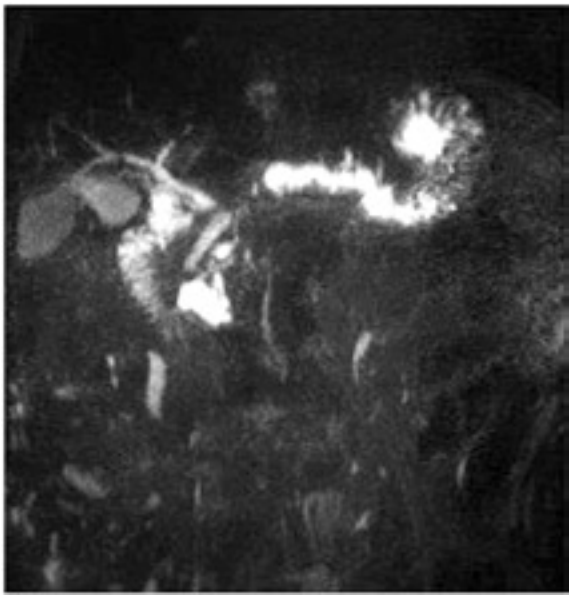
IPMN is a clinical entity of recent interest, presenting with cystic dilation of either branch or main pancreatic duct or both. Papillary proliferation of mucinous epithelium gradually and slowly grows up and shows malignant transformation. The presence of mural nodules, thickening of the cystic wall, and main pancreatic duct dilation indicates the possibility of malignancy.

In addition to its own malignant transformation the high prevalence of extrapancreatic malignancy is recently drawing attention. Yamaguchi et al. [14] reported that 30% of patients with IPMN resected had synchronous or metachronous extrapancreatic malignancy, i.e. 5 gastric cancers, 3 colon cancers, one cancer each in oral cavity, breast, prostate, and uterine cervix in three of 10 patients with main duct IPMN and 10 of 36 patients with branch duct IPMN. Sugiyama et al. [15] also found extrapancreatic malignant tumors synchronously or metachronously in 15 (32%) of 42 patients with IPMN, including 5 colon cancers, 4 gastric cancers, and one cancer each in the bile duct, lung, breast, bladder, prostate, and uterus. Adsay et al. [16] showed that 8 (28%) of 28 patients with IPMN had a history of extrapancreatic malignancy. Furthermore, even in a large series of 148 patients with IPMN reported by Osanai et al. [17], there was a 24% prevalence of extrapancreatic malignancy.

The exact cause and mechanism of the high prevalence of extrapancreatic malignancy in patients with IPMN are unknown. The fact previously noted that IPMN is more frequent in elderly population may be one of possible explanations. However, the prevalence of extrapancreatic malignant tumors in patients with pancreatic cancer which is similarly more frequent in the aged people is significantly lower than that in those with IPMN, being approximately 7% [14]. This observation indicates that all patients with IPMN including those after surgical resection need to undergo periodic checks of these extrapancreatic organs.

The possibly high prevalence of pancreatic cancer in patients with IPMN is a relatively new finding [18,19]. Benign branch duct IPMN may be associated with synchronous or meta-

Figure 1. **Magnetic resonance cholangiopancreatogram in a 72--year-old man with a 40 years' history of diabetes which showed acute exacerbation two months ago. A branch duct intraductal papillary mucinous neoplasm (arrow) is present in the uncinate process and concomitant cancer in the head of the pancreas (arrow heads). Note marked dilation of the main pancreatic duct proximal to the stenosis due to cancer. This patient has all three important clues to the diagnosis of pancreatic cancer, i.e. exacerbation of diabetes, intraductal papillary mucinous neoplasm, and dilation of the pancreatic duct**



chronous pancreatic cancer in the other portion of the pancreas (*Fig. 1*). In our series of 94 patients who underwent resection of branch duct IPMN for its possibly malignant change or the concomitant presence of pancreatic cancer, 9 patients (9.5%) were found to have pancreatic cancer synchronously or metachronously. In particular, two of the 9 patients were diagnosed as having carcinoma in situ by pancreatic juice cytology during work-up for branch duct IPMN [18]. Although MRCP seems to be replacing ERCP in the diagnosis and evaluation of IPMN, ERCP cytology of the pancreatic juice should not be negated for early detection of pancreatic cancer associated with IPMN.

Main duct IPMN is frequently malignant, the percentage being more than 60-70% [20-28]. Branch duct IPMN is also known to show malignant transformation and Uehara et al. [29] emphasized the important role of pancreatic juice cytology for the diagnosis of the malignant change. Nakaizumi et al. [30] diagnosed carcinoma in situ in patients with pancreatic duct dilation and/or small cystic dilation of branch ducts by enthusiastic use of ERCP cytology. The small cystic dilation of branch pancreatic ducts might have included branch duct IPMN. It seems that pancreatic cancer concomitant with IPMN tends to occur in those with small branch duct IPMN [19]. Therefore, it can be said that small cystic lesions including branch duct IPMN are the only clue to the early diagnosis of pancreatic cancer, especially carcinoma in situ, at the moment. The advances in modern molecular biology are expected to yield more sensitive markers for the diagnosis of pancreatic cancer, either by ERCP aspirates or by blood examinations.

References

1. Paulino-Netto A, Dreiling DA, Baronofsky ID. The relationship between pancreatic calcification and cancer of the pancreas. Ann Surg, 1960; 151: 530-7.
2. Suzuki T, Hayakawa T, Noda A, Aoki I, Inoue J, Horiguchi Y, Kamiya N, Toda Y. Carcinoma of the pancreas in association with pancreatolithiasis. Jap J Gastroenterol, 1975; 79: 1563-8.
3. Farrow B, Evers BM. Inflammation and the development of pancreatic cancer. Surg Oncol, 2002; 10: 153-69.
4. Gambill EE. Pancreatitis associated with pancreatic carcinoma – a study of 26 cases. Mayo Clin Proc, 1971; 46: 174-7.
5. Oguchi H, Nagata A, Hirabayashi H, Takeuchi K, Tamura Y, Ueno K, Shimakura K, Kawa S, Sasaki Y, Monno S, Homma T, Furuta S. A study on the complication of malignancy in pancreatic lithiasis. Jap J Gastroenterol, 1982; 79: 851-7.
6. Lowenfels AB, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, DiMagno EP, Andren-Sandberg A, Domellof L, for The International Pancreatitis Study Group. Pancreatitis and the risk of pancreatic cancer. New Engl J Med, 1993; 328: 1433-7.
7. Bansal P, Sonnenberg A. Pancreatitis is a risk factor for pancreatic cancer. Gastroenterology, 1995; 109: 247-51.
8. Karlson BM, Ekbom A, Josefsson S, McLaughlin JK, Fraumeni JF Jr, Nyren O. The risk of pancreatic cancer following pancreatitis: an association due to confounding? Gastroenterology, 1997; 113: 587-92.
9. DiMagno EP. Pancreatic cancer: clinical presentation, pitfalls and early clues. Ann Oncol, 1999; 10(Suppl. 4): 140-2.
10. Maruchi N. Epidemiology of pancreatic cancer. Internal Medicine, 1976; 43: 726-9.
11. Permert J, Larsson J, Westermark GT, Herrington MK, Christmanson L, Pour PM, Westermark P, Adrian TE. Islet amyloid polypeptide in patients with pancreatic cancer and diabetes. N Engl J Med, 1994; 330: 313-8.
12. Ding X, Flatt PR, Permert J, Adrian TE. Pancreatic cancer cells selectively stimulate islet beta cells to secrete amylin. Gastroenterology, 1998; 114: 130-8.
13. Ogawa Y, Tanaka M, Inoue K, Yamaguchi K, Chijiiwa K, Mizumoto K, Tsutsu N, Nakamura Y. A prospective pancreatographic study on the prevalence of pancreatic carcinoma in patients with diabetes mellitus. Cancer, 2000; 94: 2344-9.
14. Yamaguchi K, Yokohata K, Noshiro H, Chijiiwa K, Tanaka M. Mucinous cystic neoplasm of the pancreas or intraductal papillary-mucinous tumor of the pancreas. Eur J Surg, 2000; 166: 141-8.
15. Sugiyama M, Atomi Y. Extrapancreatic neoplasms occur with unusual frequency in patients with intraductal papillary mucinous tumors of the pancreas. Am J Gastroenterol, 1999; 94: 470-3.
16. Adsay NV, Conlon KC, Zee SY, Brennan MF, Klimstra DS. Intraductal papillary-mucinous neoplasms of the pancreas. An analysis of in situ and invasive carcinomas in 28 patients. Cancer, 2002; 94: 62-77.
17. Osanai M, Tanno S, Nakano Y, Koizumi K, Habiro A, Kohgo Y. Extrapancreatic neoplasms in patients with intraductal papillary mucinous tumors of the pancreas: analysis in surgical and follow-up series. J Jap Pancreas Soc, 2003; 18: 565-9.
18. Yamaguchi K, Nakamura K, Yokohata K, Shimizu S, Chijiiwa K, Tanaka M. Pancreatic cyst as a sentinel of in situ carcinoma of the pancreas. Report of two cases. Int J Pancreatol, 1997; 22: 227-31.
19. Yamaguchi K, Ohuchida J, Ohtsuka T, Nakano K, Tanaka M. Intraductal papillary-mucinous tumor of the pancreas concomitant with ductal carcinoma of the pancreas. Pancreatology, 2002; 2: 484-90.
20. Kobari M, Egawa S, Shibuya K, Shimamura H, Sunamura M, Takeda K, Matsuno S, Furukawa T. Intraductal papillary mucinous tumors of the pancreas comprise 2 clinical subtypes: differences in clinical characteristics and surgical management. Arch Surg, 1999; 134: 1131-6.
21. Terris B, Ponsot P, Paye F, Hammel P, Sauvanet A, Molas G, Bernades P, Belghiti J, Ruszniewski P, Flejou JF. Intraductal papillary mucinous tumors of the pancreas confined to secondary ducts show less aggressive pathologic features as compared with those involving the main pancreatic duct. Am J Surg Pathol, 2000; 24: 1372-7.
22. Doi R, Fujimoto K, Wada M, Imamura M. Surgical management of intraductal papillary mucinous tumor of the pancreas. Surgery, 2002; 132: 80-5.
23. Matsumoto T, Aramaki M, Yada K, Hirano S, Himeno Y, Shibata K, Kawano K, Kitano S. Optimal management of the branch duct type intraductal papillary mucinous neoplasms of the pancreas. J Clin Gastroenterol, 2003; 36: 261-5.
24. Choi BS, Kim TK, Kim AY, Kim KW, Park SW, Kim PN, Ha HK, Lee MG, Kim SC. Differential diagnosis of benign and malignant intraductal papillary mucinous tumors of the pancreas: MR cholangio-

pancreatography and MR angiography. Korean J Radiol, 2003; 4:157-62.

25. Kitagawa Y, Unger TA, Taylor S, Kozarek RA, Traverso LW. Mucus is a predictor of better prognosis and survival in patients with intraductal papillary mucinous tumor of the pancreas. J Gastrointest Surg, 2003; 7: 12-9.

26. Sugiyama M, Izumisato Y, Abe N, Masaki T, Mori T, Atomi Y. Predictive factors for malignancy in intraductal papillary-mucinous tumors of the pancreas. Br J Surg, 2003; 90: 1244-9.

27. Sohn TA, Yeo CJ, Cameron JL, Hruban RH, Fukushima N, Campbell KA, Lillemoe KD. Intraductal papillary mucinous neoplasms of the pancreas: an updated experience. Ann Surg, 2004; 239: 788-99.

28. Salvia R, Fernández-del Castillo C, Bassi C, Thayer SP, Falconi M, Mantovani W, Pederzoli P, Warshaw AL. Main duct intraductal papillary mucinous neoplasms of the pancreas: clinical predictors of malignancy and long-term survival following resection. Ann Surg, 2004; 239: 678-87.

29. Uehara H, Nakaizumi A, Iishi H, Tatsuta M, Kitamra T, Okuda S, Ohigashi H, Ishikawa O, Takenaka A, Ishiguro S. Cytologic examination of pancreatic juice for differential diagnosis of benign and malignant mucin-producing tumors of the pancreas. Cancer, 1994; 74: 826-33.

30. Nakaizumi A, Tatsuta M, Uehara H, Takenaka A, Ohigashi H, Ishikawa O, Ishiguro S. Usefulness of simple endoscopic aspiration cytology of pancreatic juice for diagnosis of early pancreatic neoplasm. A prospective study. Dig Dis Sci 42, 1997; 1796-803.

Hereditary pancreatitis and secondary screening for early pancreatic cancer

Vitone LJ, Greenhalf W, Howes NR, Neoptolemos JP*

Division of Surgery & Oncology, The University of Liverpool, United Kingdom

Abstract

Hereditary pancreatitis is an autosomal dominant disease with incomplete penetrance (80%) [1-10], accounting for approximately 1% of all cases of pancreatitis. It is characterized by the onset of recurrent attacks of acute pancreatitis in childhood and frequent progression to chronic pancreatitis [11-13]. Whitcomb et al. identified the cationic trypsinogen gene (PRSS1) on chromosome 7q35 as the site of the mutation that causes hereditary pancreatitis [14]. The European registry of hereditary pancreatitis and familial pancreatic cancer (EUROPAC) aims to identify and make provisions for those affected by hereditary pancreatitis and familial pancreatic cancer. The most common mutations in hereditary pancreatitis are R122H, N29I and A16V but many families have been described with clinically defined hereditary pancreatitis where there is no PRSS1 mutation [1].

It is known that the cumulative lifetime risk (to age 70 years) of pancreatic cancer is 40% in individuals with hereditary pancreatitis [15]. This subset of individuals form an ideal group for the development of a screening programme aimed at detecting pancreatic cancer at an early stage in an attempt to improve the presently poor long-term survival. Current screening strategies involve multimodality imaging (computed tomography, endoluminal ultrasound) and endoscopic retrograde cholangiopancreatography for pancreatic juice collection followed by molecular analysis of the DNA extracted from the juice. The potential benefit of screening (curative resection) must be balanced against the associated morbidity and mortality of surgery. Philosophically, the individual's best interest must be sought in light of the latest advances in medicine and science following discussions with a multidisciplinary team in specialist pancreatic centres.

Key words: hereditary pancreatitis, cationic trypsinogen gene (PRSS1), R122H, N29I, A16V, EUROPAC, pancreatic cancer, secondary screening, Ca19-9, CT, EUS, ERCP, K-ras, p53, p16.

* CORRESPONDING AUTHOR:
The University of Liverpool
Division of Surgery & Oncology
5th Floor UCD
Daulby Street, Liverpool
L69 3GA, United Kingdom
Tel: +44 151 706 4175; Fax: +44 151 706 5826
e-mail: j.p.neoptolemos@liverpool.ac.uk (John P. Neoptolemos)

Received 15.07.2005 Accepted 15.07.2005

Hereditary pancreatitis

Hereditary pancreatitis was first described by Comfort et al. in 1952 when working at the Mayo clinic [16]. Comfort et al. described a family with four definite and two suspected cases of relapsing chronic pancreatitis in childhood/adolescence. The observed pattern of inheritance appeared to follow an autosomal dominant mode but with an incomplete penetrance. This observation has since been confirmed by other groups in Europe and North America [2-10]. Indeed, the incomplete penetrance has been noted by various groups with a figure of 80% penetrance being widely accepted [1]. However, as affected individuals are more likely to be tested for the mutation than unaffected individuals and families with few affected members are less likely to be recruited, estimates of penetrance may be overestimated.

Hereditary pancreatitis accounts for approximately 1% of all cases of pancreatitis. It is characterized by the onset of recurrent attacks of acute pancreatitis in childhood and frequent progression to chronic pancreatitis [11-13]. The classic clinical and demographic characteristics include recurrent episodes of pancreatitis during childhood, equal gender distribution, the frequent presence of pancreatic duct stones, a positive family history, and the absence of other known causes of pancreatitis [13,17,18]. The EUROPAC definition of hereditary pancreatitis is two or more first-degree relatives, or three or more second-

degree relatives, in two or more generations with recurrent acute pancreatitis and/or chronic pancreatitis in the absence of other precipitating or causative factors such as gallstones, tropical pancreatitis or excess ethanol consumption [1].

The aetiology of hereditary pancreatitis remained obscure for almost 50 years since first described by Comfort et al. in 1952 [16], until the application of modern molecular genetic techniques. Linkage analysis using microsatellite markers, established cosegregation between the disease phenotype and the long arm of chromosome 7 [19-21]. Once the hereditary pancreatitis gene was mapped to 7q35, positional cloning using a candidate gene approach was employed, whereby genes already known to be in that region were sequenced. Soon afterwards Whitcomb et al. identified the third exon of the protease serine 1 or cationic trypsinogen gene (PRSS1) on chromosome 7q35 as the site of the mutation that causes hereditary pancreatitis [14]. The PRSS1 protein contains 247 amino acids with an eight amino acid activation peptide and a 15 amino acid signal sequence [22].

The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer

The European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC) was established in 1997 following the realization by a group of European pancreatologists of the need to identify and make provisions for individuals and families affected by inherited diseases of the pancreas, specifically hereditary pancreatitis and familial pancreatic cancer.

The EUROPAC study (www.liv.ac.uk/surgery/europac.html) was established as a European collaboration and in 2002, a formal collaboration was established with a similar research based registry, the Nationale Fallsammlung Familiäres Pankreaskarzinom (FaPaCa or the German National Case Collection of Familial Pancreatic Cancer) of Marburg, Germany (www.med.uni-marburg.de/e-einrichtungen/fapaca/).

The aims of the EUROPAC study have evolved with advances identified in published scientific research and with the identification of new areas of key interest in hereditary pancreatitis and familial pancreatic cancer.

The aims of the study include:

(I) to study and establish the phenotypic and genotypic relations with respect to hereditary pancreatitis (HP) and familial pancreatic cancer (FPC),

(II) to stratify the risk to family members of developing cancer and other clinical manifestations of the inherited condition,

(III) to identify pancreatic cancer susceptibility genes,

(IV) to develop a robust, evidence based secondary screening programme for the detection of early pancreatic cancer in these high risk groups with emphasis on the development and identification of molecular based techniques and markers,

(V) to provide a support service for individuals, their families and for physicians through a comprehensive, multidisciplinary network system of specialists including pancreatologists (surgeons and gastroenterologists), clinical geneticists, and other affected individuals across Europe,

(VI) to provide recommendations, with an accredited molecular genetics service on germline gene testing for genetic mutations that might predispose an individual to pancreatic cancer or pancreatitis,

(VII) to collaborate with other research groups across Europe and beyond in advancing pancreatic research through the exchange of data and materials through both national and international meetings and to publish high quality data in journals that will impact clinical practice across the globe in these disease groups.

Mutations in the cationic trypsinogen gene and variants

Since identification of PRSS1 as a disease gene, a number of different mutations have been identified. The two most frequently occurring mutations in HP are R122H and N29I. These two mutations have been identified in families with hereditary pancreatitis from Europe [23-27], Asia [28] and the Americas [14,29,30].

R122H

The R122H mutation is a single guanine (G) to adenine (A) transition mutation in the third exon of PRSS1 that results in an arginine (CGC) to histidine (CAC) missense substitution at amino acid residue 122. Note that originally residue 122 was referred to as position 117 according to the consensus position with chymotrypsinogen; hence the mutation was referred to as R117H.

The trypsin molecule contains a calcium binding pocket near the side chain connecting the two globular domains of the molecule. This side chain (the autolysis loop) contains amino acid position 122, which is a target for attack by other trypsin molecules. Enzymatic cleavage of the side chain at arginine 122 (R122) by the second trypsin leads to rapid destruction of the first trypsin molecule (autolysis). The autolysis loop is flexible and R122 may come near to the calcium binding pocket. As the concentration of soluble calcium rises, calcium enters the calcium binding pocket and limits exposure of R122 to enzymatic attack by another trypsin [31]. It is widely assumed, with some biochemical support [32-35], that the substitution of histidine for arginine results in a reduction in the destruction of autoactivated trypsinogen in a calcium dependent fashion.

The R122H mutation was easily identified as it created a novel recognition site for the restriction endonuclease AflIII. However, Howes et al. demonstrated that a neutral polymorphism within this enzyme recognition site may produce a false negative result [36]. An alternative mutation specific polymerase chain reaction approach was therefore developed for detection of the mutation even in the presence of the polymorphism [36].

N29I

A second mutation in PRSS1 was subsequently discovered a year later in two affected families without the R122H mutation [37]. A single adenine (A) to thymine (T) transversion mutation (N29I) was identified in exon 2 which results in a change from asparagine (AAC) to isoleucine (ATC) at amino

acid 29, the mutation was previously known as N21I according to the chymotrypsinogen consensus numeration.

The mechanism accounting for how N29I causes pancreatitis is uncertain, although in light of the assumed mechanism of action of R122H and the clinical similarities between R122H and N29I phenotypes, it was suggested that the mechanism must involve increased trypsin activity [37]. This may be due to enhanced autoactivation of trypsinogen, alteration of the binding of pancreatic secretory trypsin inhibitor (PSTI/SPINK1) or impairment of trypsin inactivation by altering the accessibility of the initial hydrolysis site to trypsin. Whitcomb et al. predicted conformational changes in the crystallographic structure of trypsin [38] which could explain a reduced accessibility to the calcium binding pocket. An alternative model was proposed by Nishimori et al., who suggested that the N29I mutation alters the native structure of the PRSS1 gene to a sheet structure [28]. It was implied that this conformational alteration might impair trypsin activation. Sahin-Toth and collaborators used direct biochemical approaches to investigate the mechanism rather than structural modelling and concluded that the N29I mutation increased autoactivation under acidic conditions [33]. This is the most widely accepted mechanism at the time of writing and contrasts with the perceived model for R122H pathology (i.e., reduced inactivation following autoactivation). Despite the apparently significant difference between the pathological mechanisms of N29I and R122H, initial reports from the EUROPAC registry indicate a remarkably similar pathophysiology of the disease in patients with the two mutations [1].

A16V

A third mutation where there is a cytosine (C) to thymine (T) missense mutation has been identified in exon 2 that leads to an alanine (GCC) to valine (GTC) substitution at codon 16 (A16V) [27]. This mutation affects the first amino acid of the trypsinogen molecule and thus directly the cleavage site for the signal peptide. The mechanism by which pancreatitis is initiated remains speculative, but given the position of the mutation at the edge of the signal peptide it is widely believed to involve defects in secretion.

The A16V mutation was identified during a study to determine the spectrum and frequency of mutations in the PRSS1 gene in 44 children/adolescents with chronic pancreatitis [23]. Thirty of these individuals were found to have idiopathic pancreatitis and fourteen hereditary pancreatitis. R122H was identified in one individual; A16V was found in three individuals with presumed idiopathic pancreatitis and in one said to have hereditary pancreatitis. The A16V mutation was also identified in seven first-degree relatives of these patients but only one had clinically apparent pancreatitis, suggesting low penetrance of this mutation.

Variants

The main mutations (N29I and R122H) have exclusively been found in patients with hereditary pancreatitis and, although A16V mutations were originally identified in patients with no clear family history, this mutation has not yet been identified in individuals with ethanol-induced or tropical pancreatitis [1,39-43]. In addition to these three principle mutations, there are multiple variants of the PRSS1 gene as detailed in a recent review by Howes et al. [1]. These include: -28delTCC (a three base pair deletion 28 base pairs upstream from start codon) [25], D19A [44], D22G [45], K23R [25], N29T [46], P36R [40], Y37X [47], G83E [40], K92N [40], L104P [39], R116C [39,48], V123M [40] and C139F [39]. All these variants are rare and in some cases the link with inherited pancreatitis is only suggestive. Two neutral polymorphisms (D162D [39] and N246N [39]) have also been described.

Other disease genes

Although mutations in the Kazal type 1 serine protease inhibitor (SPINK1/PSTI trypsin inhibitor) [49-58] and cystic fibrosis transmembrane conductance regulator (CFTR) [52, 59-62] genes have been associated with cases of pancreatitis of various aetiology, no other gene apart from PRSS1 has been shown to have mutations that cause hereditary pancreatitis. However, many families have been described with clinically defined hereditary pancreatitis where there is no PRSS1 mutation [1]. This indicates that there is at least one more disease gene left to be identified.

Presentation of hereditary pancreatitis

It is crucial to note that data on individuals and their families with hereditary pancreatitis, such as that collated by the EUROPAC study group are hierarchical in structure on account of the nesting of affected individuals within their families and thus they are not completely independent [1]. Howes et al. demonstrated the variation distributed within a family and between families by way of multi-level modelling [1]. This paper was the first that was large enough to use hierarchical statistical analyses in studying the relationship between biological and demographic factors of individuals with hereditary pancreatitis [1].

Howes et al. found that their cohort of patients (n=418 affected) presented with symptoms of pancreatitis at an early age, with a median onset of symptoms at 12 years (95% Confidence Intervals /CI/: 10,13), with over 70% of individuals developing symptomatic pancreatitis by the age of 20 years [1]. Lowenfels et al. looked at a large cohort of individuals with hereditary pancreatitis (n=412 affected) from 16 countries and found the mean age of symptom onset to be 14.1 years with an equal sex ratio but with a slightly more common paternal inheritance pattern (57%) [63].

Howes et al. demonstrated that individuals with R122H mutations presented 10 years earlier (95% CI: 8,12) in comparison to those individuals with the N29I mutation or compared with individuals with no PRSS1 mutation, who had a median age of presentation of 14 years (95% CI: 11,18) and 14.5 years (95% CI: 10,21), respectively [1], although not reported in the paper the data set also showed no evidence for any preference for paternal transmission (unpublished observation).

These findings of early disease onset are fairly consistent with the published literature [1,6-8,13,27,28,37,63-68] reporting significant earlier symptom onset in R122H mutation carriers. Interestingly, Keim et al. studied 101 individuals and failed to

demonstrate any significant difference in age of symptom onset between R122H and N29I mutation carriers [6]. This may be accountable to the small study number and the hierarchical structure of individuals nested within families. Bias arises given that members of families are similar in contrast to random selection of individuals from a population.

Amann et al. provides one of the few studies looking at identical twins in hereditary pancreatitis [10]. They found that the median age of symptom onset of hereditary pancreatitis in concordant twins was almost identical, with similar ages of onset seen in matched siblings and a significantly different age of symptom onset from individuals from age-, sex-, and mutation-matched controls.

Such observations suggest an important role for genetic background, aside from the causative mutations, in determining disease progression. However, bias is probable as siblings are likely to share a common environment as well as a common genetic profile.

Symptoms of hereditary pancreatitis

Howes et al. of the EUROPAC Study group have provided the largest detailed study of hereditary pancreatitis to date [1]. At the time of their guillotine, 527 individuals had been recruited from 14 countries of which 418 individuals from 112 families were affected. This was 58 (52%) families of whom 222 individuals (53%) were characterised by R122H mutations, 24 (21%) of families had N29I mutations (94 individuals, 22%) and 21 (19%) of families had no PRSS1 mutation (72 patients, 17%). Howes et al. demonstrated an overall median of 1.88 attacks (interquartile range: 0.63-3.0) per year, which was unrelated to the type of PRSS1 gene mutation or gender (multi-level modelling) [1]. Not all of the symptomatic episodes of pancreatitis were severe enough to warrant hospital admission, with the median number of admissions to hospital for complications of pancreatitis being 0.3 (interquartile range: 0.08-1.0); other attacks were managed at home or by their General Practitioner (personal physician) [1,69]. The number of hospital admissions was unaffected by gender, however, individuals with PRSS1 mutations did have a tendency for fewer hospital admissions than those with no identified causative mutation. This reached significance when comparing patients with the N29I mutation and those who did not carry a PRSS1 mutation [1]. Approximately, 90% (158/176) of individuals reported that symptomatic episodes lasted no more than one week; the remaining 10% (18/176) had attacks over one week. The duration of acute pancreatitis was not influenced by either gender or PRSS1 mutation status [1]. Prior to the Howes et al. study [1], Gorry et al. reported on two large families with PRSS1 mutations [37]. They found that 86% (24/28) of individuals in the R122H family (n=28) had more than five hospital admissions in contrast to the N29I family (n=15) where there were just 47% (7/15). In a larger study by Keim et al. the clinical characteristics of 30 families with hereditary pancreatitis consisting of six families with the N29I mutation (n=25) and 21 families with the R122H mutation (n=76) were examined [6]. In the N29I group, 24% (6/25) had no symptoms or atypical symptoms and 40% (10/25) mild symptoms. In the R122H group, 26% (20/76) had no symptoms or atypical symptoms and 42% (32/76) mild symptoms. Keim et al. admit that their sample size was small and that the clinical scoring system to classify chronic pancreatitis was not validated [6].

Secondary screening for early pancreatic cancer in high risk groups

It is estimated that 5-10% of pancreatic cancers are attributable to genetic factors [70,71]. Bartsch identified three clinical settings where there may be an inherited predisposition to pancreatic cancer [72]. Firstly, as an adjunct to a familial cancer syndrome associated with an increased risk of pancreatic cancer, as in familial atypical multiple mole melanoma (FAMMM) syndrome [73] and Peutz-Jeghers syndrome [74]. Secondly, as an inherited predisposition to pancreatic cancer linked to another condition; genetic disorders known to predispose to cancer of the pancreas include: hereditary pancreatitis [15,16] and cystic fibrosis [75]. Finally, there are a group of families with apparent autosomal dominant inheritance and a predisposition for pancreatic cancer with no known causative gene (familial pancreatic cancer) [70]. For the purposes of this article, only hereditary pancreatitis will be dealt with.

Hereditary pancreatitis and pancreatic cancer risk

Lowenfels et al. on behalf of the International Hereditary Pancreatitis Study Group estimated that the cumulative lifetime risk (to the age of 70 years) of cancer of the pancreas to be 40% in patients with hereditary pancreatitis [15]. This was supported by Howes et al. in a larger study [1] (see *Fig. 1*, *Tab. 1* and *2*). Lowenfels et al. also reported that paternal transmission of hereditary pancreatitis was associated with a much greater lifetime risk of developing pancreatic cancer [15] but the EUROPAC study group showed that there was no significant difference between paternal and maternal transmission [76].

In cancer syndromes where the gene is unknown it is not clear which individuals are at risk as many family members will not be gene carriers. This is not an issue with hereditary pancreatitis as it is likely that the pancreatic cancer in these families relates to the pancreatitis rather than directly from the gene mutation, therefore only individuals with pancreatitis would be screened.

The justification for secondary screening

Pancreatic cancer is an aggressive disease with a poor prognosis, representing 2% of all new cases of cancer but leading to 5% of all cancer deaths [77]. The median survival is approximately 4-6 months with only 5-10% of individuals being candidates for a surgical resection [78].

The prevalence of pancreatic cancer in the general population (8-12 per 100,000) is too low even in high-prevalence areas such as Northern Europe and North America to permit screen-

Figure 1. **Time to pancreatic cancer showing no significant differences by mutation status. (Reprinted form "Clinical and Genetic Characteristic of Hereditary Pancreatitis in Europe" by Howes et al. with permission from the American Gastroenterological Association** [1])

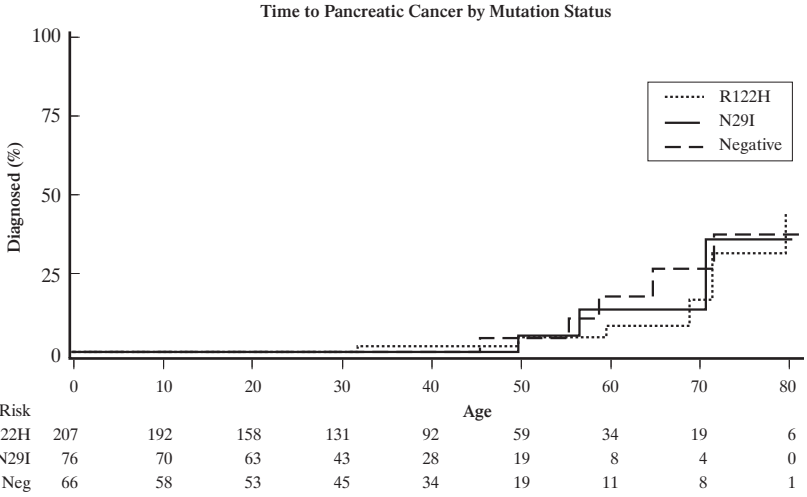


Table 1. **Cumulative risk for the development of pancreatic cancer in hereditary pancreatitis in the EUROPAC Study (n=375)** [1]

Age of risk (Years)	Cumulative Risk of Pancreatic Cancer (%)	95% Confidence Intervals
40	0.5	0.0-1.3
50	3.4	0.4-6.5
60	9.8	3.6-16.0
70	18.8	8.6-29.0
80	33.3	19.0-47.5

Table 2. **Estimates for the development of pancreatic cancer in three large studies of hereditary pancreatitis**

Study	Lifetime Risk	Number of Cancer Cases	One Cancer per Person Years
Lowenfels et al. [15]	40%	8	1066
Keim et al. [6]	-	3	1200
Howes et al. [1]	33%	26	703

ing of the asymptomatic population, given the diagnostic accuracy of present detection methods [79]. However, in the case of hereditary pancreatitis secondary screening can be justified – the primary screen would be to identify the family and the individual with pancreatitis. The secondary screen would attempt to identify those patients with an early asymptomatic cancer which was amenable to curative surgical resection. The diagnostic tests used should provide a high positive predictive value to avoid missing any surgically resectable cancers and a high negative predictive value to prevent unnecessary surgery.

The greatest concern when carrying out screening is the harm that could be caused to individuals with no malignancy. This could result from unnecessary surgery, although with hereditary pancreatitis this would involve resection of a diseased rather than a healthy pancreas. Harm may also be inflicted on a patient directly as a result of the screening modality, but this concern is reduced if the modality is applied as part of the normal management of pancreatitis. The presence of pancreatitis is an indication for screening, but distinguishing a pancreas with a small tumour from a diseased pancreas is more difficult than distinguishing a small pancreatic tumour in an otherwise healthy organ.

Imaging modalities such as endoluminal ultrasound scanning (EUS), and endoscopic retrograde cholangiopancreatography (ERCP) have been employed to distinguish patients with pancreatic cancer from patients with symptoms routinely mistaken for pancreatic cancer, such as pancreatitis [80]. The EUROPAC study group also employs molecular screening of pancreatic juice obtained at ERCP as adjuncts to imaging modalities to stratify risk, reducing the frequency of screening in lower risk patients and increasing the positive predictive value of the imaging [81].

Management of high risk individuals

A screening programme can only be justified if a positive result will offer some possibility of treatment; primary screening, by classifying individuals as high risk for pancreatic cancer is therefore, controversial. Arguments can be made that lifestyle changes may reduce risk and that advice on prevention including the avoidance of smoking are therefore, beneficial. Smoking has been suggested to increase risk of cancer in hereditary pancreatitis [82]. On the other hand, there is the issue of increased anxiety for the family unit and lack of clear evidence that such lifestyle changes will overcome the genetic risk [81]. Thus, having identified individuals at high risk, there is an ethical requirement to offer enrolment on a secondary screening programme, which would allow tumours to be identified at a treatable stage.

Guidelines were established during the third international

symposium on inherited diseases of the pancreas in Milan in 2001 for the secondary screening of patients with hereditary pancreatitis. These included patients being given the opportunity to discuss the variability in the penetrance of the pancreatic susceptibility gene(s) with a clinical geneticist, who would also address issues of psychological stress, insurance and employment discrimination [83].

The strategy of secondary screening is based on the assumption that one can detect pancreatic cancer at an early stage, at worst as pancreatic carcinoma in situ [84]. There is some evidence to suggest that those patients with pancreatic tumours of <1.0 cm can be cured. Ariyama et al. reported a 100% 5-year survival rate for seven individuals with tumours <1.0 cm and limited to the epithelium [85,86].

Certainly there is evidence that increasing tumour size correlates with an increasing rate of unresectability and decreasing survival rate underpins the need to detect tumours while they are small and have not spread locally [85]. There is also an increasingly attractive argument that the presence of high-grade dysplasia (pre-cancerous lesion) is in itself enough to justify surgery [87]. The decision to undertake surgery will be based on the risk of developing cancer outweighing the risk of an operation.

Careful characterisation of families with hereditary pancreatitis may allow trends to be established in the age of onset of pancreatic cancer. This in turn would allow the age at which pre-test risk would be enough to justify secondary screening.

Imaging of the pancreas

The most common imaging modalities at present are computed tomography (CT) and ultrasound (US) followed by endoluminal ultrasound (EUS) and positron emission tomography (PET) [88]. Alternatives are magnetic resonance imaging (MRI), endoscopic retrograde cholangiopancreatography (ERCP) and magnetic resonance cholangiopancreatography (MRCP). Little data exists on the sensitivity of these techniques in detecting lesions in asymptomatic individuals. It is clear that despite significant strides in technology, no individual imaging technique has achieved sufficient accuracy to precisely assess tumour resectability in pancreatic cancer; therefore, combinations of imaging modalities are employed. To date no consensus about the best approach to assess tumour stage or resectability has been achieved; reliable data on their combined efficacy is limited to a few prospective trials [89].

Screening modalities

Computed tomography

Traditionally, the purpose of CT has been to diagnose and stage pancreatic cancer once clinically suspected or once a patient has developed suspicious symptoms [90-92]. It has generally not been considered useful for screening asymptomatic individuals because of the belief that CT is less sensitive than EUS [93,94]. In spite of this, CT remains the most widely available and best validated tool for pancreatic imaging [95]. The sensitivity for detection of pancreatic cancer was investigated by Gangi et al. [96]. Two 'blinded' radiologists reported CT scans from patients subsequently diagnosed with pancreatic cancer. Signs of pancreatic cancer, either definitive or suspicious were identified in 93-100% of scans obtained 0-2 months before clinical diagnosis. However, with scans obtained 2-6 months and 6-18 months before diagnosis, detection was 67-83% and 63%, respectively. Only 7% of scans taken 18 months or more before diagnosis were suggestive of cancer [96]. The sensitivity of helical CT in the detection of small adenocarcinomas of the pancreas (≤2 cm) at pathological examination was evaluated by Bronstein et al. [97]. They found a sensitivity of 77% (2 observers) and 72% (10 observers) in small pancreatic masses; this group also looked at scans from patients with no adenocarcinoma and obtained a specificity of 100% (all observers). However, this high specificity is of little relevance to hereditary pancreatitis as no patients in their study had chronic pancreatitis, which may mimic carcinoma on imaging [98].

The earliest finding consistently identified by radiologists was pancreatic duct dilatation, followed by pancreatic duct cut-off [93]. These features would be expected in nearly all patients with hereditary pancreatitis, certainly with older patients (who are at most risk of cancer). Ishikawa et al. found that almost 60% of small adenocarcinomas (<1 cm) showed pancreatic duct dilatation without a mass on CT or EUS, whereas <15% showed a mass [86].

With the availability of multidetector spiral computed tomography (MDCT) scanners with narrow slice thickness and biphasic technique, the accuracy for the detection of pancreatic cancer before development has improved and despite the limitations in this group of patients it should be employed in any secondary screening programme.

Endoscopic retrograde cholangiopancreatography and molecular screening of pancreatic juice

Endoscopic retrograde cholangiopancreatography (ERCP) has a played a significant role in the diagnosis of pancreatic diseases since its development in the 1960s. According to the Japan Pancreas Society in 2003, ERCP is ranked as the third most frequent diagnostic modality employed in detecting cancers of the pancreas [99]. ERCP allows the anatomic visualisation of the hepatobiliary tree and provides a mechanism of collection of pancreatic juice for genetic analyses, brush cytology, and biopsy. Niederau and Grendell combined data from almost twenty studies and found a sensitivity of 92% and specificity of 96% for diagnosing cancer of the pancreas by ERCP [100], however, this analysis relied heavily on detection of fairly late stage tumours and the relevance to secondary screening must therefore be treated with caution.

ERCP-directed brush cytology can be used to investigate and evaluate lesions of the pancreato-hepatobiliary systems including the ampulla of Vater [101,102]. This technique requires an experienced cytopathologist and has a sensitivity, which ranges from 33-57%; the specificity ranging from 97-100% [101,103-110]. The low sensitivity may be related to technical problems and difficulties in sampling or visualisation

[111]. The role of ERCP is evolving into a therapeutic modality; its role in diagnostics is slowly being superseded by endosonographic modalities, however, the development of molecular screening models such as that developed by EUROPAC are likely to improve the sensitivity and specificity for the detection of early pancreatic cancer in high risk groups [81]. Nonetheless, the potential benefit of ERCP for pancreatic juice sampling and molecular analysis must be carefully considered against the risks involved, most significantly the risk of acute pancreatitis. Estimates of the risk of ERCP induced pancreatitis vary from 4 to 7% [112-114]. Mortality associated with post ERCP pancreatitis is approximately 0.2% [112]. These risks will depend on many factors relating to the patient and the nature of the procedure; patients with existing chronic pancreatitis would be likely to have a lower risk.

In order to improve specificity without significantly compromising sensitivity, molecular changes occurring during tumour progression are being exploited. Mutations in K-Ras occur at an early stage of development and can be found in 85% of patients with pancreatic cancer [115]. The detection of these mutations in stool and duodenal or pancreatic juice has been proposed as an early detection strategy [116]. However, K-Ras mutations can be identified in pancreatic juice from patients with chronic pancreatitis or even biliary tract stones as well as patients with cancer [81], making the specificity of this test very low. The $p16^{INK4a}$ tumour suppressor gene is inactivated in around 95% of pancreatic cancers, but this occurs later in cancer progression than K-Ras mutation [117,118]. Although, promoter hypermethylation is only involved in approximately 16% of $p16^{INK4a}$ inactivation [118], promoter methylation of DNA extracted from pancreatic juice appears to be elevated in most patients with pancreatic cancer, reflecting a change in the non-tumour cells of the diseased pancreas [81]. Detection of $p16^{INK4a}$ promoter CpG island methylation has been examined as a screening modality for pancreatic cancer [81,119-121]. Initial reports of no promoter methylation in cancer patients probably reflected low sensitivity of the assay and subsequent analysis indicated some level of promoter methylation in all pancreatic juice samples, from cancer patients or from controls [81]. Quantification rather than detection was therefore used to distinguish cancer patients, raising the threshold for the methylation level considered as positive allowed specificity to be increased but at the expense of sensitivity; a compromise threshold of 12% promoter methylation gave nearly 90% specificity with over 60% sensitivity [81].

The p53 tumour suppressor gene is mutated in about 50% of pancreatic ductal adenocarcinomas [122]. Immunocytology detects mutant p53 indirectly as a result of the accumulation of mutant p53 protein in cells. This technique however, will miss mutations that lead to loss or truncation of p53 protein [123]. Mutations have also been detected in pancreatic juice using single stranded conformational polymorphism (SSCP). In the largest of these studies, 11/26 patients (42%) with pancreatic cancer had a detectable p53 mutation in comparison to 0/16 patients with chronic pancreatitis [124]. SSCP lacks sensitivity (detecting approximately one mutant copy per 100 wild type copies) and cannot distinguish between polymorphisms, functionally silent mutations and inactivating mutations. A yeast functional assay which acts by detecting the essential transcriptional activation function of p53 has also been applied to pancreatic juice. In this technique, human p53 expressed in Saccharomyces cerevisiae activates transcription of the ADE2 gene. Yeast colonies containing wild-type p53 are white, while colonies containing mutant p53 are red as a result of the accumulation of a metabolic intermediate [81]. Using this technique 42% of 48 cancer patients were correctly identified, with no mutant p53 being identified in 49 patients with biliary tract stones (although p53 mutations were detected in 2/49 patients with chronic pancreatitis).

Recently, Yan et al. of the EUROPAC study group published data on stratification of cancer risk using p53 and K-Ras mutation status combined with $p16^{INK4a}$ promoter methylation [81]. They concluded that for individuals in a population with a 1% incidence of cancer, risk could be stratified between negligible and over 50%; exceeding 90% when discriminating patients with malignancy from patients with no pancreatic disease. The authors admit that their analysis (a Bayesian approach using the specificity and sensitivity of the three tests as independent) was based on patients with a presumed diagnosis prior to molecular analysis, which would tend to lead to an overestimate of the power of the screening modalities. Work is ongoing to clarify the sensitivity of the modalities in asymptomatic patients.

Clearly, such molecular screening models have enormous potential as adjuncts to pre-existing screening tools in the clinical management of high risk patients and are already implemented in centres like Liverpool (EUROPAC) as part of the multidisciplinary, multimodality screening programme already in place.

Endoluminal ultrasound

Endoluminal ultrasound (EUS) is high frequency, real-time ultrasonography combined with endoscopy. EUS is associated with a very low risk of adverse effects (0-0.5%) and very high sensitivity (>90%) for the detection of early, non-metastatic, pancreatic cancer [88,125,126]. As a modality, EUS can display small pancreatic lesions undetectable by CT and MRI. Some centres in the United States recommend screening for pancreatic cancer by performing yearly EUS, followed by ERCP, EUS guided fine-needle aspiration or CT to further investigate abnormalities [127]. This is in accordance with the American Gastroenterological Association recommendations for those with familial syndromes [128]. This approach has been tested with 38 high risk patients, none of whom had symptoms of pancreatic cancer. None of these patients had hereditary pancreatitis. Pancreatic masses were identified in seven patients. All seven were operated on and one of them (a 45 year old female with a history of breast cancer) was found to have an invasive ductal adenocarcinoma, this patient is still alive five years after surgery. Rulyak et al. suggested that the use of EUS to screen members of a familial pancreatic kindred was cost-effective, however, the benefit is limited to populations with a pre-test probability of pancreatic dysplasia >16% [129]. According to Rulyak et al. screening should begin at 50 years of age, or 10 years before the earliest age of onset of pancreatic cancer

in a family member, beginning with yearly examinations in a pancreatic specialist centre [130].

Given the risk of pancreatitis with ERCP, it may be reasonable to perform an EUS prior to an ERCP in patients with a family history of cancer [131]. Therefore, at the University of Washington Medical Centre, the first phase of screening in high risk patients involves EUS, which if abnormal is followed by ERCP [132]. If both are normal then they are repeated annually or per patient's choice [132]. However, it is questionable whether EUS can detect a small lesion on a background of pancreatitis and so for hereditary pancreatitis the additional modality of CT is indicated.

Tumour markers

Many of the imaging techniques previously described have the disadvantage that they are invasive or involve morbidity as a result of exposure to radiation. Therefore, a simple serum based test has advantages if adequate specificity and sensitivity can be achieved. A number of proteins have been identified that have raised levels in patients with pancreatic cancer; the question remains whether this increase occurs early enough to give the required sensitivity and whether this increase is specific to pancreatic cancer or whether levels may be elevated in high risk patients even in the absence of tumours. In addition to a high sensitivity and specificity, tumour marker testing should be cheap and reproducible.

Carbohydrate antigen 19-9 in serum

Carbohydrate antigen 19-9 (Ca19-9) is a cell surface glycoprotein (a monosialoganglioside) expressed on the surface of pancreatic cancer cells as well as by normal human pancreatic and biliary duct cells, and gastric, colonic, endometrial and salivary epithelia. It is elevated above 100 U/ml in the serum of patients with hepatocellular carcinoma, ovarian carcinoma, bronchial, colon and gastric cancers as well as pancreatic cancer. It has been found to be a useful tumour marker in diagnosis, a prognostic indicator and provides an overall evaluation of therapeutic efficacy and recurrent disease status [111,133].

Only 50% of cancers <2 cm are associated with a rise in Ca19-9 [111]. The limitations of Ca19-9 were well demonstrated in a study by Kim et al., who found a positive predictive value of less than 1% for patients undergoing ultrasonography who were described as asymptomatic; they tested 71,000 individuals using a cut-off of 37 U/ml [134]. Another important limitation of Ca19-9 relates to patients with negative Lewis blood group antigen (Lewis a-, b-). This group of patients representing 4-15% of the population are unable to synthesize Ca19-9 and so its use in this population should clearly be avoided [133,135-137].

In an early publication by Malesci et al., a Ca19-9 greater than 40 U/ml was found in 90% (57/63) of pancreatic cancer patients and in only 10% (5/50) of patients with chronic pancreatitis [138]. In 4/5 patients with chronic pancreatitis, repeat testing when the patients were in a non-relapse state revealed normal levels of Ca19-9. This study highlights that a progressive upward trend seems to be more indicative of pancreatic cancer than fluctuating levels, which may be associated with the degree of active inflammation in patients with pancreatitis [138].

Forsmark et al. retrospectively reviewed 53 patients with Ca19-9 values >90 U/ml in whom the test had been done because of clinical suspicion of pancreatic malignancy [139]. Pancreatic cancer was found in 85% (45/53) of patients. When a cut-off value of Ca19-9 >200 U/ml was used, 97% (36/37) of patients had pancreatic cancer. Thirty patients with pancreatic cancer and no radiographic criteria of unresectability underwent attempted resection; five of these patients were judged to be potentially resectable; four underwent attempted resection. In only one patient with a Ca19-9 value >300 U/ml was resection possible. Forsmark et al. concluded that pancreatic malignancy was highly suggestive in patients with suspected pancreatic cancer and a Ca19-9 >90 U/ml, while a Ca19-9 >200 U/ml was considered virtually diagnostic. In those with a Ca19-9 >300 U/ml, resection was rarely possible.

As high levels only appear to be found in late disease, use of tumour markers like Ca19-9 as a serum screening modality for the early diagnosis of pancreatic cancer is extremely limited. It should only be utilised in combination with radiological imaging and endoscopy, and molecular screening methods such as the molecular mutational analysis of pancreatic juice employed by the EUROPAC group.

Conclusions

In the last decade great strides have been made in our understanding of the molecular biology and pathophysiology of hereditary pancreatitis. This has been transcribed to the clinical setting resulting in better clinical management of those individuals with such inherited diseases of the pancreas. Indeed, the emphasis has been to identify such high risk groups for the development of pancreatic cancer through secondary screening programmes such as that offered by the EUROPAC study group in the hope that early diagnosis will lead to a 'cure' through a surgical resection. However, the benefits to the patient of embarking on such a screening programme must be considered carefully given the definite risk of morbidity and mortality associated with a pancreatic resection.

Hence, the quest for the ideal imaging and molecular modalities for the purpose of secondary screening for the diagnosis of early pancreatic cancer remains both challenging and unresolved. Philosophically, the individual's best interest must be sought in light of the latest advances in medicine and science following discussion with a multidisciplinary team inclusive of genetic counselling.

The identification of precursor lesions within pancreatic ducts has led to the formulation of a progression model of pancreatic cancer and subsequent identification of early- and late-stage changes leading to invasive cancer [93,140-145]. Ultimately, understanding the genetic events underlying the development of pancreatic cancer may serve as a useful adjunct in the screening and treatment of patients suffering from, or at risk for, pancreatic cancer. Conceptually, identification of a point on the progression, based on the appearance of molecu-

lar markers, would allow rational evaluation of the risk that cancer development is inevitable. This can only be confirmed by long-term prospective follow-up of patients from an asymptomatic state to confirmed pancreatic cancer. Prospective and repeated multimodality mutation testing of pancreatic juice in tandem with conventional imaging modalities like CT, EUS and ERCP, will further stratify the risk of pancreatic cancer in high risk groups, and thus facilitate clinical decision making.

The growth and expansion of the EUROPAC registry over the last eight years in bettering our understanding of inherited pancreatic diseases would not be possible without the collaborative efforts of both scientists and clinicians. It is only through such collaborative efforts that we may further advance our scientific knowledge of hereditary pancreatitis thus improving the management of these individuals who have an estimated lifetime risk of 40% (to the age of 70 years) for the development of pancreatic cancer [1,15]. At present, it is only through secondary screening programmes that early lesions in such high risk groups may be identified, in the hope that a curative surgical resection may be offered.

References

1. Howes N, Lerch MM, Greenhalf W, Stocken DD, Ellis I, Simon P, Truninger K, Ammann R, Cavallini G, Charnley RM, Uomo G, Delhaye M, Spicak J, Drumm B, Jansen J, Mountford R, Whitcomb DC, Neoptolemos JP. Clinical and genetic characteristics of hereditary pancreatitis in Europe. Clin Gastroenterol Hepatol, 2004; 2: 252-61.
2. Gerber BC. Hereditary pancreatitis. Arch Surg, 1963; 87: 70-80.
3. Gross J. Some recent developments pertaining to the pancreas. Ann Int Med, 1958; 49: 796-836.
4. Gross JB, Comfort MW. Hereditary pancreatitis: report on two additional families. Gastroenterology, 1957; 32: 829-54.
5. Gross J, Ulrich J, Maher F. Further observations on the hereditary form of pancreatitis, in CIBA foundation symposium. 1962, Churchill: London, p. 278-309.
6. Keim V, Bauer N, Teich N, Simon P, Lerch MM, Mossner J. Clinical characterization of patients with hereditary pancreatitis and mutations in the cationic trypsinogen gene. Am J Med, 2001; 111: 622-6.
7. Sossenheimer MJ, Aston CE, Preston RA, Gates LK Jr., Ulrich CD, Martin SP, Zhang Y, Gorry MC, Ehrlich GD, Whitcomb DC. Clinical characteristics of hereditary pancreatitis in a large family, based on high-risk haplotype. The Midwest Multicenter Pancreatic Study Group (MMPSG). Am J Gastroenterol, 1997; 92: 1113-6.
8. Sibert JR. Hereditary pancreatitis in England and Wales. J Med Genet, 1978; 15: 189-201.
9. Le Bodic L, Schnee M, Georgelin T, Soulard F, Ferec C, Bignon JD, Sagniez M. An exceptional genealogy for hereditary chronic pancreatitis. Dig Dis Sci, 1996; 41: 1504-10.
10. Amann ST, Gates LK, Aston CE, Pandya A, Whitcomb DC. Expression and penetrance of the hereditary pancreatitis phenotype in monozygotic twins. Gut, 2001; 48: 542-7.
11. Perrault J. Hereditary pancreatitis. Gastroenterol Clin North Am, 1994; 23: 743-52.
12. Moir CR, Konzen KM, Perrault J. Surgical therapy and long-term follow-up of childhood hereditary pancreatitis. J Pediatr Surg, 1992; 27: 282-6.
13. Konzen KM, Perrault J, Moir C, Zinsmeister AR. Long-term follow-up of young patients with chronic hereditary or idiopathic pancreatitis. Mayo Clin Proc, 1993; 68: 449-53.
14. Whitcomb DC. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nat Genet, 1996; 14: 141-5.
15. Lowenfels AB, Maisonneuve P, DiMagno EP, Elitsur Y, Gates LK Jr., Perrault J, Whitcomb DC. Hereditary pancreatitis and the risk of pancreatic cancer. International Hereditary Pancreatitis Study Group. J Natl Cancer Inst, 1997; 89: 442-6.
16. Comfort MW, Steinberg AG. Pedigree of a family with hereditary chronic relapsing pancreatitis. Gastroenterology, 1952; 21: 54-63.
17. Kattwinkel J, Lapey A, Di Sant'Agnese PA, Edwards WA. Hereditary pancreatitis: three new kindreds and a critical review of the literature. Pediatrics, 1973; 51: 55-69.
18. Sato T, Saito Y. Familial chronic pancreatitis associated with pancreatic lithiasis. Am J Surg, 1974; 127: 511-7.
19. Le Bodic L, Bignon JD, Raguenes O, Mercier B, Georgelin T, Schnee M, Soulard F, Gagne K, Bonneville F, Muller JY, Bachner L, Ferec C. The hereditary pancreatitis gene maps to long arm of chromosome 7. Hum Mol Genet, 1996; 5: 549-54.
20. Whitcomb DC, Preston RA, Aston CE, Sossenheimer MJ, Barua PS, Zhang Y, Wong-Chong A, White GJ, Wood PG, Gates LK Jr., Ulrich C, Martin SP, Post JC, Ehrlich GD. A gene for hereditary pancreatitis maps to chromosome 7q35. Gastroenterology, 1996; 110: 1975-80.
21. Pandya A, Blanton SH, Landa B, Javaheri R, Melvin E, Nance WE, Markello T. Linkage studies in a large kindred with hereditary pancreatitis confirms mapping of the gene to a 16-cM region on 7q. Genomics, 1996; 38: 227-30.
22. Rowen L, Koop BF, Hood L. The complete 685-kilobase DNA sequence of the human beta T cell receptor locus. Science, 1996; 272: 1755-62.
23. Bell SM, Bennett C, Markham AF, Lench NJ. Evidence for a common mutation in hereditary pancreatitis. Mol Pathol, 1998; 51: 115-7.
24. Creighton J, Lyall R, Wilson DI, Curtis A, Charnley R. Mutations of the cationic trypsinogen gene in patients with chronic pancreatitis. Lancet, 1999; 354: 42-3.
25. Ferec C, Raguenes O, Salomon R, Roche C, Bernard JP, Guillot M, Quere I, Faure C, Mercier B, Audrezet MP, Guillausseau PJ, Dupont C, Munnich A, Bignon JD, Le Bodic L. Mutations in the cationic trypsinogen gene and evidence for genetic heterogeneity in hereditary pancreatitis. J Med Genet, 1999; 36: 228-32.
26. Teich N, Mössner J, Keim V. Mutations of the cationic trypsinogen in hereditary pancreatitis. Hum Mutat, 1998; 12: 39-43.
27. Witt H, Luck W, Becker M. A signal peptide cleavage site mutation in the cationic trypsinogen gene is strongly associated with chronic pancreatitis. Gastroenterology, 1999; 117: 7-10.
28. Nishimori I, Kamakura M, Fujikawa-Adachi K, Morita M, Onishi S, Yokoyama K, Makino I, Ishida H, Yamamoto M, Watanabe S, Ogawa M. Mutations in exons 2 and 3 of the cationic trypsinogen gene in Japanese families with hereditary pancreatitis. Gut, 1999; 44: 259-63.
29. Dasouki MJ, Cogan J, Summar ML, Neblitt W, 3rd, Foroud T, Koller D, Phillips JA, 3rd. Heterogeneity in hereditary pancreatitis. Am J Med Genet, 1998; 77: 47-53.
30. Elitsur Y, Chertow BC, Jewell RD, Finver SN, Primerano DA. Identification of a hereditary pancreatitis mutation in four West Virginia families. Pediatr Res, 1998; 44: 927-30.
31. Simon P, Weiss FU, Sahin-Toth M, Parry M, Nayler O, Lenfers B, Schnekenburger J, Mayerle J, Domschke W, Lerch MM. Hereditary pancreatitis caused by a novel PRSS1 mutation (Arg-122 → Cys) that alters autoactivation and autodegradation of cationic trypsinogen. J Biol Chem, 2002; 277: 5404-10.
32. Sahin-Toth M. The pathobiochemistry of hereditary pancreatitis: studies on recombinant human cationic trypsinogen. Pancreatology, 2001; 1: 461-5.
33. Sahin-Toth M, Toth M. Gain-of-function mutations associated with hereditary pancreatitis enhance autoactivation of human cationic trypsinogen. Biochem Biophys Res Commun, 2000; 278: 286-9.
34. Sahin-Toth M. Human cationic trypsinogen. Role of Asn-21 in zymogen activation and implications in hereditary pancreatitis. J Biol Chem, 2000; 275: 22750-5.
35. Sahin-Toth M, Graf L, Toth M. Trypsinogen stabilization by mutation Arg117 → His: A unifying pathomechanism for hereditary pancreatitis? Biochem Biophys Res Commun, 1999; 264: 505-8.
36. Howes N, Greenhalf W, Rutherford S, O'Donnell M, Mountford R, Ellis I, Whitcomb D, Imrie C, Drumm B, Neoptolemos JP. A new polymorphism for the RI22H mutation in hereditary pancreatitis. Gut, 2001; 48: 247-50.
37. Gorry MC, Gabbaizedeh D, Furey W, Gates LK Jr., Preston RA, Aston CE, Zhang Y, Ulrich C, Ehrlich GD, Whitcomb DC. Mutations in the cationic trypsinogen gene are associated with recurrent acute and chronic pancreatitis. Gastroenterology, 1997; 113: 1063-8.
38. Whitcomb DC. Hereditary pancreatitis: new insights into acute and chronic pancreatitis. Gut, 1999; 45: 317-22.
39. Teich N, Bauer N, Mössner J, Keim V. Mutational screening of

patients with nonalcoholic chronic pancreatitis: identification of further trypsinogen variants. Am J Gastroenterol, 2002; 97: 341-6.

40. Chen JM, Piepoli Bis A, Le Bodic L, Ruszniewski P, Robaszkiewicz M, Deprez PH, Raguenes O, Quere I, Andriulli A, Ferec C. Mutational screening of the cationic trypsinogen gene in a large cohort of subjects with idiopathic chronic pancreatitis. Clin Genet, 2001; 59: 189-93.

41. Teich N, Mössner J, Keim V. Screening for mutations of the cationic trypsinogen gene: are they of relevance in chronic alcoholic pancreatitis? Gut, 1999; 44: 413-6.

42. Rossi L, Whitcomb DC, Ehrlich GD, Gorry MC, Parvin S, Sattar S, Ali L, Azad Khan AK, Gyr N. Lack of R117H mutation in the cationic trypsinogen gene in patients with tropical pancreatitis from Bangladesh. Pancreas, 1998; 17: 278-80.

43. Simon P, Weiss FU, Zimmer KP, Rand S, Brinkmann B, Domschke W, Lerch MM. Spontaneous and sporadic trypsinogen mutations in idiopathic pancreatitis. JAMA, 2002; 288: 2122-9.

44. Chen JM, Kukor Z, Le Marechal C, Toth M, Tsakiris L, Raguenes O, Ferec C, Sahin-Toth M. Evolution of trypsinogen activation peptides. Mol Biol Evol, 2003; 20: 1767-77.

45. Teich N, Ockenga J, Hoffmeister A, Manns M, Mössner J, Keim V. Chronic pancreatitis associated with an activation peptide mutation that facilitates trypsin activation. Gastroenterology, 2000; 119: 461-5.

46. Pfutzer R, Myers E, Applebaum-Shapiro S, Finch R, Ellis I, Neoptolemos J, Kant JA, Whitcomb DC. Novel cationic trypsinogen (PRSS1) N29T and R122C mutations cause autosomal dominant hereditary pancreatitis. Gut, 2002; 50: 271-2.

47. Chen JM, Le Marechal C, Lucas D, Raguenes O, Ferec C. "Loss of function" mutations in the cationic trypsinogen gene (PRSS1) may act as a protective factor against pancreatitis. Mol Genet Metab, 2003; 79: 67-70.

48. Le Marechal C, Bretagne JF, Raguenes O, Quere I, Chen JM, Ferec C. Identification of a novel pancreatitis-associated missense mutation, R116C, in the human cationic trypsinogen gene (PRSS1). Mol Genet Metab, 2001; 74: 342-4.

49. Bhatia E, Choudhuri G, Sikora SS, Landt O, Kage A, Becker M, Witt H. Tropical calcific pancreatitis: strong association with SPINK1 trypsin inhibitor mutations. Gastroenterology, 2002; 123: 1020-5.

50. Chandak GR, Idris MM, Reddy DN, Mani KR, Bhaskar S, Rao GV, Singh L. Absence of PRSS1 mutations and association of SPINK1 trypsin inhibitor mutations in hereditary and non-hereditary chronic pancreatitis. Gut, 2004; 53: 723-8.

51. Hassan Z, Mohan V, Ali L, Allotey R, Barakat K, Faruque MO, Deepa R, McDermott MF, Jackson AE, Cassell P, Curtis D, Gelding SV, Vijayaravaghan S, Gyr N, Whitcomb DC, Khan AK, Hitman GA. SPINK1 is a susceptibility gene for fibrocalculous pancreatic diabetes in subjects from the Indian subcontinent. Am J Hum Genet, 2002; 71: 964-8.

52. Perri F, Piepoli A, Andriulli A. On the role of CFTR, PSSR1 and PTS1/SPINK1 in idiopathic chronic pancreatitis. Eur J Hum Genet, 2003; 11: 107-8.

53. Pfutzer RH, Barmada MM, Brunskill AP, Finch R, Hart PS, Neoptolemos J, Furey WF, Whitcomb DC. SPINK1/PTS1 polymorphisms act as disease modifiers in familial and idiopathic chronic pancreatitis. Gastroenterology, 2000; 119: 615-23.

54. Rossi L, Pfutzer RH, Parvin S, Ali L, Sattar S, Kahn AK, Gyr N, Whitcomb DC. SPINK1/PSTI mutations are associated with tropical pancreatitis in Bangladesh. A preliminary report. Pancreatology, 2001; 1: 242-5.

55. Threadgold J, Greenhalf W, Ellis I, Howes N, Lerch MM, Simon P, Jansen J, Charnley R, Laugier R, Frulloni L, Olah A, Delhaye M, Ihse I, Schaffalitzky de Muckadell OB, Andren-Sandberg A, Imrie CW, Martinek J, Gress TM, Mountford R, Whitcomb D, Neoptolemos JP. The N34S mutation of SPINK1 (PSTI) is associated with a familial pattern of idiopathic chronic pancreatitis but does not cause the disease. Gut, 2002; 50: 675-81.

56. Weiss FU, Simon P, Witt H, Mayerle J, Hlouschek V, Zimmer KP, Schnekenburger J, Domschke W, Neoptolemos JP, Lerch MM. SPINK1 mutations and phenotypic expression in patients with pancreatitis associated with trypsinogen mutations. J Med Genet, 2003; 40: 40.

57. Witt H, Hennies HC, Becker MFC. SPINK1 mutations in chronic pancreatitis. Gastroenterology, 2001; 120: 1060-1.

58. Witt H, Luck W, Becker M, Bohmig M, Kage A, Truninger K, Ammann RW, O'Reilly D, Kingsnorth A, Schulz HU, Halangk W, Kielstein V, Knoefel WT, Teich N, Keim V. Mutation in the SPINK1 trypsin inhibitor gene, alcohol use, and chronic pancreatitis. JAMA, 2001; 285: 2716-7.

59. Arduino C, Gallo M, Brusco A, Garnerone S, Piana MR, Di Maggio S, Gerbino Promis G, Ferrone M, Angeli A, Gaia E. Polyvariant mutant CFTR genes in patients with chronic pancreatitis. Clin Genet, 1999; 56: 400-4.

60. Cohn JA, Noone PG, Jowell PS. Idiopathic pancreatitis related to CFTR: complex inheritance and identification of a modifier gene. J Investig Med, 2002; 50: 247S-55.

61. Feng J. Comprehensive detection of CFTR mutations in idiopathic chronic pancreatitis. Am J Human Gen, 2002; 71: 1833.

62. Reboul MP, Laharie D, Amouretti M, Lacombe D, Iron A. Isolated idiopathic chronic pancreatitis associated with a compound heterozygosity for two mutations of the CFTR gene. Gastroenterol Clin Biol, 2003; 27: 821-4.

63. Lowenfels AB, Maisonneuve P, Whitcomb DC. Risk factors for cancer in hereditary pancreatitis. International Hereditary Pancreatitis Study Group. Med Clin North Am, 2000; 84: 565-73.

64. Creighton JE, Lyall R, Wilson DI, Curtis A, Charnley RM. Mutations of the cationic trypsinogen gene in patients with hereditary pancreatitis. Br J Surg, 2000; 87: 170-5.

65. Truninger K, Kock J, Wirth HP, Muellhaupt B, Arnold C, von Weizsacker F, Seifert B, Ammann RW, Blum HE. Trypsinogen gene mutations in patients with chronic or recurrent acute pancreatitis. Pancreas, 2001; 22: 18-23.

66. Paolini O, Hastier P, Buckley M, Maes B, Demarquay JF, Staccini P, Bellon S, Caroli-Bosc FX, Dumas R, Delmont J. The natural history of hereditary chronic pancreatitis: a study of 12 cases compared to chronic alcoholic pancreatitis. Pancreas, 1998; 17: 266-71.

67. Elitsur Y, Hunt JA, Chertow BS. Hereditary pancreatitis in the children of West Virginia. Pediatrics, 1994; 93: 528-31.

68. Lowenfels AB, Maisonneuve P, Cavallini G, Ammann RW, Lankisch PG, Andersen JR, Dimagno EP, Andren-Sandberg A, Domellof L. Pancreatitis and the risk of pancreatic cancer. International Pancreatitis Study Group. N Engl J Med, 1993; 328: 1433-7.

69. Howes N, Greenhalf W, Stocken DD, Neoptolemos JP. Cationic trypsinogen mutations and pancreatitis. Clin Lab Med, 2005; 25(1): 39-59.

70. Lynch HT, Smyrk T, Kern SE, Hruban RH, Lightdale CJ, Lemon SJ, Lynch JF, Fusaro LR, Fusaro RM, Ghadirian P. Familial pancreatic cancer: a review. Semin Oncol, 1996; 23: 251-75.

71. Silverman DT, Schiffman M, Everhart J, Goldstein A, Lillemoe KD, Swanson GM, Schwartz AG, Brown LM, Greenberg RS, Schoenberg JB, Pottern LM, Hoover RN, Fraumeni JF Jr. Diabetes mellitus, other medical conditions and familial history of cancer as risk factors for pancreatic cancer. Br J Cancer, 1999; 80: 1830-7.

72. Bartsch DK. Familial pancreatic cancer. Br J Surg, 2003; 90(4): 386-7.

73. Bergman W, Watson P, de Jong J, Lynch HT, Fusaro RM. Systemic cancer and the FAMMM syndrome. Br J Cancer, 1990; 61: 932-6.

74. Su GH, Hruban RH, Bansal RK, Bova GS, Tang DJ, Shekher MC, Westerman AM, Entius MM, Goggins M, Yeo CJ, Kern SE. Germline and somatic mutations of the STK11/LKB1 Peutz-Jeghers gene in pancreatic and biliary cancers. Am J Pathol, 1999; 154: 1835-40.

75. Malats N, Casals T, Porta M, Guarner L, Estivill X, Real FX. Cystic fibrosis transmembrane regulator (CFTR) DeltaF508 mutation and 5T allele in patients with chronic pancreatitis and exocrine pancreatic cancer. PANKRAS II Study Group. Gut, 2001; 48: 70-4.

76. Lerch MM, Ellis I, Whitcomb DC, Keim V, Simon P, Howes N, Rutherford S, Domschke W, Imrie C, Neoptolemos JP. Maternal inheritance pattern of hereditary pancreatitis in patients with pancreatic carcinoma. J Natl Cancer Inst, 1999; 91: 723-4.

77. Jemal A, Murray T, Samuels A, Ghafoor A, Ward E, Thun MJ. Cancer statistics, 2003. CA Cancer J Clin, 2003; 53: 5-26.

78. Neoptolemos JP, Cunningham D, Friess H, Bassi C, Stocken DD, Tait DM, Dunn JA, Dervenis C, Lacaine F, Hickey H, Raraty MG, Ghaneh P, Buchler MW. Adjuvant therapy in pancreatic cancer: historical and current perspectives. Ann Oncol, 2003; 14: 675-92.

79. Parkin DM, Muir CS. Cancer incidence in five continents. comparability and quality of data. IARC Sci Publ, 1992; 120: 45-73.

80. Rosch T, Schusdziarra V, Born P, Bautz W, Baumgartner M, Ulm K, Lorenz R, Allescher HD, Gerhardt P, Siewert JR, Classen M. Modern imaging methods versus clinical assessment in the evaluation of hospital in-patients with suspected pancreatic disease. Am J Gastroenterol, 2000; 95: 2261-70.

81. Yan L, McFaul C, Howes N, Leslie J, Lancaster G, Wong T, Threadgold J, Evans J, Gilmore I, Smart H, Lombard M, Neoptolemos J, Greenhalf W. Molecular analysis to detect pancreatic ductal adenocarcinoma in high-risk groups. Gastroenterology, 2005; 128: 2124-30.

82. Lowenfels AB, Maisonneuve P, Whitcomb DC, Lerch MM, DiMagno EP. Cigarette smoking as a risk factor for pancreatic cancer in patients with hereditary pancreatitis. JAMA, 2001; 286: 169-70.

83. Ellis I, Lerch MM, Whitcomb DC. Genetic testing for hereditary pancreatitis: guidelines for indications, counselling, consent and privacy issues. Pancreatology, 2001; 1: 405-15.

84. Goggins M, Canto M, Hruban R. Can we screen high-risk individuals to detect early pancreatic carcinoma? J Surg Oncol, 2000; 74: 243-8.

85. Ariyama J, Suyama M, Satoh K, Sai J. Imaging of small pancreatic ductal adenocarcinoma. Pancreas, 1998; 16: 396-401.

86. Ishikawa O, Ohigashi H, Imaoka S, Nakaizumi A, Uehara H, Kitamura T, Kuroda C. Minute carcinoma of the pancreas measuring 1 cm or less in diameter – collective review of Japanese case reports. Hepatogastroenterology, 1999; 46: 8-15.

87. Brentnall TA. Cancer surveillance of patients from familial pancreatic cancer kindreds. Med Clin North Am, 2000; 84: 707-18.

88. Kalra MK, Maher MM, Mueller PR, Saini S. State-of-the-art imaging of pancreatic neoplasms. Br J Radiol, 2003; 76: 857-65.

89. Soriano A, Castells A, Ayuso C, Ayuso JR, de Caralt MT, Gines MA, Real MI, Gilabert R, Quinto L, Trilla A, Feu F, Montanya X, Fernandez-Cruz L, Navarro S. Preoperative staging and tumor resectability assessment of pancreatic cancer: prospective study comparing endoscopic ultrasonography, helical computed tomography, magnetic resonance imaging, and angiography. Am J Gastroenterol, 2004; 99: 492-501.

90. Imbriaco M, Megibow AJ, Camera L, Pace L, Mainenti PP, Romano M, Selva G, Salvatore M. Dual-phase versus single-phase helical CT to detect and assess resectability of pancreatic carcinoma. AJR Am J Roentgenol, 2002; 178: 1473-9.

91. Nishiharu T, Yamashita Y, Abe Y, Mitsuzaki K, Tsuchigame T, Nakayama Y, Takahashi M. Local extension of pancreatic carcinoma: assessment with thin-section helical CT versus with breath-hold fast MR imaging – ROC analysis. Radiology, 1999; 212: 445-52.

92. Sheridan MB, Ward J, Guthrie JA, Spencer JA, Craven CM, Wilson D, Guillou PJ, Robinson PJ. Dynamic contrast-enhanced MR imaging and dual-phase helical CT in the preoperative assessment of suspected pancreatic cancer: a comparative study with receiver operating characteristic analysis. AJR Am J Roentgenol, 1999; 173: 583-90.

93. Hruban RH, Adsay NV, Albores-Saavedra J, Compton C, Garrett ES, Goodman SN, Kern SE, Klimstra DS, Klöppel G, Longnecker DS, Luttges J, Offerhaus GJ. Pancreatic intraepithelial neoplasia: a new nomenclature and classification system for pancreatic duct lesions. Am J Surg Pathol, 2001; 25: 579-86.

94. Mertz HR, Sechopoulos P, Delbeke D, Leach SD. EUS, PET, and CT scanning for evaluation of pancreatic adenocarcinoma. Gastrointest Endosc, 2000; 52: 367-71.

95. Smith SL, Rajan PS. Imaging of pancreatic adenocarcinoma with emphasis on multidetector CT. Clin Radiol, 2004; 59: 26-38.

96. Gangi S, Fletcher JG, Nathan MA, Christensen JA, Harmsen WS, Crownhart BS, Chari ST. Time interval between abnormalities seen on CT and the clinical diagnosis of pancreatic cancer: retrospective review of CT scans obtained before diagnosis. AJR Am J Roentgenol, 2004; 182: 897-903.

97. Bronstein YL, Loyer EM, Kaur H, Choi H, David C, DuBrow RA, Broemeling LD, Cleary KR, Charnsangavej C. Detection of small pancreatic tumors with multiphasic helical CT. AJR Am J Roentgenol, 2004; 182: 619-23.

98. Kim T, Murakami T, Takamura M, Hori M, Takahashi S, Nakamori S, Sakon M, Tanji Y, Wakasa K, Nakamura H. Pancreatic mass due to chronic pancreatitis: correlation of CT and MR imaging features with pathologic findings. AJR Am J Roentgenol, 2001; 177: 367-71.

99. Fujita N, Noda Y, Kobayashi G, Kimura K, Ito K. Endoscopic approach to early diagnosis of pancreatic cancer. Pancreas, 2004; 28: 279-81.

100. Niederau C, Grendell JH. Diagnosis of pancreatic carcinoma. Imaging techniques and tumor markers. Pancreas, 1992; 7: 66-86.

101. Duggan MA, Brasher P, Medlicott SA. ERCP-directed brush cytology prepared by the Thinprep method: test performance and morphology of 149 cases. Cytopathology, 2004; 15: 80-6.

102. De Bellis M, Sherman S, Fogel EL, Cramer H, Chappo J, McHenry L, Jr., Watkins JL, Lehman GA. Tissue sampling at ERCP in suspected malignant biliary strictures (Part 1). Gastrointest Endosc, 2002; 56: 552-61.

103. Foutch PG, Kerr DM, Harlan JR, Kummet TD. A prospective, controlled analysis of endoscopic cytotechniques for diagnosis of malignant biliary strictures. Am J Gastroenterol, 1991; 86: 577-80.

104. Lee JG, Leung JW, Baillie J, Layfield LJ, Cotton PB. Benign, dysplastic, or malignant-making sense of endoscopic bile duct brush cytology: results in 149 consecutive patients. Am J Gastroenterol, 1995; 90: 722-6.

105. Ponchon T, Gagnon P, Berger F, Labadie M, Liaras A, Chavaillon A, Bory R. Value of endobiliary brush cytology and biopsies for the diagnosis of malignant bile duct stenosis: results of a prospective study. Gastrointest Endosc, 1995; 42: 565-72.

106. Pugliese V, Conio M, Nicolo G, Saccomanno S, Gatteschi B. Endoscopic retrograde forceps biopsy and brush cytology of biliary strictures: a prospective study. Gastrointest Endosc, 1995; 42: 520-6.

107. Glasbrenner B, Ardan M, Boeck W, Preclik G, Moller P, Adler G. Prospective evaluation of brush cytology of biliary strictures during endoscopic retrograde cholangiopancreatography. Endoscopy, 1999; 31: 712-7.

108. Mansfield JC, Griffin SM, Wadehra V, Matthewson K. A prospective evaluation of cytology from biliary strictures. Gut, 1997; 40: 671-7.

109. Jailwala J, Fogel EL, Sherman S, Gottlieb K, Flueckiger J, Bucksot LG, Lehman GA. Triple-tissue sampling at ERCP in malignant biliary obstruction. Gastrointest Endosc, 2000; 51: 383-90.

110. Macken E, Drijkoningen M, Van Aken E, Van Steenbergen W. Brush cytology of ductal strictures during ERCP. Acta Gastroenterol Belg, 2000; 63: 254-9.

111. Riker A, Libutti SK, Bartlett DL. Advances in the early detection, diagnosis, and staging of pancreatic cancer. Surg Oncol, 1997; 6: 157-69.

112. Loperfido S, Angelini G, Benedetti G, Chilovi F, Costan F, De Berardinis F, De Bernardin M, Ederle A, Fina P, Fratton A. Major early complications from diagnostic and therapeutic ERCP: a prospective multicenter study. Gastrointest Endosc, 1998; 48: 1-10.

113. Vandervoort J, Soetikno RM, Tham TC, Wong RC, Ferrari AP Jr., Montes H, Roston AD, Slivka A, Lichtenstein DR, Ruymann FW, Van Dam J, Hughes M, Carr-Locke DL. Risk factors for complications after performance of ERCP. Gastrointest Endosc, 2002; 56: 652-6.

114. Suissa A, Yassin K, Lavy A, Lachter J, Chermech I, Karban A, Tamir A, Eliakim R. Outcome and early complications of ERCP: a prospective single center study. Hepatogastroenterology, 2005; 52: 352-5.

115. Almoguera C, Shibata D, Forrester K, Martin J, Arnheim N, Perucho M. Most human carcinomas of the exocrine pancreas contain mutant c-K-ras genes. Cell, 1988; 53: 549-54.

116. Howes N, Greenhalf W, Neoptolemos J. Screening for early pancreatic ductal adenocarcinoma in hereditary pancreatitis. Med Clin North Am, 2000; 84: 719-38.

117. Caldas C, Hahn SA, da Costa LT, Redston MS, Schutte M, Seymour AB, Weinstein CL, Hruban RH, Yeo CJ, Kern SE. Frequent somatic mutations and homozygous deletions of the p16 (MTS1) gene in pancreatic adenocarcinoma. Nat Genet, 1994; 8: 27-32.

118. Schutte M, Hruban RH, Geradts J, Maynard R, Hilgers W, Rabindran SK, Moskaluk CA, Hahn SA, Schwarte-Waldhoff I, Schmiegel W, Baylin SB, Kern SE, Herman JG. Abrogation of the Rb/p16 tumor-suppressive pathway in virtually all pancreatic carcinomas. Cancer Res, 1997; 57: 3126-30.

119. Fukushima N. Diagnosing pancreatic cancer using methylation specific PCR analysis of pancreatic juice. Cancer Biol Ther, 2003; 2: 78-83.

120. Matsubayashi H, Sato N, Fukushima N, Yeo CJ, Walter KM, Brune K, Sahin F, Hruban RH, Goggins M. Methylation of cyclin D2 is observed frequently in pancreatic cancer but is also an age-related phenomenon in gastrointestinal tissues. Clin Cancer Res, 2003; 9: 1446-52.

121. Klump B, Hsieh CJ, Nehls O, Dette S, Holzmann K, Kiesslich R, Jung M, Sinn U, Ortner M, Porschen R, Gregor M. Methylation status of p14ARF and p16INK4a as detected in pancreatic secretions. Br J Cancer, 2003; 88: 217-22.

122. Rozenblum E, Schutte M, Goggins M, Hahn SA, Panzer S, Zahurak M, Goodman SN, Sohn TA, Hruban RH, Yeo CJ, Kern SE. Tumor-suppressive pathways in pancreatic carcinoma. Cancer Res, 1997; 57: 1731-4.

123. Kirsch DG, Kastan MB. Tumor-suppressor p53: implications for tumor development and prognosis. J Clin Oncol, 1998; 16: 3158-68.

124. Yamaguchi Y, Watanabe H, Yrdiran S, Ohtsubo K, Motoo Y, Okai T, Sawabu N. Detection of mutations of p53 tumor suppressor gene in pancreatic juice and its application to diagnosis of patients with pancreatic cancer: comparison with K-ras mutation. Clin Cancer Res, 1999; 5: 1147-53.

125. Goldstein AM, Fraser MC, Struewing JP, Hussussian CJ, Ranade K, Zametkin DP, Fontaine LS, Organic SM, Dracopoli NC, Clark WH Jr., et al. Increased risk of pancreatic cancer in melanoma-prone kindreds with p16INK4 mutations. N Engl J Med, 1995; 333: 970-4.

126. Harewood GC, Wiersema LM, Halling AC, Keeney GL, Salamao DR, Wiersema MJ. Influence of EUS training and pathology interpretation on accuracy of EUS-guided fine needle aspiration of pancreatic masses. Gastrointest Endosc, 2002; 55: 669-73.

127. Canto MI, Goggins M, Yeo CJ, Griffin C, Axilbund JE, Brune K, Ali SZ, Jagannath S, Petersen GM, Fishman EK, Piantadosi S, Giardiello FM, Hruban RH. Screening for pancreatic neoplasia in high-risk individuals: an EUS-based approach. Clin Gastroenterol Hepatol, 2004; 2: 606-21.

128. American Gastroenterological Association. American gastroenterological association medical position statement: epidemiology, diagnosis, and treatment of pancreatic ductal adenocarcinoma. Gastroenterology, 1999; 117: 1463-84.

129. Rulyak SJ, Kimmey MB, Veenstra DL, Brentnall TA. Cost-effectiveness of pancreatic cancer screening in familial pancreatic cancer kindreds. Gastrointest Endosc, 2003; 57: 23-9.

130. Rulyak SJ, Brentnall TA. Inherited pancreatic cancer: improvements in our understanding of genetics and screening. Int J Biochem Cell Biol, 2004; 36: 1386-92.

131. Brentnall TA, Bronner MP, Byrd DR, Haggitt RC, Kimmey MB. Early diagnosis and treatment of pancreatic dysplasia in patients with a family history of pancreatic cancer. Ann Intern Med, 1999; 131: 247-55.

132. Charpentier KP, Brentnall TA, Bronner MP, Byrd D, Marsh C. A new indication for pancreas transplantation: high grade pancreatic dysplasia. Clin Transplant, 2004; 18: 105-7.

133. Ritts RE, Pitt HA. CA 19-9 in pancreatic cancer. Surg Oncol Clin N Am, 1998; 7: 93-101.

134. Kim JE, Lee KT, Lee JK, Paik SW, Rhee JC, Choi KW. Clinical usefulness of carbohydrate antigen 19-9 as a screening test for pancreatic cancer in an asymptomatic population. J Gastroenterol, Hepatol, 2004; 19: 182-6.

135. Safi F, Schlosser W, Falkenreck S, Beger HG. CA 19-9 serum course and prognosis of pancreatic cancer. Int J Pancreatol, 1996; 20: 155-61.

136. Tian F, Appert HE, Myles J, Howard JM. Prognostic value of serum CA 19-9 levels in pancreatic adenocarcinoma. Ann Surg, 1992; 215: 350-5.

137. Mann DV, Edwards R, Ho S, Lau WY, Glazer G. Elevated tumour marker CA19-9: clinical interpretation and influence of obstructive jaundice. Eur J Surg Oncol, 2000; 26: 474-9.

138. Malesci A, Tommasini MA, Bonato C, Bocchia P, Bersani M, Zerbi A, Beretta E, Di Carlo V. Determination of CA 19-9 antigen in serum and pancreatic juice for differential diagnosis of pancreatic adenocarcinoma from chronic pancreatitis. Gastroenterology, 1987; 92: 60-7.

139. Forsmark CE, Vogel SB. Diagnosis of pancreatic cancer and prediction of unresectability using the tumor-associated antigen CA19-9. Pancreas, 1994; 9: 731-4.

140. Moskaluk CA, Hruban RH, Kern SE. p16 and K-ras gene mutations in the intraductal precursors of human pancreatic adenocarcinoma. Cancer Res, 1997; 57: 2140-3.

141. Brat DJ, Lillemoe KD, Yeo CJ, Warfield PB, Hruban RH. Progression of pancreatic intraductal neoplasias to infiltrating adenocarcinoma of the pancreas. Am J Surg Pathol, 1998; 22: 163-9.

142. Wilentz RE, Iacobuzio-Donahue CA, Argani P, McCarthy DM, Parsons JL, Yeo CJ, Kern SE, Hruban RH. Loss of expression of Dpc4 in pancreatic intraepithelial neoplasia: evidence that DPC4 inactivation occurs late in neoplastic progression. Cancer Res, 2000; 60: 2002-6.

143. Sohn TA, Yeo CJ, Cameron JL, Koniaris L, Kaushal S, Abrams RA, Sauter PK, Coleman J, Hruban RH, Lillemoe KD. Resected adenocarcinoma of the pancreas-616 patients: results, outcomes, and prognostic indicators. J Gastrointest Surg, 2000; 4: 567-79.

144. Hruban RH, Wilentz RE, Kern SE. Genetic progression in the pancreatic ducts. Am J Pathol, 2000; 156: 1821-5.

145. Hruban RH, Goggins M, Parsons J, Kern SE. Progression model for pancreatic cancer. Clin Cancer Res, 2000; 6: 2969-72.

Extent of lymphadenectomy in the resection of pancreatic cancer. Analysis of the existing evidence

Pedrazzoli S, Pasquali C*, Sperti C

Department of Medical and Surgical Sciences, University of Padua, Padova, Italy

Abstract

Pancreaticoduodenectomy is considered the standard procedure for the surgical treatment of the pancreatic head cancer. However, the extent of lymph node clearance associated to the procedure is still largely debated. Arguments in favour of an extended lymphadenectomy are the regular progression of lymph node invasion, without skip metastases, and the removal of the extrapancreatic neural plexus that is invaded in 52-72% of patients. Arguments against the extended lymphadenectomy are the failure of extended lymphadenectomy to improve survival in other cancers, and the severe diarrhoea that follows the skeletonisation of the superior mesenteric artery. After Ishikawa's paper, several retrospective studies supported a longer survival after an extended than after a standard lymphadenectomy, but as much retrospective studies failed to demonstrate any difference.

Only three prospective randomised controlled trials have been performed so far. Unfortunately all are underpowered, and the substantial differences in the surgical procedures, in the adjuvant treatment, and in the length of follow-up make the comparison impossible. Only one study reports a significantly longer survival for lymph node positive patients who underwent an extended lymphadenectomy, but adjuvant treatment was not performed. Furthermore, the difference was of minimal clinical impact.

At least two adequately powered prospective Randomised Controlled Trials including a true extended lymphadenectomy, and a standardised adjuvant treatment, would be required to answer the question. Unfortunately, we have not yet a standardised adjuvant (or neoadjuvant) treatment, and we do not know the impact of such treatment on the expected statistical difference in the survival after a standard or extended lymphadenectomy. The lot of work required to perform such trials probably doesn't worth the expected results.

Key words: pancreatic cancer, pancreaticoduodenectomy, lymphadenectomy.

* CORRESPONDING AUTHOR:
Department of Medical and Surgical Sciences
University of Padua
Via Giustiniani, 2
35123 Padova, Italy
Tel: +39 049 8218832; Fax: +39 049 8764671
e-mail: claudio.pasquali@unipd.it (Claudio Pasquali)

Received 01.03.2005 Accepted 26.04.2005

Introduction

Although incidence of pancreatic cancer ranks tenth among the leading cancer types, it is the fourth or fifth cause of cancer related deaths in the Western Countries [1,2]. Surgical resection is possible only in 10-20% of all patients. However, it is the only chance for long term survival, although patients cured of this disease are very rare [3]. Lymph node metastases are among the predictors of shorter survival together with the size of the primary tumour, the degree of tumour differentiation, the status of the resection margins (R0, R1, and R2), and the DNA ploidy. Several surgical procedures were proposed to increase the radicality of surgery and, hopefully, the number of long-term survivors. Total pancreatectomy was performed with increasing frequency since the first procedure for pancreatic cancer was reported in 1943 by Rockey [4], and a longer survival was reported by ReMine et al. [5] and Brooks and Coulebras [6]. A steep decline started during eighties, when the expected longer survival failed to be demonstrated [7-9]. The worsening of the quality of life due to the brittle diabetes and to the complete exocrine insufficiency, limited the use of total pancreatectomy to patients with positive resection margins at frozen section or to cancers not resectable with partial pancreatectomy [8-9]. The so-called "regional pancreatectomy", including vascular resections together with wide lymphadenectomy, was proposed by

Fortner in 1973 [10], but was followed by very few Surgeons and then abandoned in its full complexity. Apart of the very important contribution to the knowledge of the tumour spread, some aspects of the procedure were resumed later on. While arterial resections are now considered useless and high risk procedures, resection of the mesenteric-portal vein, as proposed by Fortner, is considered useful if performed to obtain clear margins without increasing the mortality rate. The sixth edition of the AJCC cancer staging manual includes the mesenteric-portal vein invasion among the resectable stage IIA (N-) or IIB (N+) lesions [11].

Also the radical lymphadenectomy was resumed by Ishikawa who reported in 1988, a significantly longer survival for patients who underwent an extended lymphadenectomy when compared to those who received only a standard lymphadenectomy. The study opened a wide debate among surgeons, with many supporting extended lymphadenectomy [12-25], while many others considering it useless [26-34]. However, the actual extent of lymphadenectomy to be associated with pancreatic resection is different among different Authors. The terms Palliative, Standard, Traditional, Extended, D1, D2, D3, Regional, Extended Retro-Peritoneal, Standard Radical, and Extended Radical are only some of those used to indicate the extent of lymph node dissection. Furthermore, the same word was also used to describe quite different procedures. To try to overcome the problems created by this Babel of terms, a Consensus Conference took place in Castelfranco Veneto, Italy on May 30 1998. The definitions, based on the lymph node classification of the Japan Pancreas Society, have been published [35,36]. Unfortunately no prospective study based on those definitions was performed. However, the prospective ESPAC-3 study (European Study Group for Pancreatic Cancer) on adjuvant treatment of pancreatic cancer included those definitions in the report of the surgical procedure performed.

Theoretical basis for an extended lymphadenectomy

Lymph node metastases of pancreatic cancer follow a strict sequential rule of invasion. Hermanek reports an incidence of 3% of skip metastases, although the lymphatic drainage may be uni- or multidirectional [37]. Skip metastases were very rare also in the study of Pedrazzoli et al. [22]. The variability of the routes followed by lymph node metastases may explain why Kocher et al. failed to find a sentinel node in pancreatic cancer [38]. In 1992, Kayahara reported that the main lymphatic pathway from the head of the pancreas to the para-aortic lymph nodes was through the lymph nodes around the superior mesenteric artery [39]. Furthermore, Japanese Authors demonstrated that the extrapancreatic neural plexus was invaded in 52-72% of patients with pancreatic cancer [40-44] and the mesenteric lymph node invasion was significantly more frequent (52% vs 8%) in patients with extrapancreatic neural plexus invasion [44]. Therefore the resection of a pancreatic head cancer without a lymphadenectomy encompassing all mesenteric nodes and leaving behind the extrapancreatic neural plexus was considered inadequate by most of the Japanese Authors. Another advantage for an extended lymphadenectomy is a better classification of the extent of the disease.

Table 1. **Morbidity and mortality rate after a "Standard" or "Extended lymphadenectomy"** [22,34,52]

	Standard PD (n=209)	Extended PD (n=213)	P value
Complication Yes/No	34/66	42/58	NS
Pancreatic fistula %	8.6	12.2	NS
Intra-abdominal abscess %	3.8	3.3	NS
Bile leak %	3.8	4.7	NS
Delayed gastric emptying %	5.9	15.1*	NS
Wound infection %	4.3	11.1	NS
Reoperation %	4.3	4.2	NS
Perioperative mortalita %	4.3	4.2	NS

* Mainly due to distal gastrectomy [34]

Theoretical basis against an extended lymphadenectomy

Extended lymphadenectomy is still under scrutiny for the surgical treatment of breast, oesophageal, gastrointestinal cancers [45-50]. This means, that in spite of hundred of published papers on these topics, a definite conclusion was not reached. On the other side, lymph nodes should be considered for their unique function: "They are neither Millipore filters, nor open lymph channels, but porous filters that temporarily hold up antigens entering through the afferent lymphatic. Lymph node lymphocytes have the opportunity to identify antigens and begin antibody production, an essential component of the immunocompetence of the host" [50]. Lymphadenectomy of non metastatic lymph nodes may eliminate also lymphocytes that are immunocompetent against pancreatic cancer cells. Based on the incidence of an R0 resection no higher than 80%, on the frequency of 10% of pathologic involvement of the second-echelon lymph nodes (N2 disease), on the frequency of truly M0 pancreatic adenocarcinoma of 5%, Pisters et al. [51] estimated that to detect a difference in a randomised trial with 80% power 238,000 patients would be required.

Evaluation of the risk of the procedure

Extended lymphadenectomy increases the morbidity, and in some studies also the mortality rate, of the surgical treatment of oesophageal and gastric cancer [47-49]. It is obvious that an increased morbidity and/or mortality rate is expected also after a pancreaticoduodenectomy with extended lymphadenectomy. *Tab. 1* reports the results of three studies [22,34,52] pooled altogether. Although some differences were present, both for the standard and the extended lymphadenectomy, within the three studies, the complication and mortality rates were very similar. This means that, in experienced institutions, an extended lymphadenectomy can be performed safely.

***Table 2*. Published reports on standard and extended lymphadenectomy in pancreaticoduodenectomy**

	Patients per group		Mortality %		Morbidity %		5-year survival %		Study type, evidence level ¶
	Standard	Extended	Standard	Extended	Standard	Extended	Standard	Extended	
Henne-Bruns et al. [20]		26 46		3.8 6.5		n.d. n.d.		35.0 17.6	Prospective, non randomised/2b
Fernandez-Cruz et al. [23] †		34		0		50		n.d.	Prospective/2c
Gazzaniga et al. [32]	48	45+31#	8.3	3.9	29	26	6.8	13.1	Prospective, non randomised/2b
Iacono et al. [24]	13	17	0	0	46	47	n.d.	n.d.	Prospective, non randomised/2b
Popiela et al. [33] ‡	65	136	6.9	6.9	43	43	16.7 67.6 †	16.7 67.6 †	Prospective, non randomised/2b
Capussotti et al. [25] ‡	37	112	5.4	6.3	35	38	8.4	8.4	Prospective, non randomised/2b
Pedrazzoli et al. [22]	40	41	5	5	45	34	7.5 ¥	0 ¥	Prospective, randomised/1b
Yeo et al. [34] ‡ Pancreatic cancer	146 84	148 83	4.1	2	29	43	23 10	29 25	Prospective, randomised/1b
Nimura et al. [55]	51	50	0	2	10	20	29.3	15.1	Prospective, randomised/1b

¶ – Oxford Centre for Evidence-based Medicine Levels of Evidence (May 2001)
† – Ampullary cancers
# – Adjuvant chemotherapy
‡ – Pancreatic and ampullary cancers
¥ – Four-year actual survival
* – P<0.01

Evaluation of the Quality of Life (QOL) after the procedure

Severe diarrhoea is the main patient's complaint after an extended lymphadenectomy. Ishikawa reported a watery diarrhoea "which necessitates daily administration of both opium (for 6-30 months) and intravenous infusion (for 1-4 months)" [53]. Furthermore, "these patients stayed in the hospital for 2.5 postoperative months on average, and more than half of them needed re-hospitalization for the treatment of oedema caused by poor nutrition" [53]. Severe diarrhoea was also reported by Henne-Bruns [20] in 76% of the 46 patients who underwent extended radical retroperitoneal lymphadenectomy, by Mosca and Boggi [54] in 33% of patients, and by Nimura et al. [55] in 48% of patients. The presence of severe diarrhoea was not evaluated during the prospective study of Pedrazzoli et al. [22]. However, a 15-20% of motility disorders, mainly diarrhoea, were reported during the discussion of the paper. Fortner reported only 1 patient with profuse diarrhoea among 97 patients who underwent regional pancreatectomy [56]. On the other hand, Yeo et al. [34] did not found any difference in QOL among patients who underwent standard or extended lymphadenectomy. Therefore we must admit that there are several important differences in the surgical technique of an extended lymphadenectomy that can explain the different incidence of severe diarrhoea.

Evidence based data comparing standard vs radical pancreaticoduodenectomy

First of all we need to clarify that the extent of the so-called standard and radical (extended) procedures are different among both retrospective and prospective studies. Some of those differences are reported in a previous review [57]. We have already published a detailed analysis of retrospective studies published prior to 1999 [58]. We will complete the analysis of papers published after that review (*Tab. 2*). Henne-Bruns et al. [20] compared a regional lymphadenectomy (RLA) performed in 26 patients with an extended radical retroperitoneal lymphadenectomy (ELA) performed in 46 patients. The RLA was similar to the radical pancreaticoduodenectomy and ELA was similar to the extended radical pancreaticoduodenectomy as defined in Castelfranco Veneto in 1998 [35]. Therefore, both procedures should be considered as "extended" lymphadenectomies. There was no difference in morbidity, mortality rate and survival between the two groups of patients. Fernandez-Cruz et al. [23] performed a pylorus preserving pancreaticoduodenectomy with radical lymphadenectomy in 34 consecutive patients with ampullary cancer. On the basis of the documented lymph node spread, the Authors concluded for a need of a wide extensive lymph node dissection. Gazzaniga et al. [32] reported on 124 patients that underwent pancreaticoduodenectomy with D1 (n=48), or D2 (n=76) lymphadenectomy. Thirty-one D2 patients received also adjuvant treatment. The morbidity and mortality rate, as well as long-term survival, were the same for the three groups of patients. Iacono et al. [24] reported on 30 patient that underwent standard (n=13) or extended (n=17) lymphadenectomy. The morbidity and mortality rate were the same for the two groups of patients, while the long-term survival was prolonged after an extended lymphadenectomy. Popiela et al. resected 201 patients with pancreatic or ampullary cancer. Sixty-five underwent standard and 136 extended lymphadenectomy. Curiously, patients with lymph node negative pancreatic cancer had a significantly higher 5-year survival after an extended procedure. Capussotti et al. [25] reported on a consecutive series of 149 periampullary adenocarcinoma. A standard resection was performed in 112 patients, an extended lymphadenectomy

in 37 patients. Although the 5-year survival was the same, the extended lymphadenectomy "was the most powerful determinant of 2-year survival by multivariate analysis". In 2002 Yeo et al. reported the results of the largest RCT on standard versus extended lymphadenectomy. Overall 299 patients were enrolled in the study; 5 were subsequently excluded leaving 294 patients for analysis: 167 pancreatic and 132 periampullary cancers. The overall survival, the disease specific survival and the lymph node positive pancreatic cancer patient's survival was the same after the standard or the extended procedure. More recently, Yuji Nimura [55] presented the preliminary results of a prospective randomised controlled trial performed by 14 Centres in Japan between March 2000 and May 2003. Patient's accrual was stopped after 112 enrolled cases with 11 drop-out. The decision was taken on the basis of the very preliminary results showing no difference in survival. The only significant difference reported by Prof. Nimura was the worse QOL at 3 months after an extended lymphadenectomy, mainly due to the severe diarrhoea in 48% of patients.

Table 3. **Characteristics of the three prospective RCT**

	Pedrazzoli [22]	Yeo [34]	Nimura [55]
Period	1991–1994	1996–2001	2000–2003
Partecipating Centres	6	1	14
Enrolled patients	83	299	112
Drop-out patients	2	5	11
Histology reviewer	External	Internal	n.r.
Skeletonization celiac axis and SMA	Yes	Partial	Yes
Harvested lymph nodes:			
Standard	13.3	17	13.3
Extended	19.8	28.5	40.1
Adjuvant treatment	No	Yes (78%)	No
Survival report	Actual	Actuarial	Actuarial
Minimum follow-up	4 years	2 years	1 year

n.r. – not reported

Discussion

Is it possible, from the data reported in the literature, to draw any conclusion about the usefulness of an extended lymphadenectomy associated to the pancreaticoduodenectomy in patients with pancreatic cancer?

If we follow Pisters' conclusion [51], the demonstration of a hypothetical difference of 0.4% in a randomised trial with 80% power, would require 238,000 patients randomised to each of the two arms. If this is true, the conclusion should be that extended lymphadenectomy is useless.

However, Pisters' statement based on two wrong assumptions. In fact, if the first assumption that the incidence of an R0 resection may be no higher than 80% is correct, the second assumption about the frequency of pathologic involvement of the further tissue removed with an extended lymphadenectomy and the third assumption that only M0 patients benefit from an extended lymphadenectomy are incorrect:

1. The statement that "only patients who have pathologically involved second-echelon lymph nodes (N2 disease) (invaded in 10% of patients) can benefit …" should be reviewed, because an extended lymphadenectomy removes also the extrapancreatic nerve plexus that is invaded in 52-72% of patients with pancreatic cancer [40-44]. Therefore the frequency of involved second echelon lymph nodes and/or of the extrapancreatic nerve plexus is estimated to be 60% (0.60) and not 10% (0.10).

2. The statement that "only patients who have involved lymph nodes without visceral metastatic disease are likely to derive a survival benefit…" is wrong both because it is very rare to find patients with N2 disease that are truly M0 (long-term survivors), and because the reported difference in survival rate for N+ patients is restricted to the second year [22,15,25]. The percentage of patients that will benefit from the removal of the small amount of more tumour cells removed together N2 lymph nodes and the extrapancreatic nerve plexus is difficult to evaluate. Patients who die within one year after surgery have enough M1 undetectable disease to benefit from the removal of the small amount of more tumour cells included in an extended lymphadenectomy. On the other hand, very few of the long-term survivors are N2+ and/or extrapancreatic nerve plexus +. Therefore, it is impossible to define how many patients will actually benefit from this point of view.

In 2001 I was requested to prepare a prospective randomised study based on the previous experience [22], on the results of the consensus conference of Castelfranco Veneto [35], and on the survival curves of the ESPAC-1 study [60]. 15 European Centres gave their consent to participate. The primary endpoint was the 2-year survival proportion. The hypothesis to be tested using the log rank test was an improvement in the 2-year survival from 20% to 40% when lymph node positive patients undergo a "Radical" pancreaticoduodenectomy. To detect this difference with 80% power and alpha equal to 0.05 level of significance (two-sided), a total of 284 patients were needed to be confident that at least 75 lymph node positive patients (53% of the total) were included in each surgical arm ("Standard" or "Radical" pancreatoduodenectomy). The sample size was also able to detect an improvement in the 3-year survival from 7 to 20% with the same power and alpha levels [61]. The possible improvement after a "Radical" pancreaticoduodenectomy of the 2 and 3-year survival rate of the N-0 patients in whom lymph node micro metastases were detected (50-70% of the N-0 patients) was also included in the evaluation. Unfortunately the study aborted.

Therefore we have only three prospective randomised controlled trials. They show no survival advantage from an extended lymphadenectomy [22,34,55]. However, when subgroups of patients were analyzed, using an a posteriori analysis that was not planned at the time of study design, the first trial [22] reported a statistically significant ($p<0.05$), although clinically modest, longer survival rate in node positive patients after an extended rather than a standard lymphadenectomy. The differences among the three trials are reported in *Tab. 3*. Some aspects should be underlined. The trial of Pedrazzoli et al. [22] has been criticized for the small number of lymph nodes harvested in both arms. The problem was not due to the extent

of lymphadenectomy, but to the inadequate experience of the pathologists. In fact, the standard lymphadenectomy of the Japanese study [55] harvested the same number of lymph nodes as the Italian study [22] that removed several more lymph node groups (12b1, 12b2, 12c, 8a, 14a, 14b). An adjuvant treatment was performed in 78% of the Johns Hopkins' patients and in none of the other two studies. Furthermore, the skeletonisation of the celiac trunk and of the SMA was included only in the Italian and Japanese studies.

We must remember that every well designed study has the 20% of probability of missing a significant difference. This means that at least two prospective adequately powered RCT with concordant results are needed to confirm or exclude the hypothesis. Now we have only two RCT completely comparable for the extent of surgery [22,55] because the third-one includes adjuvant treatment and less extensive surgery for the "Radical" group. The two trials give discordant results about the survival of LN+ patients. Therefore, a definitive conclusion from the statistical point of view can't be drown. Furthermore, we do not know if the failure of the Johns Hopkins study to demonstrate a different survival after a standard or an extended lymphadenectomy was due to the inadequate extent of the lymphadenectomy or to the effect of the adjuvant treatment.

Are further prospective studies needed to clarify the actual usefulness of an extended lymphadenectomy? In spite of the discordant results of the several studies for and against the extended procedure, the answer should be no. The advantage of patient's survival of the extended procedure, provided it does exist, is clinically negligible.

Conclusions

The extended lymphadenectomy can be performed safely by experienced surgeons, ameliorates the tumour staging in a significant number of patients, and, chiefly, does not worsen long-term survival, but it is not the treatment of choice of pancreatic cancer.

Acknowledgement

This study has been supported by the Ministero Università e Ricerca Scientifica (Cofin 2003063754_001), Rome, Italy.

References

1. Bray F, Sankila R, Ferlay J, Parkin DM. Estimates of cancer incidence and mortality in Europe in 1995. Eur J Cancer, 2002; 38: 99-166.
2. Jemal A, Murray T, Ward E, Samuels A, Tiwari RC, Ghafoor A, Feuer EJ, Thun MJ. Cancer statistics, 2005. CA Cancer J Clin, 2005, Jan-Feb; 55(1): 10-30.
3. Yeo CJ. Pancreatic cancer: 1998 update. J Am Coll Surg, 1998; 187: 429-42.
4. Rockey EW. Total pancreatectomy for carcinoma. Case report. Ann Surg, 1943; 118: 603-11.
5. ReMine WH, Priestly JT, Judd ES, King JN. Total pancreatectomy. Ann Surg, 1970; 172: 595-604.
6. Brooks JR, Culebras JM. Cancer of the pancreas. Palliative operation, Whipple procedure, or total pancreatectomy? Am J Surg, 1976; 131: 516-9.
7. Niederhuber JE, Ensminger WD, Campbell DA. An integrated approach to the treatment of pancreatic adenocarcinoma. In: Pancreatic disease. Diagnosis and therapy. Dent TL, Eckhauser FE, Vinik A, Turcotte JG eds. Grune & Stratton, Inc, New York, 1981.
8. Ihse I, Anderson H, Andrén-Sandberg Å. Total pancreatectomy for cancer of the pancreas: Is it appropriate? World J Surg, 1996; 20: 288-94.
9. Karpoff HM, Klimstra DS, Brennan MF, Conlon KC. Results of total pancreatectomy for adenocarcinoma of the pancreas. Arch Surg, 2001;136: 44-7.
10. Fortner JG. Regional resection of cancer of the pancreas: a new surgical approach. Surgery, 1973; 73: 307-20.
11. Greene FL, Page DL, Fleming ID, Fritz AG, Balch CM, Haller DG, Morrow M, Eds. AJCC Cancer Staging Handbook, Springer-Verlag, New York, Berlin, Heidelberg; 2002.
12. Ishikawa O, Ohhigashi H, Sasaki Y, Kabuto T, Fukuda I, Furukawa H, Imaoka S, Iwanaga T. Practical usefulness of lymphatic and connective tissue clearance for the carcinoma of the pancreas head. Ann Surg, 1988; 208: 215-20.
13. Manabe T, Ohshio G, Baba N, Miyashita T, Asano N, Tamura K, Yamaki K, Nonaka A, Tobe T. Radical pancreatectomy for ductal cell carcinoma of the head of the pancreas. Cancer, 1989; 64: 1132-7.
14. Hiraoka T, Uchino R, Kanemitsu K, Toyonaga M, Saitoh N, Nakamura I, Tashiro S, Miyauchi Y. Combination of intraoperative radiation with resection of cancer of the pancreas. Int J Pancreatol, 1990; 7: 201-7.
15. Gall FP, Kessler H, Hermanek P. Surgical treatment of ductal pancreatic carcinoma. Eur J Surg Oncol, 1991; 17: 173-81.
16. Nagakawa T, Konishi I, Higashino Y, Ueno K, Ohta T, Kayahara M, Ueda N, Maeda K, Miyazaki I. The spread and prognosis of carcinoma in the region of the pancreatic head. Jpn J Surg, 1989; 19: 510-8.
17. Nagakawa T, Kayahara M, Ohta T, et al. Patterns of neural and plexus invasion of human pancreatic cancer and experimental cancer. Int J Pancreatol, 1991; 10: 113-9.
18. Satake K, Nishiwaki H, Yokomatsu H, Kawazoe Y, Kim K, Haku A, Umeyama K, Miyazaki I. Surgical curability and prognosis for standard versus extended resection for T1 carcinoma of the pancreas. Surg Gynecol Obstet, 1992; 175: 259-65.
19. Henne-Bruns D, Kremer B, Meyer-Pennwitt U. Partial duodenopancreatectomy with radical lymphadenectomy in patients with pancreatic and periampullary carcinomas: initial results. Hepatogastroenterol, 1993; 40: 145-9.
20. Henne-Bruns D, Vogel I, Luttges J, Kloppel G, Kremer B. Surgery for ductal adenocarcinoma of the pancreatic head: staging, complications, and survival after regional versus extended lymphadenectomy. World J Surg, 2000; 24: 595-602.
21. Naganuma T, Isaji S, Kawarada Y. Staging and extended resection for pancreatic cancer. Pancreas, 1998; 16: 355-62.
22. Pedrazzoli S, DiCarlo V, Dionigi R, Mosca F, Pederzoli P, Pasquali C, Kloppel G, Dhaene K, Michelassi F. Standard versus extended lymphadenectomy associated with pancreatoduodenectomy in the surgical treatment of adenocarcinoma of the head of the pancreas: a multicenter, prospective, randomized study. Lymphadenectomy Study Group. Ann Surg, 1998; 228: 508-17.
23. Fernandez-Cruz L, Sabater L, Ardid J, Navarro S, Kuriansky J, Cardona V. Radical lymphadenectomy for patients with carcinoma of the Ampulla of Vater: evaluation of the extent of lymph node metastasis. HPB, 1999; 1: 79-83.
24. Iacono C, Accordini S, Bortolasi L, Facci E, Zamboni G, Montresor E, Marinello PD, Serio G. Results of pancreaticoduodenectomy for pancreatic cancer: extended versus standard procedure. World J Surg, 2002; 26: 1309-14.
25. Capussotti L, Massucco P, Ribero D, Vigano L, Muratore A, Calgaro M. Extended lymphadenectomy and vein resection for pancreatic head cancer: outcomes and implications for therapy. Arch Surg, 2003; 138: 1316-22.
26. Geer RJ, Brennan MF. Prognostic indicators for survival after resection of pancreatic adenocarcinoma. Am J Surg, 1993; 165: 68-73.
27. Masson B, Loze S, Bercovici D. Is extended lymphadenectomy justified in surgery for pancreatic adenocarcinoma? Apropos of a series of 59 cases. Chirurgie, 1994-95; 120(4): 216-8.
28. Nakao A, Harada A, Nonami T, Kaneko T, Takagi H. Regional vascular resection using catheter bypass procedure for pancreatic cancer. Hepatogastroenterology, 1995; 42: 734-9.
29. Hirata K, Sato T, Mukaiya M, Yamashiro K, Kimura M, Sasaki K, Denno R. Results of 1001 pancreatic resections for invasive ductal adenocarcinoma of the pancreas. Arch Surg, 1997; 132: 771-7.

30. Mukaiya M, Hirata K, Satoh T, Kimura M, Yamashiro K, Ura H, Oikawa I, Denno R. Lack of survival benefit of extended lymph node dissection for ductal adenocarcinoma of the head of the pancreas: retrospective multi-institutional analysis in Japan. World J Surg, 1998; 22: 248-53.

31. Imaizumi T, Hanyu F, Harada N, Hatori T, Fukuda A. Extended radical whipple resection for cancer of the pancreatic head: operative procedure and results. Dig Surg, 1998; 15: 299-307.

32. Gazzaniga GM, Cappato S, Papadia F, Mori L, Filauro M. D1 versus D2 pancreatoduodenectomy in surgical therapy of pancreatic head cancer. Hepatogastroenterology, 2001; 48: 1471-8.

33. Popiela T, Kedra B, Sierzega M. Does extended lymphadenectomy improve survival of pancreatic cancer patients? Acta Chir Belg, 2002; 102: 78-82.

34. Yeo CJ, Cameron JL, Lillemoe KD, Sohn TA, Campbell KA, Sauter PK, Coleman J, Abrams RA, Hruban RH. Pancreaticoduodenectomy with or without distal gastrectomy and extended retroperitoneal lymphadenectomy for periampullary adenocarcinoma, part 2: randomized controlled trial evaluating survival, morbidity, and mortality. Ann Surg, 2002; 236: 355-68.

35. Pedrazzoli S, Beger H, Obertop H, Andrén-Sandberg Å, Fernandez Cruz L, Henne Bruns D, Lüttges J, Neoptolemos JP. A Surgical and Pathological Based Classification of Resective Treatment of Pancreatic Cancer: Summary of an International Workshop on Surgical Procedures in Pancreatic Cancer. Digestive Surgery, 1999; 16 (4): 337-45.

36. General Rules for the study of pancreatic cancer, 4th ed. Japan Pancreas Society, Kanehara Co., 1993; P25-35.

37. Hermanek P. Lymph nodes and malignant tumors. Zentralbl Chir, 2000; 125: 790-5.

38. Kocher HMK, Sohail M, Benjamin IS, Patel AG. Lymph node mapping in pancreatic cancer: a feasibility study. DDW, San Francisco, May 19-22, 2002 Abstr. T1638.

39. Kayahara M, Nagakawa T, Kobayashi H, Mori K, Nakano T, Kadoya N, Ohta T, Ueno K, Miyazaki I. Lymphatic flow in carcinoma of the head of the pancreas. Cancer, 1992; 70: 2061-6.

40. Kayahara M, Nagakawa T, Konishi I, Ueno K, Ohta T, Miyazaki I. Clinicopathological study of pancreatic carcinoma with particular reference to the invasion of the extrapancreatic neural plexus. Int J Pancreatol, 1991; 10: 105-11.

41. Nakao A, Harada A, Nonami T, Kaneko T, Takagi H. Clinical significance of carcinoma invasion of the extrapancreatic nerve plexus in pancreatic cancer. Pancreas, 1996; 12: 357-61.

42. Nagakawa T, Kayahara M, Ueno K, Ohta T, Konishi I, Ueda N, Miyazaki I. A clinicopathologic study on neural invasion in cancer of the pancreatic head. Cancer, 1992; 69: 930-5.

43. Takahashi T, Ishikura H, Motohara T, Okushiba S, Dohke M, Katoh H. Perineural invasion by ductal adenocarcinoma of the pancreas. J Surg Oncol, 1997; 65: 164-70.

44. Hirai I, Kimura W, Ozawa K, Kudo S, Suto K, Kuzu H, Fuse A. Perineural invasion in pancreatic cancer. Pancreas, 2002; 24: 15-25.

45. Kepple J, Van Zee KJ, Dowlatshahi K, Henry-Tillman RS, Israel PZ, Klimberg VS. Minimally invasive breast surgery. J Am Coll Surg, 2004; 199: 961-75.

46. Taat CW, van Laschot JJ, Gouma DJ, Obertop H. Role of extended lymph node dissection in the treatment of gastrointestinal tumours: a review of the literature. Scand J Gastroenterol, 1995; 212 (Suppl): 109-16.

47. Lerut T, Nafteux P, Moons J, Coosemans W, Decker G, De Leyn P, Van Raemdonck D, Ectors N. Three-field lymphadenectomy for carcinoma of the esophagus and gastroesophageal junction in 174 R0 resections: impact on staging, disease-free survival, and outcome: a plea for adaptation of TNM classification in upper-half esophageal carcinoma. Ann Surg, 2004; 240: 962-74.

48. Siewert JR, Bottcher K, Stein HJ, Roder JD. Relevant prognostic factors in gastric cancer: ten-year results of the German Gastric Cancer Study. Ann Surg, 1998; 228: 449-61.

49. Hartgrink HH, van de Velde CJ, Putter H, Bonenkamp JJ, Klein Kranenbarg E, Songun I, Welvaart K, van Krieken JH, Meijer S, Plukker JT, van Elk PJ, Obertop H, Gouma DJ, van Lanschot JJ, Taat CW, de Graaf PW, von Meyenfeldt MF, Tilanus H, Sasako M. Extended lymph node dissection for gastric cancer: who may benefit? Final results of the randomized Dutch gastric cancer group trial. J Clin Oncol, 2004; 22: 2069-77.

50. Cady B. Fundamentals of contemporary surgical oncology: biologic principles and the threshold concept govern treatment and outcomes. J Am Coll Surg, 2001; 192: 777-92.

51. Pisters PW, Evans DB, Leung DH, Brennan MF. Re: Surgery for ductal adenocarcinoma of the pancreatic head. World J Surg, 2001; 25: 533-4.

52. Iacono C, Bortolasi L, Facci E, Falezza G, Prati G, Mangiante G, Serio G. Does Extended pancreaticoduodenectomy increase operative morbidity and mortality vs. standard pancreaticoduodenectomy? J Gastrointest Surg, 1997; 1: 446-53.

53. Ishikawa O. Surgical technique, curability and postoperative quality of life in an extended pancreatectomy for adenocarcinoma of the pancreas. Hepatogastroenterol, 1996; 43: 320-25.

54. Boggi U, Del Chiaro M, Massa M, Croce C, Gremmo F, Sgambelluri A, Vistoli F, Signori S, Campatelli A, Di Candio G, Campani D, Bevilacqua G, Mosca F. Five-year actual survival following extended or standard lymphatic clearance in cancer of the head of the pancreas. Pancreas Cancer 2004 –Tirrenia Pisa, Italy, April 24-26, 2004.

55. Nimura Y. Japanese results of multicenter RCT on extended lymphadenectomy. Pancreas Cancer 2004 –Tirrenia Pisa, Italy, April 24-26, 2004.

56. Fortner JG, Klimstra DS, Senie RT, Maclean B. Tumor size is the primary prognosticator for pancreatic cancer after regional pancreatectomy. Ann Surg, 1996; 223: 147-53.

57. Pedrazzoli S, Pasquali C, Sperti C. Role of surgery in the treatment of bilio-pancreatic cancer: the European experience. Semin Oncol, 2002; 29(Suppl 20): 23-30.

58. Pedrazzoli S, Pasquali C, Sperti C. General aspects of surgical treatment of pancreatic cancer. Dig Surg, 1999; 16(4): 265-75.

59. Schafer M, Mullhaupt B, Clavien PA. Evidence-based pancreatic head resection for pancreatic cancer and chronic pancreatitis. Ann Surg, 2002; 236: 137-48.

60. Neoptolemos JP, Stocken DD, Dunn JA, Almond J, Beger HG, Pederzoli P, Bassi C, Dervenis C, Fernandez-Cruz L, Lacaine F, Buckels J, Deakin M, Adab FA, Sutton R, Imrie C, Ihse I, Tihanyi T, Olah A, Pedrazzoli S, Spooner D, Kerr DJ, Friess H, Buchler MW. European Study Group for Pancreatic Cancer. Influence of resection margins on survival for patients with pancreatic cancer treated by adjuvant chemoradiation and/or chemotherapy in the ESPAC-1 randomized controlled trial. Ann Surg, 2001; 234: 758-68.

61. Freedman LS. Tables of the number of patients required in clinical trials using Log rank test. Statistics in Medicine, 1982; 1: 121-9.

Application of specific cytologic, cytogenetic and molecular-cytogenetic techniques for the characterization of solid tumors

Schwanitz G*, Raff R

Institute of Human Genetics, University of Bonn, Germany

Abstract

Investigations of solid tumors have shown that a very specific characterization of aberrant tissues can best be performed using a combination of cytologic, cytogenetic and molecular-cytogenetic methods. Thus, cytological analyses may serve to examine various features of tumors cultivated *in vitro*, e.g. growth peculiarities, cell morphology, specific details of cell division and mitotic rates, and anomalies of the spindle apparatus. Besides, chromosomal diagnostics characterizing non-specific aberrations focuses on the pathological karyotype and its evolution and heterogeneity, as well as on the development of secondary chromosomal aberrations. In the field of molecular-cytogenetic diagnostics we emphasize particularly the combination of metaphase and interphase analyses and the investigation of specific structural aberrations by fluorescence in situ hybridization (FISH). In contrast to the method of comparative genomic hybridization (CGH), the spectrum of applications for both methods is discussed. The findings described in this paper were obtained primarily from the analysis of 68 tumors of the urogenital tract (20 kidney tumors, 33 bladder tumors, 15 testis tumors).

Key words: tumor cytogenetics, tumor growth in vitro, polykaryotic cells, micronucleus formation, multipolar spindle apparatus, composite karyotype, meta- and interphase-FISH, CGH, cell cycle abnormalities.

* CORRESPONDING AUTHOR:
Institute of Human Genetics
University of Bonn
Wilhelmstrasse 31
D-53111 Bonn, Germany
Tel: +49 228 2872338; Fax: +49 228 2872380
email: raff@uni-bonn.de (Prof. Gesa Schwanitz)

Received 22.04.2005 Accepted 26.04.2005

Introduction

Solid tumors can be analysed *in vitro* by a combination of cytologic and cytogenetic parameters. At the same time, these methods permit the differentiation between tumor cells and neighbouring normal tissue in cases where non-transformed cells may have contaminated the probe.

The object of this paper is a characterization of different cytologic and cytogenetic parameters relevant to the analysis of such tumors, in which both the potential and the limits of these methods will be discussed.

The *in vitro* solid tumor cultures described below were for the most part derived from tumors of the urogenital tract (kidney [1,2], bladder [3], and testis [4-6]). In some few cases findings obtained from head/neck tumors were included [7,8].

Cell growth and morphology

1. Cell growth

Unlimited growth is a characteristic feature of all tumor cells. Control mechanisms which, i.e. coordinate processes of proliferation in normal meristematic cells are absent.

Furthermore, a break-down of contact inhibition occurs in cell culture. Cells tend to grow in a criss-cross manner, covering each other, so that instead of a monolayer the result will be three-dimensional cell groups (*Fig. 1a-c*).

2. Cell morphology

Variable cell shape and differing nucleus-plasma ratios are a conspicuous phenomenon in tumor cells. Cytoplasmatic deposits which develop mainly around the nucleus may be attributed to a changed cell metabolism. So-called micronuclei also occur and are caused by abnormalities in the cell cycle which lead to an aberrant distribution of single chromosomes in anaphase of mitosis and the appearance of acentric fragments in the cytoplasma. These chromosomes and fragments are stimulated by the main nucleus to form an additional small nucleus.

Figure 1. **Growth abnormalities of testicular tumor cells *in vitro* (vital cell culture, phase contrast microscopy): a) criss-cross-growth, b) star-like growth, c) three-dimensional growth**

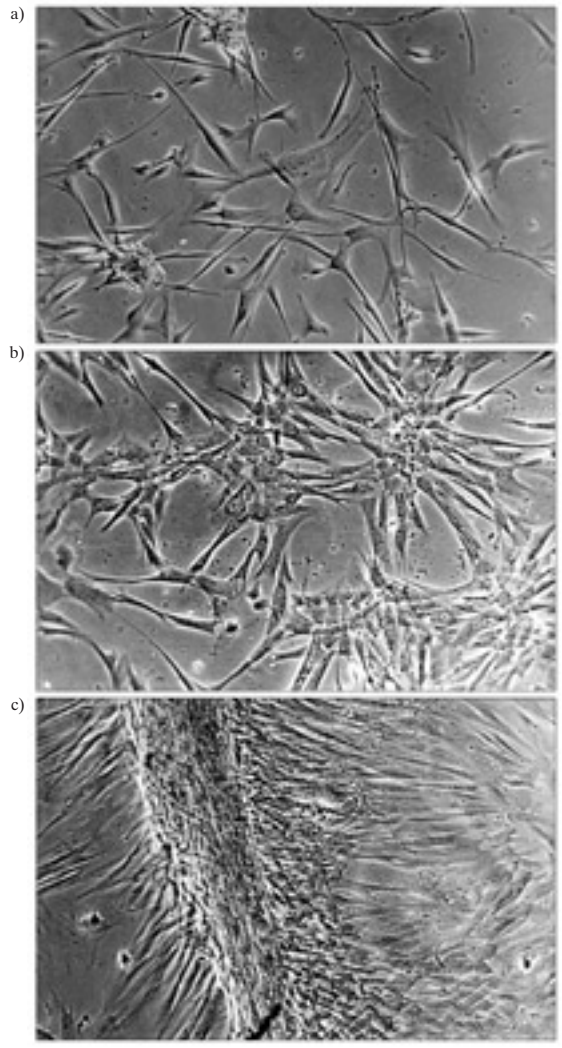
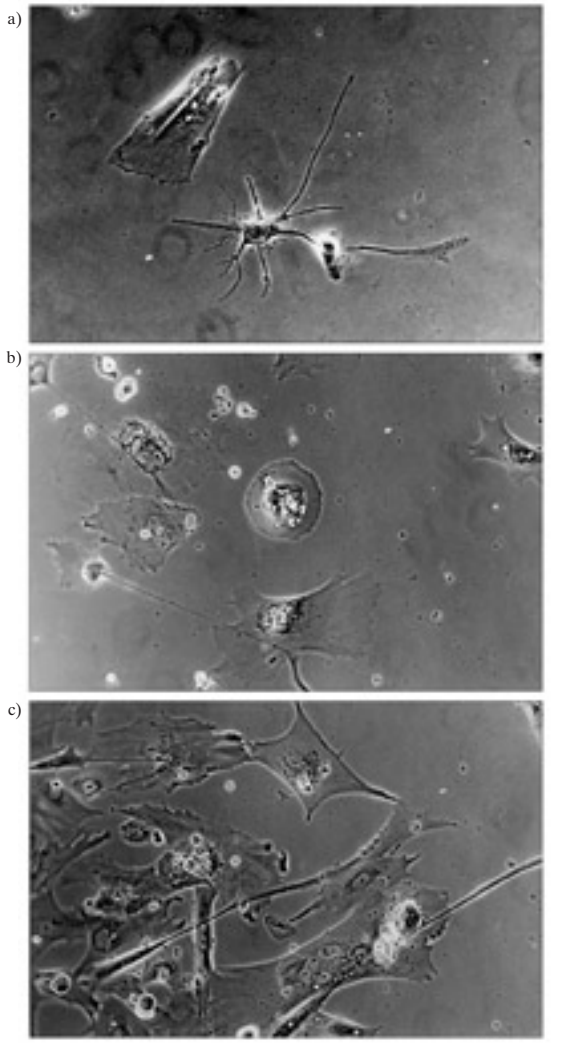


Figure 2. **Morphologic changes in testicular tumor cells *in vitro* (vital cell culture, phase contrast microscopy): a) and b) variation in cell shape, c) polykaryotic cells with perinuclear deposits**



Also multinucleated (2-8 nuclei) cells with a distinctly enlarged or diminished nucleus occur (*Fig. 2a-c*).

3. Morphology of the nucleus

Nuclei are often polymorphic, exhibiting constrictions and cytoplasmic inclusions as well as more complex structures (*Fig. 3a-c*).

4. Changes in tumor growth during prolonged or specific cultivation

Prolonged *in vitro* cultivation of tumor cells as well as the use of specific cell culture media in combination with the frequently heterogeneous nature of tumor cells may lead to the selective proliferation of particular subpopulations which may be especially well-adapted to the *in vitro* conditions, but which are not representative of the original tumor. Therefore, cultures of several different biopsy samples should be set up for each tumor culture and the results of these long-term cultures should be compared with those of a direct preparation [9,10].

5. Mitotic index and polyploidy rates

In our own studies, the mitotic index as a measure of the rate of cell division fluctuated greatly in different tumors of the same type. The mitotic index was determined two hours after addition of colcemid; the resulting arrest of cells in metaphase for an extended period of time automatically leads to an artificially increased value. Normal fibroblast cultures were used as a comparison. While controls of fibroblasts usually amount to a mitotic index of approximately 10‰, the values for tumor cells, even for the same type of tumor, varied strongly, ranging from 1-180‰. This was as well observed for the polyploidy rates, with the majority of aberrations being tetraploid mitoses. The normal reference value was 0.5-1.6%, whereas the tumor cultures showed rates of 5-10%. Isolated tumors were primarily triploid, which made their polyploidy level 6n.

6. Analysis of the spindle apparatus

The spindle apparatus may be analysed via indirect immunofluorescence, and the nucleus or rather the chromosomes are

Figure 3. **Nuclear abnormalities of testicular tumor cells *in vitro* (Acridin orange staining)**

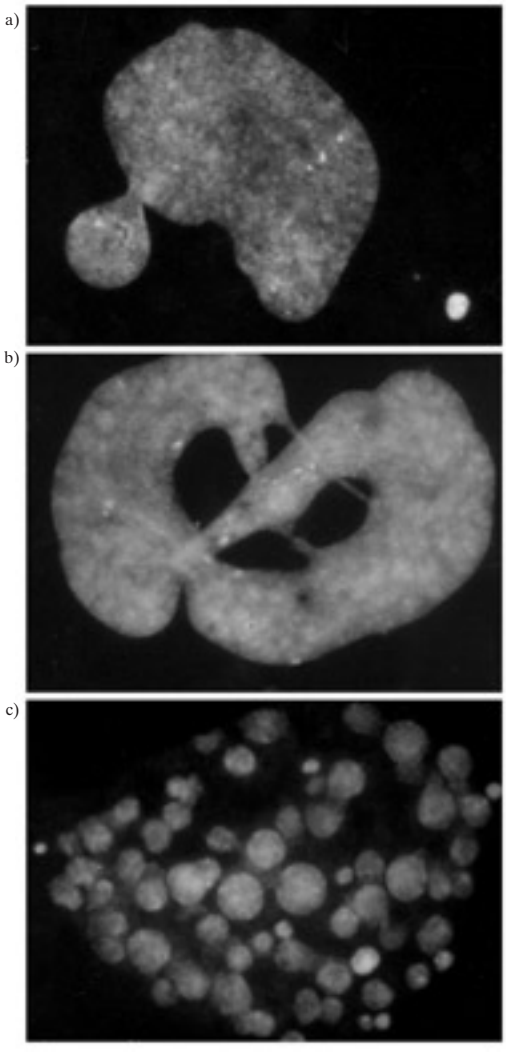


Figure 4. **Multipolar spindle apparatus (human lung carcinoma cells, cell line A549)**

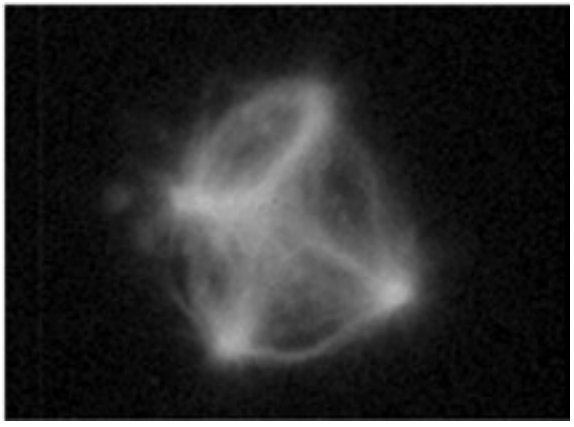
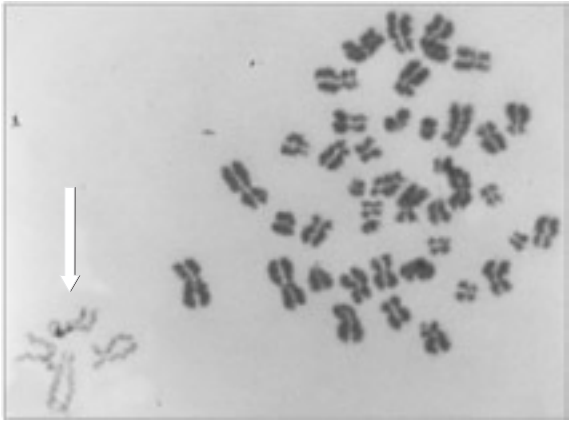


Figure 5. **Asynchronous cell cycle leading to mitosis with chromosomes in pro- and metaphase stage (Giemsa staining)**



counterstained with propidium iodid or DAPI. Typical anomalies we found in our own investigations were multipolar and degenerated spindles [11]. As before, analyses of untransformed cells served as controls. We found aberration rates of 1-2% for normal fibroblast cultures as compared to 15-40% for various tumor cultures (*Fig. 4*).

These aberrations induce a secondary increase of aneuploidy in cultured tumor cells.

Chromosome analyses

1. Non-specific chromosome aberrations

In general, chromosomes in tumor preparations exhibit a tendency to reduced condensation and aberrant spiralisation. Condensation abnormalities may be limited to isolated chromosomes. This phenomenon occurs when the chromosomes do not pass through the cell cycle in a synchronised manner. For example, individual chromosomes have not yet reached metaphase but have remained in late prophase (*Fig. 5*).

2. Karyotype analyses

In tumor cytogenetics, various banding techniques may be used, and may often be combined in order to obtain as much information as possible. This is especially necessary in cases where complex rearrangements of the chromosomes have occurred. A first analysis provides details about the modal number of chromosomes, conspicuous marker chromosomes, rearrangements and amplifications (*Fig. 6*).

3. Heterogeneity and tumor evolution

The analysis of a large number of metaphases is absolutely essential for the assessment of the heterogeneity of a cell population. Solid tumors often exhibit highly increased karyotype instability. This inherent characteristic exists *in vivo*, and persists in the *in vitro* culture as well, where new mutations can also occur. The ability of a unique karyotype to be passed on to daughter cells *in vitro* depends on the specific selection advantages and disadvantages in each case.

For tumor karyotyping it is therefore necessary to register the entirety of various characteristic aberrations and to record the tumor evolution as well.

Figure 6. **Composite karyotype of a permanent cell line (T966) of a head-neck tumor (GTG-banding)**

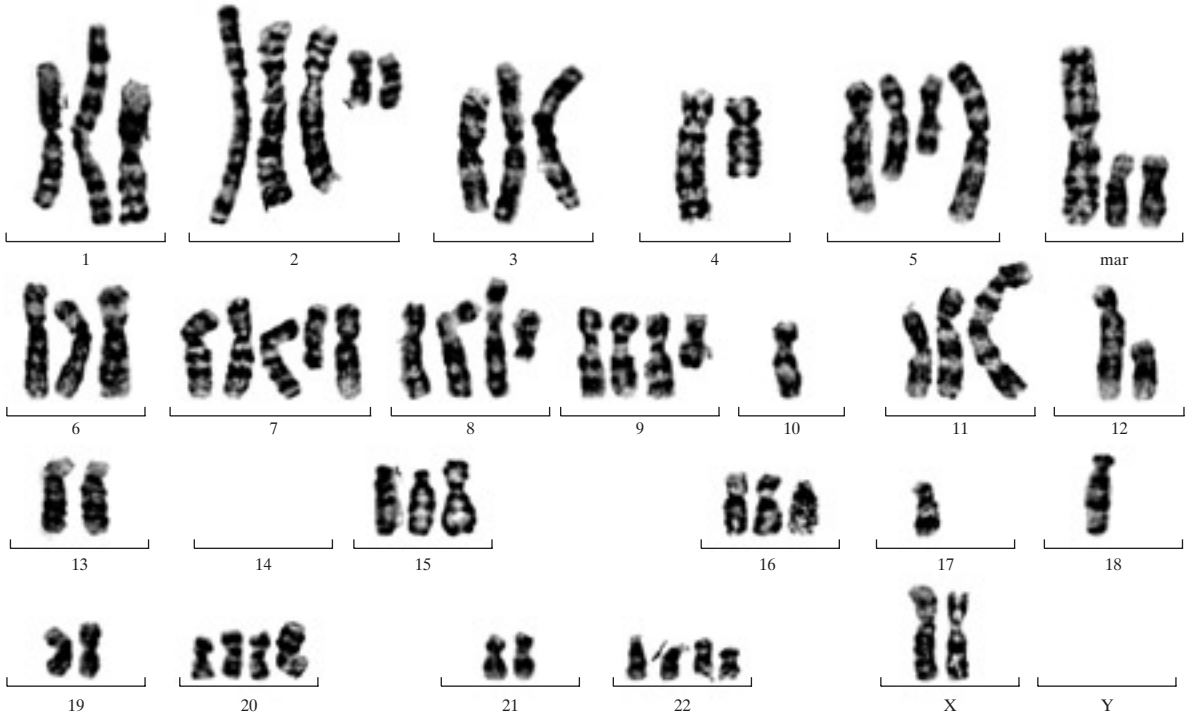


Table 1. **Results of karyotype analysis after long-term cell culture of a bladder tumor (number of analysed cells in square brackets)**

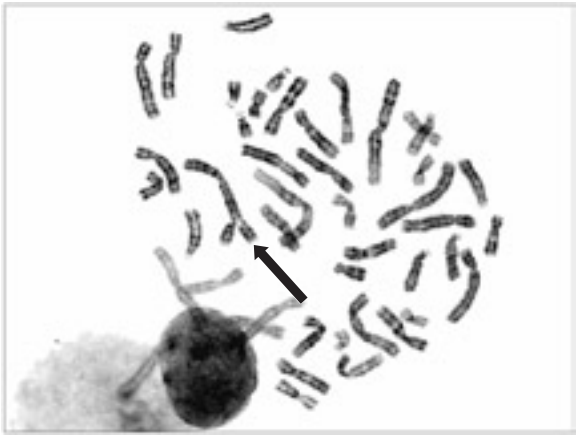
Normal karyotype:
46, XY [12]

Single karyotypes:
38, XY,-?5,-7,-11,-12,-15,-19,-19,-20
45, Y,-X
45, XY,-21 [2]
45, XY,-11
45, XY,-19
45, XY,-22
45, XY,-2,-19,+mar1
45, XY,-21,+?
46, XY,-7,+mar2
46, XY,-8,+mar3
46, XY,der(12)add(12(p13)
46, Y,-X,+7,-16,+17
85, XY,-3,-8,-14,-?15,-18
88, XXY,-6,-7,-8
89, XXYY,-?8,-13,-18
90, X,+del(4)(p?23),-5,-5,-8,+11,+11,+?14,+16,-17,t(19;?)(q?13.3;?)x2

Composite karyotypes:
Mainline: 38~90, XY,-7,-8,-19 [8]
Sideline: 45, XY,-21 [3]

A so-called "composite karyotype" contains all the clonal alterations that occur (*Tab. 1*). These can subsequently be analysed regarding their interchromosomal and intrachromosomal distribution for both numerical and structural aberrations. Furthermore, various types of aberrations (e.g. translocations, isochromosomes, deletions, duplications, monosomies, trisomies) are characteristic for distinct tumor types and for individual stages. The characterization of complex chromosomal rearrangements may require additional molecular cytogenetic analysis (*Tab. 1*).

Figure 7. **Partial endoreduplication of chromosome 2 (GTG banding)**



4. Failure of chromosomal replication and secondary aberrations

Tumor cells exhibit an increased tendency to abnormal replication of the chromosomes during S-phase of the cell cycle. This can result in complete or partial endoreduplications in the following mitosis (*Fig. 7*). Moreover, metaphases of tumor cells may show elevated rates of chromosomal breakage. In parallel, interphase cells in these cultures often display an increased rate of micronuclei (*Fig. 8*). Usually it is not possible to differentiate

Figure 8. **Interphase cell with micronucleus formation (Giemsa staining)**

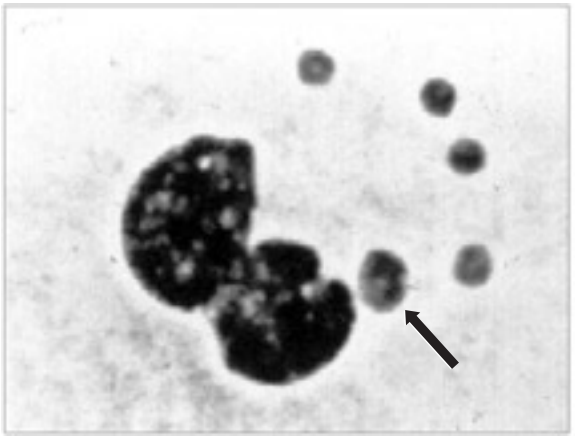
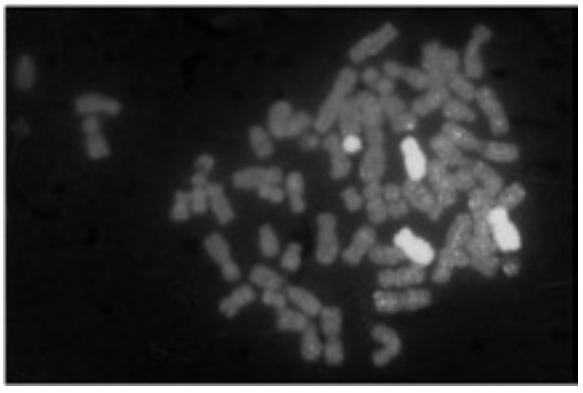


Figure 9. **Testicular tumor with an additional isochromosome of the short arms of chromosome 12 (fluorescence *in situ* hybridisation, FISH, with whole chromosome paint for chromosome 12)**



between exogenous induced breaks caused by cytostatic therapy on the one hand, and breaks caused by a higher mutation rate in the tumor on the other hand. The latter explanation appears to be more relevant, as tumor cells of patients not undergoing therapy also exhibit higher breakage rates.

Molecular cytogenetic analyses

The following is a short description of the two methods most often utilised for diagnostic purposes in tumor cytogenetics.

1. Fluorescence *in situ* hybridization (FISH)

Hybridization with defined DNA probes can be performed using metaphase or interphase spreads [12]. This technique is frequently used to characterise structurally altered chromosomes in more detail and for additional quantitative analyses (*Fig. 9a-c*). FISH is especially suited for the analysis of complex balanced rearrangements and in cases where the same chromosome occurs monosomic, trisomic or in higher amplification numbers (*Fig. 10*).

Figure 10. **Interphase analyses showing simultaneous gains and losses of chromosome X (FISH with a centromeric probe for the X chromosome)**

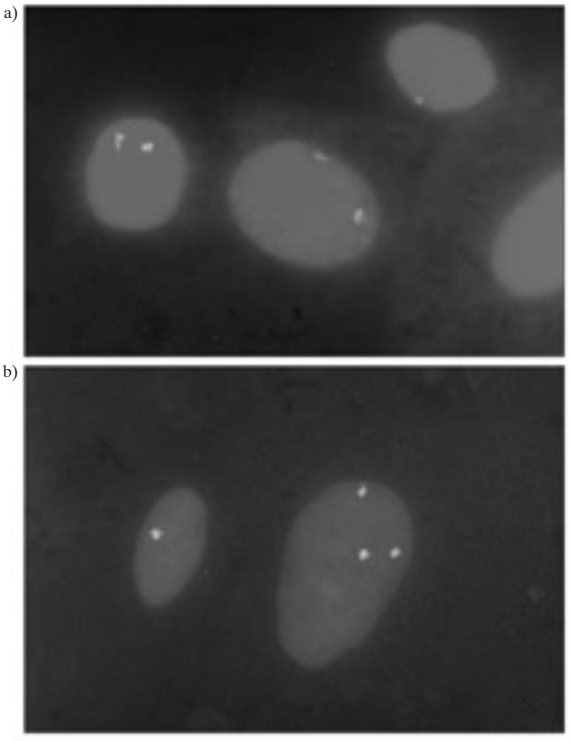
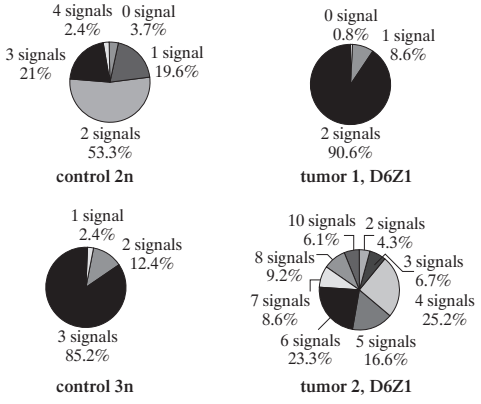


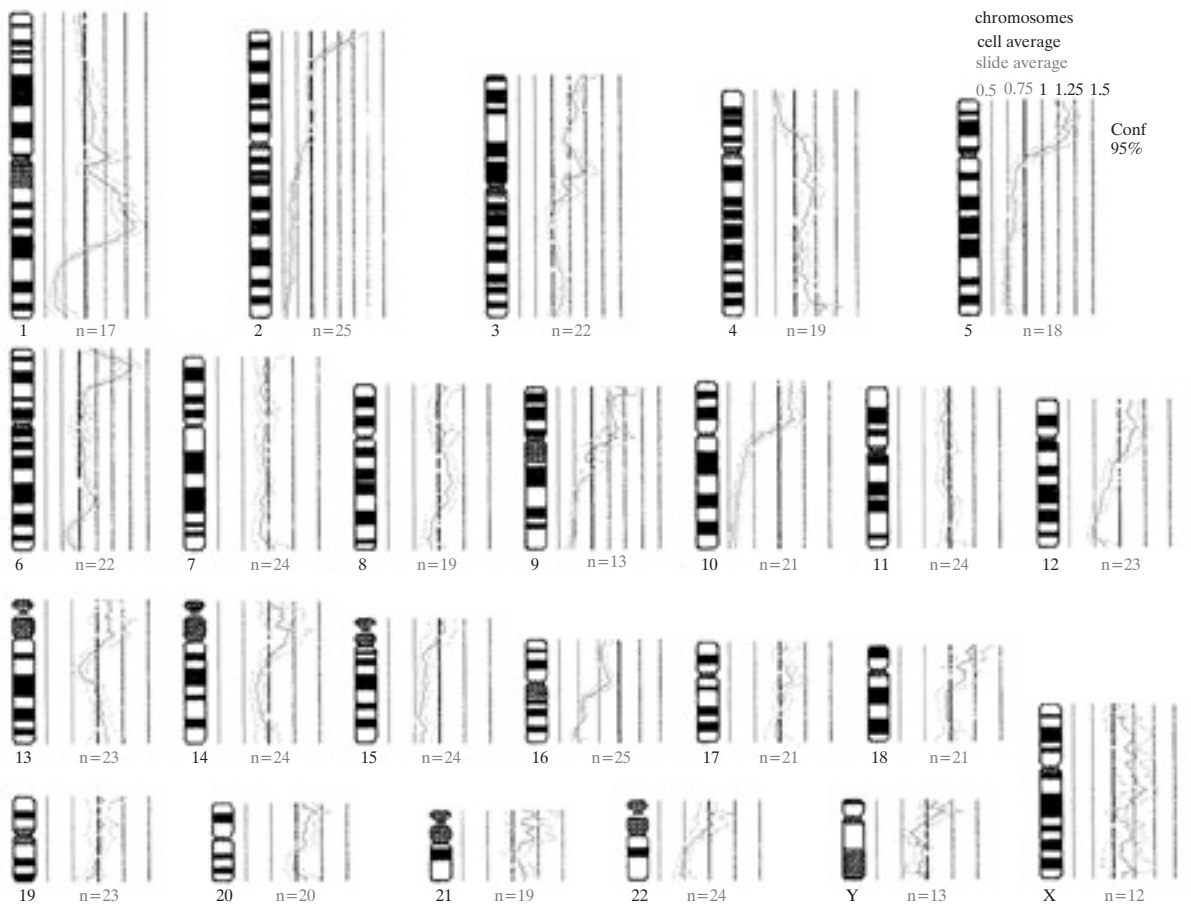
Figure 11. **Results of Interphase-FISH in head-neck tumors and control cultures demonstrating the quantitative analyses of gains and losses of chromosome 6 (FISH with a centromeric probe for chromosome 6)**



2. Comparative genomic hybridization (CGH)

This method has the great advantage to be independent from cell division stages for chromosome analysis. DNA extraction of the tumor which is to be examined can even be performed with a sample in fixative. During hybridization, chromosomal gains and losses can be determined in one single step (*Fig. 11*).

Figure 12. **CGH-profile of bladder tumor. More losses than gains of chromosomes 8, 9, 15, 17, 19, 20, 22, X and Y. More gains of chromosomes 4, 5, 11, 14, 16 and 18**



The disadvantage of this method lies in the fact that complex aberrations, the simultaneous presence of duplications and deletions and the karyotype complexity of main and side lines cannot be assessed.

References

1. Petersen V, Brühl P, Schwanitz G, Vogel J. Zytogenetische Analysen am Nierenzellkarzinom. Hamburg: Dr. Kovać; 1994.

2. Petersen V, Brühl P, Schwanitz G, Vogel J. Vergleichende Analyse von pathologischen, zytologischen und zytogenetischen Befunden beim Nierenzellkarzinom. Urologe, 1992; 32: 2-6.

3. Sleegers, E. Cytogenetische Charakterisierung von urothelialen Blasencarcinomen: Vergleichende Untersuchung von Karyotypanalyse, Fluoreszenz in situ Hybridisierung (FISH) und vergleichende genomische Hybridisierung (CGH). Dissertation, Bonn, Germany, 1999.

4. Viersbach R, Roitzheim B, Schwanitz G, Brühl P. Zytogenetische Untersuchungen zur Charakterisierung von Tumorzellen am Beispiel des Seminoms. I. Mitteilung: Anomalien des Spindelapparates. Akt Urol, 1994; 25: 181-5.

5. Roitzheim B, Viersbach R, Brühl P. Zytogenetische Untersuchungen zur Charakterisierung von Tumorzellen am Beispiel des Seminoms. II. Mitteilung: Fehlverteilung der Chromosomen und Mikronukleusbildung in Abhängigkeit von Anomalien des Spindelapparates. Akt Urol, 1995; 26: 195-9.

6. Vogel J, Roitzheim R, Jaeger N, Schwanitz G. Vergleichende histologische, zytologische und zytogenetische Untersuchungen an Keimzelltumoren. Verh Dtsch Ges Path, 1991; 75: 264.

7. Spitz, R. Molekularzytogenetische Charakterisierung von Kopf-Hals-Tumoren durch kombinierte Analyse aus Fluoreszenz in situ Hybridisierung (FISH) und vergleichender genomischer Hybridisierung (CGH). Dissertation, Bonn, Germany, 2000.

8. Hildebrandt, B. Cytogenetische Charakterisierung von Kopf-Hals-Tumorzelllinien unter besonderer Berücksichtigung der Effekte von Taxol. Dissertation, Bonn, Germany, 1997.

9. Schubert R, Schwanitz G. Solid Tissues. In: Wegner RD, editor. Diagnostic Cytogenetics. Berlin, Heidelberg, New York: Springer; 1999: p. 132-41.

10. Schwanitz G, Schubert R. Kulturen tierischer und menschlicher Zellen. In: Angewandte Biologie. Weinheim: VCH Press; 1990, p. 439-55.

11. Kochendörfer U, Stammberger I, Mayer D, Schwanitz G. A new possible parameter for the detection of aneuploidy inducing substances: the analysis of qualitative und quantitative abnormalities of the spindle apparatus. Mutat Res, 1996; 361: 55-66.

12. Schwanitz G, Schubert R. Fluorescence in situ hybridisation. In: Wegner RD, editor. Diagnostic Cytogenetics. Berlin, Heidelberg, New York: Springer; 1999: p. 305-34.

Progresses in the medical treatment of advanced colorectal cancer

Sultan-Amar V, Fabre-Guillevin E*, Piedbois P

Department of Medical Oncology, Assistance Publique – Hôpitaux de Paris, Henri Mondor Hospital, Créteil, France

Abstract

In the last 2 decades, major progresses have been made in the management of patients with advanced colorectal cancer (ACC). The modulation of 5-fluorouracil (5-FU) by folinic acid (LV), followed by the introduction of irinotecan and oxaliplatin have significantly improved the outcome of these patients. New strategies consist of oral fluoropyrimidines, and of targeted agents to inhibit cancer signalisation.

Key words: advanced colorectal cancer, 5-fluorouracil, oxaliplatin, irinotecan, targeted therapy, cetuximab, bevacizumab.

Introduction

Chemotherapy of advanced colorectal cancer (ACC) has considerably evolved since the time bolus 5-fluorouracil (5-FU) was the only treatment. The efficacy of 5-FU has been largely improved by folinic acid (LV), continuous infusion and oral fluoropyrimidines. In the last years, therapeutic options have broadened considerably with the introduction of oxaliplatin and irinotecan. More recently, promising results have been reported with the development of biologically targeted agents which aim to inhibit cancer signalisation and represent a significant step forward.

* CORRESPONDING AUTHOR:
Department of Medical Oncology, Hôpital Henri Mondor,
5I avenue du Maréchal de Lattre de Tassigny,
94010 Créteil, France.
Tel: 33 1 49812578; Fax: 33 1 4981 2569,
e-mail: elizabeth.fabre-guillevin@hmn.ap-hop-paris.fr (Elizabeth Fabre-Guillevin)

Received 07.12.2004 Accepted 27.04.2005

Cytotoxic agents

The last 10 years have seen considerable changes in the management of ACC with the emergence of novel cytotoxic agents like irinotecan, oxaliplatin and oral fluoropyrimidines.

For a long time, ACC have been considered as resistant to chemotherapy, and 5-FU was the only drug used in these patients. In the 80's, fluoropyrimidines' efficacy was increased by their biomodulation and their administration in continuous infusion. Modulation of 5-FU by LV or 5-FU continuous infusion double the tumor response rate achieved with 5-FU bolus [1-3]. The biweekly LV5FU2 regimen takes advantage of these findings [4], but overall best 5-FU regimens led to only small improvements in survival.

The introduction of irinotecan and oxaliplatin opened new perspectives. Globally, 5-FU/LV/irinotecan or 5-FU/LV/oxaliplatin lead to tumor response of 50%, progression–free survival around 9 months, and overall survival around 18 months (*Tab. 1*) [5-9]. Clearly, a step forward had been done.

Oral fluoropyrimidines

Oral fluoropyrimidines, such as tegafur/uracil (UFT) and capecitabine, mimic infusional 5-FU and make easier drug administration to preserve as far as possible the quality of life.

UFT and capecitabine have demonstrated their efficacy in large randomized phase III trials in which they were compared to bolus 5FU/LV [10-13]. Both oral fluoropyrimidines are interesting alternatives to 5-FU/LV bolus regimens and have recently been approved in Europe for first-line treatment. Furthermore, the development of oral fluoropyrymidines in metastatic cancers has opened new perspectives in the field of adjuvant chemotherapy [14-16].

Table 1. **Advances in first line treatment**

Endpoint	5-FU bolus	5-FU/LV or CI 5FU	5-FU/LV + CPT-11 or 5-FU/LV + oxaliplatine
Tumor response rate	~ 10%	~ 25%	~ 50%
Median progression-free Survival	~ 4 months	~ 6 months	~ 9 months
Median overall survival	~ 12 months	~ 12 months	~ 18 months

CI: continuous infusion

Targeted therapies

A better understanding of oncogenesis has allowed the development of highly efficient agents able to target critical pathways involved in cancer progression. Targeted therapies have also the potential to be better tolerated and therefore may be alternatives to conventional chemotherapy. Recent studies have shown their ability to increase cytotoxics efficiency when administered in association, and even to reverse acquired drug resistance in some cases. At present time, treatment of advanced colorectal cancers represents a promising field of clinical investigations.

Targeting epidermal growth factor receptor

The Epidermal Growth Factor Receptor (EGFR) family consists of 4 members: HER-1 (erb-B1/EGFR), HER-2 (Erb--B2), HER-3 (Erb-B3) and HER-4 (Erb-B4). Each of these proteins is composed of an extracellular binding domain, a transmenbrane segment and an intracellular protein kinase domain. The binding of epidermal growth factor (EGF) drives to the dimerization of ligand-receptors and therefore the triggering of intrinsic protein kinase activity. Several intracellular substrates are phosphorylated, leading to the activation of two major signalling pathways (Ras-Raf-Map-kinase, PI3K-AKT and Jak/Stat) which regulate transcription of molecules involved in oncogenesis [17]. The EGFR is frequently deregulated in colorectal carcinoma [18], and overexpression of the receptor confers a poor prognosis.

The crucial role of EGFR in tumor proliferation and its frequent overexpression provide the rationale for the treatment of ACC.

The targeting of EGFR and its downstream signalling pathways include inhibitors of tyrosine kinase activity (ZD1839, OSI-774) and monoclonal antibodies directed against the extracellular domain of EGFR (C225, ABX-EGF).

Up to now, studies using EGFR tyrosine kinase inhibitors remain disappointing. However, a phase II study evaluating the efficacy of gefitinib (ZD1839, Iressa) associated with FOLFOX yielded 75% partial response if patients were previously untreated, and 23% otherwise [19].

Several monoclonal antibodies to the EGFR (EGFRMabs) have already been used in phase II or phase-III clinical trials with interesting results. Among them, the one furthest ahead in clinical development is the IMC-C225 also called C225 (cetuximab). C225 is a murine chimeric monoclonal antibody which binds selectively to EGFR with a higher affinity than either EGF or TGF α. Cetuximab is usually administered as a 400 mg/m^2 intravenous loading dose followed by a weekly dose of 250 mg/m^2. Most common side-effects consist of acneiform skin rash (60%) and anaphylactic reactions (2%) which may be prevented by prophylactic antihistamin therapy. Cetuximab is effective in single-agent administration, but the major innovation brought by this drug is the potential to reverse resistance to cytotoxic agent.

Cetuximab has been tested in different phase II trials alone or associated to ironotecan or oxaliplatin. Cetuximab was first used in patients with refractory metastatic colorectal cancer and progressing on an irinotecan based regimen [20]. Combination of cetuximab to irinotecan yielded a 23% response rate and a 6 months median duration in a non-comparative trial enrolling 121 patients; 31% patients presented a stable disease [21]. These results were confirmed in a large randomized phase II trial (474 patients) [22] demonstrating a 23% response rate. Median time to progression was 126 days (versus 45 days for cetuximab alone) proving the efficiency of cetuximab in heavely pretreated patients. Toxicity appeared manageable; grade 3-4 toxicity included diarrhea (20%), asthenia (13%), neutropenia (11%), acne (7%) and vomiting (6%).

On the basis of these results, cetuximab was approved in numerous countries for patients progressive under irinotecan regimen.

Three other trials used cetuximab associated to irinotecan in first line therapy. They respectively enrolled 21, 25 and 19 evaluable patients. Response rate were comprised between 43 and 58% [23-25].

More recently, cetuximab has been associated to oxaliplatin in three studies; two of them are still going on. First results of the EXPLORE study [26] has been presented at the 2004 ASCO meeting. This trial aims to include 1100 patients and to compare FOLFOX 4 to FOLFOX 4+ cetuximab in second line therapy. First analysis showed that the regimen seems feasible and safe. The ACROBAT study [27] evaluated FOLFOX4 + cetuximab as first line treatment in 61 patients. Efficacy results are very encouraging with 81% response rate (including 2 complete responses) and 7 stable diseases (12%). Data suggest that cetuximab is safe and effective when combined to FOLFOX 4. Grade 3-4 toxicity included diarrhea (26%), acne (21%), neutropenia (14%), mucositis (9%), asthenia (9%), vomiting (5%) and neurotoxicity (2%).

Futures directions of cetuximab development include its combination with other cytotoxic or targeted therapies.

Targeting vascular endothelial growth factor

The development of new vessels (angiogenesis) is essential for tumoral growth, invasion and metastasis. Several mechanisms and molecules are involved in the angiogenesis, and each of them represents a potential target. Currently vascular endothelial growth factor (VEGF) is thought to be the major growth factor. Therefore, targeting VEGF appears as a promising strategy. VEGF acts via two receptors (VEGF-R1 and VEGF-R2) which are located on endothelial cells and present

a protein kinase activity. Overexpression of VEGF is associated with metastatic phenotype and poor prognosis [28-29].

Rhu-mab-VEGF (bevacizumab, AVASTIN®) is a humanized monoclonal antibody against VEGF which has been furthermore investigated in a 3 arms randomized phase II trial [30] enrolling 104 patients. Bevacizumab (5 mg/kg or 10 mg/kg) combined to 5-FU/LV obtained better response rate (40 and 24%), median time to progression (9.2 and 7.2) and median survival (21.5 and 16.1 months) than 5-FU/LV.

These results were confirmed in 2003, when for the first time, a phase III trial demonstrated an angiogenic agent can improve overall survival [31]. In this trial involving 815 previously untreated patients, the addition of bevacizumab (5 mg/kg/two weeks) to irinotecan/5-FU/LV increased response rate (45% versus 25%, p=0.0029), progression-free survival time (10.6 versus 6.2 months, p<0.00001) and overall survival time (20.3 versus 3.2 months, p=0.00003). Toxicity was equivalent except hypertension. In 2004, bevacizumab received approval for use in first-line treatment in combination with 5-FU based chemotherapy.

Currently, the oral angiogenesis PTK/ZK222584 which selectively targets the VEGF-R tyrosine kinase inhibitor is evaluated in association with 5-FU/LV plus irinotecan or oxaliplatin [32-33].

Conclusion

Irinotecan and oxaliplatin have widely expanded the options available for the management of patients with ACC, with consequent improvements in survival. More recently, promising results have been reported with new agents such as angiogenic inhibitors and anti-EGF receptors which represent another step forward. The association of targeted therapies with cytotoxic chemotherapies exploit their complementary mechanism of actions. The use of these new strategies such as maintenance therapy after achieving best response must be further explored. Ongoing and future trials will demonstrate the optimal schedules of drug administrations, and will better quantify their benefit upon standard approaches. One must also keep in mind that these advances are an economic challenge. For example 8 weeks of 5-FU/LV Mayo Clinic regimen cost $63 whereas for the same duration, FOLFIRI costs $9.381 and FOLFIRI + cetuximab $30.675 [34]. Cost-effectiveness analyses are wanted to evaluate the real financial implications of these drugs.

References

1. Advanced Colorectal Cancer Meta-Analysis Project. Modulation of fluorouracil by leucovorin in patients with advanced colorectal cancer: evidence in terms of response rate. J Clin Oncol, 1992; 10(6): 896-903.
2. Meta-Analysis Group In Cancer. Modulation of 5-fluorouracil by leucovorin in patients with advanced colorectal cancer: an updated meta-analysis based on 3,300 patients. J Clin Oncol, 2004; 22: 3766-75.
3. Meta-analysis Group In Cancer. Efficacy of intravenous continuous infusion of fluorouracil compared with bolus administration in advanced colorectal cancer. J Clin Oncol, 1998; 16(1): 301-8.
4. de Gramont A, Bosset JF, Milan C. Randomized trial comparing monthly low-dose leucoverin and fluorouracil bolus with bimonthly high-dose leucovorin and fluorouracil bolus plus continuous infusion for advanced colorectal cancer: a French intergroup study. J Clin Oncol, 1997; 15(2): 808-15.
5. Rougier P, Van Cutsem E, Bajetta E, Niederle N, Possinger Kurt, Labianca R, Navarro M, Morant R, Bleiberg H, Wils J, Awad L, Herait P, Jacques C. Randomised trial of irinotecan versus fluorouracil by continuous infusion after fluorouracil failure in patients with metastatic colorectal cancer. Lancet, 1998; 352(9138): 1407-12.
6. Douillard JY, Cunningham D, Roth AD, Navarro M, James RD, Karasek P, Jandik P, Iveson T, Carmichael J, Alakl M, Gruia G, Awad L, Rougier P. Irinotecan combined with fluorouracil compared with fluorouracil alone as first-line treatment for metastatic colorectal cancer: a multicentre randomised trial. Lancet, 2000; 355(9209): 1041-7.
7. Saltz LB, Cox JV, Blanke C. The Irinotecan Study Group. Irinotecan plus fluourouracil and leucoverin for metastatic colorectal cancer. N Engl J Med, 2000; 343(13): 905-14.
8. de Gramont A, Figer A, Seymour M, Homerin M, Hmissi A, Cassidy J, Boni C, Cortes-Funes H, Cervantes A, Freyer G, Papamichael D, Le Bail N, Louvet C, Hendler D, de Braud F, Wilson C, Morvan F, Bonetti A. Leucovorin and fluorouracil with or without oxaliplatin as first-line treatment in advanced colorectal cancer. J Clin Oncol, 2000; 18(16): 2938-47.
9. Giacchetti S, Perpoint B, Zidani R. Phase III multicenter randomized trial of oxaliplatin added to chronomodulated fluorouracil-leucovorin as first-line treatment of metastatic colorectal cancer. J Clin Oncol, 2000; 18(1): 136-47.
10. Douillard JY, Hoff PM, Skillings JR. Multicenter phase III study of uracil/tegafur and oral leucovorin versus fluorouracil and leucovorin in patients with previously untreated metastatic colorectal cancer. J Clin Oncol, 2002; 20(17): 3605-16.
11. Carmichael J, Popiela T, Radstone D, Falk S, Borner M, Oza A, Skovsgaard T, Munier S, Martin C. Randomized comparative study of tegafur/uracil and oral leucovorin versus parenteral fluorouracil and leucovorin in patients with previously untreated metastatic colorectal cancer. J Clin Oncol, 2002; 20(17): 3617-27.
12. Hoff PM, Ansari R, Batist G, Cox J, Kocha W, Kuperminc M, Maroun J, Walde D, Weaver C, Harrison E, Burger HU, Osterwalder B, Wong AO, Wong R. Comparison of oral capecitabine versus intravenous fluorouracil plus leucovorin as first-line treatment in 605 patients with metastatic colorectal cancer: results of a randomized phase III study. J Clin Oncol, 2001; 19(8): 2282-92.
13. van Cutsem E, Twelves C, Tabernero J. XELOX: Mature results of a multinational, phase II trial of capecitabine plus oxaliplatin, an effective 1st line option for patients (pts) with metastatic colorectal cancer (MCRC). Proc Am Soc Clin Oncol, 2003; 1023.
14. Wolmark N, Wieand B, Lembersky L, Colangelo R, Smith R, Pazdur N, Pittsburgh P, Rockville MD. A phase III trial comparing oral UFT to FULV in stage II and III carcinoma of the colon: Results of NSABP Protocol C-06. Proc Am Soc Clin Oncol, 2004; 3508.
15. Cassidy J, Scheithauer W, MC Kendrick J. Capecitabine (X° vs bolus 5FU/leucoverin (LV)) as adjuvant therapy for colon cancer (the X-ACT study): efficacy results of a phase III trial. Proc Am Soc Clin Oncol, 2004; 3509.
16. Sakamoto J, Ohashi Y, Hamada C, Buyse M, Burzykowski T, Piedbois P; Meta-Analysis Group of the Japanese Society for Cancer of the Colon and Rectum; Meta-Analysis Group in Cancer. Efficacy of oral adjuvant therapy after resection of colorectal cancer: 5-year results from three randomized trials. J Clin Oncol, 2004; 22(3): 484-92.
17. Mendelsohn J, Baselga J. Status of epidermal growth factor receptor antagonists in the biology and treatment of cancer. J Clin Oncol, 2003; 21(14): 2787-99.
18. Rosen LS. Biologic approaches to the treatment of gastrointestinal malignancy. Curr Opin Oncol, 2002; 14(4): 431-8.
19. Cho CD, Fisher GA, Halsey J. A phase II study of gefitinib in combination with FOLFOX-4 (IFOX) in patients with unresectable or metastatic colorectal cancer. Proc Am Soc Clin Oncol, 2003; 1062.
20. Saltz LB, Meropol NJ, Loehrer PJ Sr. Phase II trial of cetuximab in patients with refractory colorectal cancer that expresses the epidermal growth factor receptor. J Clin Oncol, 2004; 22(7): 1201-8.
21. Saltz L, Rubin M, Hochster H. Cetuximab (IMC-C225) Plus Irinotecan (CPT-11) is Active in CPT-11-Refractory Colorectal Cancer (CRC) that expresses Epidermal Growth Factor Receptor (EGFR). Proc Am Soc Clin Oncol, 2001; 7.
22. Cunningham D, Humblet Y, Siena S. and al. Cetuximab (C225) alone or in combination with irinotecan (CPT-11) in patients with epidermal growth factor receptor (EGFR)-positive, irinotecan-refractory metastatic colorectal cancer (MCRC). Proc Am Soc Clin Oncol, 2003; 1012.

23. Raoul JL, Van Laetem JL, Mitry E. Phase II study of cetuximab combined with FOLFIRI (bi-weekly irinotecan plus infusional 5-FU and folinic acid (FA) in patients (pts) with metastatic, Epidermal Growth Factor Receptor (EGFR)-expressing colorectal cancer (CRC). Eur J Cancer Suppl, 2003; 1(5): S89.

24. Rosenberg AH, Loehere PJ, Needle MN. Erbitux (IMC-C225) plus weekly irinotecan (CPT-11), luorouracil and leucoverin in colorectal cancer that expresses the epidermal growth factor receptor. Proc Am Soc Clin Oncol, 2002; 536.

25. Schöffski P, Lutz C-H, Folprecht G. Cetuximab (C225, Erbitux) in combination with irinotecan, infusional 5-fluorouracil and folinic acid is safe and active in patients with metastatic colorectal cancer expressing epidermal growth factor receptor; Results of a phase I study. Eur J Cancer, 2002, 495.

26. Badarinath S, Mitchell EP, Jennis A. Cetuximab plus FOLFOX for colorectal cancer (EXPLORE): Preliminary safety analysis of a randomized phase III trial. Proc Am Soc Clin Oncol, 2004; 3531.

27. Tabernero JM, van Cutsem E, Sastre J. An international phase II study of cetuximab in combination with oxaliplatin/5-fluorouracil (5-FU)/folinic acid (FA) (FOLFOX-4) in the first-line treatment of patients with metastatic colorectal cancer (CRC) expressing Epidermal Growth Factor Receptor (EGFR). Preliminary results. Proc Am Soc Clin Oncol, 2004; 3512.

28. Arii S, Mori A, Uchida S, Fujimoto K, Shimada Y, Imamura M. Implication of vascular endothelial growth factor in the development and metastasis of human cancers. Hum Cell, 1999; 12(1): 25-30.

29. Harada Y, Ogata Y, Shirouzu K. Expression of vascular endothelial growth factor and its receptor KDR (kinase domain-containing receptor)/Flk-1 (fetal liver kinase-1) as prognostic factors in human colorectal cancer. Int J Clin Oncol, 2001; 6(5): 221-8.

30. Bergsland E, Hurwitz H, Fehrenbacher L. A Randomized Phase II Trial Comparing rhuMAb VEGF (Recombinant Humanized Monoclonal Antibody to Vascular Endothelial Cell Growth Factor) Plus 5-Fluorouracil/Leucovorin (FU/LV) to FU/LV Alone in Patients with Metastatic Colorectal Cancer. Proc Am Soc Clin Oncol, 2000; 939.

31. Hurwitz H, Fehrenbacher L, Novotny W. Bevacizumab plus irinotecan, fluorouracil, and leucoverin for metastatic colorectal cancer. N Engl J Med, 2004; 350(23): 2335-42.

32. Steward W.P., Thomas L, Morgan B. Extended phase I study of the oral vascular endothelial growth factor (VEGF) receptor inhibitor PTK787/ZK 222584 in combination with oxaliplatin/5-fluorouracil (5-FU)/leucovorin as first line treatment for metastatic colorectal cancer. Prco Am Soc Clin Oncol, 2003; 1098.

33. Trarbach T., Schleucher N, Riedel U. Phase I study of the oral vascular endothelial growth factor (VEGF) receptor inhibitor PTK787/ZK 222584 (PTK/ZK) in combination with irinotecan/5-fluorouracil/leucovorin in patients with metastatic colorectal cancer. Proc Am Soc Clin Oncol, 2003; 1144.

34. Schrag D. The price tag on progress-chemotherapy for colorectal cancer. N Engl J Med, 2004; 351(4): 317-9.

New strategy for acute necrotizing pancreatitis: Continuous Regional Arterial Infusion (CRAI) therapy

Mikami Y*, Takeda K, Omura N, Abe H, Fukuyama S, Motoi F, Egawa S, Sunamura M, Matsuno S

Division of Gastroenterological Surgery, Tohoku University, Graduate School of Medicine, Sendai, Japan

Abstract

Acute pancreatitis is an autodigestive disease, of which protease inhibition has been the focus of experimental and clinical research. Different from Europe and the United States, protease inhibitors are often applied in the treatment of acute pancreatitis in Japan. However, in clinical settings, the effect of protease inhibitors on acute pancreatitis is still controversial. Continuous Regional Arterial Infusion (CRAI) of protease inhibitors and antibiotics therapy were developed in Japan and it has been demonstrated that CRAI therapy has beneficial effects on severe acute necrotizing pancreatitis. In the Japanese clinical guidelines for the treatment of acute pancreatitis, published in 2003, CRAI therapy is still classified as a special therapy. However, a Randomized Controlled Trial for CRAI therapy has started and CRAI therapy is expected to become a new standard therapy for severe acute pancreatitis. CRAI therapy is aimed at preventing the progression of pancreatic inflammation and pancreatic infection. CRAI therapy can decrease the mortality rate and the frequency of pancreatic infection in severe acute pancreatitis, but it should be started as soon as possible after the onset of acute pancreatitis.

Key words: acute pancreatitis, Continuous Regional Arterial Infusion (CRAI) therapy, protease inhibitor, nafamostat mesilate, contrast enhanced CT, angiography.

* CORRESPONDING AUTHOR:
Division of Gastroenterological Surgery,
Tohoku University, Graduate School of Medicine
Aobaku, Seiryo-machi 1-1
Sendai 980-8574, Japan
Tel: +81-22-717-7205; Fax: +81-22-717-7209
e-mail: y-mikami@surg1.med.tohoku.ac.jp (Yukio Mikami)

Received 07.07.2005 Accepted 14.07.2005

Abbreviations: CRAI – Continuous Regional Arterial Infusion; RCT – Randomized Controlled Trial; CEA – celiac artery; SMA – supramesenteric artery; ICU – intensive care unit.

Introduction

Acute pancreatitis is an autodigestive disease and protease inhibitors have been expected to be a key factor in the therapeutic approach to its treatment. However, in clinical settings, the effect of protease inhibitors in the treatment of acute pancreatitis is still controversial and a new regimen of anti protease therapy is required to establish the role of protease inhibitors in treating acute pancreatitis.

In 2003, evidence based clinical guidelines for treating acute pancreatitis were published in Japan [1], assessing all proposed therapies and examination techniques for acute pancreatitis, which were then categorized and their efficacies re-evaluated. In these guidelines, fluid resuscitation, intensive care and surgical therapy for pancreatic infection are recommended as the basic therapies for acute pancreatitis. Additionally, Continuous Regional Arterial Infusion (CRAI) of protease inhibitor and antibiotics were proposed as special therapy for severe acute pancreatitis. CRAI of protease inhibitor and antibiotics, which markedly increases the tissue concentration of administered drugs in acute pancreatitis, was pioneered in Japan and has been demonstrated to reduce the mortality rate and the frequency of infected pancreatic necrosis.

In this review, this new strategy for acute pancreatitis – CRAI of protease inhibitor and antibiotics therapy – is summarized and discussed.

Protease inhibitors

Protease inhibitors were developed to inhibit pancreatic proteases, such as trypsin, which have an important role in

Figure 1. **Angiographic appearance of acute pancreatitis. A) normal pancreas; B) acute necrotizing pancreatitis. Diffuse narrowing of the splenic and common hepatic arteries and impaired visualization of the ramifications are shown in acute necrotizing pancreatitis. (9-year-old boy at 56 hours after the onset)**

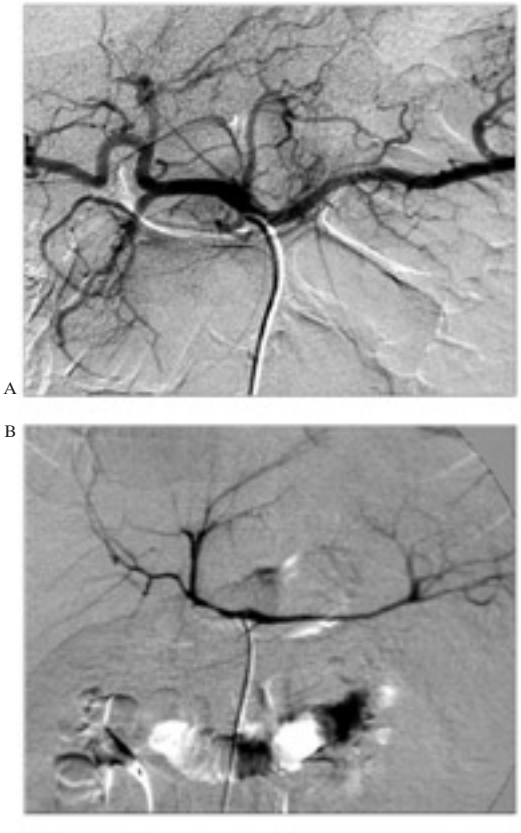
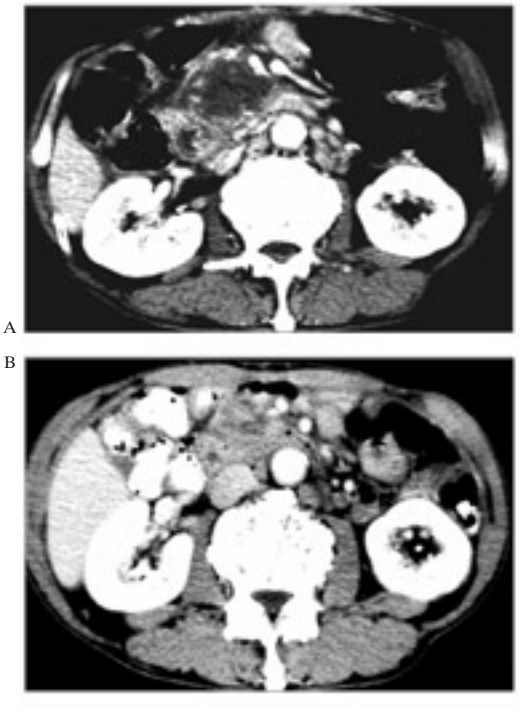


Figure 2. **Contrast enhanced CT finding in a patient with acute necrotizing pancreatitis (68-year-old male). A) before CRAI therapy (at 65 hours after onset); B) after CRAI therapy (at 2 weeks after onset). The inflammation of the pancreatic head (arrow) improved after CRAI therapy**

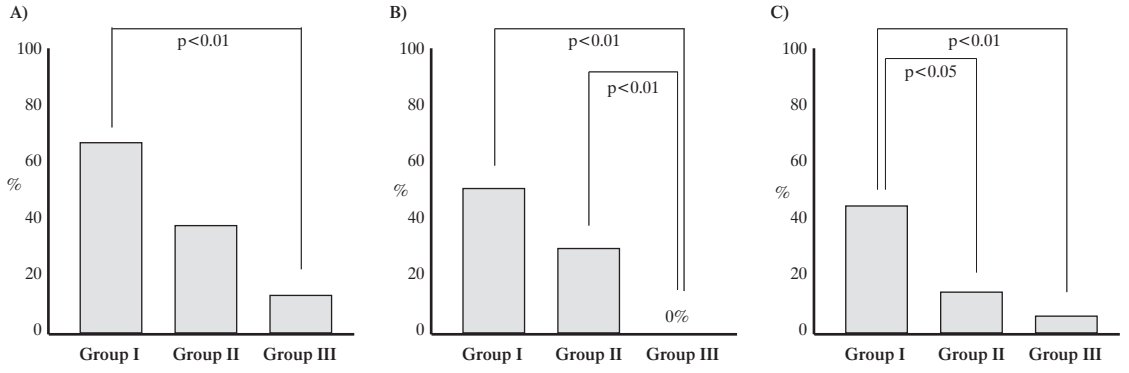


pancreatic inflammation. Aprotinin, which was often used in 1960's-1970's for the treatment of acute pancreatitis, has not shown any beneficial effects on acute pancreatitis in its Randomized Controlled Trials (RCTs) [2-4]. Gabexate mesilate was developed in Japan and some RCTs of gabexate mesilate have been performed [5-7]. However, most of them failed to demonstrate any significant beneficial effects on acute pancreatitis. Especially, in a German RCT of gabexate mesilate [5], more than 200 patients were included and more than 4 g/day of gabexate mesilate was administered for the treatment of acute pancreatitis. However, the RCT did not show any significant beneficial effects on mortality or morbidity of complications in acute pancreatitis. As a result of these RCTs, the European and American guidelines for the treatment of acute pancreatitis do not admit any beneficial effects of protease inhibitors [8-9].

In 2000 Chen et al. reported that the continuous intravenous administration of gabexate mesilate (2400 mg/day for 7 days) significantly decreased the morbidity of complications and mortality rate in severe acute pancreatitis [10]. The meta-analysis [11-13], which does not contain the RCT reported by Chen et al., did not show any clinical beneficial effects of gabexate mesilate on mild and moderate acute pancreatitis. Although the analysis also did not show the beneficial effects of gabexate mesilate on the mortality rate and the frequency of surgical intervention in severe acute pancreatitis, they showed that gabexate mesilate (900-2400 mg/day for 4-12 days) decreased the morbidity of complications in severe acute pancreatitis. In consideration of these results, the effect of protease inhibitors can not be neglected completely, but in Europe and United States, their effects on acute pancreatitis are not accepted. In Japan, there has been no RCT of protease inhibitors which has evaluated the mortality rate for acute pancreatitis and there is no positive evidence for the treatment of acute pancreatitis with protease inhibitors. However, based on the report of Chen et al. and the results of the meta-analysis, the intravenous administration of gabexate mesilate (2400 mg/day) is recommended in Japan. Additionally, the double-blind controlled studies have shown that nafamostat mesilate and urinastatin have nearly the same effect as gabexate mesilate on acute pancreatitis [14-16] and, therefore, nafamostat mesilate and urinastatin are also often used in the treatment of acute pancreatitis in Japan.

As stated above, the clinical effects of protease inhibitors for the treatment of acute pancreatitis are still controversial. In Japan, although the effect of protease inhibitors on acute pancreatitis is recognized, the route, period of treatment and the infusion dose of protease inhibitors are still under investigation.

Figure 3. **Infectious complications and mortality in patients with acute necrotizing pancreatitis. A) frequency of intra-abdominal infection; B) frequency of infected pancreatic necrosis; C) mortality rate. All patients received the protease inhibitor, nafamostat mesilate, and antibiotics. Group I (n=16) received both medications intravenously. Group II (n=22) received nafamostat, via Continuous Regional Arterial Infusion (CRAI), and antibiotics intravenously. Group III (n=15) received both medications via CRAI. (From Reference 24, with permission)**



CRAI of protease inhibitors and antibiotics

It is characteristic that the half-life of the protease inhibitors clinically used is very short because they are promptly broken down by elastase in the blood and metabolized in the liver [17]. Moreover, ischemia and disturbance of the pancreatic microcirculation occur in severe acute pancreatitis and the concentration of protease inhibitor in the pancreas is considered to be decreased [18]. Therefore, CRAI was developed as a new drug delivery system to the pancreas which infuses drugs directly to the pancreatic artery. Kakugawa and Yamauchi et al. [19] investigated the concentration of nafamostat mesilate in the pancreas according to the route of infusion and they reported that the concentration of nafamostat mesilate in the pancreas infused by CRAI was five times higher than that infused intravenously in dog acute pancreatitis. Mikami et al. [20] reported that the concentration of nafamostat mesilate in the pancreas infused by CRAI was ten times higher than that infused intravenously in rat severe acute pancreatitis. In both reports, CRAI of nafamostat mesilate significantly improved the severity of acute pancreatitis compared with the intravenous infusion of nafamostat mesilate. As mentioned above, although more than 4 g/day of gabexate mesilate was infused for the treatment of acute pancreatitis in the German RCT [5], there were no significant differences in the mortality rate and morbidity of complications. This result might imply that the intravenous administration of 4 g/day gabexate mesilate did not increase the concentration of the drug in the pancreas and the amount of the drug was not enough to prevent the spread of inflammation.

Regarding antibiotics, a RCT performed in Italy showed that imipenem significantly prevented pancreatic infection in acute pancreatitis [21]. Büchler et al. [22] investigated the concentration of imipenem in pancreatic operations involving acute necrotizing pancreatitis and reported that the concentration of imipenem in the pancreas was very high. However, the specimen they investigated was not the pancreatic necrotic tissue itself, but the edematous tissue around the pancreatic necrotic tissue. Therefore, although they showed that the concentration of imipenem in the pancreas is very high, the concentration of imipenem in the pancreatic necrotic tissue is unclear. In consideration of the complicated pathology of acute necrotizing pancreatitis, CRAI aiming to obtain a high pancreatic concentration of drugs is considered to be an ideal drug delivery system and limiting the extent of pancreatic inflammation and pancreatic infection is expected.

Methodology of CRAI of protease inhibitor and antibiotics

After the admission of a patient with acute pancreatitis, contrast enhanced CT examination and fluid resuscitation are performed as soon as possible. Then, according to the findings of the contrast enhanced CT, clinical findings and laboratory data, the severity of the acute pancreatitis is diagnosed. Patients for whom CRAI therapy is indicated then undergo angiography examination. The femoral artery is punctured by the Seldinger method and a 4Fr or 5Fr catheter is inserted from the femoral artery and angiography of the celiac artery (CEA) and the supramesenteric artery (SMA) is performed. It is important that the artery flowing to the region of inflammation is selected for the route of administration in CRAI therapy. For example, if the main region of inflammation is in the pancreatic head, the tip of the catheter is located in the common hepatic artery or gastroduodenal artery or SMA. If inflammation is located in the pancreatic body-tail, the tip of the catheter is manipulated into the splenic artery, or sometimes the dorsal pancreatic artery. If inflammation extends throughout the whole pancreas, the tip of the catheter is located in the CEA, and in some institutes a separate catheter is located in each of the splenic and gastroduodenal arteries. The catheter for the infusion of drugs is the same as that used for angiography. The catheter is fixed to the femoral skin with silk strings. Before returning to the intensive care unit (ICU) or the ward, the catheter is filled with saline containing

heparin. Nafamostat mesilate (240 mg) dissolved in 500 ml 5% glucose solution is infused through the catheter continuously at 20 ml/h. Imipenem (0.5 g) is dissolved in 100 ml saline and infused intra-arterially every 12 hours. The period of CRAI therapy is 5 days, following which only antibiotics are administrated for 7 days to prevent pancreatic infection.

The efficacy of CRAI therapy

Imaizumi et al. [23] investigated the efficacy of CRAI therapy for severe acute pancreatitis in 51 patients admitted to ICU. They reported that CRAI therapy significantly decreased the rate of surgery and that the survival rate of the patients with CRAI therapy was significantly higher than that of the patients without CRAI therapy.

One aim of CRAI therapy is to prevent the progression of inflammation from pancreatic ischemia to pancreatic necrosis and to prevent early pancreatic infection. Therefore, the indication for CRAI therapy is a patient with severe acute pancreatitis whose pancreas is not enhanced homogenously by contrast enhanced CT on admission. In acute necrotizing pancreatitis, the necrotic, hemorrhagic, ischemic and edematous regions coexist in the early phase of the disease and the necrotic and ischemic regions appear to be heterogeneous low density areas in contrast enhanced CT but not all of these heterogeneous low density areas are actually necrotic. Generally, in acute necrotizing pancreatitis, the necrotic regions are clearly distinguished from the non-necrotic regions in contrast enhanced CT during the first one/two weeks after the onset of pancreatitis and there is no clear demarcation between these two regions within 4-5 days after the onset of pancreatitis [18]. Therefore, the window for the start of CRAI therapy is during this 4-5 days after onset.

Takeda et al. [24] reported that the mortality rates of patients with acute necrotizing pancreatitis who were started on CRAI therapy within 48 hours, during 48-72 hours and after 72 hours after onset were 3.2%, 9.1% and 26.3%, respectively. The mortality rate of patients on CRAI therapy within 48 hours after onset was significantly lower than that after 72 hours after onset. Similarly, the cooperative survey of CRAI therapy in Japan has shown that the mortality rate of patients who beginning CRAI within 48 hours and after 48 hours after onset was 11.9% and 23.6%, respectively, and there was a significant difference between them [25]. In our department, CRAI therapy was performed only for the patients with acute necrotizing pancreatitis within seven days after onset, and we have recognized that it is important to begin CRAI therapy as soon as possible after onset.

The characteristic effect of CRAI therapy on acute pancreatitis is improvement in abdominal pain. Takeda et al. reported that abdominal pain was improved in all patients on whom abdominal pain could be evaluated during CRAI therapy [26]. Similarly, the cooperative survey of CRAI therapy in Japan has reported that abdominal pain disappeared in 76% of the patients at 48 hours after initiation of CRAI therapy and the pain had disappeared in 87% of patients at 72 hours after initiation of the therapy [25]. This may be an effect of the protease inhibitor, which inhibits the inflammatory process in the pancreatic parenchyma caused by activation of pancreatic enzymes.

In Japan, both protease inhibitor and antibiotics are infused during CRAI therapy. Takeda et al. compared retrospectively the effect of CRAI therapy among (1) patients with both intravenous infusion of protease inhibitor and antibiotics, (2) patients with intra-arterial infusion of protease inhibitor and intravenous infusion of antibiotics and (3) patients with intra-arterial infusion of both protease inhibitor and antibiotics. They reported that the mortality rate of patients with both intra-arterial infusions was significantly lower than that of the patients with both intravenous infusions. They also reported that there was no significant difference between patients with intra-arterial infusion of protease inhibitor and intravenous infusion of antibiotics and patients with both intra-arterial infusions in the mortality rate, but the frequency of the infected pancreatic necrosis in patients with both intra-arterial infusions was significantly lower than for patients with intra-arterial infusion of protease inhibitor and intravenous infusion of antibiotics [26]. Therefore, CRAI of both protease inhibitor and antibiotics is effective both for the improvement of mortality rate and the prevention of infected pancreatic necrosis and especially, CRAI of protease inhibitor may have an effect on the prevention of infected pancreatic necrosis.

Recently, Hirota et al. [27] reported enteric necrosis combined with severe acute pancreatitis due to non-occlusive mesenteric ischemia and they proposed a modified CRAI therapy via infusion through the SMA in addition to the pancreatic artery to prevent enteric necrosis. The indication of this therapy is patients with vascular spasm of the SMA. Takagi and Isaji et al. reported that the intra-arterial infusion of protease inhibitors to the SMA has an effect on the prevention of pancreatic infection in dog acute necrotizing pancreatitis [28]. They also reported that the infusion of antibiotics through the SMA is effective for the prevention of gut bacterial translocation. However, there is still little clinical evidence for the effectiveness of their method and further clinical study is required. The protease inhibitors used in CRAI therapy are also applied in the treatment of disseminated intravascular coagulation. In acute necrotizing pancreatitis, patients with vascular spasm are often observed in the early phase of the disease and the blood circulation is likely to be disturbed. Although there is still no method to dramatically improve vascular spasm in acute pancreatitis, the effect of protease inhibitor, which also has an anticoagulant effect, is being re-evaluated with a view to the prevention of embolization when the blood circulation is disturbed [18].

Conclucions

This review discussed the role, method and the results of new therapy for acute necrotic pancreatitis – CRAI of protease inhibitor and antibiotics therapy. Although CRAI therapy is still one of the special therapies for acute pancreatitis in Japan, the efficacy of this therapy on the early phase of acute severe pancreatitis is widely recognized. Currently, RCTs of CRAI therapy for acute pancreatitis are in progress and it is expected that the efficacy of this therapy will eventually be recognized internationally.

References

1. Hirata K, Mayumi T, Ohtsuki M, Matsuno S, Takada T. Clinical guideline of acute pancreatitis based on evidences. Nippon Shokakibyo Gakkai Zasshi, 2003; 100: 965-73.
2. Skyring A, Singer A, Tornya P. Treatment of acute pancreatitis with Trasylol: Report of a controlled therapeutic trial. Br Med J, 1965; 5462: 627-9.
3. Baden H, Jordal K, Lund F, Zachariae F. A double-blind controlled clinical trial of Trasylol. Preliminary results in acute pancreatitis and in prophylaxis against postoperative pancreatitis. Acta Chir Scand, 1967; 378(Suppl.): 97-102.
4. Imrie CW, Benjamin IS, Ferguson JC, McKay AJ, Mackenzie I, O'Neill J, Blumgart LH. A single-centre double-blind trial of Trasylol therapy in primary acute pancreatitis. Br J Surg, 1978; 65: 337-41.
5. Büchler M, Malfertheiner P, Uhl W, Schölmerich J, Stockmann F, Adler G, Gaus W, Rolle K, Beger HG. Gabexate mesilate in human acute pancreatitis. German Pancreatitis Study Group. Gastroenterology, 1993; 104: 1165-70.
6. Valderrama R, Perez-Mateo M, Navarro S, Vazquez N, Sanjose L, Adrian MJ, Estruch J. Multicenter double-blind trial of gabexate mesilate (FOY) in unselected patients with acute pancreatitis. Digestion, 1992; 51: 65-70.
7. Yang CY, Chang-Chien CS, Liaw YF. Controlled trial of protease inhibitor gabexate mesilate (FOY) in the treatment of acute pancreatitis. Pancreas, 1987; 2: 698-700.
8. Banks PA. Practice guidelines in acute pancreatitis. Am J Gastroenterol, 1997; 92: 377-86.
9. Working Party of the British Society of Gastroenterology; Association of Surgeons of Great Britain and Ireland; Pancreatic Society of Great Britain and Ireland; Association of Upper GI Surgeons of Great Britain and Ireland. UK guidelines for the management of acute pancreatitis. Gut, 2005; 54(Suppl. 3): 1-9.
10. Chen HM, Chen JC, Hwang TL, Jan YY, Chen MF. Prospective and randomized study of gabexate mesilate for the treatment of severe acute pancreatitis with organ dysfunction. Hepatogastroenterology, 2000; 47: 1147-50.
11. Freise J, Melzer P, Schmidt FW, Horbach L. Gabexate mesilate in the treatment of acute pancreatitis. Results of a Hannover multicenter double-blind study with 50 patients. Z Gastroenterol, 1986; 24: 200-11.
12. Pederzoli P, Cavallini G, Falconi M, Bassi C. Gabexate mesilate vs aprotinin in human acute pancreatitis (GA.ME.P.A.). A prospective, randomized, double-blind multicenter study. Int J Pancreatol, 1993; 14: 117-24.
13. Andriulli A, Leandro G, Clemente R, Festa V, Caruso N, Annese V, Lezzi G, Lichino E, Bruno F, Perri F. Meta-analysis of somatostatin, octreotide and gabexate mesilate in the therapy of acute pancreatitis. Aliment Pharmacol Ther, 1998; 12: 237-45.
14. Watanabe S. Acute pancreatitis: overview of medical aspects. Pancreas, 1998; 16: 307-11.
15. Takeuchi T, Takebe T, Sato T. Clinical evaluation of FUT-175 (nafamostat mesilate) on pancreatitis. A double-blind controlled study with gabexate mesilate. Digest Med, 1984; 1: 255-70.
16. Yamamura H, Tamakuma S, Nakashima M. Clinical evaluation of MR-20 on shock. A double-blind controlled study with aprotinin. J Clin Exp Med, 1984; 129: 730-8.
17. Tsukagoshi S. Pharmacokinetics studies of nafamostat mesilate (FUT), a synthetic protease inhibitor, which has been used for the treatments of DIC and acute pancreatitis, and as an anticoagulant in extracorporeal circulation. Jpn J Cancer Chemother, 2000; 27: 767-74.
18. Takeda K, Mikami Y, Fukuyama S, Egawa S, Sunamura M, Ishibashi T, Sato A, Masamune A, Matsuno S. Pancreatic ischemia associated with vasospasm in the early phase of human acute necrotizing pancreatitis. Pancreas, 2005; 30: 40-9.
19. Yamauchi J, Takeda K, Shibuya K, Sunamura M, Matsuno S. Continuous regional application of protease inhibitor in the treatment of acute pancreatitis. An experimental study using closed duodenal obstruction model in dogs. Pancreatology, 2001; 1: 662-7.
20. Mikami Y, Takeda K, Matsuda K, Qiu-Feng H, Fukuyama S, Egawa S, Sunamura M, Matsuno S. Rat experimental model of continuous regional arterial infusion of protease inhibitor and its effects on severe acute pancreatitis. Pancreas, 2005; 30: 248-53.
21. Pederzoli P, Bassi C, Vesentini S, Campedelli A. A randomized multicenter clinical trial of antibiotic prophylaxis of septic complications in acute necrotizing pancreatitis with imipenem. Surg Gynecol Obstet, 1993; 176: 480-3.
22. Büchler M, Malfertheiner P, Friess H, Isenmann R, Vanek E, Grimm H, Schlegel P, Friess T, Beger HG. Human pancreatic tissue concentration of bactericidal antibiotics. Gastroenterology, 1992; 103: 1902-8.
23. Imaizumi H, Kida M, Nishimaki H, Okuno J, Kataoka Y, Kida Y, Soma K, Saigenji K. Efficacy of continuous regional arterial infusion of a protease inhibitor and antibiotic for severe acute pancreatitis in patients admitted to an intensive care unit. Pancreas, 2004; 28: 369-73.
24. Takeda K, Yamauchi J, Shibuya K, Sunamura M, Mikami Y, Matsuno S. Benefit of continuous regional arterial infusion of protease inhibitor and antibiotic in the management of acute necrotizing pancreatitis. Pancreatology, 2001; 1: 668-73.
25. Takeda K, Matsuno S, Ogawa M, Watanabe S, Atomi Y. Continuous regional arterial infusion (CRAI) therapy reduces the mortality rate of acute necrotizing pancreatitis: results of a cooperative survey in Japan. Hepatobiliary Pancreat Surg, 2001; 8: 216-20.
26. Takeda K, Matsuno S, Sunamura M, Kakugawa Y. Continuous regional arterial infusion of protease inhibitor and antibiotics in acute necrotizing pancreatitis. Am J Surg, 1996; 171: 394-8.
27. Hirota M, Inoue K, Kimura Y, Mizumoto T, Kuwata K, Ohmuraya M, Ishiko T, Beppu T, Ogawa M. Non-occlusive mesenteric ischemia and its associated intestinal gangrene in acute pancreatitis. Pancreatology, 2003; 3: 316-22.
28. Takagi K, Isaji S. Therapeutic efficacy of continuous arterial infusion of an antibiotic and a protease inhibitor via the superior mesenteric artery for acute pancreatitis in an animal model. Pancreas, 2000; 21: 279-89.

Anti-cytokine strategies in acute pancreatitis: pathophysiological insights and clinical implications

Rau BM*, Krüger CM, Schilling MK

Department of General, Visceral and Vascular Surgery, University of the Saarland, Homburg/Saar, Germany

Abstract

The clinical presentation of acute pancreatitis varies significantly from mild self-limiting discomfort to a severe life-threatening condition. Once the disease process is initiated, the severity of the disease is largely determined by a complex network of activated inflammatory mediators such as cytokines, proteolytic enzymes, reactive oxygen species, and many more which render the local injury to a systemic disease with multiple organ dysfunction, sepsis, and considerable mortality. Remarkable progress in diagnostic modalities, intensive care technologies, and organ preserving surgical techniques have decreased mortality of severe acute pancreatitis during the past decades. However, the treatment of acute pancreatitis still remains largely supportive and no specific approach exists to prevent evolving complications. A large body of clinical and experimental evidence suggests that cytokines are key factors in the pathomechanism of local and systemic complications of acute pancreatitis. Targeting cytokine activity as therapeutic approach to acute pancreatitis is a challenging concept and the results of modulating activation of TNF-α, IL-1β, IL-2, IL-10, PAF and various chemokines has indeed been promising in the experimental setting even if tested under therapeutic conditions. However, experience from a limited number of clinical trials on anti cytokine strategies in acute pancreatitis has remarkably emphasized that translating successful experimental observations into reproducible clinical associations seems to be difficult.

* CORRESPONDING AUTHOR:
Department of General, Visceral and Vascular Surgery
University of the Saarland
Kirrberger Strasse
66421 Homburg/Saar, Germany
Tel: +49 6841 162 2630; Fax: +49 6841 162 3132
e-mail: bettina.rau@uniklinikum-saarland.de (Bettina M. Rau)

Received 21.06.2005 Accepted 21.06.2005

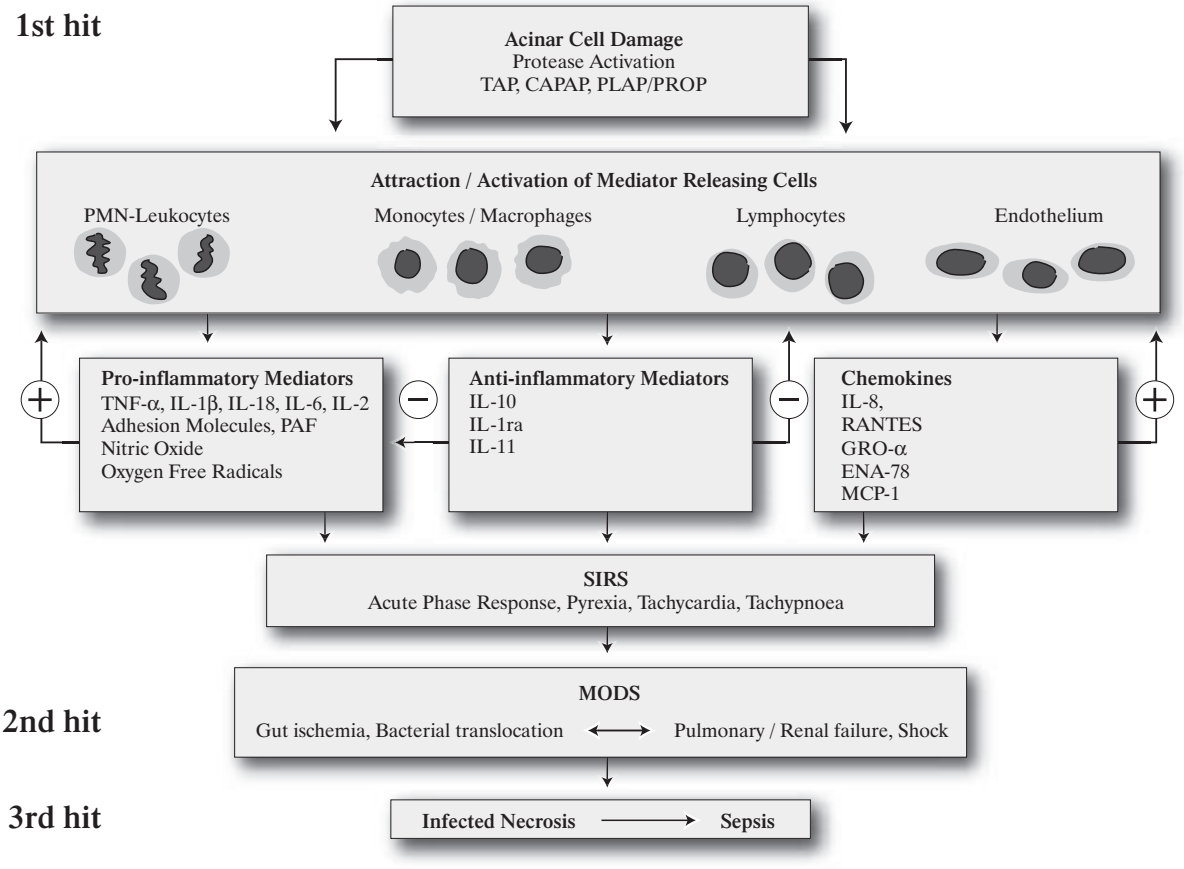
Key words: cytokines, acute pancreatitis, anti-cytokine approaches.

Introduction

Acute pancreatitis usually takes an uneventful course with complete restitutio ad intergrum and mortality rates of less than 1%. In contrast, in about 25% of all patients the disease takes a severe course evolving to a potentially life-threatening condition with considerable morbidity and mortality [1]. It has been largely shown that the hallmark of severe acute pancreatitis is the development of pancreatic necrosis which is still the essential determinant of further complications [2,3]. Depending on the extent of intra- and extrapancreatic necrosis pancreatic infections and remote organ failure frequently arise as major complications in the subsequent course of the disease and considerably add to mortality [2-6]. Nowadays, a multidisciplinary approach of prolonged intensive care management and delayed organ sparing surgical protocols has decreased the mortality of severe acute pancreatitis to about 20% to 30% in the past 20 years [7,8]. Despite the introduction of novel therapeutic concepts such as early ERCP in biliary acute pancreatitis, prophylactic antibiotics, and enteral nutrition since the early 90ies [7] the management of acute pancreatitis still remains largely supportive and despite all efforts, a break-trough in lowering mortality in patients with severe attacks has not been achieved [3-6]. Currently, no disease-specific medical treatment has ever been proven to overcome relevant complications such as pancreatic necrosis, infection of necrosis or organ failure effectively.

Dissatisfaction with persistently high mortality rates along with an improved understanding of the underlying pathomechanism of acute pancreatitis have made pancreatologists to pursue the search for alternative therapeutic approaches. More than 100 years ago Chiari proposed the first pathophysiological concept of acute pancreatitis by “autodigestion” of the gland via its own enzymes [9]. Since the mid 80ies, the pancreatic proteinase-antiproteinase imbalance was thought

Figure 1. **Schematic overview of the inflammatory cascade in acute pancreatitis. Activation of various leukocyte subsets and endothelium at the site of injury release various pro- and antiinflammatory cytokines, chemokines, and other mediators. An overt and sustained activation of proinflammatory mediators leads to systemic inflammatory response syndrome (SIRS) which may further proceed to multi organ dysfunction syndrome (MODS), infection of necrosis and sepsis**



to play a key role in the pathogenesis of acute pancreatitis. Hence, trypsinogen activation is believed to be one of the earliest pathophysiological events which triggers a cascade of other pancreatic proenzymes such as chymotrypsinogen, type I prophospholipase A_2, procarboxypeptidase B, or proelastase [10]. According to the "autodigestion" theory by Chiari, premature trypsinogen activation within the acinar cells has been found in various experimental models of acute pancreatitis [10,11]. Subsequently, significant amounts of trypsinogen and other proteases have been measured in the interstitial space as well as in the systemic circulation with a positive correlation to the extent of pancreatic tissue destruction and overall disease severity [11]. However, trypsinogen activation is only a temporary event in acute pancreatitis and most recent experimental studies have questioned the prevailing opinion of its dominating pathophysiological role [12]. These recent findings would at least in part explain the failure of antiproteinase therapy [13] or inhibiting pancreatic enzyme secretion [14] in decreasing complications and associated mortality in human acute pancreatitis.

An alternative pathophysiological concept was proposed by Rinderknecht in 1988. According to his theory, a complex network of inflammatory mediators released by activated leukocytes was suggested as key factor for rendering the local pancreatic insult into a systemic disease with distant organ failure [15]. In the subsequent years a growing number of clinical studies convincingly showed that acute pancreatitis is reflected by a large array of circulating inflammatory variables such as cytokines, chemokines, reactive oxygen species, adhesion molecules, acute phase proteins, and others [16,17]. In patients with an overt systemic inflammatory response and subsequent organ failure the quantitative release of nearly all mediators measured was significantly higher than that observed in patients with mild disease [18,19]. As a clinical consequence, measurement of specific inflammatory mediators offered a new interesting alternative to an easier severity stratification of acute pancreatitis compared with expensive imaging procedures or clinical staging scores [20]. Beyond the diagnostic and prognostic implications the "mediator hypothesis" was further substantiated by a growing number of experimental studies which ultimately lead to the establishment of the so-called "three" hit theory (*Fig. 1*). Herein, various pro- and antiinflammatory mediators are considered as important link between the initial local insult (first hit), the systemic host response and organ failure (second hit), and subsequent septic complications (third hit) in acute pancreatitis. By either inhibiting leukocyte activation or directly targeting leukocyte derived inflammatory mediators cytokines

Table 1. **Therapeutic effects of cytokine modulating approaches in experimental acute pancreatitis**

Author	Target	Model	Delay of drug administration	Intrapancreatic damage	Distat organ damage	Mortality
Norman et al. [35]	TNF-α	CDE, mouse	1.5 days	edema ↓	no effect	decrease
Norman et al. [43]	IL-1β	Cerulein, mouse	1 hour	necrosis, edema ↓	ND	ND
Norman et al. [45]	IL-1β	CDE, mouse	1.5 days	necrosis, edema ↓	lung ↓	decrease
Paszkowski et al. [48]	IL-1β/ICE	TC, rat	12 hours	necrosis ↓	lung ↓	decrease
Kusske et al. [75]	IL-10	CDE, mouse	33 hours	histologic score ↓	ND	decrease
Rongione et al. [76]	IL-10	Cerulein, rat	2 hours	histologic score ↓	ND	ND
Zou et al. [80]	IL-10	TC, rat	30 minutes	necrosis ↓	lung ↓, liver ↓	decrease
Mayer et al. [89]	IL-2	Cerulein, mouse	6 hours	histologic score ↓	lung ↓	ND
Formela et al. [99]	PAF	Ischemia, rat	30 minutes	histologic score ↓	ND	ND
Hofbauer et al. [101]	PAF	Duct ligation, opossum	2 days	necrosis ↓	lung ↓	ND
Foitzik et al. [102]	PAF	GDOC, rat	6 hours	microcirculation ↓	lung, kidney function ↓	decrease
Bhatia et al. [117]	CINC	Cerulein, rat	1 hour	no effect	lung ↓	ND
Bhatia et al. [118]	RANTES	Cerulein, mouse	1 hour	no effect	lung ↓	ND
Bhatia et al. [120]	MCP-1	Cerulein, mouse	1 hour	necrosis ↓	ND	ND

CDE – choline deficient ethionine supplemented; TC – Taurocholate; GDOC – Glycodeoxycholic acid; ND – not determined

have been recognized as central determinants of severity in acute pancreatitis and emerged as interesting targets for a potential therapeutic approach (*Tab. 1*).

The Role of Cytokines

Cytokines are a family of low molecular weight proteins (16-28 kDa), which have been extensively investigated in inflammatory conditions including acute pancreatitis. More than 30 different cytokines have been identified so far. With few exceptions, cytokines are not constitutively expressed in normal tissues and upregulation is usually initiated following external stimuli such as injury or stress in various cell types [16,17]. All cytokines cause their effects via highly specific membrane bound cell-surface receptors and have pleiotropic activities on a variety of target cells. On a functional basis cytokines can be divided into two groups: the pro- and the anti-inflammatory cytokines. There is immense redundancy within the system in such that many cytokines share similar biological effects and in the absence of another, they can fill the gap. Currently, there is no more doubt about the detrimental role of many cytokines in promoting local tissue destruction and mediating distant organ complications in acute pancreatitis and inflammatory disorders in general.

Tumor necrosis factor-α (TNF-α) and Interleukin-1β

Tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) belong to the so-called "first order" proinflammatory cytokines. Activated macrophages and polymorphonuclear leukocytes have been thought to be the primary site and major source of TNF-α and IL-1β synthesis for years [18,21]. However, more recent studies have emphasized the impact of acinar cells in contributing to the synthesis and release of TNF-α and other cytokines [22-25]. A primary involvement of TNF-α in acute pancreatitis was shown in both clinical [26] and experimental studies [27-30] already since the early 90ies. Besides an early rise of systemic TNF-α concentrations [27-29] organ-specific and time dependent upregulation of TNF-α mRNA and protein levels in the pancreas but also in distant organs such as lung and liver provided the first evidence that cytokines are important mediators of systemic complications and survival [29-31]. In the experimental setting TNF-α antagonism by either anti TNF-α antibodies or TNF-α receptor blockade almost uniformly revealed protective effects on local intrapancreatic damage, systemic severity, and mortality [32-36]. An effective amelioration of pancreatitis associated pulmonary damage could be shown by alternative anti TNF strategies using inhibitors of p38 mitogen-activated protein (MAP) kinases of nuclear factor κB (NFκB) [23,37,38]. Interestingly, the protective effect of TNF-α antagonism on disease severity and mortality was still observed in a therapeutic study design after the systemic effects already had fully developed [35].

Similar observations have been made for IL-1β, the second of the "first order" cytokines. As observed for TNF-α, organ-specific expression of IL-1β is an early feature in experimental acute pancreatitis and is found in both the pancreas and distant organs [21,31] and correlates with the severity of the model studied. However, in contrast to an overt local overexpression, systemic IL-1β concentrations remain relatively low [19,39]. Unlike TNF-α, IL-1β synthesis and release has been mainly demonstrated by activated leukocyte populations [21,18] and no direct effect of this cytokine on acinar cell viability or function has ever been demonstrated [40,41]. Blockade of the IL-1 receptor by pharmacological agents or targeted genetic disruption revealed a significant reduction of intrapancreatic damage, systemic severity, and mortality in every established pancreatitis model similar to that observed by blocking TNF-α [36,42-45]. A recent interesting alternative approach to inhibit IL-1β activation in acute pancreatitis included the inhibition of caspase-1, formerly termed interleukin 1β-converting enzyme

(ICE). Targeting ICE activity by a specific synthetic inhibitor led to a dramatic amelioration of severity and mortality irrespective of the model used [46-48]. Of specific interest is the fact that the protective effects on overall severity and mortality were still present after a therapeutic window of 12 hours following induction of severe acute pancreatitis [48]. By comparing anti TNF-α and anti IL-1β strategies, it becomes clearly evident that both cytokines share striking similarities in their pathophysiological functions and closely control the regulation of their own and each other [44,49]. This was convincingly shown by Denham et al. [36] who could not demonstrate any additive protective effects by combined genetic disruption of TNF-α and the IL-1 receptor in two models of murine acute pancreatitis.

Surprisingly, in contrast to their outstanding pathophysiological importance both cytokines play no role as biochemical markers for a reliable severity assessment of acute pancreatitis in the clinical setting. Is has been largely shown that TNF-α measurements are difficult, because they are substantially hampered by intermittent TNF-α release and a short plasma half-life of less than 20 minutes. Similar observations have been made for IL-1β, which shows an early and transicient increase in most severe cases only [19,50-52]. The soluble TNF receptor complex as well as the IL-1 receptor antagonist (IL-1RA) are more stable than the cytokines itself and thus easier to measure. Although TNF receptors [50,53] and IL-1ra [19,50,51,54] were found to correlate with severe acute pancreatitis and associated organ failure they are no candidate parameters for a meaningful clinical application.

In contrast to the extensive investigations of both cytokines in experimental respect no study has ever been conducted investigating TNF-α or IL-1 antagonism in clinical acute pancreatitis. However, interesting insights can be drawn from a number of sepsis trials. Acute pancreatitis, especially in its severe form, shares striking similarities with sepsis and septic shock. The clinical feature of multi system organ failure and the inflammatory mediator profile are indistinguishable in each of these conditions and suggest a common pathogenic mechanism, albeit as a result of different inflammatory stimuli. Some large randomized multicenter trials on anti TNF-α and IL-1 antagonism in patients with sepsis have demonstrated overall disappointing results. The use of an anti TNF-α antibody in patients with sepsis failed to reduce 28 day mortality in two phase III trials, the North American Sepsis Trial (NORASEPT) and the International Sepsis Trial. Likewise, fusion proteins for p75 TNF-R and p55 TNF-R were ineffective as well [55,56]. A *post hoc* analysis of a controlled trial of human recombinant IL-1 RA in patients with sepsis syndrome revealed an increase in survival time in a subgroup of patients with multi organ failure [57]. However, this observation again could not be confirmed by a subsequent trial [58].

Interleukin-18

Interleukin-18 (IL-18), formerly called interferon-γ-inducing factor, is a novel proinflammatory cytokine playing an important role in the Th-1 response, primarily due to its ability to induce IFN-γ production in T-cells and natural killer cells [59]. IL-18 shares striking similarities with IL-1β concerning structure and function. Both are synthesized as biologically inactive precursors requiring proteolytic cleavage into their mature form by caspases-1/ICE. Moreover, the biological activity of IL-18 is closely related to that of IL-1β: IL-18 induces the gene expression and synthesis of TNF, IL-1, and several chemokines by means of a putative IL-18 receptor complex which is a member of the IL-1R family as well [59]. IL-18 has gained considerable attention since the striking protective effects of caspase-1 inhibition have been reported by a large number of experimental studies in various inflammatory conditions [60] including acute pancreatitis [46-48]. Under therapeutic conditions, caspase-1 antagonism has been more effective in reducing pancreatitis related severity and mortality [48] than has IL-1 antagonism [43,45] in severe experimental models. Therefore, caspase-1 mediated activation of IL-18 may well explain the better results of blocking caspases-1 activity [61]. In fact, by comparing the dynamics of systemic IL-1β and IL-18 concentrations an interesting observation became evident supporting this theory: IL-1β revealed a temporary and moderate increase during the very first days after onset of symptoms in severe disease only [19]. In contrast, IL-18 was released in much higher concentrations with maximum levels during the second week after disease onset in patients with persisting multiorgan system failure [62,63]. The effectiveness of delayed ICE treatment could therefore be a result of inhibited generation of mature IL-18 rather than IL-1β. Interestingly, neutralizing IL-18 activity by monoclonal antibodies has indeed proven to decrease intrapancreatic damage more effectively than neutralizing IL-1β activity in cerulein-induced pancreatitis in mice [64]. Since a therapeutic inhibition of IL-18 has not been investigated in any model of acute pancreatitis so far this interesting cytokine will need further investigation.

Interleukin-6

Interleukin-6 (IL-6) is produced by a wide range of cells including monocytes/macrophages, endothelial cells, and fibroblasts in response to potent proinflammatory stimuli such as endotoxin, IL-1β or TNF-α. IL-6 is the primary inducer of the acute-phase response in various inflammatory conditions [65]. The clinical value of IL-6 for an early and accurate severity stratification of acute pancreatitis has been recognized since the very first reports on cytokine measurements in human acute pancreatitis appeared in the literature [66,67]. Hence, a large number studies have addressed this issue which uniformly confirmed that IL-6 is an earlier marker of severity than the currently established "gold standard" C-reactive protein [19,67]. In terms of predicting pancreatitis associated complications, IL-6 was found to be an excellent predictor of remote organ failure [18,19,53]. In contrast to the exhaustive clinical investigation of IL-6, only few studies have ever addressed the role of this cytokine as potential target for modulating disease severity. From the limited number of experimental studies prophylactic inhibition or genetic deletion of IL-6 had a deleterious [68,69] rather than a protective effect [70] on disease severity and mortality [68-70]. Therefore, the presence of a physiologic IL-6 mediated inflammatory response seems to be necessary for local

and systemic damage control in acute pancreatitis. However, in an overall sense, these observations preclude IL-6 as driving force for the initiation or propagation of organ-specific complications of acute pancreatitis.

Interleukin-10

Interleukin-10 (IL-10) is a potent anti-inflammatory cytokine expressed by almost all cells but primarily released by activated monocytes/macrophages and Th-2 lymphocytes. This cytokine exerts its antiinflammatory properties through inhibition of various proinflammatory cytokines and adhesion molecules on the transcriptional and post-transcriptional level. In addition, IL-10 induces the synthesis of natural cytokine antagonists such as IL-1RA and TNF-α receptors [71]. In human acute pancreatitis, circulating levels of IL-10 were found to correlate with the severity of the disease [19,51] and with organ failure or death in some, but not all of the studies [72,73]. Interesting pathophysiological aspects of this cytokine arose from a number of experimental studies which uniformly demonstrated a protective effect of IL-10 in several models of acute pancreatitis [74-80]. Irrespective whether IL-10 activity was blocked [79], the IL-10 gene was genetically disrupted [78] or the cytokine activity was augmented [74-77,80] organ specific damage in the pancreas, the lung and the liver as well as mortality were significantly reduced. Of specific interest was the observation that the protective effects were still observed when active IL-10 or genetic transfection was started in a therapeutic fashion after acute pancreatitis had been induced [75,76,80].

Convincing experimental evidence of IL-10 modulating approaches has driven the design of clinical trials with the aim to reduce the occurrence of post-ERCP pancreatitis by prophylactic recombinant IL-10 administration. In 2001 Deviere et al. [81] published a single center, double blind controlled study in patients undergoing ERCP, which showed that IL-10 was able to decrease the incidence of post-ERCP pancreatitis independently from other risk factors as well as the length of hospital stay. Inconclusive results came from an US-american trial in which only a trend toward a reduced incidence of post-ERCP pancreatitis and hospital stay was found [82]. A recent meta-analysis including four randomized clinical trials in 294 patients receiving recombinant IL-10 and 259 patients receiving placebo before ERCP could show that IL-10 significantly reduces the risk of post-ERCP pancreatitis [83]. However, the ultimate benefit of IL-10 treatment in preventing post-ERCP pancreatitis is not definitely proven and still needs further evaluation.

Interleukin-2

Increasing clinical evidence suggests that an impaired immune function contributes to the progression of acute pancreatitis. However, cellular immune functions constitute a complex network and seem to have distinct roles in the early toxic and the late septic stages of acute pancreatitis [19,84-86]. Interleukin-2 (IL-2) is a product of activated Th-1 lymphocytes and plays a central role in normal immune function. Clinical and experimental observations have pointed out that the activation of the T-cell system within the inflammatory cascade of acute pancreatitis enhances pancreatic tissue injury [87], the inflammatory response [88-90], and mortality [88]. The release of the soluble IL-2 receptor shows a close correlation with persisting organ complications during the later stages of the disease [19,86] with peak levels predicting a lethal outcome [19]. In diet induced acute pancreatitis in mice a significant reduction of IL-2 production with a consecutively enhanced susceptibility to endotoxin-induced mortality was found during the later stages of the disease which could be reversed by *in vivo* therapy with recombinant IL-2 [90]. These experimental data are well in line with clinical observations and strongly suggest that an impaired immune function increases the risk of subsequent septic complications. Interestingly, the administration on the immunostimulant Levamisole, which is known to potentiate IL-2 production, effectively decreased the incidence of pancreatic infections in a cat model of severe acute pancreatitis [91]. In contrast to the late effects of IL-2 deficiency and immunoparalysis the deleterious consequences of an overt IL-2 mediated T-cell response during the early course of the disease could be emphasized in moderate to severe models of murine acute pancreatitis [87-89]. T and B-cell deficient mice with acute pancreatitis exhibit significantly lower pulmonary damage [88]. The immunosuppressant FK506 which inhibits IL-2 production on the transcriptional level effectively decreased early local and systemic disease severity [89,92], even if given therapeutically after induction of pancreatitis [89]. However, opposite results were shown by another study in which FK506 significantly worsened survival in diet-induced murine pancreatitis [93]. As a result of the obvious controversies even in the experimental setting and the diverse effects of IL-2 during different stages of acute pancreatitis the general concept of immunomodulation as a potential therapeutic target is yet attractive but remains inconclusive and is not ready to be transferred to clinical application.

Platelet-Activating Factor

Platelet-activating factor (PAF) is a lipid that functions as a proinflammatory cytokine since it induces platelet activation and aggregation, neutrophil and monocyte activation, chemotaxis, and vascular effects in terms of vasodilatation and increased vascular permeability [94]. Upon activation leukocytes, platelets, and endothelial cells are major sources of PAF release. PAF synthesis and secretion is closely related to TNF-α and IL-1β in a synergistic manner [94].

A pathophysiological implication of PAF in acute pancreatitis could be first demonstrated by an Italian group in 1989. Administration of PAF into the superior pancreaticoduodenal artery of rabbits induced classical morphologic and biochemical changes of acute pancreatitis in a dose dependent manner within 24-72 hours of injection [95]. These exciting observations have made a number of groups to pursue the role of this novel cytokine in acute pancreatitis [96-103]. Surprisingly, the course of PAF levels has never been investigated in human acute pancreatitis, however, an increase of PAF concentrations in pancreas, lung, ascites, and plasma was found in experimen-

tal models [97,98]. Except one study [103] PAF antagonism has been shown to reduce nearly all pathophysiological changes of acute pancreatitis in established experimental models [99-102]. Besides a significant amelioration of local intrapancreatic damage and microcirculatory derangements a considerable decrease of distant organ involvement and mortality was observed, if PAF antagonists were applied in a therapeutic fashion [99,101,102].

On the basis of the almost uniformly positive experimental results PAF antagonism is one of the few pharmaceutical approaches to acute pancreatitis which have passed the threshold from the experimental setting to clinical application. Lexipafant, one of the most powerful PAF antagonists has been tested in two phase II trials encountering 133 patients with acute pancreatitis [104] or predicted severe acute pancreatitis [105]. In both studies a significant improvement of organ failure or organ failure scores was observed and justified the subsequent initiation of a randomized, double-blind, placebo controlled multicenter trial in 290 patients with predicted severe acute pancreatitis [106]. As with other previous multicenter trials assessing pharmacological agents [13,14] lexipafant did not show any clear benefit in reducing complications, new onset organ failure, or mortality in acute pancreatitis. However, apart from providing interesting insights to the clinical pathophysiology of acute pancreatitis some positive aspects of this approach need to be underscored. Systemic sepsis, development of pseudocysts, and systemic IL-8 and E-selectin levels were significantly lower in the treated than in the non-treated group. A *post hoc* logistic regression analysis showed that initiation of lexipafant treatment within 48 hours of disease onset was related to a lower mortality rate.

Chemokines

Chemokines are a family of small (8-10 kDa), inducible, secreted cytokines with chemotactic and activating effects on different leukocyte subsets thus providing a key stimulus for directing leukocytes to the areas of injury [107]. Over 50 different chemokines and more than 20 receptors with overlapping functions have been characterized. Chemokines can be subdivided on a structural basis into the CXC-subfamily in which the first two of four conserved cysteine residues are separated by another amino acid and the CC-subfamily in which the first two cysteine residues are adjacent. The structural classification of the chemokines also determines their biological activity: while a subgroup of the CXC-chemokines, such as interleukin-8, are potent neutrophil chemoattractants and activators, the CC-chemokines comprising monocyte chemoattractant protein (MCP)-1, -2, -3, macrophage inflammatory protein (MIP)-1α and -1β, regulated on activation, normal T-cell expressed and secreted (RANTES), and eotaxin predominantly affect monocytes [107,108]. Although the importance of chemokines in inflammatory conditions has been well recognized they have only recently become the focus of interest in acute pancreatitis. So far, only a small number of experimental and clinical studies have pointed out that chemokine blockade may be at least as effective as cytokine blockade because of their more proximal position within the inflammatory mediator cascade.

Interleukin-8

Interleukin-8 (IL-8) is the most well known and best characterized member of the chemokine family in acute pancreatitis. IL-8 is synthesized by a large number of different cells such as leukocyte subsets, endothelial and even pancreatic acinar cells [18,23,24]. As a chemokine-specific feature IL-8 is able to stimulate neutrophil chemotaxis and the release of proteolytic enzymes as well as reactive oxygen species thereby enhancing tissue destruction [108]. Along with IL-6, IL-8 has been payed much attention to as early prognostic biochemical variable of disease severity within the first days after onset of symptoms in acute pancreatitis [18,19,109]. An even more interesting aspect of IL-8 was described by our group [110,111]. In patients with necrotizing pancreatitis who developed septic multi organ failure during the later stages of the disease IL-8 has proven as an excellent marker for monitoring this life-threatening complication [111]. Some years later the deleterious role of IL-8 in acute pancreatitis could be nicely demonstrated in the experimental setting [112]. However, only one study has ever investigated the role of anti-IL-8 treatment in this context, yet with interesting results. In a rabbit model of acute pancreatitis Osman et al. [113] could show that prophylactic blockade of IL-8 lead to a significant reduction of systemic severity, lung injury, and mortality, whereas the local intrapancreatic damage remained unchanged. Although it remains yet unproven whether the protective effects are still observed in a therapeutic design, the study strongly supports the role of chemokines in mediating distant organ failure.

Other chemokines

Besides IL-8 other chemokines such as monocyte chemoattractant protein-1 (MCP-1), growth-related oncogene alpha (GRO-α), and epithelial neutrophil-activating protein 78 (ENA78) could be found in high concentrations during the early stages of clinical acute pancreatitis. The quantitative release of these chemokine was more related to the occurrence of systemic than of local complications, thus suggesting a pivotal role in the pathomechanism of distant organ failure [114,115]. In experimental acute pancreatitis chemokines were found to be upregulated as early as 30 minutes after cerulein hyperstimulation [116] and acinar cells were shown to be a major source of chemokine synthesis [23]. In several experimental studies pancreatitis associated pulmonary damage was effectively reduced, if activation of the CXC chemokines CINC or RANTES *via* specific antibodies or synthetic inhibitors was blocked [117,118]. Targeted disruption of the MIP-1α/RANTES receptor CCR-1 had a similar effect in the cerulein model in mice [119]. An interesting common observation of these studies was the fact that despite a significant reduction of overall severity and pulmonary damage no effects on local intrapancreatic damage were observed. So far, MCP-1 seems to be only chemokine which exerts a detrimental role on the degree of local intrapancreatic damage [120]. Unfortunately, although the protective effects were still observed after therapeutic inhibition in most studies [117,118,120], the role of chemokine antagonism on mortality has never been investigated so far.

Anti cytokine strategies in the clinical setting

A large body of experimental evidence suggests that modulation of pro- or antiinflammatory cytokine activation has favourable effects on local and systemic disease severity in acute pancreatitis. Different approaches to inhibit cytokine activation have been used in various experimental models, many of them have even proven effective, if applied in a therapeutic fashion after induction of acute pancreatitis. Despite the number of favourable experimental studies, only few clinical trials on anti-cytokine strategies have been performed. Hence, only PAF antagonism and IL-10 treatment have been investigated in acute pancreatitis by controlled studies with largely disappointing results.

Yet, cytokines are still an exciting and challenging target for potential new approaches to the treatment of acute pancreatitis. The failure of the few representative clinical studies on anti--cytokine strategies does not necessarily mean that this approach is generally ineffective since we know that those cytokine identified so far most likely represent the "tip of the iceberg" only [121]. However, before new clinical trials are started, there must be careful consideration of why previous interventions were not effective.

In fact, every "first order" cytokine known so far is a strong promoter for the progression of acute pancreatitis on its own. However, a magnitude of other potent inflammatory mediators is known to interact and control the cytokine release and vice versa. It therefore should be kept in mind that the concept of blocking a single elevated cytokine may be too simple to deal with the complex problem of acute pancreatitis. It remains largely questionable, if there is any "ultimate" target at all, and if so it still needs to be defined.

Secondly, as patients with acute pancreatitis move through different phases from sterile inflammatory response to septic organ failure, there may be intervals when it is appropriate to inhibit multiple cytokines while at other times it may be appropriate to augment them.

A third point involves the optimum timing to start cytokine antagonism. Norman et al. [16] has well described that the therapeutic window in acute pancreatitis is restricted to about 48-72 hours following the onset of symptoms until complications such as necrosis or organ failure develop. This concept is supported by at least two controlled trials using different pharmaceutical approaches: the European PAF-antagonist phase III trial [106] and the German Octreotide trial [122] in which a beneficial effect was achieved when treatment was started within 48 hours after onset of symptoms. However, clinical experience has shown that many patients with severe disease usually present to specialized centers capable to provide this kind of specific and expensive treatment beyond the 48 hour time interval after onset of symptoms.

At present, a truly optimistic view on anti-cytokine strategies is not supported by any of the large representative clinical studies. The main limitations of anti-cytokine-treatment strategies relate to the difficulties in translating successful experimental observations into reproducible clinical associations due to the complexity and individuality of the human nature [123]. Moreover, deficiencies in study design with insufficient sample sizes, inconsistent definitions and tools to stratify disease severity as well as non-comparable study endpoints have further contributed to the failure of nearly all clinical studies on new pharmacological approaches to acute pancreatitis. Despite all limitations, treatment of acute pancreatitis by cytokine modulation still remains an attractive concept. However, further work will be needed to overcome the fundamental conceptional problems as well as to accomplish our still incomplete understanding of the complex pathophysiology of this challenging disease.

References

1. Bradley EL 3rd. A clinically based classification system for acute pancreatitis. Arch Surg, 1993; 128: 586-90.
2. Beger HG, Bittner R, Block S, Büchler M. Bacterial contamination of pancreatic necrosis. A prospective clinical study. Gastroenterol, 1986, 49: 433-8.
3. Isenmann R, Rau B, Beger HG. Bacterial infection and extent of necrosis are determinants of organ failure in patients with acute necrotizing pancreatitis. Br J Surg, 1999; 86: 1020-4.
4. Isenmann R, Rau B, Beger HG. Early severe acute pancreatitis: characteristics of a new subgroup. Pancreas, 2001; 22: 274-8.
5. Buter A, Imrie CW, Carter CR, Evans S, McKay CJ. Dynamic nature of early organ dysfunction determines outcome in acute pancreatitis. Br J Surg, 2002; 89: 298-302.
6. Johnson CD, Abu-Hilal M. Persistent organ failure during the first week as a marker of fatal outcome in acute pancreatitis. Gut, 2004; 53: 1340-4.
7. Working Party of the British Society of Gastroenterology; Association of Surgeons of Great Britain and Ireland; Pancreatic Society of Great Britain and Ireland; Association of Upper GI Surgeons of Great Britain and Ireland: UK guidelines for the management of acute pancreatitis. Gut, 2005; 54 (Suppl. 3): 1-9.
8. Uhl W, Warshaw A, Imrie C, Bassi C, McKay CJ, Lankisch PG, Carter R, Di Magno E, Banks PA, Whitcomb DC, Dervenis C, Ulrich CD, Satake K, Ghaneh P, Hartwig W, Werner J, McEntee G, Neoptolemos JP, Buchler MW. IAP guidelines for the surgical management of acute pancreatitis. Pancreatology, 2002; 2: 565-73.
9. Chiari H. Über die Selbstverdaung des menschlichen Pankreas. Zeitschrift für Heilkunde, 1896; 17: 69-96.
10. Gorelick FS, Otani T. Mechanisms of intracellular zymogen activation. Baillieres Best Pract Res Clin Gastroenterology, 1999; 13: 227-40.
11. Frossard JL. Trypsin activation peptide (TAP) in acute pancreatitis: from pathophysiology to clinical usefulness. JOP, 2001; 2: 69-77.
12. Halangk W, Krüger B, Ruthenbürger M, Stürzebecher J, Albrecht E, Lippert H, Lerch MM. Trypsin activity is not involved in premature, intrapancreatic trypsinogen activation. Am J Physiol Gastrointest Liver Physiol, 2002; 282: G367-74.
13. Seta T, Noguchi Y, Shimada T, Shikata S, Fukui T. Treatment of acute pancreatitis with protease inhibitors: A meta-analysis. Eur J Gastroenterol Hepatol, 2004; 16: 1287-93.
14. Cavallini G, Frulloni L. Somatostatin and octreotide in acute pancreatitis: the never-ending story. Dig Liver Dis, 2001; 33: 192-201.
15. Rinderknecht H. Fatal pancreatitis, a consequence of excessive leukocyte stimulation? Int J Pancreatol, 1988; 3: 105-12.
16. Norman J. The role of cytokines in the pathogenesis of acute pancreatitis. Am J Surg, 1998; 175: 76-83.
17. Makhija R, Kingsnorth AN. Cytokine storm in acute pancreatitis. J Hepatobiliary Pancreat Surg, 2002; 9: 401-10.
18. McKay CJ, Gallagher G, Brooks B, Imrie CW, Baxter JN. Increased monocyte cytokine production in association with systemic complications in acute pancreatitis. Br J Surg, 1996; 83: 919-23.
19. Mayer J, Rau B, Gansauge F, Beger HG. Inflammatory mediators in human acute pancreatitis: clinical and pathophysiological implications. Gut, 2000; 47: 546-52.
20. Rau B, Schilling MK, Beger HG. Laboratory markers of severe acute pancreatitis. Dig Dis, 2004; 22: 247-57.
21. Fink GW, Norman JG. Intrapancreatic interleukin-1 beta gene expression by specific leukocyte populations during acute pancreatitis. J Surg Res, 1996; 63: 369-73.
22. Gukovskaya AS, Gukovsky I, Zaninovic V, Song M, Sandoval D, Gukovsky S, Pandol SJ. Pancreatic acinar cells produce, release, and

respond to tumor necrosis factor-alpha. Role in regulating cell death and pancreatitis. J Clin Invest, 1997; 100: 1853-62.

23. Blinman TA, Gukovsky I, Mouria M, Zaninovic V, Livingston E, Pandol SJ, Gukovskaya AS. Activation of pancreatic acinar cells on isolation from tissue: cytokine upregulation via p38 MAP kinase. Am J Physiol Cell Physiol, 2000; 279: C1993-2003.

24. Blanchard JA 2nd, Barve S, Joshi-Barve S, Talwalker R, Gates LK Jr. Cytokine production by CAPAN-1 and CAPAN-2 cell lines. Dig Dis Sci, 2000; 45: 927-32.

25. Ramudo L, Manso MA, Sevillano S, de Dios I. Kinetic study of TNF-alpha production and its regulatory mechanisms in acinar cells during acute pancreatitis induced by bile-pancreatic duct obstruction. J Pathol, 2005; 206: 9-16.

26. Exley AR, Leese T, Holliday MP, Swann RA, Cohen J. Endotoxaemia and serum tumour necrosis factor as prognostic markers in severe acute pancreatitis. Gut, 1992; 33: 1126-8.

27. Grewal HP, Kotb M, el Din AM, Ohman M, Salem A, Gaber L, Gaber AO. Induction of tumor necrosis factor in severe acute pancreatitis and its subsequent reduction after hepatic passage. Surgery, 1994; 115: 213-21.

28. Hughes CB, Gaber LW, Kotb M, Mohey el-Din AB, Pabst M, Gaber AO. Induction of acute pancreatitis in germ-free rats: evidence of a primary role for tumor necrosis factor-alpha. Surgery, 1995; 117: 201-5.

29. Norman JG, Fink GW, Franz MG. Acute pancreatitis induces intrapancreatic tumor necrosis factor gene expression. Arch Surg, 1995; 130: 966-70.

30. Hughes CB, Henry J, Kotb M, Lobaschevsky A, Sabek O, Gaber AO. Up-regulation of TNF alpha mRNA in the rat spleen following induction of acute pancreatitis. J Surg Res, 1995; 59: 687-93.

31. Norman JG, Fink GW, Denham W, Yang J, Carter G, Sexton C, Falkner J, Gower WR, Franz MG. Tissue-specific cytokine production during experimental acute pancreatitis. A probable mechanism for distant organ dysfunction. Dig Dis Sci, 1997; 42: 1783-8.

32. Grewal HP, Mohey el Din A, Gaber L, Kotb M, Gaber AO. Amelioration of the physiologic and biochemical changes of acute pancreatitis using an anti-TNF-alpha polyclonal antibody. Am J Surg, 1994; 167: 214-8.

33. Hughes CB, Gaber LW, Mohey el-Din AB, Grewal HP, Kotb M, Mann L, Gaber AO. Inhibition of TNF alpha improves survival in an experimental model of acute pancreatitis. Am J Surg, 1996; 62: 8-13.

34. Hughes CB, Grewal HP, Gaber LW, Kotb M, el-Din AB, Mann L, Gaber AO. Anti-TNF alpha therapy improves survival and ameliorates the pathophysiologic sequelae in acute pancreatitis in the rat. Am J Surg, 1996; 171: 274-80.

35. Norman JG, Fink GW, Messina J, Carter G, Franz MG. Timing of tumor necrosis factor antagonism is critical in determining outcome in murine lethal acute pancreatitis. Surgery, 1996; 120: 515-21.

36. Denham W, Yang J, Fink G, Denham D, Carter G, Ward K, Norman J. Gene targeting demonstrates additive detrimental effects of interleukin-1 and tumor necrosis factor during pancreatitis. Gastroenterol, 1997; 113: 1741-6.

37. Yang J, Murphy C, Denham W, Botchkina G, Tracey KJ, Norman J. Evidence of a central role for p38 map kinase induction of tumor necrosis factor alpha in pancreatitis-associated pulmonary injury. Surgery, 1999; 126: 216-22.

38. Jaffray C, Yang J, Carter G, Mendez C, Norman J. Pancreatic elastase activates pulmonary nuclear factor kappa B and inhibitory kappa B, mimicking pancreatitis-associated adult respiratory distress syndrome. Surgery, 2000; 128: 225-31.

39. Fink GW, Norman JG. Specific changes in the pancreatic expression of the interleukin 1 family of genes during experimental acute pancreatitis. Cytokine, 1997; 9: 1023-7.

40. Fink G, Yang J, Carter G, Norman J. Acute pancreatitis-induced enzyme release and necrosis are attenuated by IL-1 antagonism through an indirect mechanism. J Surg Res, 1997; 67: 94-7.

41. Denham W, Yang J, Fink G, Denham D, Carter G, Bowers V, Norman J. TNF but not IL-1 decreases pancreatic acinar cell survival without affecting exocrine function: a study in the perfused human pancreas. J Surg Res, 1998; 74: 3-7.

42. Tanaka N, Murata A, Uda K, Toda H, Kato T, Hayashida H, Matsuura N, Mori T. Interleukin-1 receptor antagonist modifies the changes in vital organs by acute necrotizing pancreatitis in a rat experimental model. Crit Care Med, 1995; 23: 901-8.

43. Norman J, Franz M, Messina J, Riker A, Fabri PJ, Rosemurgy AS, Gower WR Jr. Interleukin-1 receptor antagonist decreases severity of experimental acute pancreatitis. Surgery, 1995; 117: 648-55.

44. Norman JG, Fink G, Franz M, Guffey J, Carter G, Davison B, Sexton C, Glaccum M. Active interleukin 1 receptor required for maximal progression of acute pancreatitis. Ann Surg, 1996; 223: 163-9.

45. Norman JG, Franz MG, Fink GS, Messina J, Fabri PJ, Gower WR, Carey LC. Decreased mortality of severe acute pancreatitis after proximal cytokine blockade. Ann Surg, 1995; 221: 625-34.

46. Norman J, Yang J, Fink G, Carter G, Ku G, Denham W, Livingston D. Severity and mortality of experimental pancreatitis are dependent on interleukin-1 converting enzyme. J Interferon Cytokine Res, 1997; 17: 113-8.

47. Rau B, Paszkowski AS, Lillich S, Baumgart K, Möller P, Beger HG. Differential effects of caspase-1/interleukin-1beta-converting enzyme on acinar cell necrosis and apoptosis in severe acute experimental pancreatitis. Lab Invest, 2001; 81: 1001-13.

48. Paszkowski AS, Rau B, Mayer JM, Möller P, Beger HG. Therapeutic application of caspase-1/interleukin-1β-converting enzyme inhibitor decreases the death rate of severe acute experimental pancreatitis. Ann Surg, 2002; 235: 68-76.

49. Denham W, Fink G, Yang J, Ulrich P, Tracey K, Norman J. Small molecule inhibition of tumor necrosis factor gene processing during acute pancreatitis prevents cytokine cascade progression and attenuates pancreatitis severity. Am Surg, 1997; 63: 1045-9.

50. Dugernier TL, Laterre PF, Wittebole X, Roeseler J, Latinne D, Reynaert MS, Pugin J. Compartmentalization of the inflammatory response during acute pancreatitis. Correlation with local and systemic complications. Am J Resp Crit Care Med, 2003; 168: 148-57.

51. Brivet FG, Emilie D, Galanaud P. Pro- and anti-inflammatory cytokines during acute severe pancreatitis: an early and sustained response, although unpredictable of death. Crit Care Med, 1999; 27: 749-55.

52. Chen CC, Wang SS, Lee FY, Chang FY, Lee SD. Proinflammatory cytokines in early assessment of the prognosis of acute pancreatitis. Am J Gastroenterol, 1999; 94: 213-8.

53. de Beaux AC, Goldie AS, Ross JA, Carter DC, Fearon KC. Serum concentrations of inflammatory mediators related to organ failure in patients with acute pancreatitis. Br J Surg, 1996; 83: 349-53.

54. Mentula P, Kylanpaa ML, Kemppainen E, Jansson SE, Sarna S, Puolakkainen P, Haapiainen R, Repo H. Plasma anti-inflammatory cytokines and monocyte human leucocyte antigen-DR expression in patients with acute pancreatitis. Scand J Gastroenterol, 2004; 39: 178-87.

55. Cohen J, Carlet J. INTERSEPT: an international, multicenter, placebo-controlled trial of monoclonal antibody to human tumor necrosis factor-alpha in patients with sepsis. International Sepsis Trial Study Group. Crit Care Med, 1996; 24: 1431-40.

56. Abraham E, Anzueto A, Gutierrez G, Tessler S, San Pedro G, Wunderink R, Dal Nogare A, Nasraway S, Berman S, Cooney R, Levy H, Baughman R, Rumbak M, Light RB, Poole L, Allred R, Constant J, Pennington J, Porter S. Double-blind randomised controlled trial of monoclonal antibody to human tumor necrosis factor in treatment of septic shock. NORASEPT II Study Group. Lancet, 1998; 351: 929-33.

57. Fisher CJ Jr, Dhainaut JF, Opal SM, Pribble JP, Balk RA, Slotman GJ, Iberti TJ, Rackow EC, Shapiro MJ, Greenman RL. Recombinant human interleukin 1 receptor antagonist in the treatment of patients with sepsis syndrome. Results from a randomized, double-blind, placebo-controlled trial. Phase III rhIL-1ra Sepsis Syndrome Study Group. JAMA, 1994; 271: 1836-43.

58. Opal SM, Fisher CJ Jr, Dhainaut JF, Vincent JL, Brase R, Lowry SF, Sadoff JC, Slotman GJ, Levy H, Balk RA, Shelly MP, Pribble JP, LaBrecque JF, Lookabaugh J, Donovan H, Dubin H, Baughman R, Norman J, DeMaria E, Matzel K, Abraham E, Seneff M. Confirmatory interleukin-1 receptor antagonist trial in severe sepsis: a phase III, randomized, double-blind, placebo-controlled, multicenter trial. The Interleukin-1 Receptor Antagonist Sepsis Investigator Group. Crit Care Med, 1997; 25: 1115-24.

59. Dinarello CA. IL-18: a Th1-inducing, proinflammatory cytokine and new member of the IL-1 family. J Allergy Clin Immunol, 1999; 103: 11-24.

60. Siegmund B. Interleukin-1beta converting enzyme (caspase-1) in intestinal inflammation. Biochem Pharmacol, 2002; 64: 1-8.

61. Dinarello CA, Fantuzzi G. Interleukin-18 and host defense against infection. J Infect Dis, 2003; 187: S370-84.

62. Rau B, Baumgart K, Paszkowski AS, Mayer JM, Beger HG. Clinical relevance of caspase-1 activated cytokines in acute pancreatitis: High correlation of serum interleukin-18 with pancreatic necrosis and systemic complications. Crit Care Med, 2001; 29: 1556-62.

63. Wereszczynska-Siemiatkowska U, Mroczko B, Siemiatkowski A. Serum profiles of interleukin-18 in different severity forms of human acute pancreatitis. Scand J Gastroenterol, 2002; 37: 1097-102.

64. Rau B, Zhuang Y, Schöler C, Hans J, Tilton B, Schilling M. Caspase-1 activated cytokines in experimental acute pancreatitis: synergistic effects of IL-1ß and IL-18 on the development of disease severity. Pancreas, 2003; 27: 403.

65. Gadient RA, Patterson PH. Leukemia inhibitory factor, Interleukin 6, and other cytokines using the GP130 transducing receptor: roles in inflammation and injury. Stem Cells, 1999; 17: 127-37.

66. Leser HG, Gross V, Scheibenbogen C, Heinisch A, Salm R, Lausen M, Ruckauer K, Andreesen R, Farthmann EH, Scholmerich J. Elevation of serum interleukin-6 concentration precedes acute-phase response and reflects severity in acute pancreatitis. Gastroenterol, 1991; 101: 782-5.

67. Heath DI, Cruickshank A, Gudgeon M, Jehanli A, Shenkin A, Imrie CW. Role of interleukin-6 in mediating the acute phase protein response and potential as an early means of severity assessment in acute pancreatitis. Gut, 1993; 34: 41-5.

68. Liu Q, Djuricin G, Nathan C, Gattuso P, Weinstein RA, Prinz RA. The effect of interleukin-6 on bacterial translocation in acute canine pancreatitis. Int J Pancreatol, 2000; 27: 157-65.

69. Cuzzocrea S, Mazzon E, Dugo L, Centorrino T, Ciccolo A, McDonald MC, de Sarro A, Caputi AP, Thiemermann C. Absence of endogenous interleukin-6 enhances the inflammatory response during acute pancreatitis induced by cerulein in mice. Cytokine, 2002; 18: 274-85.

70. Suzuki S, Miyasaka K, Jimi A, Funakoshi A. Induction of acute pancreatitis by cerulein in human IL-6 gene transgenic mice. Pancreas, 2000; 21: 86-92.

71. Morre KW, de Waal Malefyt R, Coffman RL, O'Garra A. Interleukin-10 and the interleukin-10 receptor. Annu Rev Immunol, 2001; 19: 683-765.

72. Simovic MO, Bonham MJ, Abu-Zidan FM, Windsor JA. Anti-inflammatory cytokine response and clinical outcome in acute pancreatitis. Crit Care Med, 1999; 27: 2662-5.

73. Mentula P, Kylanpaa ML, Kemppainen E, Jansson SE, Sarna S, Puolakkainen P, Haapiainen R, Repo H. Early prediction of organ failure by combined markers in patients with acute pancreatitis. Br J Surg, 2005; 92: 68-75.

74. Van Laethem JL, Marchant A, Delvaux A, Goldman M, Robberecht P, Velu T, Deviere J. Interleukin 10 prevents necrosis in murine experimental acute pancreatitis. Gastroenterology, 1995; 108: 1917-22.

75. Kusske AM, Rongione AJ, Ashley SW, McFadden DW, Reber HA. Interleukin-10 prevents death in lethal necrotizing pancreatitis in mice. Surgery, 1996; 120: 284-8.

76. Rongione AJ, Kusske AM, Kwan K, Ashley SW, Reber HA, McFadden DW. Interleukin 10 reduces the severity of acute pancreatitis in rats. Gastroenterology, 1997; 112: 960-7.

77. Osman MO, Jacobsen NO, Kristensen JU, Deleuran B, Gesser B, Larsen CG, Jensen SL. IT 9302, a synthetic interleukin-10 agonist, diminishes acute lung injury in rabbits with acute necrotizing pancreatitis. Surgery, 1998; 124: 584-92.

78. Gloor B, Todd KE, Lane JS, Rigberg DA, Reber HA Mechanism of increased lung injury after acute pancreatitis in IL-10 knockout mice. J Surg Res, 1998; 80: 110-4.

79. Van Laethem JL, Eskinazi R, Louis H, Rickaert F, Robberecht P, Deviere J. Multisystemic production of interleukin 10 limits the severity of acute pancreatitis in mice. Gut, 1998; 43: 408-13.

80. Zou WG, Wang DS, Lang MF, Jin DY, Xu DH, Zheng ZC, Wu ZH, Liu XY. Human interleukin 10 gene therapy decreases the severity and mortality of lethal pancreatitis in rats. J Surg Res, 2002; 103: 121-6.

81. Deviere J, Le Moine O, Van Laethem JL, Eisendrath P, Ghilain A, Severs N, Cohard M. Interleukin 10 reduces the incidence of pancreatitis after therapeutic endoscopic retrograde cholangiopancreatography. Gastroenterology, 2001; 120: 498-505.

82. Dumot JA, Conwell DL, Zuccaro G Jr, Vargo JJ, Shay SS, Easley KA, Ponsky JL. A randomized, double blind study of interleukin 10 for the prevention of ERCP-induced pancreatitis. Am J Gastroenterol 2001; 96: 2098-102.

83. Singh et al. DDW 2002, abstract T 1726.

84. Curley PJ. Endotoxin, cellular immune dysfunction and acute pancreatitis. Ann R Coll Surg Engl, 1996; 78: 531-5.

85. Kylanpaa-Back ML, Takala A, Kemppainen E, Puolakkainen P, Kautiainen H, Jansson SE, Haapiainen R, Repo H. Cellular markers of systemic inflammation and immune suppression in patients with organ failure due to severe acute pancreatitis. Scand J Gastroenterol, 2001; 36: 1100-7.

86. Kylanpaa-Back ML, Takala A, Kemppainen EA, Puolakkainen PA, Leppaniemi AK, Karonen SL, Orpana A, Haapiainen RK, Repo H. Procalcitonin, soluble interleukin-2 receptor, and soluble E-selectin in predicting the severity of acute pancreatitis. Crit Care Med, 2001; 29: 63-9.

87. Demols A, Le Moine O, Desalle F, Quertinmont E, Van Laethem JL, Deviere J. DC4(+) T cells play an important role in acute experimental pancreatitis in mice. Gastroenterology, 2000; 118: 582-90.

88. Mayer J, Laine VJO, Rau B, Hotz HG, Foitzik T, Nevalainen TJ, Beger HG. Systemic lymphocyte activation modulates the severity of diet-induced acute pancreatitis in mice. Pancreas, 1999; 19: 62-8.

89. Mayer JM, Laine VJ, Gezgin A, Kolodziej S, Nevalainen TJ, Storck M, Beger HG. Single doses of FK506 and OKT3 reduce severity in early experimental acute pancreatitis. Eur J Surg, 2000; 166: 734-41.

90. Curley P, Nestor M, Collins K, Saporoschetz I, Mendez M, Mannick JA, Rodrick ML. Decreased interleukin-2 production in murine acute pancreatitis: potential for immunomodulation. Gastroenterology, 1996; 110: 583-8.

91. Widdison AL, Karanjia ND, Alvarez C, Reber HA. Influence of levamisole on pancreatic infection in acute pancreatitis. Am J Surg, 1992; 163: 100-3.

92. Rau B, Paszkowski AS, Lillich S, Mayer JM, Möller P, Beger HG. Effects of FK506 on pancreatic acinar cell damage and mortality in severe acute experimental pancreatitis. Pancreas, 2001; 23: 457.

93. Echigo Y, Inoue K, Kogire M, Doi R, Higashide S, Sumi S, Kaji H, Imamura M. Effects of Cyclosporine and Tacrolimus (FK 506) on acute pancreatitis in mice. Arch Surg, 1995; 130: 64-8.

94. Johnson CD. Platelet-activating factor and platelet-activating factor antagonists in acute pancreatitis. Dig Surg, 1999; 16: 93-101.

95. Emanuelli G, Montrucchio G, Gaia E, Dughera L, Corvetti G, Gubetta L. Experimental acute pancreatitis induced by platelet activating factor in rabbits. Am J Pathol, 1989; 134: 315-26.

96. Dabrowski A, Gabryelewicz A, Chyczewski L. The effect of platelet activating factor antagonist (BN 52021) on cerulein-induced acute pancreatitis with reference to oxygen radicals. Int J Pancreatol, 1991; 8: 1-11.

97. Zhou W, McCollum MO, Levine BA, Olson MS. Role of platelet-activating factor in pancreatitis-associated acute lung injury in the rat. Am J Pathol, 1992; 140: 971-9.

98. Kald B, Kald A, Ihse I, Tagesson C. Release of platelet-activating factor in acute experimental pancreatitis. Pancreas, 1993; 8: 440-2.

99. Formela LJ, Wood LM, Whittaker M, Kingsnorth AN. Amelioration of experimental acute pancreatitis with a potent platelet-activating factor antagonist. Br J Surg, 1994; 81: 1783-5.

100. Wang X, Sun Z, Borjesson A, Haraldsen P, Aldman M, Deng X, Leveau P, Andersson R. Treatment with lexipafant ameliorates the severity of pancreatic microvascular endothelial barrier dysfunction in rats with acute hemorrhagic pancreatitis. Int J Pancreatol, 1999; 25: 45-52.

101. Hofbauer B, Saluja AK, Bhatia M, Frossard JL, Lee HS, Bhagat L, Steer ML. Effect of recombinant platelet-activating factor acetylhydrolase on two models of experimental acute pancreatitis. Gastroenterology, 1998; 115: 1238-47.

102. Foitzik T, Hotz HG, Eibl G, Hotz B, Kirchengast M, Buhr HJ. Therapy for microcirculatory disorders in severe acute pancreatitis: effectiveness of platelet-activating factor receptor blockade vs. endothelin receptor blockade. J Gastrointest Surg, 1999; 3: 244-51.

103. Rivera JA, Werner J, Warshaw AL, Lewandrowski KB, Rattner DW, Fernandez del Castillo C. Lexipafant fails to improve survival in severe necrotizing pancreatitis in rats. Int J Pancreatol, 1998; 23: 101-6.

104. Kingsnorth AN, Galloway SW, Formela LJ. Randomized, double-blind phase II trial of Lexipafant, a platelet-activating factor antagonist, in human acute pancreatitis. Br J Surg, 1995; 82: 1414-20.

105. McKay CJ, Curran F, Sharples C, Baxter JN, Imrie CW. Prospective placebo-controlled randomized trial of lexipafant in predicted severe acute pancreatitis Br J Surg, 1997; 84: 1239-43.

106. Johnson CD, Kingsnorth AN, Imrie CW, McMahon MJ, Neoptolemos JP, McKay C, Toh SK, Skaife P, Leeder PC, Wilson P, Larvin M, Curtis LD. Double blind, randomised, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. Gut, 2001; 48: 62-9.

107. Rollins BJ. Chemokines. Blood, 1997; 90, 909-28.

108. Baggiolini M, Loetscher P, Moser B. Interleukin-8 and the chemokine family. Int J Immunopharmacol, 1995; 17: 103-8.

109. Gross V, Andreesen R, Leser H-G, Ceska M, Liehl E, Lausen M, Farthmann EH, Schölmerich J. Interleukin-8 and neutrophil activation in acute pancreatitis. Eur J Clin Invest, 1992; 22: 200-3.

110. Rau B, Steinbach G, Gansauge F, Mayer JM, Grünert A, Beger HG. The potential role of procalcitonin and interleukin-8 in the

prediction of infected necrosis in acute pancreatitis. Gut, 1997; 41: 832-40.

111. Rau B, Steinbach G, Gansauge F, Mayer JM, Grünert A, Beger HG. The role of Interleukin-8 in the severity assessment of septic complications in necrotizing pancreatitis. Digestion, 1997; 58: 11.

112. Osman MO, Lausten SB, Jakobsen NO, Kristensen JU, Deleuran B, Larsen CG, Jensen SL. Graded experimental acute pancreatitis: monitoring of a renewed rabbit model focusing on the production of interleukin-8 (IL-8) and CD11b/CD18. Eur J Gastroenterol Hepatol, 1999; 11: 137-49.

113. Osman MO, Kristensen JU, Jacobsen NO, Lausten SB, Deleuran B, Deleuran M, Gesser B, Matsushima K, Larsen CG, Jensen SL. A monoclonal anti-interleukin 8 antibody (WS-4) inhibits cytokine response and acute lung injury in experimental severe acute necrotising pancreatitis in rabbits. Gut, 1998; 43: 232-9.

114. Shokuhi S, Bhatia M, Christmas S, Sutton R, Neoptolemos JP, Slavin J. Levels of the chemokines growth-related oncogene alpha and epithelial neutrophil-activating protein 78 are raised in patients with severe acute pancreatitis. Br J Surg, 2002; 89: 566-72.

115. Rau B, Baumgart K, Kruger CM, Schilling M, Beger HG. CC-chemokine activation in acute pancreatitis: enhanced release of monocyte chemoattractant protein-1 in patients with local and systemic complications. Intensive Care Med, 2003; 29: 622-9.

116. Grady T, Liang P, Ernst SA, Logsdon CD. Chemokine gene expression in rat pancreatic acinar cells is an early event associated with acute pancreatitis. Gastroenterol, 1997; 113, 1966-75.

117. Bhatia M, Brady M, Zagorski J, Christmas SE, Campbell F, Neoptolemos JP, Slavin J. Treatment with neutralizing antibody against cytokine-induced neutrophil chemoattractant (CINC) protects rats against acute pancreatitis associated lung injury. Gut, 2000; 47: 838-44.

118. Bhatia M, Proudfoot AE, Wells TN, Christmas S, Neoptolemos JP, Slavin J. Treatment with Met-RANTES reduces lung injury in caerulein-induced pancreatitis. Br J Surg, 2003; 90: 698-704.

119. Gerard C, Frossard JL, Bhatia M, Saluja A, Gerard NP, Lu B, Steer ML. Trageted disruption of the β-chemokine receptor CCR-1 protects against pancreatitis associated lung injury. J Clin Invest, 1997; 100: 2022-7.

120. Bhatia M, Ramnath RD, Chevali L, Guglielmotti A. Treatment with bindarit, a blocker of MCP-1 synthesis, protects mice against acute pancreatitis. Am J Physiol Gastrointest Liver Physiol, 2005; 288: G1259-65.

121. Matuschak GM. Circulating cytokine concentrations and outcome prediction in intensive care unit patients: still the tip of the iceberg? Crit Care Med, 1996; 24: 1769-71.

122. Uhl W, Büchler MW, Malfertheiner P, Beger HG, Adler G, Gaus W. A randomised, double blind, multicentre trial of octreotide in moderate to severe acute pancreatitis. Gut, 1999; 45: 97-104.

123. Imahara SD, O'Keefe GE. Genetic determinants of the inflammatory response. Curr Opin Crit Care, 2004; 10: 318-24.

Antibiotic treatment in acute pancreatitis

Uomo G*

Internal Medicine Department, 3rd Division, Cardarelli Hospital, Napoli, Italy

Abstract

Severe acute pancreatitis is characterized by a poor prognosis with local and systemic complications, high morbidity and mortality. From the morphological standpoint, almost all patients suffering from severe forms of acute pancreatitis present various degree of pancreatic necrosis. In these patients the occurrence of infection of pancreatic necrosis certainly represents a very important prognostic factor as it has worldwide accepted as the leading cause of death. In addition, the discovery of an infected necrosis represents a crucial point in the treatment of these patients as it is the only clear-cut shift from medical to surgical treatment in necrotizing pancreatitis. Over the last years, earlier and more precise identification of pancreatic necrosis together with availability of new classes of antibiotics with documented activity against the most commonly involved bacteria and able to reach in therapeutic concentration the pancreatic necrosis give us the opportunity to perform some important controlled clinical trials on antibiotic prophylaxis in necrotizing acute pancreatitis. The great majority of these studies showed the usefulness of a prophylactic regimen (using antibiotics such as fluoroquinolones and carbapenems) in terms of reduction of pancreatic and extrapancreatic infections in comparison with untreated controls. Nevertheless, some questions on this topic still present controversial aspects such as the antibiotic of choice, the duration of treatment, the possible opportunistic infections with fungi and/or resistant strains. Antibiotics may prove very useful in patients with documented infected necrosis and high anaesthesiological risk unfit for surgical debridement and drainage; some initial experiences show the possibility that antibiotic treatment may be curative without surgery in these selected cases.

Key words: acute pancreatitis, pancreatic necrosis, antibiotic prophylaxis, antibiotic treatment, fluoroquinolones, imipenem, meropenem.

Introduction

When we are talking about acute pancreatitis (AP) we are facing with two complete different diseases, i.e. mild and severe AP. Almost all patients with mild disease recovery within few days and they do not require any specific treatment, including antibiotics. For these patients, presenting with *edematous* form of AP, we can observe a spontaneous resolution of the disease; the main clinical problem consist of the correction of the etiological factor to avoid recurrences. On the contrary, severe AP presents a poor prognosis with local and systemic complications, high morbidity and mortality [1]. From the morphological standpoint, severe AP shows various degree of *pancreatic necrosis* in almost all cases. In a recent survey on AP in Italy (1184 patients prospectively enrolled in 2 years), severe forms represent the 14% only of all AP, but mortality (20%) and morbidity (47%) are almost completely confined in this form. Data from other series coming from Europe and USA shows a percentage of severe forms little bit superior – 15 to 25% – with a related mortality up to 50% [2-5]. In these patients the occurrence of pancreatic infection, that means *infection of pancreatic necrosis*, certainly represents a very important prognostic factor as it has worldwide accepted as the leading cause of death. On this context, infection of necrosis accounts for a major cause of death in the late phase of the disease, in general after the second week, when most deaths are the sequel of ongoing sepsis and septic multiple organ failure [6].

* CORRESPONDING AUTHOR:
Internal Medicine Department, Cardarelli Hospital
Via Cardarelli 9, 80131 Napoli, Italy
Tel: 0039 081 7472101; Fax: 0039 081 7474042
e-mail: generoso.uomo@ospedalecardarelli.it (Generoso Uomo)

Received 15.03.2005 Accepted 25.04.2005

Infection of pancreatic necrosis in severe AP

Pancreatic infection basically occurs in patients with AP presenting pancreatic or peripancreatic necrosis and/or fluid collections. Pancreatic necrosis become infected in a percentage ranging from 20 to 40% and, as a rule, a time dependent increase of the infection rate with the duration of the disease is registered (*Fig. 1*) [6,7]. Patients suffering from AP develop pancreatic infection mainly after the second week of the disease, whereas the most important complications within the first two weeks are the systemic complications related to the organ(s) failure. Our recent data based upon a prospective evaluation of 210 patients with AP observed in four years [8] showed that infection of necrosis developed in 18 of 75 patients (24%) with necrotizing forms. The occurrence of infection of necrosis represents a crucial point in the treatment of these patients as it is the only clear-cut shift from medical to surgical treatment in necrotizing pancreatitis [7,9]. The extent of pancreatic necrosis correlates with the incidence of its infection. As a consequence, strict monitoring of necrotic process, by means of contrast-enhanced computed-tomography and of fine-needle percutaneous aspiration of necrosis for bacteriological examination when clinical suspicion of infection arises, is required [10]. Recognition of bacterial strains at fresh-microscopy of the aspirated material or positive results of the cultural exam indicates surgical debridement as soon as possible.

Several pathways of bacteria into pancreatic necrosis have been described: a) hematogenous, *via* the blood circulation; b) ascending infection from the duodenum via the pancreatic duct; c) from the portal vein and the liver *via* the biliary duct system; d) transcolonic migration *via* the lymphatics [11]. The latter pathway is the most important with many *in vitro* and *in vivo* studies which clearly support this mechanism [1,6]. In AP a reduced gut motility secondary to some mediators such as nitric oxide is reported; this lead to alteration of intestinal microflora and to damage of mucosal barrier with increase of gut permeability (*Fig. 2*). In addition, the impairment of gut microcirculation, local ischemia, and a decrease of immune system response related to cytokine release enforce the mucosal barrier damage, thus leading to the translocation of intestinal bacteria into the bloodstream and to a secondary colonization of pancreatic necrosis. The occurrence that bacteria most frequently isolated from infected necrosis (*Tab. 1*) are mainly Gram-negative strains, typical of intestinal flora, strongly support this pathway. In the great majority of patients the infection is monomicrobial with anaerobes bacteria accounting for 15% of cases. In a recent paper [12] – *Tab. 2* – we reported monomicrobial flora in the

Figure 1. **Infection of pancreatic necrosis in severe acute pancreatitis: the incidence rate is a time dependent, and nearly 70% of this event occurs after the second week of the onset of the disease**

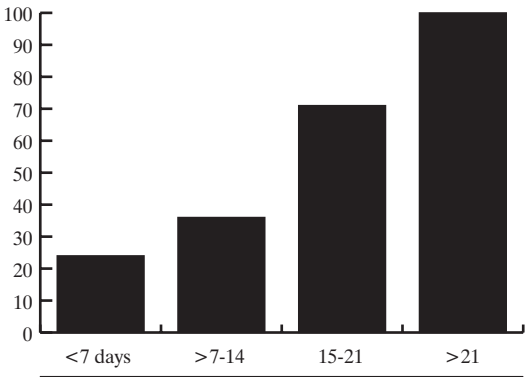


Table 1. **Spectrum of bacteria isolate in infected pancreatic necrosis: mean value from three large series** [16-18]

Monomicrobial flora	60-87%
Escherichia coli	25.9%
Pseudomonas aeruginosa	15.9%
Staphylococcus aeureus	15.3%
Klebsiella spp	10.1%
Proteus mirabilis	10.1%
Streptococcus faecalis	4.4%
Various anaerobes	15.8%

Figure 2. **Bacterial translocation pathway determines colonization of pancreatic necrosis during necrotizing acute pancreatitis (AP) from colon microorganisms (see text)**

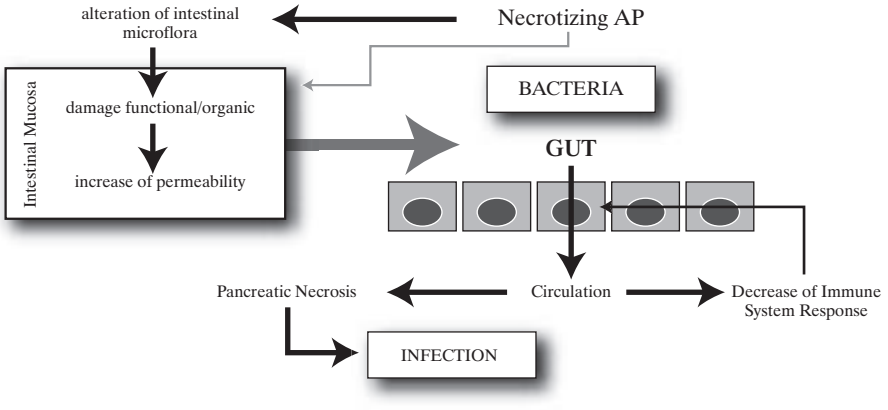


Table 2. **Microbiological isolates in 22 patients presenting severe acute pancreatitis complicated by infection of necrosis – personal experience** [12]

Monomicrobial flora	**17/22 (77.3%)**	
Polimicrobial flora	**5/22 (22.7%)**	
	• Escherichia coli	6
	• Pseudomonas aeruginosa	5
	• Enterococcus faecalis	4
	• Staphylococcus aeureus	3
	• Xanthomonas maltophilia	3
	• Klebsiella oxytocica	2
	• Enterobacteriacee	2
	• Proteus mirabilis	1
	• Streptococcus mitis	1
	• Bacillus species	1

Table 3. **Efficacy factor of various antibiotics in the prophylaxis of infection of pancreatic necrosis in severe acute pancreatitis (AP); efficacy factor represents the ratio between the bacterial spectrum covered and the pancreatic penetration, at least at the minimal inhibitory concentration; the maximum efficacy factor is 1**

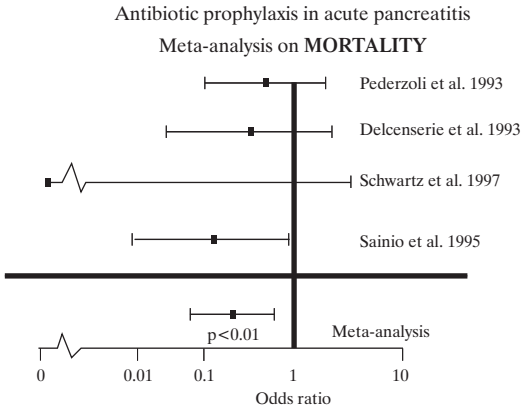
Antibiotics in AP	efficacy factor
Netilmicin	0.14
Tobramycin	0.12
Mezlocillin	0.71
Piperacillin	0.71
Cefotiam	0.75
Ceftizoxime	0.76
Cefotaxime	0.78
Ceftriaxone	0.79
Ciprofloxacin	0.86
Ofloxacin	0.87
Imipenem	0.98
Meropenem	0.98

77% of microbiological isolates from 22 patients with infected necrosis and stressed the clinical relevance of the occurrence of *Xanthomonas malthophilia* pancreatic infection (mortality of 100% in our experience). This Gram-negative organism belongs to the *Pseudomonas* family and it has the peculiarity of growth in plastic devices and of resistance to carbapenem antibiotics we currently use in the prophylaxis of infected necrosis.

Antibiotic prophylaxis to prevent infection of pancreatic necrosis

The history of prophylaxis of pancreatic infection starts near 40 years ago. Earlier studies do not indicate favourable effects on the outcome of AP [13-15]. We can today identify at least three reasons for these negative results: 1) all studies were carried out before the CECT era; as a consequence, no clear criteria were adopted to ascertain the presence of necrosis ant its stratification into two categories, i.e. sterile and infected, was lacking; 2) many of the included patients had edematous pancreatitis; 3) the authors utilize ampicillin that subsequent studies showed

Figure 3. **Meta-analysis on mortality resulted from data of four studies of antibiotic prophylaxis in severe acute pancreatitis** (see text for references)



to be unable to reach pancreatic necrosis. Starting from the 90' years a less empiric approach was utilised, on the basis of more precise identification of pancreatic necrosis, stratification of the disease severity, and deeper knowledge of prognostic relevance of pancreatic infection. In addition, and utmost important, new advances in antibiotic pancreatic penetration, especially during the acute phase of the disease, became available together with broad-spectrum antibiotics with documented pancreatic penetration at therapeutic *minimal inhibitory concentration* (MIC). On this context, today we have to talk about the **efficacy factor** of a specific antibiotic, that means a ratio between the bacterial spectrum covered and the pancreatic penetration, at least in MIC [16-18]. *Tab. 3* shows the efficacy factor of several antibiotics; keeping in mind that the maximum efficacy factor is 1, fluoroquinolones and imipenem present an efficacy factor clearly more advantageous than cephalosporin, ureidopenicillin, and aminoglucosides.

Over the last ten years many studies on antibiotic prophylaxis in AP have been performed. Some of these are single-centre studies [12,19-22], others are multicenter studies [23-29], others are meta-analysis researches [30-32]. Significant advantages on mortality in treated patients in comparison with controls were observed in the meta-analysis studies (*Fig. 3*). In particular, the recent *Cochrane review*, updated March 2003, concluded that there is "strong evidence that intravenous antibiotic prophylactic therapy for 10 to 14 days decreases the risk of superinfection of pancreatic necrosis (Odds ratio 0.51, p=0.04) and decreases mortality (Odds ratio 0.32, p=0.02)" [32]. A general consensus on the usefulness of antibiotic treatment in prophylaxis of infection of pancreatic necrosis in AP appeared in the recent literature as national or international recommendations or guidelines [33-40] – *Tab. 4*. Nevertheless, some questions on this topic require additional comments. The first one concerns the antibiotic of choice. The efficacy factor already discussed shows that the classes of carbapenems (*imipenem*, *meropenem*) and fluorochinolones (*pefloxacin*, *levofloxacin*) represent the best options. Within these two classes, one study from Bassi and co-workers [25] showed significant better results on infected necrosis and extrapancreatic infection rate and lower mortality

Table 4. **International guidelines and recommendations for the antibiotic prophylaxis in Acute Pancreatitis (Cat.: category of evidence)**

American College of Gastroenterology 1997 [33]
…it is reasonable to initiate antibiotic therapy in severe acute pancreatitis
British Society of Gastroenterology 1998 [34]
…there is some evidence to support the use of prophylactic antibiotics
Santorini Consensus Conference 1999 [35]
…prophylactic antibacterial treatment is strongly recommended in severe pancreatitis (Cat. A)
Italian Guidelines 1999 [36]
…antibiotic treatment is indicated in severe acute pancreatitis
German Guidelines 2000 [37]
…antibiotic prophylaxis is not generally recommended; indication could be necrotizing pancreatitis, severe acute pancreatitis (Cat. B)
World Congress Gastroenterology 2002 [38]
…antibiotic prophylaxis is advised in patients with greater 30% necrosis and imipenem is recommended currently (Cat. A)
Japan Guidelines 2002 [39]
…in severe and possibly severe acute pancreatitis broad-spectrum antibiotics should be used prohylactically (Cat. A)
International Association of Pancreatology 2002 [40]
…the use of prophylactic broad-spectrum antibiotics reduces infection rates in CT-proven necrotizing pancreatitis (Cat. A)

Table 5. **Results of a recent randomized trial of our group in which comparison of prophylactic treatment with meropenem vs imipenem was performed; all patients (n=176) suffering from severe necrotizing acute pancreatitis** [12]

	Meropenem	**Imipenem**
patients (n)	**88**	**88**
daily dosage (i.v.)	**500 mg x 3**	**500 mg x 4**
Necrosis <30/30-50/>50%	51/25/12	54/21/13
Infection of necrosis	10 (11.4%)	12 (13.6%)
Extrapancreatic sepsis	19 (21.6%)	13 (23.9%)
Multi-organ-failure	6 (6.8%)	8 (9%)
Systemic complications	30 (34.1%)	33 (37.5%)
Local complications	28 (31.8%)	30 (34.1%)
Surgery	15 (17%)	16 (18.1%)
Deaths	12 (13.6%)	10 (11.3%)
Hospitalization (days)	24 (7-90)	23.3 (6-80)

in patients treated with imipenem (29 cases) in comparison with the group of patients treated with pefloxacin (27 cases). Our group have recently published the results of another trial [12] in which imipenem treatment (2000 mg i.v./day) was compared with meropenem schedule (1500 mg i.v./day). Considering all series of patients (176 cases), we found subsequent infection of necrosis in a percentage of 12% only. This strongly confirms the opportunity of the antibiotic prophylaxis in these patients. No difference was observed between patients treated with meropenem and those treated with imipenem in terms of incidence of pancreatic infection, extrapancreatic infection and clinical outcome – *Tab. 5*. Meropenem resulted as effective as imipenem in preventing septic complications of patients with severe AP; one advantage resulted from the financial analysis as meropenem treatment, at the time of the study, resulted cheaper than imipenem one [12]. The second question about antibiotic prophylaxis in AP is related to the duration of treatment. No doubt exists as regards the opportunity to start the treatment as soon as possible but the its duration is less clear. In the clinical practice, treatment period isn't hardly ever shorter than three or four weeks, but some patients can require a longer time [35,41]. Another question arising on this topic concerns the possible occurrence of complications of antibiotic prophylaxis. Generally speaking, presumptive risk and problems of prophylactic antibiotic application still remain a controversial issue [42]. One aspect is related to a change into the bacterial spectrum and to a selection of resistant strains induced by a prolonged antibiotic treatment. Resistance of carbapenems was recently reported in limited series of patients with AP and it yielded a significant risk factor for a fatal outcome [43]. Another point regards a presumptive increase of the incidence of fungal infection, in particular of Candida infection [44,45]. The clinical significance in terms of severity of this occurrence has been likely overestimated [46]; the recent *Cochrane review*, updated March 2003 [32], concluded that there is not an increased preponderance of fungi infection with antibiotics (Odds 0.83, p=0.7). In our recent experience [12], we did not find fungi in 22 patients with documented infected pancreatic necrosis, also in 3 of them with *Candida albicans* colonization of central venous catheter. The last point requiring an additional comment on this field is related to the possibility of the enhancement of the power of antibiotic treatment by other medications. Olah and co-workers [47] have recently published the results of a randomised trial in whom early jejunal feeding combined with prophylactic imipenem showed significant better results (in terms of reduction of the septic complication rate) when compared with parenteral nutrition plus the same antibiotic regimen in patients with necrotizing AP. All the same, one can strongly speculate on the potential role of probiotics coupled with antibiotic prophylaxis and a randomised large trial on this topic seems to be timely today [48].

Antibiotic for the treatment of infected pancreatic necrosis

To the best of our present knowledge the discovery of infection of pancreatic necrosis in severe AP indicates a surgical approach as soon as possible; in general this means necrosectomy, debridement and multiple drainage catheters [1,3,5,9]. This option is efficacious and relatively safe but it can result less feasible in some patients presenting high anesthesiological risk for advanced age and/or severe co-morbidities [49,50]. On this context, the rising question could be: can the antibiotic therapy be *curative* in pancreatic necrosis already infected? While there is a general consensus regarding the usefulness of antibiotics for the prophylaxis in patients with severe AP, the role of antibiotics in the treatment of infection of pancreatic necrosis represents a controversial issue. Very few data are available in the literature [7,32,41] regarding this argument. Nordback and co-workers [22] report a very interesting results in a recent trial on the utilization of antibiotics (imipenem 3 g/day) for the prophylaxis

of pancreatic infection. They found a significant reduction of the pancreatic infection rate in the treated group (8% vs 42% of the control group) but, more interesting, 9 out of 14 patients without prophylaxis who develop an infection were cured with imipenem without surgical debridement. Our group also made a favourable experience on this topic. During the period January 1998 – December 2003 we observed 101 patients suffering from necrotizing AP with a mortality of 12.9% (13 patients). All patients were treated with prophylactic antibiotic; 24 of them (23.8%) develop infected necrosis and 20 out of 24 were operated on. So, four patients because of high anaesthesiological risk underwent medical treatment only, including three weeks of imipenem (two patients, 2 g/day i.v.) or meropenem (two patients, 1.5 g/day i.v.) treatment; favourable outcome was observed and morphological resolution registered in all patients at various interval time.

Conclusions

Many recent data indicates that patients with necrotizing AP may benefit from the application of a strict cardio-respiratory monitoring, sharp hydroelectrolytic and caloric supplementation and – last but not least – appropriate prophylactic antibiotic regimen. The usage of antibiotics able to reach the pancreatic necrosis and to cover the spectrum of bacteria most frequently involved is becoming a mandatory part of the treatments schedule all over the world. This represents a substantial step forward in the clinical practice as the overwhelming majority of clinical studies focused on the natural history and outcome of AP shows that bacterial infection of pancreatic necrosis is the leading cause of death in patients affected by this demanding disease.

Acknowledgement

Invited lecture at the First International Congress of the European Academy of Surgical Sciences, 15-17 September 2004, Gdańsk, Poland.

References

1. Bradley EL 3rd. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, Ga, September 11 through 13, 1992. Arch Surg, 1993; 128: 586-90.

2. Baron TH, Morgan DE. Acute necrotizing pancreatitis. N Engl J Med, 1999; 340; 1412-7.

3. Rau B, Pralle U, Uhl W, Schoenberg MH, Beger HG. Management of sterile necrosis in instances of severe acute pancreatitis. J Am Coll Surg, 1995; 181: 279-88.

4. Neoptolemos JP, Rarety M, Finch M, Sutton R. Acute pancreatitis: the substantial human and financial costs. Gut, 1998; 42: 886-91.

5. Gullo L, Migliori M, Olah A, Farkas G, Levy P, Arvanitakis C, Lankisch P, Beger HG. Acute pancreatitis in five European countries: etiology and mortality. Pancreas, 2002; 24: 223-7.

6. Bassi C. Infected pancreatic necrosis. Int J Pancreatol, 1994; 16: 1-10.

7. Buchler MW, Gloor B, Muller CA, Friess H, Seller ChA, Uhl W. Acute necrotizing pancreatitis: treatment strategy according to the status of infection. Ann Surg, 2000; 232: 619-26.

8. Uomo G, Cavallera A, Esposito P, Pacelli L, Visconti M, Rabitti PG. Extrapancreatic infections in acute pancreatitis – results of a prospective clinical study. Dig Liv Dis, 2002; 34: 824.

9. Uomo G, Visconti M, Manes G, Calise F, Laccetti M, Rabitti PG. Nonsurgical treatment of acute necrotizing pancreatitis. Pancreas, 1996; 12: 142-8.

10. Balthazar EJ, Ranson JH, Naidich DP, Megibow J, Caccavale R, Cooper MM. Acute pancreatitis: prognostic value of CT. Radiology, 1985; 156: 767-72.

11. Runkel N, Eibl G. Pathogenesis of pancreatic infection. In Buchler et al. (eds): Acute Pancreatitis: novel concepts in biology and therapy. Blackwell Science, Oxford, 1999: 255-61.

12. Manes G, Rabitti PG, Menchise A, Riccio E, Balzano A, Uomo G. Prophylaxis with meropenem of septic complications in acute pancreatitis: a randomized, controlled trial versus imipenem. Pancreas, 2003; 27: 79-83.

13. Craig RM, Dordal E, Myles L. The use of ampicillin in acute pancreatitis. Ann Intern Med, 1975; 83: 831-2.

14. Howes R, Zuidema GD, Cameron JL. Evaluation of prophylactic antibiotics in acute pancreatitis. J Surg Res, 1975; 18: 197-200.

15. Finch WT, Sawyers JL, Schenker S. A prospective study to determine the efficacy of antibiotics in acute pancreatitis. Ann Surg, 1976; 183: 667-71.

16. Bassi C, Pederzoli P, Vesentini S, Falconi M, Bonora A, Abbas H. Behavior of antibiotics during human necrotizing pancreatitis. Antimicrob. Agents Chemother, 1994; 38: 830-6.

17. Beger HG, Bittner R, Block S, Buchler MW. Bacterial contamination of pancreatic necrosis. A prospective clinical study. Gastroenterology, 1987; 91: 433-8.

18. Seiler CA, Uhl W, Friess H, Buchler MW. Strategies for antibiotic usage in acute pancreatitis. In: Buchler et al. (eds): Acute Pancreatitis: novel concepts in biology and therapy. Blackwell Science, Oxford, 1999; 283-90.

19. Sainio V, Kemppainen E, Puolakkainen P, Taavitsainen M, Kivisaari L, Valtonen V. Early antibiotic treatment in acute necrotising pancreatitis. Lancet, 1995; 346: 663-7.

20. Delcenserie R, Yzet T, Ducroix JP. Prophylactic antibiotics in treatment of severe acute alcoholic pancreatitis. Pancreas, 1996; 13: 198-201.

21. Schwarz M, Isenmann R, Meyer H, Beger HG. Antibiotic use in necrotizing pancreatitis. Results of a controlled study. Dtsch Med Wochenschr, 1997; 122: 356-61.

22. Nordback I, Sand J, Saaristo R, Paajanen H. Early treatment with antibiotics reduces the need for surgery in acute necrotizing pancreatitis. A single-center randomized study. J Gastrointest Surg, 2001; 5: 113-8.

23. Pederzoli P, Bassi C, Vesentini S, Campedelli A. A randomized multicenter clinical trial of antibiotic prophylaxis of septic complications in acute necrotizing pancreatitis with imipenem. Surg Gynecol Obstet, 1993; 176: 480-3.

24. Luiten EJ, Hop WC, Lange JF, Bruining HA. Controlled clinical trial of selective decontamination for the treatment of severe acute pancreatitis. Ann Surg, 1995; 222: 57-65.

25. Bassi C, Falconi M, Talamini G, Uomo G, Papaccio G, Dervenis C, Salvia R, Bertazzoni Minelli E, Pederzoli P. Controlled clinical trial of pefloxacin versus imipenem in severe acute pancreatitis. Gastroenterology, 1998; 115: 1513-7.

26. Takeda K, Matsuno S, Ogawa M, Watanabe S, Atomi Y. Continuous regional arterial infusion (CRAI) therapy reduces the mortality rate of acute necrotizing pancreatitis: results of a cooperative survey in Japan. J Hepatobiliary Pancreat Surg, 2001; 8: 216-20.

27. Maravi-Poma E, Gener J, Alvarez-Lerma F, Olaechea P, Blanco A, Dominguez-Munoz JE. Spanish Group for the Study of Septic Complications in Severe Acute Pancreatitis. Early antibiotic treatment (prophylaxis) of septic complications in severe acute necrotizing pancreatitis: a prospective, randomized, multicenter study comparing two regimens with imipenem-cilastatin. Intensive Care Med, 2003; 29: 1974-80.

28. Spicak J, Hejtmankova S, Cech P, Hoskovec D, Kostka R, Lefler J. Antibiotic prophylaxis in severe acute pancreatitis: Randomised multicenter prospective trial with meropenem. Pancreatology, 2003; 3: 220.

29. Isenmann R, Runzi M, Kron M, Kahl S, Kraus D, Jung N, Maier L, Malfertheiner P, Goebell H, Beger HG and the German Antibiotics in Severe Acute Pancreatitis Study Group. Prophylactic antibiotic treatment in patients with predicted severe acute pancreatitis: a placebo-controlled, double-blind trial. Gastroenterology, 2004; 126: 997-1004.

30. Golub R, Siddiqi F, Pohl D. Role of antibiotics in acute pancreatitis: a meta-analysis. J Gastrointest Surg, 1998; 2: 498-502.

31. Sharma VK, Howden CW. Prophylactic antibiotic administration reduces sepsis and mortality in acute necrotizing pancreatitis: a meta-analysis. Pancreas, 2001; 22: 28-31.

32. Bassi C, Larvin M, Villatoro E. Antibiotic therapy for prophylaxis against infection of pancreatic necrosis in acute pancreatitis. Cochrane Database Syst Rev, 2003; (4): CD002941.

33. Banks PA. Practice guidelines in acute pancreatitis. Am J Gastroenterol, 1997; 92: 377-86.

34. Glazer G. Mann DW. United Kingdom Guidelines for the management of acute pancreatitis. Gut, 1998; 42 (suppl. 2): 1S-13S.

35. Dervenis C, Johnson CD, Bassi C, Bradley EL, Imrie CW, McMahon MJ, Modlin I. Diagnosis, objective assessment of severity, and management of acute pancreatitis: Santorini consensus conference. Int J Pancreatol, 1999; 25: 195-210.

36. Uomo G, Pezzilli R, Cavallini G. Management of acute pancreatitis in clinical practice. ProInf-AISP study. Ital J Gastroenterol Hepatol, 1999; 31: 635-42.

37. Runzi M, Layer P, Buchler MW, Beger HG, Ell Ch, Folsch UR, Goebell H, Hopt UT, Lankisch PG, Schmidt WE, Schniegel W, Scholmerich J. Therapie der akuten pankreatitis. Gemeinsame leitiinen. Z Gastroenterol, 2000; 38: 571-81.

38. Toouli J, Brooke-Smith M, Bassi C, Carr-Locke D, Telford J, Freeny P, Imrie C, Tandon R. Guidelines for the management of acute pancreatitis. J Gastroenterol Hepatol, 2002; 17(Suppl): S15-S39.

39. Mayumi T, Ura H, Arata S, Kitamura N, Kiriyama I, Shibuya K, Sekimoto M, Nago N, Hirota M, Yoshida M, Ito Y, Hirata K, Takada T. Evidence-based clinical practice guidelines for acute pancreatitis proposal. J Hepatobiliary Panc Surg, 2002; 9: 413-22.

40. Uhl W, Warshaw A, Imrie C, Bassi C, McKay CJ, Lankisch PG, Carter R, DiMagno E, Banks PA, Withcomb DC, Dervenis C, Ulrich CD, Stake K, Ghaneh P, Hartwig W, Werner J, McEntee G, Neoptolemos JP, Buchler MW. IAP guidelines for the surgical management of acute pancreatitis. Pancreatology, 2002; 2: 565-73.

41. Bradley EL. Guiding the reluctant. A primer on guidelines in general and pancreatitis in particular. Pancreatology, 2003; 3: 139-43.

42. Pezzilli R. Antibiotic prophylaxis in acute necrotizing pancreatitis: yes or no? JOP. J Pancreas, 2004; 5: 161-4.

43. De Waele JJ, Vogelaers D, Hoste E, Blot S, Colardyn F. Emergence of antibiotic resistance in infected pancreatic necrosis. Arch Surg, 2004; 139: 1371-5.

44. Gotzinger P, Wamser P, Barlan M, Sautner T, Jakesz R, Fugger R. Candida infection of local necrosis in severe acute pancreatitis is associated with increased mortality. Shock, 2000; 14: 320-3.

45. De Waele JJ, Vogelaers D, Blot S, Colardyn F. Fungal infections in patients with severe acute pancreatitis and the use of prophylactic therapy. Clin Infect Dis, 2003; 37: 208-13.

46. Gloor B, Muller CA, Worni M, Stahel PF, Redaelli C, Uhl W, Buchler MW. Pancreatic infection in severe pancreatitis: the role of fungus and multiresistant organisms. Arch Surg, 2001; 136: 592-6.

47. Olah A, Pardavi G, Belagyi T, Nagy A, Issekutz A, Mohamed GE. Early nasojejunal feeding in acute pancreatitis is associated with a lower complication rate. Nutrition, 2002; 18: 259-62.

48. Dervenis C. Enteral nutrition in severe acute pancreatitis: future development. JOP. J Pancreas, 2004; 5: 60-3.

49. Uomo G, Talamini G, Rabitti PG, Cataldi F, Cavallera A, Rengo F. Influence of advanced age and related comorbidity on the corse and outcome of acute pancreatitis. Ital J Gastroenterol Hepatol, 1998; 30: 616-21.

50. Uomo G. Inflammatory pancreatic diseases in older patients – recognition and management. Drugs Aging 2003; 20: 59-70.

Diabetic nephropathy and cardiovascular diseases

Czekalski S*

Chair and Department of Nephrology, Transplantology and Internal Diseases, Poznań University of Medical Sciences, Poznań, Poland

Abstract

Diabetic nephropathy is diagnosed either when persistent increase of urinary albumin excretion rate (UAER) above 30 mg/24h in a patient with diabetes was discovered (early or incipient nephropathy) or when UAER values are persistently elevated above 300 mg/24h (overt or clinical nephropathy). In both situations the additional criteria of presence of diabetic retinopathy and the absence of the evidence of other kidney or renal tract disease should be fulfilled. It was found that the excess of cardiovascular events and mortality occurs already in diabetic patients with persistent microalbuminuria, but is particularly evident in macroalbuminuric diabetic patients and results not only from end-stage renal failure (ESRF) but rather from cardiovascular disease (CVD), the latter mainly in type 2 diabetic patients. Several traditional risk factor for atherosclerosis has been identified in diabetic patients with micro- or macroalbuminuria including elevated blood pressure levels, dyslipidemia and procoagulatory state associated with endothelial dysfunction. Microalbuminuria is currently regarded as a marker of generalized endothelial damage, it reflects transvascular albumin leakage, now recognized as an early event in atherogenesis. Recently the association of microalbuminuria with the marker of chronic inflammation (C-reactive protein) and with increased production of vascular endothelial growth factor (VEGE) was described. Thus, multiple mechanisms are involved in the development and progression of cardiovascular complications both in micro- and macroalbuminuric diabetic patients and all these mechanisms should be regarded as the target for therapeutic intervention.

* CORRESPONDING AUTHOR:
ul. Przybyszewskiego 49, 60-355 Poznań, Poland
Fax: +48 061 8691 688
e-mail: sczekals@usoms.poznan.pl (Stanisław Czekalski)

Received 22.02.2005 Accepted 03.03.2005

Key words: diabetic nephropathy, microalbuminuria, macroalbuminuria, cardiovascular diseases, mortality.

There are two different criteria of diabetic nephropathy in the medical literature. First one defines that persistent albuminuria (urinary albumin excretion rate, UAER >300 mg/24 hours or 200 μg/minute) is the hall-mark of diabetic nephropathy which can be diagnosed clinically if the following additional criteria are fulfilled: presence of diabetic retinopathy and the absence of clinical or laboratory evidence of other kidney or renal tract disease [1]. Another one defines that persistently raised UAER already above arbitrary established normal range, so-called microalbuminuria (UAER >30 mg/24 hours or 20 μg/min, and less than or equal to 300 mg/24 hours or 200 μg/min), associated with identical additional criteria as in the case of first definition, is sufficient for diagnosis of early (incipient) diabetic nephropathy [2]. According to this second criterion, persistently elevated UAER values >300 mg/24 hours or >200 μg/min, should be named macroalbuminuria, which is usually associated with proteinuria exceeding 0.5 g/24 hours and is indicative for more advanced stage of diabetic nephropathy: overt diabetic nephropathy.

Irrespective of differences in the criteria, diabetic nephropathy is a major cause of illness and death in diabetes. The excess of cardiovascular events and mortality occurs already in diabetic patients with persistent microalbuminuria, but is particularly evident in proteinuric diabetic patients and results not only from end-stage renal failure (ESRF) but rather from cardiovascular disease (CVD), the latter mainly in type 2 diabetic patients.

Until recently, microalbuminuria was said to confer a 60 to 85% risk of the development of overt proteinuria within 6 to 14 years in type 1 diabetic patients, suggesting an inexorable process leading to overt proteinuria [3,4]. The prospective study of patients with persistent microalbumiuria followed for six years revealed last year [5] that regression of microalbuminuria in type 1 diabetes was frequent, with a cumulative incidence of

58 percent. The determinants of the regression of microalbuminuria (defined as a 50 percent reduction in UAER from one two-year period to the next) were: short duration of microalbuminuria, glycosylated hemoglobin levels less than 8 percent, systolic blood pressure less than 115 mmHg, serum cholesterol level below 5.12 mmol/l (198 mg/dl) and serum trigliceride level below 1.64 mmol/l (145 mg/dl). The use of angiotensin-converting-enzyme inhibitors (ACEI) was not associated with the regression of microalbuminuria. These results indicated that elevated UAER does not imply inexorably progressive nephropathy and suggested that the definition of early diabetic nephropathy should be modified. It was hypothesized that very low systemic blood pressure attenuates shear stress and may permit the recovery of glomerular integrity and this and/or other mechanisms underlying the regression of microalbuminuria are most effective in the low range of glycosylated hemoglobin and low levels of cholesterol and trigliceride [5]. This hypothesis is based on the observations that microalbuminuria is associated with impairment of renal hemodynamic autoregulation [6,7]. Other factors, including genetic predisposition [8], should however-be considered as determinants of development and further evaluation of microalbuminuria in diabetic patients.

Although currently microalbuminuria should be considered as a marker of dynamic, rather than fixed renal injury in patients with type 1 diabetes [5], it was demonstrated that microalbuminuria is a marker for increased risk of cardiovascular disease in these patients [9]. The aerobic work capacity is impaired in patients with persistent microalbuminuria [10], which suggests that microangiopathy or other pathological process is affecting myocardium. The increase in the ratio of low-density and high-density lipoprotein has been described [11], indicating atherogenic lipid profile. Impaired fibrinolytic activity and elevated plasma levels of fibrinogen and von Willebrand factor are present in patients with microalbuminuria, suggesting generalized endothelial injury [9]. Microalbuminuria is regarded as a marker of generalized endothelial damage, it reflects transvascular albumin leakage and has been proposed to indicate increased endothelial permeability, now recognized as an early event in atherogenesis [11,12]. It was demonstrated that microalbuminuria precedes the increase in arterial blood pressure, and a concomitant increase in UAER and blood pressure was observed with the estimated mean annual increase in blood pressure of 2.7 mmHg [13,14]. Elevated blood pressure is additional risk factor for development of cardiovascular complications in patients with type 1 diabetes and microalbuminuria. The prevalence of arterial hypertension (blood pressure values ≥140/90 mmHg) in adult type 1 diabetic patients with microalbuminuria is 52%, and is increased when compared to patients with normoalbuminuria in whom was estimated as 42% [15,16]. Several studies have reported that sodium and water retention play a dominant role in the initiation and maintenance of systemic hypertension in patients with microalbuminuria, whereas the contribution of the renin-angiotensin-aldosterone system is smaller [1]. A genetic predisposition to hypertension in type 1 diabetic patients developing diabetic nephropathy was suggested and confirmed recently [17]. It was also demonstrated that the D allele associated with the insertion (I)/deletion (D) polymorphism of the gene for angiotensin-converting enzyme (ACE) and DD homozygocity are risk factors for an accelerated course of diabetic nephropathy in patients with type 1 diabetes. Of particular interest is that deletion polymorphism in the ACE gene is associated with coronary heart disease (CHD) in type 1 diabetic patients with nephropathy [18] as well as in nondiabetic patients [19]. The DD genotype appeared also to increase mortality once dialysis treatment was initiated. Since plasma ACE level (and also tissue ACE activity, as demonstrated in experimental studies) in DD subjects is about twice that of II subjects, with ID subjects having intermediate levels [20], it strongly suggests that increased generation of the angiotensin II (Ang II) unfavourably influences development of both renal and vascular changes and that inhibition of Ang II activity should have important protective effects. These protective effects associated with the use of ACE inhibitors or/and the antagonists of AT_1 receptors of Ang II were confirmed in several prospective studies in diabetic patients with nephropathy.

The generalized vascular lesions, the atherogenic changes in the lipid profile and elevated blood pressure that characterize patients with type 1 diabetes and microalbuminuria may lead to increased incidence of cardiovascular events and even to death from vascular disease, but it is still not proven whether microalbuminuria in it self is associated with an excess mortality in these patients, or whether it is so only because it is a predictor of clinical nephropathy and end-stage renal disease (ESRD). It is also not directly proven whether regression of microalbuminuria is associated with the reduced incidence of cardiovascular events, although it seems very probable. It has been demonstrated microalbuminuria independently predicts all-cause and cardiovascular mortality in general population [21].

It is also well documented that microalbuminuria in patients with type 2 diabetes is a predictor of cardiovascular complications and death [22,23]. The prevalence of microalbuminuria averaged 27% in cross-sectional evaluation of type 2 diabetic patients. Several traditional cardiovascular risk factors, including elevated hemoglobin $A1_C$ (HbA_{1C}) levels, obesity and hypertension were found with increased frequency in patients with type 2 diabetes and microalbuminuria and increased frequency of the CHD was observed as well [24]. Particularly high prevalence of arterial hypertension averaging 90% of the subjects has been found in type 2 diabetic patients with microalbuminuria [16] and very often hypertension preceded the development of microalbuminuria for many years. The mean 24 h systolic blood pressure showed a significant positive correlation with UAER within the microalbuminuric patients with type 2 diabetes [25]. Persistent microalbuminuria appeared to predict and aggravate lipoprotein abnormalities in type 2 diabetic patients. In these patients, a significant increase in very-low-density lipoprotein (VLDL) cholesterol, VLDL and low-density lipoprotein (LDL) trigliceride levels and a decrease in high-density lipoprotein (HDL) cholesterol levels were seen after the 5-year follow-up [26]. In many subjects the components of metabolic syndrome (central obesity, the resistance of periferal tissues to insulin, hyperinsulinemia, hypertension and the atherogenic lipid profile) precedes the development of type 2 diabetes and recently microalbuminuria was included as an additional factor in the criteria for diagnosis of this syndrome [27]. A variety of hemostatic abnormalities have been shown to correlate significantly

with UAER in diabetic patients, including plasma fibrinogen, factor VII activity, factor VII antigen, protein C, lipid peroxides and others [28,29] indicating a tendency to hypercoagulability in microalbuminuric type 2 diabetic patients.

It is generally accepted that increased blood pressure levels, hyperlipidemia and hemostatic abnormalities constitute the set of factors with are responsible for the significantly increased risk of developing macrovascular disease in type 2 diabetic patients with microalbuminuria, which indicate the endothelial damage [9]. Deckert et al. [30] suggested a common pathogenic mechanism of microalbuminuria and premature atherosclerosis because of the similarity and functional alterations of glomeruli and large vessel walls in patients with albuminuria.

There is growing support for the suggestion that microalbuminuria may be reflection of generalized endothelial dysfunction in capillaries (e.g. glomeruli) and arteries [31,32], and that leakage of albumin though the glomerular wall might be a marker of preclinical atheriosclerosis [33]. Theoretically, such a leakiness may allow for an increased lipid insudation into the large vessel wall, thereby linking microalbuminuria to atherogenesis [21,23].

Recently, an association of cardiovascular disease with the markers of inflammation was demonstrated both in non-diabetic and diabetic populations [23]. Stehouwer et al. [34] prospectively followed markers of chronic inflammation and endothelial dysfunction in a large group of patients with type 2 diabetes for 9 years. Markers for both endothelial dysfunction and chronic inflammation, as well as microalbuminuria, were found to be interrelated, to have developed in parallel, progressed with time, and were related to death. It may be concluded that a sensitive marker of (sub)clinical inflammation, such C-reactive protein (CRP) and microalbuminuria reflect intimately related components of the atheroslcerotic disease process [35]. It was demonstrated that elevated CRP levels enhances the relationship between blood pressure (which has been shown to be the main determinant of microalbuminuria in diabetes and hypertension) and microalbuminuria. This interaction was found independently of other factors. It was suggested that CRP may be a marker of vascular disease, which indicates impaired autoregulation of glomerular pressure and/or dysfunction of glomerular endothelinum. Both of these factors may enhance microalbuminuria. It is of interest, that CRP and microalbuminuria both predict incident cases of type 2 diabetes, which underlines their role in insulin resistance [6,37].

The results of the recent study [38] have suggested that the link between cardiovascular risk factors and microalbuminuria may originate from elevated vascular endothelial growth factor (VEGF) levels. It was shown that hyperglicemia plays an important role in increasing VEGF plasma concentrations and VEGF levels are increased in patients with diabetes. Elevated circulating VEGF levels, increased additionally when hypertension is present, may cause increased vascular permeability, which results in microalbuminuria in the kidney. It was concluded that there is a relation between increased VEGF levels and subsequent occurrence of microalbuminuria and the increased cardiovascular risk. The significant association between microalbuminuria and VEGF was dependent on cardiovascular risk factors [38].

Independently of the mechanisms involved in the development of microalbuminuria in type 2 diabetic patients, several retrospective and prospective studies have shown that microalbuminuria is not only predictor for proteinuria and progressive diabetic nephropathy but also a prognostic indicator of early mortality from cardiovascular disease. In the prospective study of elderly diabetic patients, UAER was the best prognostic factor for long-term mortality [39]. In another study [40] the excess mortality in type 2 diabetic patients was highly significantly increased among those with microalbuminuria (28%), compared with those with normal UAER (5%), and the predictive power of microalbuminuria persisted after adjustment for the effect of major risk factors. It was also demonstrated that albuminuria was much strongly associated with premature death from cardiovascular diseases than with end-stage renal disease (ESRD): after 10 years of follow-up 69% of patients died from acute myocardial infarction, cardiac failure, or stroke, while only 7% patients died from ESRD [1]. It may be concluded that microalbuminuria in type 2 diabetic patients appeared to be more relevant as a marker for cardiovascular disease and death than for renal failure [9].

The patients with both type 1 and type 2 diabetes complicated by overt nephropathy with proteinuria demonstrate even higher risk of cardiovascular complications than the patients with microalbuminuria, due to the effects of the same factors influencing prognosis in patients with microalbuminuria. It was demonstrated, however, that the survival of type 1 diabetic patients with overt nephropathy improved substantially because early antihypertensive treatment. In the long-term observational follow-up study it was shown that the median survival time was 13.9 years in type 1 diabetic patients with diabetic nephropathy [39]. The study also revealed that death due to ESRD was reduced to 35%. When the cumulative death rate during the natural history of diabetic nephropathy in type 1 patients were compared before and after introduction of effective antihypertensive treatment, it appeared that the mean survival times increased from 5-7 years before to >16 years after introduction on modern hypertensive treatment [1]. The recent meta-analysis of the prospective studies evaluating the effect of the antagonist of the Ang II AT 1 receptors (ARB) in type 2 diabetic patients either with micro- or macroalbuminuria, hypertension and even elevated blood preasure levels, revealed a significant risk reduction of 15% of cardiovascular events as compared with patients treated with other hypotensive agents [1], along with marked renoprotective effect of ARBs.

There are several additional risk factors for cardiovascular complications in patients with type 1 and type 2 diabetes and nephropathy, including diabetic cardiomyopathy, autonomic neuropathy of cardiovascular system, left ventricular hypertrophy and cardiac rhythm abnormalities (which predispose to sudden death), which were not discussed in this paper. The presented data seems, however, sufficient to support the conclusion that early detection of microalbuminuria or proteinuria in a patient with diabetes indicates not only a potential risk for the development of progressive kidney function impairment, but is also a marker of high risk of cardiovascular complications. These patients should receive a multifactorial treatment and should be monitored carefully to prevent or slow down the progression of both kidney and cardiovascular complications.

References

1. Parving HH, Mauer M, Ritz E. Diabetic nephropathy, in: The Kidney, 7th ed., ed: BM Brenner, Saunders, Philadelphia, 2004; 1777--818.
2. Mogensen CE. Definition of diabetic renal disease in insulin-dependent diabetes mellitus based on renal function tests, in: The kidney and hypertension in diabetes mellitus, 2nd ed., ed: CE Mogenen, Kluver Academic Publishers, Boston, 1994; 1-14.
3. Viberti GC, Hill RD, Jarrett RJ, Argyropoulos A, Mahmud U, Keen H. Microalbuminuria as a predictor of clinical nephropathy in insulin-dependent diabetes mellitus. Lancet, 1982; 1: 1430-2.
4. Mogensen CE, Christensen CK. Predicting diabetic nephropathy in insulin-dependent patients. N Engl J Med, 1984; 311: 89-93.
5. Perkins BA, Ficociello LH, Silva KH, Finkelstein DM, Warram JH, Krolewski AS. Regression of microalbuminuria in type 1 diabetes. New Engl J Med, 2003; 348: 2285-93.
6. Remuzzi G, Ruggenenti P, Begini A. Understanding the nature of renal disease progression. Kidney Int, 1997; 51: 2-15.
7. Raptis AE, Viberti GC. Pathogenesis of diabetic nephropathy. Exp Clin Endocrinol Diabetes 2001, 109(Suppl 2): S424-S37.
8. Krolewski AS. Genetics of diabetic nephropathy: evidence for major and minor gene effects. Kidney Int, 1999; 55: 1582-96.
9. Mogensen CE (ed.): Microalbuminuria. A marker for organ damage. 2nd ed, Science Press Lim, London, 1996.
10. Jensen T. Impaired aerobic work capacity in insulin-dependent diabetics with increased urinary albumin excretion. BMJ, 1988; 296: 1352--4.
11. Ross R. Atherosclerosis – an inflammatory disease. N Engl J Med, 1999; 340: 115-26.
12. Jensen JS, Borch-Johnsen K, Jensen G, Feldt-Rasmussen B. Microalbuminuria reflects a generalized transvascular albumin leakines in clinically healthy subjects. Clin Sci (Colch), 1995; 88: 629-33.
13. Mathiesen ER. The relationship between blood pressure and urinary albumin excretion in the development of microalbuminuria. Diabetes, 1990; 39: 245-9.
14. Feldt-Rasmussen B, Mathiesen ER, Deckert T. Effects of two years of strict metabolic control on progression of incipient nephropathy in insulin-dependent diabetes. Lancet, 1986; 2: 1300-4.
15. Parving HH, Hommel E, Mathiesen ER, Skott P, Edsberg B, Bahusen M. Prevalence of microalbuminuria, arterial hypertension, retinopathy and neuropathy in patients with insulin-dependent diabetes. BMJ, 1988; 296: 156-60.
16. Tarnow L, Rossing P, Gall MA. Prevalence of arterial hypertension in diabetic patients before and after the JNC-V. Diabetes Care, 1994; 17: 1247-51.
17. Fegerudd JA, Tarnow L, Jacobsen P. Predisposition to essential hypertension and development of diabetic nephropathy in IDDM patients. Diabetes, 1998; 47: 439-44.
18. Tarnow L, Cambien F, Rossing P, Flemming FS, Hansen BV, Ricard S. Insertion/deletion polymorphism in the angiotensin-I-converting enzyme gene is associated with coronary heart disease in IDDM patients with diabetic nephropathy. Diabetologia, 1995; 38: 798-803.
19. Cambien F, Poirier O, Lecerf L. Deletion polymorphism in the gene for angiotensin – converting enzyme is a potential risk factor myocardial infarction. Nature, 1992; 359: 641-4.
20. Rigat B, Hubert C, Corrol P, Sourbier F. PCR detection of the insertion/deletion polymorphism of the human angiotensin converting enzyme gene (DCP1) (dipeptidyl-carboxy peptidase 1). Nucleid Acids Res, 1992; 20: 1433-5.
21. Yuyun MF, Khaw KT, Luben R, Welch A, Bingham S, Day NE, Wareham NJ. Microalbuminuria independently predicts all-cause and cardiovascular mortality in a British population: The European Prospective Investigation into Cancer in Norfolk (EPIC-Norfolk) population study. International J Epidemiol, 2004; 33: 189-98.
22. Mogensen CE. Microalbuminuria predicts clinical proteinuria and early mortality in maturity-onset diabetes. N Engl J Med, 1984; 310: 973-7.
23. Lane JT. Microalbuminuria as a marker of cardiovascular and renal risk in type 2 diabetes mellitus: a temporal perspective. Am J Physiol Renal Physiol, 2004; 286: F442-F50.
24. Gall MA, Rossing P, Skøtt P, Damsbo P, Vaag A, Beck K, Dejgard A, Lauritzen M, Lauritzen E, Hougaard P, Beck-Nielsen H, Parving HH. Prevalence of micro- and macroalbuminuria, arterial hypertension, retinopathy and large vessel disease in European type 2 (non-insulin-dependent) diabetic patients. Diabetologia, 1991; 34: 655-61.
25. Schmitz A, Mau Pedersen M, Hansen KW. Blood pressure by 24 h ambulatory recording in type 2 (non-insulin-dependent) diabetics. Relationship to urinary albumin excretion. Diabete Metab, 1991; 17: 301-7.
26. Niskanen L, Kusitupa M, Sarlund H, Siitonen O, Voutilainen E, Penttila I, Pyorala K. Microalbuminuria predicts the development of serum lipoprotein abnormalities favouring atherogenesis in newly diagnosed type 2 (non-insulin-dependent) diabetic patients. Diabetologia, 1990; 33: 237-43.
27. Alberti K, Zimmet P for the WHO Consultation. Definition, diagnosis and classification of diabetes mellitus and its complications. Part 1: diagnosis and classification of diabetes mellitus. Provisional raport of a WHO consultation. Diabet Med, 1998; 15: 539-53.
28. Knöbl P. Thrombogenic factors are related to urinary albumin excretion rate in type 1 and type 2 diabetic patients. Diabetologia, 1993; 36: 1045-50.
29. Murakami T, Komiyama Y, Egawa H, Murata K. Elevation of factor XIa – antitripsin complex levels in NIDDM patients with diabetic nephropathy. Diabetes, 1993; 42: 233-8.
30. Deckert T, Kofoed-Eneroldsen A, Novgaard K, Borch-Johnsen K, Feldt-Rasmussen B, Jensen T. Microalbuminuria – implications of micro- and macrovascular disease. Diabetes Care, 1992; 15: 1181-6.
31. Deckert T, Feld-Rasmussen B, Borch-Johnsen K, Jensen T, Kofoed-Eneroldsen A. Albuminuria reflects widespread vascular damage. The Steno Hypothesis. Diabetologia, 1989; 32: 219-26.
32. Stehouwer CD, Yudkin JS, Fioretto P, Nosadini R. How heterogenous is microalbuminuria in diabetes mellitus? The case for "benign" and "malignant" microalbuminuria. Nephrol Dial Transplant, 1998; 13: 2751-4.
33. Jensen JS. Microalbuminuria and the risk of atherosclerosis. Clinical, epidemiological and physiological investigations. Dan Med Bull, 2000; 47: 63-78.
34. Stehouwer C, Gall M, Twisk J, Knudsen E, Emeis J, Parving HH. Increased urinary albumin excretion, endothelial dysfunction, and chronic low grade inflammation in type 2 diabetes: progressive, interrelated, and independently associated with risk of death. Diabetes, 2002; 51: 1157-65.
35. Stuvelink EM, Bakker SJL, Hillege HL, Burgerhof JGM, de Jong PE, Gans ROB, de Zeeuw D. The PREVEND Study Group: C-reactive protein modifies the relationship between blood pressure and microalbuminuria. Hypertension, 2004; 43: 791-6.
36. Mykkanen L, Haffner SM, Kunsisto J. Microalbuminuria precedes the development of NIDDM. Diabetes, 1994; 43: 552-7.
37. Freeman DL, Norrie J, Caslake MJ. C-reactive protein is an independent predictor of risk for the development of diabetes in the West of Scotland Coronary Prevention Study. Diabetes, 2002; 51: 1596-600.
38. Asselbergs FW, de Boer RA, Diercks GFH, Langerfeld B, Tio RA, de Jong PE, van Veldkuisen DJ, van Gilst WH. Vascular endothelial growth factor: the link between cardiovascular risk factors and microalbuminuria? Int J Cardiol, 2004; 93: 211-5.
39. Rossing P, Hongaard P, Barch-Johnsen K, Parving HH. Predictors of mortality in insulin dependent diabetes: 10 years follow-up study. BMJ, 1996; 313: 779-84.
40. Pourdjabbar A, Lapointe N, Ronleau JL. Angiotensin receptor blockers: Powerful evidence with cardiovascular outcomes? Can J Cardiol, 2002; 18(Suppl A): 7A-11A.

Hemostasis in chronic renal failure

Małyszko J[1]*, Małyszko JS[1], Myśliwiec M[1], Buczko W[2]

[1] Department of Nephrology and Transplantology, Medical University of Białystok, Poland
[2] Department of Pharmacodynamics, Medical University of Białystok, Poland

Key words: hemostasis, chronic renal failure.

Introduction

Hemostasis is a process of blood clot formation at the site of vessel injury. When a blood vessel wall breaks, the hemostatic response must be quick, localized, and carefully regulated. Bleeding or a thrombosis may occur due to missing or dysfunctional moieties of the coagulation or fibrinolytic factors. The pathways of thrombin-stimulated fibrin clot formation and plasmin-induced clot lysis are linked and commonly regulated. When they work in coordinated harmony, a clot is laid down to stop bleeding, followed by eventual clot lysis and tissue repairing. Abnormal bleeding can result from diminished thrombin generation (e.g., due to factor VIII deficiency) or enhanced plasmin formation (e.g., due to alpha-2-antiplasmin deficiency). Conversely, excessive production of thrombin (e.g., due to an inherited thrombophilia) can lead to thrombosis. Clot formation and its subsequent lysis may be decided in the four steps: initiation and formation of the platelet plug, propagation by the coagulation cascade, termination of the clotting by antithrombotic control mechanisms and removal of the clot by fibrinolysis.

In a variety of slowly progressive renal diseases such as chronic glomerulonephritis, diabetic nephropathy, and polycystic kidney disease, it is at present, not possible or very difficult to correct the underlying disease. Eventual progression to renal failure is common in patients with various kidney diseases once the serum creatinine exceeds 1.5 to 2.0 mg/dL. This may occur even if the underlying disorder is "cured". After a certain point, a reduction in the number of functioning nephrons eventually leads to loss of the more normal remaining nephrons. Renal failure may be associated with a variety of signs and symptoms that are collectively referred to as the uremic state. However, the is no predictable correlation between the development of these problems and the severity of renal disease. Loss of renal function results in the accumulation of metabolic waste products and alters the normal homeostatic mechanisms. Potential consequences of these abnormalities are the signs and symptoms of uremia. Using renal replacement therapy in a form dialyses or kidney transplantation, the physician can treat these disturbances and improve the quality of life in many patients with chronic, end-stage renal disease.

Platelet dysfunction in uremia

Platelets are activated at the site of vascular injury to form a platelet plug that provides the initial hemostatic response to stop bleeding. The functional response of activated platelets involves: adhesion – a sticking of platelets to the subendothelial matrix, aggregation – platelet-platelet cohesion, secretion – the release of platelet granule proteins (serotonin, ADP, thrombospondin, fibrinogen, thromboxane A2, growth factors) and procoagulant activity – the enhancement of thrombin generation.

It is likely that multiple factors are responsible for the platelet dysfunction in uremia. Three of the factors that may contribute are the retention of uremic toxins, anemia, and nitric oxide [1]. Platelet dysfunction is observed mainly in advanced uremia before starting dialysis treatment. It is probably related to uremic toxins present in the circulation. The importance of circulating toxins is suggested by commonly seen a beneficial effect of acute dialysis on platelet dysfunction, although the bleeding time is rarely normalized [1]. Urea alone, however, is probably not the major platelet toxin and there is no correlation between blood urea nitrogen and the bleeding time in patients with renal failure [2]. Other potential toxins include guanidinosuccinic acid, phe-

* CORRESPONDING AUTHOR:
Department of Nephrology and Transplantology
Medical University of Białystok
ul. Żurawia 14
15-540 Białystok, Poland
Tel/Fax: 48 85 7434586
e-mail: jolmal@poczta.onet.pl (Jolanta Małyszko)

Received 05.05.2005 Accepted 06.05.2005

nolic acid, and middle molecules (mol wt 500 to 3000 Daltons). However, no single compound accumulating in end-stage renal failure has been unequivocally identified as being responsible for platelet dysfunction. *In vitro* studies in which normal platelets are incubated with uremic serum suggest that a dialyzable factor interferes with the binding of fibrinogen to GPIIb-IIIa [3]. Several authors reported that platelet aggregation induced by different stimuli in PRP was depressed in chronic uremia [4,5] whereas other studies provide evidence of hyperaggregability [6-8]. In our previous study, in hemodialyzed (HD) and peritoneally dialyzed (CAPD – chronic ambulatory peritoneal dialysis) patients platelet aggregation in PRP induced by 5 agonists was significantly depressed, whereas platelet aggregation in whole blood (considered as more physiological) did not differ from that in the healthy volunteers. So far, there have been no comprehensive data on platelet aggregation in CAPD subjects. In one report of Arends et al. [9] it was stated that treatment by means of peritoneal dialysis can at least partially correct platelet dysfunction and reverse the bleeding tendency. Kim et al. [10] found that there was a direct link between hypoalbuminemia and increased platelet aggregation in CAPD, which confirmed eaarlier observation of Sloand et al. [11]. Kozek-Langenecker et al. [12] found a decreased expression of fibrinogen binding sites on resting uremic platelets (from hemodialyzed patients) compared with normal platelets as measured by reduced binding of activation-independent monoclonal antibody against platelet GPIIIa. It has been hypothesized that fibrinogen fragments may occupy a percentage of platelet fibrinogen receptors, thus preventing binding of fibrinogen to platelets, a step considered to be essential for aggregation. On the other hand, Himmelfarb et al. [13] found that HD patients had a marked increase in circulating reticulated platelets compared to PD patients or controls, indicating accelerated platelet turnover. Increased platelet activation and turnover may contribute to the qualitative platelet dysfunction in uremia.

Degree of anemia appears to correlate relatively closely with the degree of prolongation of the bleeding time [14], which reflects the impaired platelet-vessel wall interactions. The prolongation of the bleeding time is a common feature of chronic renal failure. It has been proposed that rheologic factors play an important role in the relationship between anemia and platelet dysfunction [14]. At a hematocrit above 30 percent, the red cells primarily occupy the center of the vessel, while the platelets are in a skimming layer at the endothelial surface. This close proximity allows the platelets to adhere and then form a platelet plug when there is an endothelial injury. With anemia, on the other hand, the platelets are more dispersed, thereby impairing adherence to the endothelium. Correction of anemia with blood transfusions or erythropoietin often improves platelet function.

Moreover, nitric oxide (NO; endothelium-derived relaxing factor) produced by endothelial cells and platelets, is a potent inhibitor of platelet aggregation. It has been reported that platelet NO synthesis is increased in uremic patients and that uremic plasma stimulates NO production by cultured endothelial cells [15]. It may be due to elevated blood levels of guanidinosuccinic acid, a uremic toxin that may be a precursor for nitric oxide [16]. Noris et al. [17] suggested that increased NO biosynthesis may contribute to platelet dysfunction and possibly other manifestations of uremic syndrome, including hemodialysis hypotension and administration of an NO synthesis inhibitor normalizes the bleeding time in uremic rats. Nonetheless, up to date controversial results on platelet function in dialyzed patients: impaired or enhanced, have been a matter of debate.

Endothelium in uremia

In renal failure endothelial dysfunction and atherosclerosis are almost universal, as well as cardiovascular complications. Endothelial cell injury is the probable cause due to uremic toxins retention, dyslipidemia, hypertension and secondary hyperparathyroidism as well as increased levels of IL-1 and TNFα. Signs of endothelial dysfunction have been reported in dialyzed patients [18,19-21]. The assessment of endothelial cell injury *in vivo* is complex due to multifunctional nature of these cells. Endothelial damage is an injury response mechanism, which includes impaired endothelium-dependent vasodilation, increased adhesion of platelets and leukocytes. This is a putative first step in atherogenesis. Moreover, exposure of endothelial cells to oxidized LDL *in vitro* releases various adhesion molecules including VCAM (vascular cell adhesion molecule), ICAM (intercellular cell adhesion molecule), selectins and vWF [22], which are considered as markers of endothelial cell injury. On the other hand, vascular endothelium provides effective anticoagulant properties by expressing surface bound proteoglycans, such as thrombomodulin and heparan sulfate, and by releasing coagulation factors inhibitors, such as protein S and tissue factor pathway inhibitor-TFPI [23]. Thus, endothelial dysfunction may be responsible for accelerated atherosclerosis in patients with chronic renal failure. So far, many reports devoted to disturbances in hemostasis and endothelium in hemodialyzed patients [24,25] did not focus on hemostasis and endothelial function in patients on CAPD.

P-selectin, released from activated platelets and endothelial cells may play a very important role in the development of atherosclerosis. However, it has been controversial whether plasma P-selectin concentration reflects activation of platelets or endothelial cells [26]. In our previous study [27], P-selectin did not differ between patients on CAPD and healthy volunteers. Platelet glycoprotein V–GPV shed to blood is considered as a new marker of platelet function. In our previous study we found a similar concentration of GPV in CAPD patients and the healthy volunteers [28].

E-selectin has only been described on endothelial cells and may therefore represent a circulating surrogate for evaluation of endothelial cell activation or damage [29]. In our previous study [27], E-selectin did not differ significantly between all three groups studied in contrast to the study of Bonomini et al. [30], who found elevated E-selectin in undialyzed CRF patients, HD and CAPD subjects. They also showed a strong linear correlation between serum creatinine and serum levels of adhesion molecules: ICAM, VCAM, E-selectin and P-selectin. In our study, we were unable to show such correlations between kidney function and concentration of adhesion molecules [28]. However, concentrations of ICAM, VCAM, TFPI (total, full length and truncated) and thrombomodulin were significantly

higher in CAPD patients when compared to the healthy volunteers. It may be due to the inadequate clearance as well as their enhanced synthesis/release. Jacobson et al. [21] found elevated markers of endothelial dysfunction: vWF, thrombomodulin, and soluble adhesion molecules: ICAM and VCAM, as well as strong correlations between them in dialyzed and nondialyzed patients with chronic renal failure. However, correlations were found in the whole group (10 HD patients, 20 CAPD, 25 with chronic renal failure).

Recent studies indicate that vascular endothelial growth factor (VEGF), the major stimulus of angiogenesis, prompts activated endothelial cells to become prothrombotic. On VEGF stimulation, endothelial cells increase TF expression on their membranes and thereby generate thrombin activity from prothrombin [31]. Moreover, recently Blann et al. [32] suggested that increased VEGF might be evidence of the early stages of atherosclerosis, i.e. increased angiogenesis in response to early injury of the arterial wall. On the other hand, elevated VEGF may just reflect increased turnover of endothelial cells, which are damaged by the disease process [33]. In our previous study, we found elevated VEGF in CAPD patients [28].

Vascular endothelial cells express CD40 and ligation of CD40 on endothelial cell is known to upregulate expression of the inflammatory adhesion molecules: E-selectin, VCAM-1 and ICAM-1 [34]. Slupsky et al. [35] demonstrated that ligation of CD40 on endothelial cells initiated a procoagulant phenotype which included upregulation of tissue factor and down regulation of thrombomodulin. In our previous study [27], we observed a significant rise in CD40 ligand in CAPD patients. Moreover, local ligation of CD40 on endothelial cell in the presence of increased TF concentration, observed in CAPD patients [18] might play a role in the thrombotic complications in these patients.

A novel cell adhesion molecule localized at the endothelial junction is CD146. It is constitutively expressed in all human endothelial cells irrespective of anatomical site or vessel calibers [36,37]. Moreover, an increase of CD146 expression is detectable on HUVEC treated with inflammatory cytokines [37], suggesting that endothelial activation modulates its expression. Recently, Bardin et al. [38] reported an increased plasma CD146 levels in several pathophysiological settings, linked to endothelial junctional alteration, i.e. chronic renal failure. This increase was corroborated by increased expression of CD146 on kidney biopsies from 5 patients with renal failure. Bardin et al. [38] suggested that elevation of CD146 in patients with CRF could be due to an increased release or to its reduced elimination. However, they did not study CD146 correlation with renal function.

Coagulation in uremia

Classically, the intrinsic pathway is initiated by the exposure of blood to a negatively charged surface (such as glass in the aPTT clotting time) and the extrinsic pathway is activated by tissue factor-TF exposed at the site of injury or TF-like material. Both pathways converge on the activation of factor X which then activates prothrombin to thrombin, the final enzyme of the clotting cascade. Thrombin converts fibrinogen from a soluble plasma protein into an insoluble fibrin clot. It is now established that the generation or exposure of TF at the wound site is the primary physiologic event in initiating clotting [39]. TF-induced coagulation plays an important role in the pathophysiology of many diseases including thrombosis, atherosclerosis, ischemia-reperfusion injury, sepsis or glomerulonephritis [40]. Factor VII participates in the initiation of TF pathway-induced coagulation, and an increase in factor VII activity has been recognized as a risk factor for cardiovascular disease, a common finding and a potent cause of mortality in renal patients [41]. The interactions of activated platelets and the clotting cascade, with their subsequent amplification, give rise to a hemostatic response that is rapid and localized to the injury site. It is also potentially explosive, and if unchecked, could lead to thrombosis, vascular inflammation, and tissue damage. Fortunately, antithrombotic pathways are mostly anchored on vascular endothelial cells, which play an active role in maintaining the fluidity of blood. The termination phase involves two circulating enzyme inhibitors, antithrombin (formerly called antithrombin III) and tissue factor pathway inhibitor-TFPI; and, a clotting-initiated inhibitory process, the protein C pathway. Expressed primarily by the microvascular endothelium-TFPI, appears to be the major physiologic inhibitor of TF-induced coagulation [40].

Significant alterations in the plasma levels of coagulation factors and natural anticoagulants have been observed in uremics. In comparison with hemodialyzed patients there are still a limited data concerning hemostasis in CAPD subjects. Indirect evidence of hypercoagulation in HD provide the following laboratory alterations: hyperfibrinogenemia, enhanced: factor VII activity, factor VIII and von Willebrand factor concentration, low: antithrombin III, protein C and S activities, activities of factor II, IX, X and XII despite their normal or elevated plasma concentrations [42,43]. In CAPD a peculiar coagulation profile is observed: hyperfibrinogenemia, elevated activities of factors II, VII, VIII, IX, X, XII, high concentrations of protein S, normal antithrombin III and protein C [44,45]. In our previous study [18] elevated plasma markers of ongoing coagulation – prothrombin fragments 1+2 (F1+2) and thrombin-antithrombin complexes were found in HD and CAPD patients relative to healthy volunteers. Thus, a conversion of prothrombin into thrombin by factor X appears to be more accelerated in CAPD patients, leading to increased fibrin formation. Sagripanti et al. [46] found that elevated prothrombin fragments 1+2 in hemodialyzed patients reflected increased *in vivo* conversion of prothrombin into thrombin rather than impaired renal catabolism or excretion of this polypeptide. Moreover, Kario et al. [47] shown that high plasma prothrombin fragments 1+2 were accompanied by factor VII hyperactivity in hemodialyzed patients, suggesting that increase in F1+2 actually reflected hypercoagulation. Opposite results presented Tomura et al. [48]. According to Kobayashi et al. [44] hypercoagulability and secondary hyperfibrinolysis occur in CAPD patients when compared to healthy volunteers. However, among 21 patients studied by them 19 were treated with rHuEPO (3 were diabetic), therefore, their results are difficult to interprete since erythropoietin affects coagulation and fibrinolysis. As the interactions between TF and factor VII can

trigger coagulation cascade, it is conceivable that the eventual result is increased thrombin generation.

In our previous study we found that concentrations of TF were significantly higher in dialyzed patients (HD, CAPD) when compared to the healthy volunteers as well as TFPI activity [49]. Cella et al. [50] did not observe any differences in TFPI activity between chronically hemodialyzed patients and healthy subjects, whereas Kario et al. [51] reported an increased plasma TFPI activity before dialysis. It could be due to a reduced kidney catabolism or to endothelial cell damage. High TFPI activity in uremia may also reflect endothelial cell injury due to hemodialysis treatment. The increased concentrations of other markers of endothelial cell damage such as vWF and thrombomodulin are in keeping with this concept.

However, in our study [18] vWF concentrations in CAPD subjects were lower than in HD patients. On the other hand, in CAPD patients there are no systemic anticoagulation but TFPI activity is also enhanced. Thus, increased TFPI activity before dialysis session in HD patients seems unlikely to be due to the effects of residual heparin from the previous dialysis session. TFPI is a potent inhibitor of the factor VIIa/TF complex in the presence of factor Xa, as well as being a direct inhibitor of factor Xa [40]. This high level of TFPI might thus counterbalance the increased activity of factor VII in uremia and may be considered as a defence mechanism against hypercoagulable state.

Fibrinolysis in uremia

To restore vessel patency following hemostasis, the clot must be organized and removed by plasmin in conjunction with wound healing and tissue remodeling. Fibrin binds plasminogen, the precursor molecule to plasmin, and tissue plasminogen activator (tPA). It leads to formation of active, proteolytic plasmin [52,53], which cleaves the polymerized fibrin strand at multiple sites, releasing fibrin degradation products (FDPs), i.e. D-dimers. The plasminogen/plasminogen-activator system is complex, paralleling the coagulation cascade [54]. Plasmin activity is regulated by vascular endothelial cells that secrete both plasminogen activators (tissue-type plasminogen activator and urokinase-type plasminogen activator) and plasminogen activator inhibitors (PAI-1 and PAI-2). Balance between tPA and PAI is the major determinant of the overall fibrinolytic activity. When fibrin is degraded by plasmin, new carboxy-terminal lysines are exposed in the partially digested clot. These residues provide additional sites for plasminogen binding to the clot, creating a positive feedback loop in the clot lysis. The carboxy-terminal lysines are susceptible to removal by carboxypeptidases [55,56]. Thrombin Activatable Fibrinolysis Inhibitor–TAFI, a proenzyme form of carboxypeptidase-B is a newly recognized physiologic substrate for the thrombin-thrombomodulin (TTM) complex [57]. Therefore, it couples two distinct in function systems: coagulation and fibrinolysis. Activated TAFI delays clot lysis.

In dialyzed patients both impaired overall fibrinolytic activity and hyperfibrinolysis have been reported [18,44,58]. Therefore, the question arises, whether activation of fibrinolysis is primary or secondary. Tomura et al. [48] found increased tissue plasminogen activator-tPA and decreased its inhibitor-PAI-1 in 17 HD patients when compared to 17 CAPD subjects. In CAPD patients overall fibrinolytic activity as reflected by prolonged ECLT is depressed when compared to HD subjects. Moreover, plasmin-antiplasmin complexes have been found to be elevated or normal in dialyzed patients [58,59]. It may suggest only local activation of fibrinolysis. According to Lane at al. [60] and others [43-45,59,61] in hemodialyzed and peritoneally dialyzed patients, hyperfibrinolysis is secondary to activation of coagulation cascade. At the same time, overall fibrinolytic activity is impaired [5,62,63]. In our previous study [18] we found that in CAPD patients overall fibrinolytic activity as reflected by prolonged ECLT was depressed when compared to HD subjects. Moreover, plasmin-antiplasmin complexes are lower in CAPD than in HD. However, FAI (fibrinolytic activity index = fibrinogen/ECLT) was significantly higher in non-diabetic CAPD patients when compared to non-diabetic HD subjects. Nakamura et al. [64] reported that HD patients exhibited a high PAP levels but a low plasmin activity. Moreover, Tomura et al. [48] showed lack of correlation between PAP and tPA or PAI in HD. It may be due to the fact that PAP complexes are not sensitive markers of plasmin generation in dialyzed subjects. Opatrny et al. [62] reported a fibrinolysis defect manifesting after standard fibrinolytic stimulus (DDAVP-1-deamino-8-D-arginine vasopressin) by an insufficient decrease in PAI-1 concentration in patients with type 2 diabetes mellitus maintained on chronic hemodialysis. On the other hand, Babazono et al. [63] compared coagulation and fibrinolysis in 23 diabetic patients on long-term CAPD with hemodialyzed diabetic patients. They found that CAPD patients had higher fibrinogen and von Willebrand factor as well as serum lipids relative to HD patients. They concluded that CAPD was associated with more atherogenic lipid profile than were on hemodialyses and with hypercoagulable state but not with decreased fibrinolysis state. We have reported for the first time that TAFI concentration and activity in dialyzed patients with diabetic nephropathy was significantly higher that in the relevant groups of dialyzed patients without diabetic nephropathy [65]. Previously, we found that TAFI concentrations were significantly higher in CAPD [66] as well as in kidney transplant recipients [67], two populations of kidney patients with decreased fibrinolytic activity and a hypercoagulable state. Differences in TAFI concentration and activity between HD and CAPD patients were at the level of statistical significance ($p=0.09$ and $p=0.07$, respectively).

Conclusions

Disturbances in hemostasis are common complications of kidney diseases. Their occurrence and severity correlate quite well with the progressive loss of renal function to end-stage renal disease. Both bleeding diathesis and thromboembolism have been identified [14]. The principal cause of these abnormalities is the uremic state and as a rule, it is at least partially reversible with the institution of adequate renal replacement therapy. The pathogenesis of uremic bleeding is multifactorial. It has been attributed to: platelet dysfunction, abnormal platelet-vessel wall interactions and altered rheological properties of the blood flow [4,68,69]. The most important determinants

Table 1. **Some hemostatic parameters in HD and CAPD patients**

	HD	CAPD
Fibrinogen	↑	↑↑
F II activity	↓	↑
F VII activity	↑	↑
FVIII activity	↓	↑
F IX activity	↓	↑
F X activity	↓	↑
F XII activity	↓	↑
Protein C activity	↓	↔
Protein S activity	↓	↑
antithrombin	↓	↔
vWF	↑	↑
thrombomodulin	↑	↑
TF	↑	↑
TFPI	↑	↑
F1+2	↑	↑
TAT	↑	↑
PAP	↑	↑
t-PA	↑	↓
PAI-1	↓	↑
TAFI	↔	↑

of the pathogenesis of the prothrombotic state in uremia are: increased levels of clotting factors and decreased levels of clotting inhibitors, hyperfibrinogenemia, diminished fibrinolytic activity, and platelet hyperaggregability (*Tab. 1*). At present, the incidence of bleeding is apparently declining, whereas thrombotic complications have become the predominant causes of mortality [41]. The intensity of hypercoagulability is thought to be related to the degree of hypoalbuminemia, being more evident at serum albumin levels of <2g/dL, with an implicated participatory role of the associated hypertriglyceridemia and changes in arachidonic acid metabolism that accompany the metabolic response to hypoalbuminemia [70].

References

1. Rabelink, TJ, Zwaginga, JJ, Koomans, HA, Sixma, JJ. Thrombosis and hemostasis in renal disease. Kidney Int, 1994; 46: 287-96.
2. Steiner, RW, Coggins, C, Carvalho, ACA. Bleeding time in uremia: A useful test to assess clinical bleeding. Am J Hematol, 1979; 7: 107-17.
3. Gawaz MP, Dobos G, Spath M, Schollmeyer P, Gurland HJ, Mujais SK. Impaired function of platelet membrane glycoprotein IIb-IIIa in end-stage renal disease. J Am Soc Nephrol, 1994; 5: 36-46.
4. di Minno G, Martinez J, McKean M, de la Rosa J, Burke JF, Murphy S. Platelet dysfunction in uremia. Multifaceted defect partially corrected by dialysis. Am J Med, 1985; 79: 552-9.
5. Panicucci F, Sagripanti A, Pinori E, Vispi M, Lecchini L, Barsotti G, Giovanetti S. Comprehensive study of hemostasis in chronic uremia. Nephron, 1983; 33: 5-8.
6. Viener A, Aviram M, Better OS, Brook JG. Enhanced in vitro platelet aggregation in hemodialysis patients. Nephron, 1986; 43: 139-43.
7. Remuzzi G, Benigni A, Dodesini P, Schieppati A, Gotti E, Livio M, Mecca G, Donati B, de Gaetano G. Platelet function in patients on maintenance hemodialysis: Depressed or enhanced? Clin Nephrol, 1992; 17: 60-3.
8. Nakamura Y, Masui N, Nakagawa S. Evaluation of the platelet function of chronically uremic patients on regular dialysis treatment by platelet sensitivity test. J Jpn Soc Dial Ther, 1989; 22: 1341-5.
9. Arends JP, Krediet RT, Boeschoten EW, van der Lelie J, Veenhof CH, von dem Borne AE. Improvement of bleeding time, platelet aggregation and platelet count during CAPD treatment. Proc Eur Dial Transplant Assoc, 1981; 18: 280-3.
10. Kim SB, Chi HS, Park JS, Hong CD, Yang WS. Effect of increasing serum albumin on plasma D-Dimer, von Willebrand factor, and platelet aggregation in CAPD patients. Am J Kidney Dis, 1999; 33: 312-7.
11. Sloand EM, Bern MM, Kaldany A. Effect on platelet function of hypoalbuminemia in peritoneal dialysis. Thromb Res, 1986; 44: 419-25.
12. Kozek-Langenecker SA, Masaki T, Mohammad H, Green W, Mohammad SF, Cheung AK. Fibrinogen fragments and platelet dysfunction in uremia. Kidney Int, 1999; 56: 299-305.
13. Himmelfarb J, Holbrook D, McMonagle E, Ault K. Increased reticulated platelets in dialysis patients. Kidney Int, 1997; 51: 834-9.
14. Eberst E, Berkowitz LR. Hemostasis in renal disease: Pathophysiology and management. Am J Med, 1994; 96: 168-79.
15. Remuzzi G, Perico N, Zoja C, Corna D, Macconi D, Vigano G. Role of endothelium-derived nitric oxide in the bleeding tendency of uremia. J Clin Invest, 1990; 86: 1768-71.
16. Noris, M, Remuzzi, G. Uremic bleeding: Closing the circle after 30 years of controversies? Blood, 1999; 94: 2569-74.
17. Noris M, Benigni A, Boccardo P, Aiello S, Gaspari F, Todeschini M, Figliuzzi M, Remuzzi G. Enhanced nitric oxide synthesis in uremia: Implications for platelet dysfunction and dialysis hypotension. Kidney Int, 1993; 44: 445-50.
18. Małyszko J, Małyszko JS, Myśliwiec M. Comparison of hemostasis between CAPD and HD patients. Perit Dial Int, 2001; 21: 158-65.
19. Kawaguchi Y, Kubo H, Yamamoto H, Nakayama K, Shigematsu T, Sakai O. Is atherosclerosis accelerated by CAPD? Perit Dial Int, 1996; 16 (Suppl 1): S23-S30.
20. Kuriyama S, Tomonari H, Yoshida H, Hikita M, Sakai O. Endothelial cell dysfunction in patients with impaired renal function. Nippon Jinzo Gakkai Shi, 1996; 38: 372-8.
21. Jacobson SH, Egberg N, Hylander B, Lundahl J. Correlation between soluble markers of endothelial dysfunction in patients with renal failure. Am J Nephrol, 2002; 22: 42-7.
22. Bolton CH, Downs LG, Victory JG, Dwight JF, Tomson CR, Mackness MI, Pinkney JH. Endothelial dysfunction in chronic renal failure: roles of lipoprotein oxidation and proinflammatory cytokines. Nephrol Dial Transplant, 2001; 16: 1189-97.
23. Ross R. Atherosclerosis – an inflammatory disease. N Engl J Med, 1998; 340: 115-26.
24. Bombeli T, Mueller M, Haeberli A. Anticoagulant properties of the vascular endothelium. Thromb Haemost, 1997; 77: 408-23.
25. Gris JC, Branger B, Vecina F, Sabadani B, Fourcade J, Schved JF. Increased cardiovascular risk factors and features of endothelial activation and dysfunction in dialyzed uremic patients. Kidney Int, 1994; 46: 807-13.
26. Małyszko JS, Małyszko J, Pawlak D, Pawlak K, Buczko W, Myśliwiec M. Serotonergic mechanism are involed in the pathogenesis of thrombotic complication in rHuEPO-treated hemodialyzed patients. Nephron, 2000; 75: 612-8.
27. Ishiwata N, Takio K, Katayama M, Watanabe K, Titani K, Ikeda Y, Handa M. Alternatively spliced isoform of P-selectin is present in vivo as soluble molecule. J Biol Chem, 1994; 38: 23708-15.
28. Małyszko J, Małyszko JS, Myśliwiec M. Endothelial cell injury markers in chronic renal failure on conservative treatment and continuous ambulatory peritoneal dialysis (CAPD). Kidney Blood Press Res, 2004, 27: 71-7.
29. Gearing AJH, Hemingway I, Piggott R, Hughes J, Rees AJ, Cashman SJ. Soluble forms of vascular adhesion molecules. E-selectin, ICAM-1 and VCAM-1: Pathological significance. Ann NY Acad Sci, 1992; 667: 324-31.
30. Bonomini M, Reale M, Santarelli P, Stuard S, Settefrati N, Albertazzi A. Serum levels of soluble adhesion molecules in chronic renal failure and dialysis patients. Nephron, 1998; 79: 399-407.
31. Zucker S, Mirza H, Conner CE. Vascular endothelial growth factor induces tissue factor and matrix metalloproteinase production in endothelial cells: conversion of prothrombin to thrombin results in progelatinase-A activation and cell proliferation. Int J Cancer, 1998; 75: 780-5.
32. Blann AD, Belgore FM, Constans J, Conri C, Lip GYH. Plasma vascular endothelial growth factor and its receptor Flt-1 in patients with hyperlipidemia and atherosclerosis and the effects of fluvastatin and fenofibrate. Am J Cardiol, 2001; 87: 1160-3.
33. Blann AD, Lip GYH. The endothelium in atherothrombotic disease: assessment of function, mechanisms and clinical implications. Blood Coagul Fibrinol, 1998; 9: 297-306.

34. Karmann K, Min W, Fanslow WC, Pober JS. Activation and homologous desensitization of human endothelial cells by CD 40 ligand, tumor necrosis factor, and interleukin 1. J Exp Med, 1996; 184: 173-82.

35. Slupsky JR, Kalbas M, Willuweit A, Henn V, Kroczek RA, Muller-Berghaus G. Activated platelets induce tissue factor expression on human umbilical vein endothelial cells by ligation of CD 40. Thromb Haemost, 1998; 80: 1008-14.

36. Bardin N, Frances V, Lesaule G, Horschowski N, George F, Sampol J. Identification of the S-Endo 1 endothelial-associated antigen. Biochem Biophys Res Commun, 1996; 218: 210-6.

37. Bardin N, George F, Mutin M, Brisson C, Horschowski N, Frances V, Lesaule G, Sampol J. S-Endo 1, a pan-endothelial monoclonal antibody recognizing a novel human endothelial antigen. Tissue Antigens, 1996; 548: 531-9.

38. Bardin N, Moal V, Anfosso F, Daniel L, Brunet P, Sampol J, Dignat George F. Soluble CD146, a novel endothelial marker, is increased in physiopathological settings linked to endothelial junctional alteration. Thromb Haemost, 2003; 90: 915-20.

39. Rapaport, SI, Rao, LV. The tissue factor pathway: How it has become a "prima ballerina". Thromb Haemost, 1995; 74: 7-17.

40. Broze GJ Jr. Tissue factor pathway inhibitor. Thromb Haemostas, 1995; 74: 90-3.

41. Locatelli F, Del Vecchio L, Manzoni C. Morbidity and mortality on maintenance hemodialysis. Nephron, 1998; 80: 380-400.

42. Małyszko J, Małyszko JS, Pawlak D, Pawlak K, Buczko W, Myśliwiec M. Platelet function, hemostasis and serotonin in acute and chronic renal failure. Thromb Res, 1996; 83: 351-61.

43. Vaziri ND, Gonzales ED, Wang J, Said S. Blood coagulation, fibrinolytic, and inhibitory proteins in end-stge renal disease: Effect of hemodialysis. Am J Kidney Dis, 1994; 23: 828-35.

44. Kobayashi M, Yorioka N, Yamakido M. Hypercoagulability and secondary hyperfibrinolysis may be related to abnormal lipid metabolism in patients treated with continuous ambulatory peritoneal dialysis. Nephron, 1997; 76: 56-61

45. Vaziri ND, Shah GM, Winer RL, Gonzales E, Patel B, Alikhani S, Nguyen QX, Yamamoto J. Coagulation cascade, fibrinolytic system, antithrombin III, protein C and protein S in patients maintained on continuous ambulatory peritoneal dialysis. Thromb Res, 1989; 53: 173-80.

46. Sagripanti A, Cozza V, Baicchi U, Camici A, Cupisti A, Barsotti G. Increased thrombin generation in patients with chronic renal failure. Int J Clin Lab Res, 1997; 27: 72-5.

47. Kario K, Matsuo T, Yamada T, Nakao K, Shimano C, Matsuo M. Factor VII hyperactivity in chronic dialysis patients. Thromb Res, 1992; 67: 105-9.

48. Tomura S, Nakamura Y, Doi M, Ando R, Ida T, Chida Y, Ootsuka S, Shinoda T, Yanagi H, Tsuchiya S, Marumo F. Fibrinogen, coagulation factor VII, tissue plasminogen activator, plasminogen activator inhibitor-1, and lipid as cardiovascular risk factors in chronic hemodialysis and continuous ambulatory peritoneal dialysis patients. Am J Kidney Dis, 1996; 27, 848-54.

49. Małyszko J, Małyszko JS, Myśliwiec M. TF and TFPI in CAPD and HD. Nephrol Dial Transplant, 1998; 13, 2424-6.

50. Cella G, Vertolli U, Naso A, Vianello A, Rampin E, Sbarai A, Boeri G, Strauss WE. Tissue factor pathway inhibitor (TFPI) activity in uremic patients during hemodialysis. Thromb Res, 1996; 81: 671-7.

51. Kario K, Matsuo T, Yamada T, Matsuo M. Increased tissue factor pathway inhibitor levels in uremic patients on regular hemodialysis. Thromb Haemostas, 1994; 71: 275-9

52. Collen D. On the regulation and control of fibrinolysis. Edward Kowalski Memorial Lecture. Thromb Haemost, 1980; 43: 77-89.

53. Hoylaerts, M, Rijken, DC, Lijnen, HR, Collen D. Kinetics of the activation of plasminogen by human tissue plasminogen activator. Role of fibrin. J Biol Chem, 1982; 257: 2912-9.

54. Kolev K, Machovich R. Molecular and cellular modulation of fibrinolysis. Thromb Haemost, 2003; 89: 610-21.

55. Nesheim M. Fibrinolysis and the plasma carboxypeptidase. Curr Opin Hematol, 1998; 5: 309-13.

56. Redlitz A, Tan AK, Eaton DL, Plow EF. Plasma carboxypeptidases as regulators of the plasminogen system J Clin Invest, 1995; 96: 2534-8.

57. Booth NA. TAFI meets the sticky ends. Thromb Haemost, 2001; 85: 1-2.

58. Haznedaroglu IC, Erdem Y, Dundar S, Caglar S, Kirazli S. TAT and PAP in hemodialysis patients: two cats in a bag? Thromb Res, 1995; 80: 447-9.

59. Ishii Y, Yano S, Kanai H, Maezawa A, Tsuchida A, Wakamatsu R, Naruse T. Evaluation of blood coagulation-fibrinolysis system in patients receiving chronic hemodialysis. Nephron, 1996; 73: 407-12.

60. Lane DA, Ireland H, Knight I, Wolff S, Kyle P, Curtis JR. The significance of fibrinogen derivates in plasma in human renal failure. Br J Haematol, 1984; 56: 251-60.

61. Alwakeel J, Gader AM, Hurieb S, al-Momen AK, Mitwalli A, Abu Aisha H. Coagulation inhibitors and fibrinolytic parameters in patients on peritoneal dialysis and haemodialysis. Int Urol Nephrol, 1996; 28: 255-61.

62. Opatrny K Jr, Zemanova P, Mares J, Vit L, Opatrna S, Sefrna F, Hejda V, Tomsu M, Eiselt J, Massry SG. Fibrinolysis defect in long-term hemodialysis patients with type 2 diabetes mellitus and its relation to metabolic disorder. Am J Nephrol, 2002; 22: 429-36.

63. Babazono T, Miyamae M, Tomonaga O, Omori Y.Cardiovascular risk factors in diabetic patients undergoing continuous ambulatory peritoneal dialysis. Adv Perit Dial, 1996; 12: 120-5.

64. Nakamura Y, Tomura S, Tachibana K, Chida Y, Marumo F. Enhanced fibrinolytic activity during the course of hemodialysis. Clin Nephrol, 1992; 38: 90-6.

65. Małyszko J, Małyszko JS, Hryszko T, Myśliwiec M. Thrombin Activatable Fibrinolysis Inhibitor (TAFI) and markers of endothelial cell injury in dialyzed patients with diabetic nephropathy. Thromb Haemost, 2004; 91: 480-6.

66. Hryszko T, Malyszko J., Małyszko JS, Brzósko S, Myśliwiec M. Patients on peritoneal dialysis but not on hemodialysis have elevated concentration and activity of Thrombin Activatable Fibrinolysis Inhibitor. Thromb Res, 2001; 104: 322-8.

67. Hryszko T, Małyszko J, Małyszko JS, Brzósko S, Pawlak K, Myśliwiec M. A possible role of thrombin activatable fibrinolysis inhibitor in disturbances of fibrinolytic system in renal transplant recipients. Nephrol Dial Transplant, 2001; 16: 1692-6.

68. Castillo R, Lozano T, Escolar G, Revert L, Lopez J, Ordinas A. Defective platelet adhesion on vessel subendothelium in uremic patients. Blood, 1986; 68: 337-42.

69. Livio M, Marchesi E, Remuzzi G, Gotti E, Mecca G, de Gaetano G. Uremic bleeding: Role of anemia and beneficial effect of red cell transfusions. Lancet, 1982; 11: 1013-5.

70. Gandrille S, Aiach M. Albumin concentration influences fibrinolytic activity in plasma and purified systems. Fibrinolysis, 1990; 4: 225-32.

A grand challenge for research: multimodal, multilevel and multiscale systems in medicine and biology

Coatrieux JL*

Laboratoire Traitement du Signal et de l'Image, INSERM-Université de Rennes 1, Campus de Beaulieu, Rennes Cedex, France

Abstract

Computational modelling, nano-bioscience and information technology in biology and medicine will play a major role in the interdisciplinary attempts to elucidate structures and functions of living systems. Developing tools capable to integrate the new advances and make benefit of them is crucial: accumulation of data and knowledge base with only storage and retrieval capabilities will have a poor impact if they are not made "active" or "operational". This is where models will play a central role in offering, not only sound ways for representation or simulation, but also the appropriate frames to put the players in the right place, with intra- and inter-level coupling and multisource handling. This paper advocated that sequential observations of multiple and complex mechanisms will be of limited interest to understand the inter-relations that are occurring at the same time, and therefore, that designing multimodal, multilevel and multiscale experiments, matched with these models, are of major importance.

Key words: modelling, multimodal data, multilevel descriptions, multiscale processing.

Introduction

There is a large consensus today about the need for convergence of genomics, proteomics, metabolomics, biochemistry, biology and physiology with computer sciences, information technology, mathematics and automatic control. The wealth of new data and knowledge related to sub-cellular and supra-cellular mechanisms calls for smart warehouses allowing efficient queries. The same requirements can be found at individual and population level with the aim to improve the diagnosis decision and the therapeutic means, to better manage health care systems. They all deal with large scale, dynamically varying, non-linear complex systems.

If there is a general agreement on this situation, no clear definition on the ways to carry out such tasks are available. All disciplines are concerned and claim that they are the right places to drive this research while, at the same time, recognizing the need for multidisciplinary or interdisciplinary competences. New educational tracks are open, novel journals and conferences are launched, new attractive keywords are displayed, multiple reports are disseminated, and this does not help in clarifying the most relevant approaches to undertake. It may be more interesting sometimes to have a look back to science and to see if we are not reinventing the wheel by just creating "virtual worlds" with pseudomagic clothes. There is no doubt for instance that interface domains are existing for decades, and the "biomedical engineering" is one example, among many others. Some decline may have been observed in physiology, whose goal is to explore whore organs in their natural context. Structural biology has taken the lead for years and left functions away. Computational modelling has a long experience to share with other fields even if for a long time it has been considered by experimental scientists as a marginal and too abstract way to handle real problems.

This short paper does not pretend to bring "ready-to-use" solutions in this area. However, its goal is to point out some important issues that will be more and more important in the future. Section 2 reviews the last trends illustrating the views recently proposed under different headings and sketches a few important issues to deal with. An overall picture, organized around the multimodal, multilevel, multisource and multiscale concepts and illustrated through two examples, is provided Section 3 before concluding.

* CORRESPONDING AUTHOR:
Laboratoire Traitement du Signal et de l'Image,
INSERM-Université de Rennes 1,
Campus de Beaulieu, 35042 Rennes Cedex, France
Fax: +33 2 2323 6917
e-mail: jean-louis.coatrieux@univ-rennes1.fr (Jean Louis Coatrieux)

Received 25.05.2005 Accepted 01.06.2005

Some trends and key paradigms

A few recent headings

"Integrative" is certainly the most popularised term today in almost all Life Sciences and particularly in Biology and Physiology [1,2]. It is opposed to the "reductionnist" approach whose goal consists to identify molecular and cellular events studied in isolated systems (like it is performed in genomics, proteomics, biochemistry and cell biology). "Integrative" is seen as the studies targeted to the understanding of physiological functions in the context of organ or organ systems. Behind these views, there is the perception that molecular biology can not provide all the answers to understand the genetic, proteomic and cellular mechanisms involved in tissue organization, growth, differentiation, etc. It is striking to see the move from structural to functional, genomics to metabolomics... However, fundamental questions are posed at the same time by this debate. One of the key point is how to derive findings or to extrapolate the observed behaviours to global, *in vivo*, organs or systems at specific life stages. The functional properties, and the structure-to-function features, are among the most concerned.

Systems Biology is another fashionable topic even if it is loosely defined. It deals with studies of intra- and intercellular dynamics, using systems and signal-oriented approaches. One of the goal is to identify structural characteristics and variables in order to derive mathematical models and to simulate the sub-cellular, cellular and supra-cellular dynamics. The emphasis is put here on regulation, prediction and control, signals and information, theoretical modelling, predictive behaviour, all terms referring to engineering and applied mathematical sciences or physics. The cell has been already widely explored: an example of the mammalian cell can be found in [3] where the main circuitry is identified with growth, differentiation and apoptosis controls. The fascinating features of such modelling, and consequently the challenges to face, are related to the sensing capabilities, catalyse reactions, switches, actuators and to the number of distinct inputs/outputs that are present, some being known, others being only approximated or assumed. Many questions for Systems Biology arise about information processing, the transduction pathways, the types of reactions, the non-linear relations involved, the robustness, the role of multiple loops, the mix of discrete and continuous components, etc. These issues are central to Systems Biology and call for advances in mathematical modelling (the recent paper by Sontag [4] argues that automatic control should, in turn, benefit of biological problems by identifying new theoretical problems).

Nanomedicine has emerged very recently and several surveys have been published to analyse their potential opportunities for health [5-8]. Nanomedicine, as defined in [8], aims at "the comprehensive monitoring, repair and improvement of all human biological systems, working from the molecular level using engineered devices and nanostructures to achieve medical benefit". The same report identified nanomaterials and devices, nanoimaging and analytical tools, novel therapeutic and drug delivery systems, as the major technological components to address. It also emphasized the importance of regulatory issues for clinical applications and the need of in-depth toxicological studies, either environmental and clinical. Taking nanoimaging as example (refer to [9] for more details), there is no clear frontiers with what is called "molecular imaging" even if the initial views where mainly directed to medical modalities. In both cases the objective is the *in vivo* measurement and characterization of biological processes at the cellular and molecular level and, beyond the standard anatomical and functional mapping, the *in vivo* detection and quantification of molecular disease markers or therapeutic agents via specific probes. It is expected that early disease manifestations will be detected by enzymes or signalling molecules. Succeeding in such challenges should take time of course and should address many faces among which patient-specific patterns and adverse drug reactions.

All these topics are of course inter-related and represent the many attempts to understand the overall levels of living bodies. The "flags" they display express the search for new paths. They lack sometimes of basic links to major theoretical disciplines and may try to rebuild them on their own, for their specific purpose. The convergence mentioned above aims at merging technology, informatics with mathematics, statistical physics to deal with the many facets to jointly address. Historically, truly pluridisciplinary fields can not be away of this effort for several reasons. First, learning or simply understanding the key concepts coming from other disciplines requires time before really bringing significant contributions. Second, these concepts are confronted to very different practices which also must be acquired. Third, the techniques already in hands may be not sufficient to face the new problems to be solved. Fourth, the amount of data and knowledge is such that it may be difficult to properly adjust the trade-off between the many elementary components and the global properties to take into account. The next section will exemplify a few points in this direction.

Basic questions

Determinist versus stochastic views

Most of the molecular mechanisms involved in gene expression and cellular processes have relied on the paradigm of determinism. In this classical view of a genetic program, a cell differentiates during embryo development upon an input signal and no variability (all cells must react identically to the stimulus) can be expected. The stochastic aspects of cell physiology have been widely discarded. However, there is more and more evidence that, instead to consider on-off schemes, stochastic behaviours have an important role. The notion of "average cell" has been recently discussed [10] and a model was proposed based on a probability for each gene of a single cell to be activated at any time, probability depending of the concentration of transcriptional regulators. Fluctuations at a macrolevel in heartbeats, in regulatory networks, in the activity of neurons or in proteins and nucleic acids, that can in certain cases look like noise, have been recognized as major features. Statistical physics point out that many stochastic processes (say stochastic disorder at the molecular level) can lead to organized structures (e.g. macroscopic level, tissues). There are many problems to be solved before capturing, describing, modelling these fluctuations and understanding how the biological entities control them during normal growth and pathological disorders. We need further

in vivo experiments to elucidate the stochastic rules governing cellular and supra-cellular mechanisms but in-silico models can provide some insights on their plausibility.

From genes to biological organizations

If it is true that genetics has brought many major highlights within the last decades, it can also be questioned. It may be important to consider, for cell differentiation, phenotypic auto-stabilization (differentiated cells stabilizing their own phenotype) and interdependence for proliferation (differentiated cells stimulating the proliferation of alien phenotypes). It has been shown in embryogenesis for instance that these two mechanisms can generate an organized cellular bi-layer structure from two cell types with finite growth, and that their imbalance leads to tissue disorganization and cancer-like growth [11]. These elements suggest that the molecular theory where cells rest or proliferate according to the input signals they receive can be challenged by other views based on quantitative equilibrium between a set of factors involved in tissue organization, including the cellular microenvironment, the tissue structure and, in other words, the whole organism is concerned. The consequence of such view is that both genomic and cellular interactions are involved in tissue organization. The assumptions about our accurate descriptions of elements are perhaps overestimated because much remains to discover at nano-, micro- and macrolevels in living systems. But, it is true, that the study of entire ensembles has to be conducted. The next challenging task is to reassemble them into global pictures in order to capture their proper roles and their key collective properties. This is the objective of the Physiome project [12,13].

Complex systems and complexity

The convergent feeling that we have to understand complex systems is not enough. Complexity must be defined in a more precise way. Von Neumann already in 1966 said about complexity that “none of this can get out of the realm vague statement until one has defined the concept of complication correctly... the simplest mechanical and thermodynamical systems had to be discussed a long time before the correct concepts of energy and entropy could be extracted from them”. Oxford dictionary defines complexity as “comprehending various parts connected together; composite, compound, involved, intricate”. Parts, that can not so easily be separated, are both distinct (large variety and heterogeneity, highly variable behaviours) and connected (with constraints, redundancy and strong dependency). Roughly speaking, we may say that complexity increases when the variety (distinction) and dependency (connection) of parts increase in space, time and function.

However, the number of parts left out the connectivity, what may be of utmost importance, “organisation” and “levels of organisation” (molecules, proteins, cells, assemblies, tissues, organs, systems, etc.). Complexity is sometimes specified as the way in which the whole is different from the composition of its parts. In other words, a complex system should show collective properties that can not be apprehended from their elementary components.

There are two approaches debated on complex systems, either by questioning a given object from multiple disciplines, or, by tackling a specific question transversal to different objects. The latter being the essence of a complex system science, its purpose is to model biological objects, ecological systems, social organizations and also the highly sophisticated man-made systems. It is very likely that both views will be continuing in the future. What is at our mathematical reach today? What can be built on our present physiological/biological knowledge? What generic questions may traverse the all fields? The answers are not much.

Mode, level, source, scale and models

This section is devoted to three major issues that should bring new highlights to undertake some of the biological and the medical problems above mentioned. They are not of course the only topics of interest to address in the future: in each area of engineering science (information processing in its wide sense), in all biomedical and clinical disciplines (understanding of disease and the underlying mechanisms), there are significant advances that may be foreseen. There is no in our mind a hierarchy that should justify to consider these problems as less important than those reported and discussed below. All are participating to the search of answers to basic questions or, equally, to better care of human beings.

Modelling, processing and sensing

The tight connection between data acquisition and data processing is well established. It includes the design of innovative sensors providing vectorial (multichannel or multilead) signals capable to provide observations at nano-, micro- or macroscales. Their main advantage is to achieve a very high time resolution, their drawback being a poor spatial sampling. Conversely, macro-imaging modalities (ranging from the new Multi Slice Computer Tomography – MSCT, high field Magnetic Resonance Imaging to Nuclear Modalities like Positron Emission Tomography – PET) lead to relatively well sampled volume data sets but with still limited time resolution (the exceptions being ultrasound techniques and perhaps the emerging optical devices). A major feature between these two sources of data remains perhaps the relative lack of innovations for devices devoted to physiological signal sensing while the imaging modalities are improving every day and can significantly change our access to pathological patterns. Another trend in clinical devices (mainly in imaging but also through the development of micro-technologies) is the emphasis put on multimodal techniques with either post-registration of data sets or a direct coupling of sources in the same system (for instance CT and PET).

When surveying the recent advances in processing techniques, it is worth noting that a number of new resources are at our disposal: wavelets, time-frequency, blind source separation, particle filtering for signal processing, Kernel methods in data mining, deformable models and level sets in image analysis are among the most well known [14]. All these tools, when applied to data to detect, separate mixture components, extract quantitative features, are aimed at improving decisions, the ultimate goal being early and better diagnosis. They belong to what I call the “surface approach” which means that no explicit

Figure 1. **The basic loop providing the capability from hypothesis generation, to design biological experiments or medical protocols, with the appropriate data acquisition resources, the most efficient information processing means in order to feed, refine and evaluate the relevance of model and the most plausible interpretations. Additional loops can be added (processing to sensing, for instance) if the analysis does not introduce formal representations of physiological knowledge)**

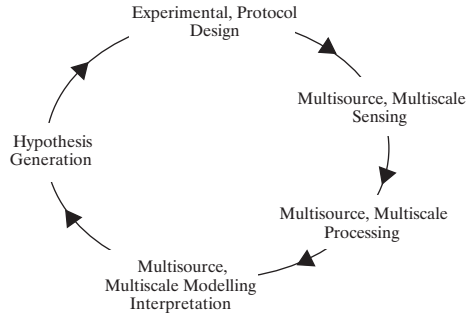
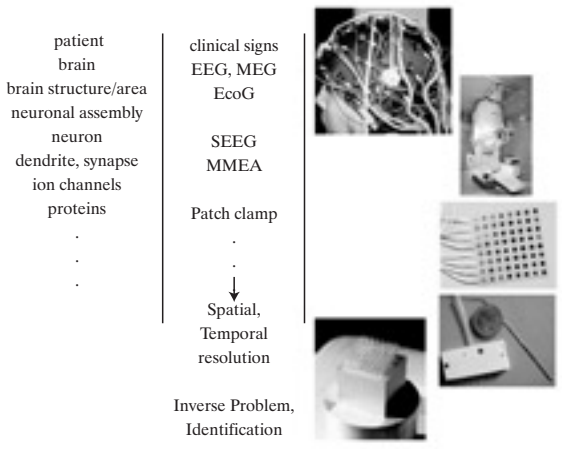


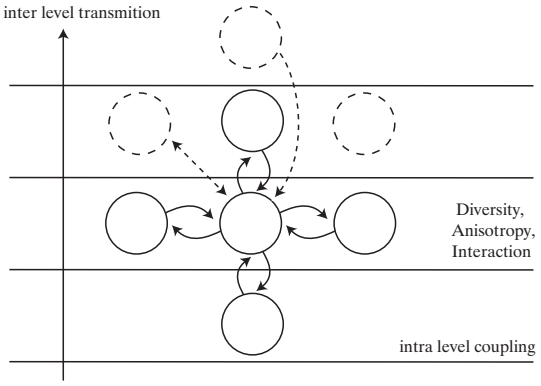
Figure 2. **The multilevel signals that can be recorded during in-vitro and in-vivo observations of cells, neuronal assemblies and main cerebral structures. Pictures from top to bottom display the dense EEG setting, a MEG platform, an electrode array in EcoG, depth electrodes for SEEG and a Multiple Micro Electrode Arrays (MMEA)**



formulation of the pathophysiological mechanisms originating the observed patterns is carried out. I oppose that to the "deep approach" which tries to establish the link, at a given detail level, between these patterns and the underlying mechanisms. The latter requires to design a model of these mechanisms, allowing a physical interpretation of the information conveyed by the data.

These remarks represent the foundation of *Fig. 1*. It is my feeling that the fundamental loop, iterated over time, must include both sensing, processing and modelling. In addition this loop has to integrate both the multimodal, multiscale, multilevel and multisource dimensions. Multimodal means that a specific object, at a given scale, must be observed simultaneously in most, if not all, the physical components (electrical, mechanical, chemical, etc.) that drive its behaviour. We are far from that and such approach requires the design, not only of the appropriate

Figure 3. **A schematic representation of the multilevel challenge. Rows depict the intra-level interactions between similar entities, columns the inter-level connections with close and distant loops**



devices, but also of new experimental protocols, either in biology or in medicine. Multilevel concerns the ability to derive relations between very elementary entities with macrosets constituted by these entities leading to different, collective behaviours (an example of that is the jump from neurons to populations of neurons, see next paragraph). Multisource refers, for instance, to the several features that can be extracted from the data: it is in some way related to multiparametric approaches or the so-called fusion problems. When dealing with image sequence, it will combine both motion information, boundary and region features, intensity-based or topology-based. Multiscale methods call for innovating views to jointly acquired and processed data at fine and large scale. It can be sometimes close to the multilevel concept but when applied to time, it discriminates the long-term dependences to the immediate responses to local events.

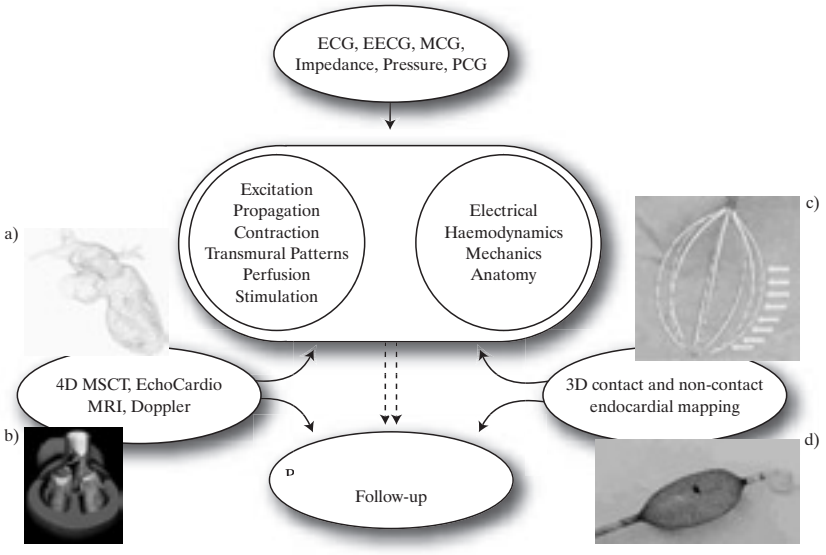
It is the conjunction of these all dimensions with the coupling to physiologically-founded models which is, in our views, very challenging.

An example of multilevel, monomodal, multitimescale system

Let us take the epilepsy case to illustrate our purpose. The data that we may access are identified *Fig. 2*. They go from membrane properties with the ion channels that can observed through patch clamp techniques, to neuronal *in vivo* characteristics available by means of multiple micro arrays (MMA), still limited populations of neurons using stereo-electro-encephalography signals (SEEG), brain structures with electro-corticography (EcoG) up to the brain activities with high density EEG and magneto-encephalography (MEG). These multilevel data represent of course important jumps over description levels, e.g. too rough and sparse to reflect the continuum we are looking for, among which synaptic delays, excitation and inhibition, afferent and efferent connections and loops, conduction paths, etc.

They remain monomodal, e.g. electrically-based mechanisms are observed. However, they provide a first step of the frame required to model and to understand the intra-level coupling and the inter-level transitions (*Fig. 3*). From the multiscale standpoint, we are also interested in short impulses (submil-

Figure 4. **Major measurements and components intervening in clinical diagnosis of cardiac disorders with focus on Cardiac Resynchronization Therapy. Picture (a) is from [Garreau, 2004]** [21]**. Picture (b) is from [Schleich, 2002]** [22]**. Pictures (c) and (d) are from [de Boer, 2000]** [23]



liseconds), potentiation effects over long-range time horizon (i.e. seconds, minutes or more) but also on the immediately adjacent interactions or very distant ones (from nanometers up to millimeters or more). Models here should play a major role to simulate and provide insights on the plausible roles of neuronal network topologies (chains, lattices, fully-connected graphs), on the mutual synchronization of cells (uniform or non-uniform pulse-coupled oscillators), on travelling waves and non-linear dynamics, etc. A lot of models have been developed to render the functional behaviours at different levels. The reader interested in this area can refer to the compartmental model [15], recently investigated in networks with axo-axonal gap junctions [16], the work of Nunez [17] putting emphasis on the delays due to axonal conduction and long-range interactions, population models [18,19] and the efforts devoted to link micro and macro behaviours by Wright and Liley [20].

An example of multimodal, monolevel approach

The key dimensions for further advances in clinical diagnosis and therapy of cardiac disorders are reported *Fig. 4*. Only a few of them, that we consider as major issues to deal with, will be discussed here. The integration of multimodal imaging data is a critical issue [24]. It starts with the diagnosis tools providing the 2D, 3D and 4D elements to capture local, regional and global characteristics required to determine the morphological and functional patterns of the heart, either normal or abnormal. The progress in ultrasound techniques, and in Multi Slice CT allows now to acquire 3D time image sequences with high contrast and spatio-temporal resolutions. The major problems, beyond spatio-temporal registration methods aimed at deriving a common coordinate system, are to extract quantitative features that can be physically and physiologically interpreted with a proper anatomical reference. Accurate and reliable segmentation methods, fulfilling the time computation constraints in clinical practice, with robust motion estimation algorithms and perfusion parameters have to be combined in a sound information processing frame in order to get a full view of the status of the heart. Electrical catheter-based mapping [25-27] is a relevant complement of the imaging sources for electrophysiological tracking but they have the inconvenient to be invasive, expensive and to increase the time duration of the exploration, and as such put more clinical demands.

The physiopathological in-silico modelling of the heart capable to fuse together the patient specific features (i.e. electrical, mechanical and perhaps more importantly the electromechanical, mechanochemical, etc.) with the corresponding anatomical structures into generic models integrating the last data obtained through *in vitro*, *ex vivo* and *in vivo* experiments is perhaps the grand challenge for tomorrow. A lot of efforts have been devoted to the restitution of the electrophysiological activity of the heart and two main model families can be distinguished (refer to [28, 29] for full references): 1. simplified models, which are limited to the simulation of an action potential waveform, without taking into account any sub-cellular process, such as the Fitzugh-Nagumo's model (which was later improved by Aliev and Panfilov) or the model proposed by van Capelle and Durrer and 2. electrophysiologically detailed models: which are based on the Hodgkin-Huxley approach for modelling ionic currents. A variety of models have been proposed for the later type, with increasing levels of detail and for specific myocardial tissues (i.e. ventricular, atrial, or Purkinje myocites). Large-scale electrical models have been developed [30,31], some being mapped to 3D anatomical data [32,33] but the key issue remains the inverse problem, i.e. the identification of the system from the current observed data. However, even if it is not out of reach, we are still far to deal with the full complexity of cardiac mechanisms. To just take an example, the excitation-contraction coupling, which refers to the physiological processes linking myocite depolarisa-

tion and contraction, involves many structural and regulatory proteins whose nature and function are just emerging [34].

Merging the multifunctional models we need to face electrical, mechanical, haemodynamic facets, at different scales, distinct supports, time dynamics with the multimodal data that we have at our disposal, would directly impact our capability to diagnose and care. Further clinical advances should rely on the design of intelligent devices, implantable or not, able to handle the several variables required, with both real-time recording, processing, stimulation capabilities.

Conclusions

The topics addressed in this paper are a few among many fields where major emerging research is carried out. They are at the exact convergence point between biology, medicine, physics, mathematics and engineering science. The future is open due to the amount of knowledge that are currently acquired and the challenging work to perform before really achieving significant breakthroughs. Engineering – with competences in computer science (database management), automatic control (modelling and control), information processing (recognition and fusion for signals and images), microtechnology (sensing devices) – must be fully part of this future. Moreover, biomedical engineering and medical informatics are key players because they are used to interdisciplinary research, to design pertinent experiments and also to take care of the all aspects involved between a research finding and its transformation into a product with an health care impact.

Acknowledgement

The author is indebted to many colleagues and friends for the active discussions and exchanges both in the LTSI (A Bardou, JJ Bellanger, A Hernandez, F Wendling) and outside (P Bourgine, P Chauvel, J Demongeot, Y Meyer).

References

1. Coatrieux JL. Integrative science: A modelling challenge. IEEE Eng Med Biol, 2004; 23(1): 12-4.

2. Coatrieux JL. Integrative science: place and future of the model-based information processing. IEEE Eng Med Biol, 2004; 23(4): 19-21.

3. Hanahan D, Weinberg RA. The Hallmarks of cancer. Cell, 2000; 100: 57-70.

4. Sontag ED. Some directions in control theory inspired by systems biology. Syst Biol, 2004; 1(1): 9-18.

5. Commission of the European Communities Communication: Towards a European Strategy for nanotechnology, EU, DG Research, Brussels, 2004.

6. NIH Roadmap. Nanomedicine, NIH, USA, 2004. Newman MEJ, Models of the small-world: a review. J Stat Phys, 2000; 101: 819-41

7. UK Royal Society and Royal Academy of Engineering: Report on Nanoscience and Nanotechnologies, Opportunities and Uncertainties, UK, 2004.

8. ESF Scientific Forward Look on Nanomedicine, European Science Foundation, Strasbourg, France, 2005.

9. Li KCP Ed. Molecular Imaging I-II. Special Issues, IEEE Eng Med Biol Magazine, 2004: 23: 4-5.

10. Levsky JM, Singer RH. Gene expression and the myth of the average cell. Trends Cell Biol, 2003; 13: 4-6

11. Laforge B, Guez D, Martinez M, Kupiec JJ. Modeling embryogenesis and cancer: an approach based on an equilibrium between the autostabilization of stochastic gene expression and the interdependence of cells for proliferation. Prog Biophys Mol Biol, 2004.

12. Crampin EJ, Halstead M, Hunter P, Nielsen P, Noble D, Smith N, Tawhai M. Computational physiology and the Physiome Project. Exp Physiol, 2004; 89: 1-26.

13. Demongeot J, Bezy-Wendling J, Mattes J, Haigron P, Glade N, Coatrieux JL. Multiscale modeling and imaging: the challenges of biocomplexity. Procedings of IEEE, 2003; 91(10): 1723-37.

14. Coatrieux JL. Integrative science: biosignal processing and modelling. IEEE Eng Med Biol, 2004; 23(3): 9-12.

15. Traub RD, Jefferys JGR, Miles R, Whittington MA, Toth K. A branching dentritic model of a rodent CA3 pyramidal neurone. J Physiol, 1994; 481: 79-95.

16. Traub RD, Schmitz D, Jefferys JG, Draghun A. High frequency population oscillations are predicted to occur in hippocampal pyramidal neuronal networks interconnected by axoaxonal gap junctions. Neuroscience, 1999; 92(2): 407-26.

17. Nunez PL. Neocortical dynamics and human EEG rhythms. New York-London, Oxford University Press, 1994.

18. Wendling F, Bellanger JJ, Bartolomei F, Chauvel P. Relevance of nonlinear lumped-parameter models in the analysis of depth-EEG epileptic signals. Biological Cybernetics, 2000; 83: 367-8.

19. Wendling F, Bartolomei F, Bellanger JJ, Chauvel P. Epileptic fast activity can be explained by a model of impaired GABAergic dendritic inhibition. Eur J Neurosc, 2002; 15(9): 1499-508.

20. Wright JJ, Liley DTJ. Dynamics of the brain at global and microscopic scales: neural networks and the EEG. Behavioral and Brain Sciences, 1996; 19: 285-320.

21. Garreau M, Simon A, Boulmier D, Guillaume H. Cardiac Motion Extraction in Multislice Computed Tomography by using a 3D Hierarchical Surface Matching Process. IEEE computers in Cardiology, Chicago, 2004.

22. Schleich JM, Dillenseger JL, Andru S, Coatrieux JL, Almange C. Understanding normal cardiac development using animated models. IEEE Computer Graphics and Applications, 2002; 22: 14-9.

23. De Boer IH, Sachse FB, Mang S, Dössel O. Methods for determination of electrode positions in tomographic images. International Journal of Bioelectromagnetism, 2000: 2-2.

24. Roux C, Coatrieux JL. Contemporary perspectives on Three Dimensional Biomedical Imaging. IOS Press, Amsterdam, 1997.

25. Gepstein L, Hayan G, Ben-Haim SA. A novel method for non-fluoroscopic catheter-based electroanatomical mapping of the heart: in vitro and in vivo accuracy results. Circulation, 1997; 95: 1611-22.

26. Schilling RJ, Peters S, Davies DW. Simultaneous endocardial mapping in the left ventricle using a noncontact catheter: comparison of contact and reconstructed electrograms during sinus rhythm. Circulation, 1998; 98: 887-98.

27. De Bakker JMT, Hauer RNW, Simmens TA. Activation mapping: unipolar versus bipolar recording. In: Zipes DP, Jalife J Eds, Cardiac Electrophysioloy: from cell to bedsite. 3rd Edition Philadelphia, Saunders, 2000; 1068-78.

28. Bardou A, Auger P, Birkui P, Chasse JL. Modeling of cardiac electrophysiological mechanisms: from action potential genesis to its propagation in myocardium. Crit Rev Biomed Eng, 1996; 24: 141-221.

29. Virag N, Blanc O, Kappenberger L. Computer simulation and experimental assessment of cardiac electrophysiology. Futura Publishing, 2001.

30. Noble D, Winslow RL. Reconstruction of the heart: network models of SA node-atrial interaction, in Computational Biology of the Heart. Wiley & Sons, 1997; 49-64.

31. Quan W, Evans SJ, Hastings HM. Efficient integration of a realistic two-dimensional cardiac tissue model by domain decomposition. IEEE Trans Biomed Eng, 1998; 45(3): 372-85.

32. Hunter PJ, Nash MP, Sands GB. Computational electromechanics of the heart, in Computational Biology of the Heart. Wiley & Sons, 1996; 345-407.

33. Rudy Y. Insights from theoretical simulations in a fixed pathway. J Cardiovasc Electrophysiology, 1995; 6: 294-312.

34. Bers D. Cardiac excitation-contraction coupling. Nature, 2002; 415: 198-205.

New approaches to health promotion and informatics education using Internet in the Czech Republic

Zvárová J*

European Center for Medical Informatics, Statistics and Epidemiology, Institute of Computer Science,
Academy of Sciences of the Czech Republic, Prague, Czech Republic

Abstract

The paper describes nowadays information technology skills in the Czech Republic. It focuses on informatics education using Internet, ECDL concept and the links between computer literacy among health care professionals and quality of health care. Everyone understands that the main source of wealth of any nation is information management and the efficient transformation of information into knowledge. There appear completely new decisive factors for the economics of the near future based on circulation and exchange information. It is clear that modern health care cannot be build without information and communication technologies. We discuss several approaches how to contribute to some topics of information society in health care, namely the role of electronic health record, structured information, extraction of information from free medical texts and sharing knowledge stored in medical guidelines.

Key words: e-health, e-learning, ECDL, medical guidelines, electronic health record.

Introduction

Modern information and communication technologies have strongly influenced health care. The health care sector has to face enormous acceleration in appearance of new knowledge, in development of new technologies and technical devices, new drugs, as well as spread of new diseases. The patient benefit, professional competence and service excellence can be facilitated by the achievements of information and communication technology. Consequently, the ability to use this technology – computer literacy, seems to become an imperative for health care professionals and other staff involved in health care delivery [1].

What are information technology skills in the Czech Republic? At the end of the year 2003, about 30% of the population was using the Internet. However, one tenth of all Internet users were students, and more than half were less than 25 years old. We are speaking in the European programs about e-accessibility. It is the basic assumption for the future application of e-Health. In the period of 2002-2004 the Czech Republic run domestic online programs as a part of the Action Realization Plan of the State Information Politics. The programs were designed to introduce new information and communication technologies in Czech health care. However, coming from the figures, we can see a local portion of PC-equipped households in the Czech Republic. Now about 13% of the households in the Czech Republic are equipped with a PC. That means a low possibility to introduce the advanced concepts of health telematics to the home care applications.

Nowadays, informatics education using Internet is strongly increasing in Czech universities and other institutions. They organize graduate, postgraduate (doctoral) and long-life education in the field of biomedical and health informatics to fill in the gap. The links between computer literacy and quality of health care as well as the need for computer literacy among health care professionals is obvious. Health care in general is not the fastest in utilizing information technology to its full benefit. Due to new achievements in electronic heath record and telemedicine we can expect great changes in health care services.

Telemedicine contributes very strongly to a new model of health care. Information technologies (IT) start to be a part of professional practice in health care. Patients also have easy access to vast amounts of information and they are encouraged to use it. Use of information technology greatly enhances the

* CORRESPONDING AUTHOR:
European Center for Medical Informatics, Statistics and Epidemiology,
Institute of Computer Science,
Academy of Sciences of the Czech Republic,
Pod Vodarenskou vezi 2
182 07 Prague, Czech Republic
Fax: +42 28 6581453
e-mail: zvarova@euromise.cz (Jana Zvárová)

Received 25.05.2005 Accepted 31.05.2005

access to and flexibility of education; e-learning has become an attractive and cost-effective addition to traditional methods of education and training. Informed and empowered patients are able to be involved in decision making about own treatment. To this environment we introduced some concepts that are actively promoting e-accessibility among patients and health professionals. For example in the Czech Republic the ECDL concept has been applied, new courses, electronic books, web pages and evaluation systems using internet (e.g. ExaME system) have been developed and applied. The research and development under the National Research Plan have been strongly focused on information society in health care. Further we will give more details on some new approaches to health promotion and informatics education using Internet connected with research and education in the Czech Republic and we mention several activities managed by the EuroMISE Center (European Centre for Medical Informatics, Statistics and Epidemiology) in the health oriented research and education (www.euromise.cz).

Informatics Education using Internet

Links between computer literacy and quality of health care as well as the need for computer literacy among health care professionals is obvious. Nowadays, informatics education using Internet is strongly increasing in Czech schools and other institutions. The concept of ECDL has been introduced as one of the tools to assure the quality of informatics education.

The need for computer literacy among professionals has been acknowledged and has been recognised as one of the priorities for developments in the European countries. IT professionals joined in the Council of European Professional Informatics Societies (CEPIS, www.cepis.com) defined the concept of the European Computer Driving Licence (ECDL, www.ecdl.com) based on the results of the European project. The underlying intention was to offer the means of certifying knowledge of essential concepts of IT, and competence in the use of a personal computer and of common computer applications. To reach this goal the ECDL Syllabus defines the term 'computer literacy' and classifies 7 domains (modules) within this term. These are: basic concepts of IT, using the computer and file management, word processing, spreadsheets, databases, presentations, and information and communication. Each module lists the facts to be known and the skills to be mastered for a candidate to achieve the ECDL certification. The ECDL testing was launched in 1997 and has quickly attracted the attention of both the general public and employers in Europe and oversees. In the Czech Republic the ECDL testing started in the year 2001 and has been supervised by the Czech Society of Cybernetics and Informatics (www.cski.cz) [2]. In the year 2004 the number of the issued certificates increased to 5330. Total numbers of indexes, certificates, accredited centres and testers are given in *Tab. 1*.

The ability to manage modern communication tools such as Internet services is one of the most important prerequisites for the development and use of e-education, especially in the lifelong learning. Now we describe the Czech program of postgraduate doctoral studies in Biomedical informatics where

Table 1. **Total numbers of indexes, certificates, accredited centres and testers in the years 2001-2004**

Year	Absolute number	Cumulative number			
	Issued Indexes	Issued Indexes	Issued Certificates	Testing Terms	Accredited Centres
2001	3880	3880	863	966	6
2002	1980	5860	1887	1806	40
2003	3232	9092	3471	2916	65
2004	3905	12997	5330	3281	97

Internet is widely used in education and training activities [3]. The agreement on cooperation of Charles University in Prague and Academy of Sciences of the Czech Republic in postgraduate doctoral studies was signed on April 23rd, 1997. The main goal of this agreement has been cooperation in development and running of joint education and training of young researchers. Based on this agreement the system of postgraduate doctoral studies in biomedicine has been opened (www.kav.cas.cz/pdsb). There is now 19 boards of scientific disciplines in postgraduate doctoral studies in biomedicine, one of them the board of Biomedical Informatics. EuroMISE Centre is the joint workplace of Charles University in Prague, Institute of Computer Science of the Academy of Sciences of the Czech Republic, University of Economy in Prague, General University Hospital in Prague and Municipal Hospital in Caslav participates highly in the biomedical informatics education and training. New teaching methods and tools, based on nowadays information and communication technologies using Internet, have been developed. New books, their electronic versions and corresponding knowledge bases for evaluation of students' knowledge were published in two opened editions Biomedical Informatics and Biomedical Statistics [4-7]. Their electronic versions are available on Internet (www.euromise.cz). Moreover, advanced system ExaMe for evaluation of knowledge using Internet in distant and open education was introduced [8]. Since 2001 the system ExaMe has been regularly used in different courses developed by the EuroMISE Centre. *Fig. 1* shows the screen of the ExaMe test for evaluation of knowledge in the course "Statistics in Biomedical Research". More details about the system ExaMe can be found in [9].

Telemedicine contribution to a new model of health care

The national program of research (NPR) and development in the Czech Republic was elaborated in two foresights organized in the years 2001 and 2003. They significantly helped to formulate the content and structure of the key research topics for NPR. More then 500 experts evaluated various topics independently and set-up their preferences. The detailed SWOT analysis was applied to quantify the results of expert reports and evaluation. The Czech government approved the proposal of the first foresight for NPR in April 2003. The research priorities were implemented in the call for proposals opened in February

Figure 1. **The screen of the ExaMe test in the course "Statistics in Biomedical Research"**

Figure 3. **Application of the MUDRlite in stomatology**

2004 for the period 2004-2008. Several of the core areas covered by IT disciplines were focused on the health care issues directly or the health care domain, where Internet and telemedicine play a very important role.

Let us mentioned objectives of the project "Information technologies for development of continuous shared health care" selected in the first call for proposals of NPR. The project deals with development of methods and technologies for continous shared health care in information society. Remote approach to information (data, knowledge) stored in patient health documentation is the central issue of the project. Electronic Health Record (EHR) is a basic assumption for telemedical applications. The research results on universal multimedia electronic health record have been implemented. In the Czech Republic results of the research on MUltimedia Distributed electronic health Record (MUDR) have started to be exploited [10] in different forms, including applications of PDA. Basic features of the MUDR EHR are:

- Tree level architecture
- Separation of values from description of features (knowledge base)
- Representation using both knowledge base and real values by graph structure
- Unified view on data of any type
- Presentation of data in many languages
- Integration of systems for decision support, e.g. medical guidelines.

Figure 2. **The screen of the MUDR EHR showing a structured way of storing data**

However, the main objective of the research is to cover the widest area of patient data by structured form, see *Fig. 2*. For applied research it is very important to verify developed methods and technologies in health care practice. MUDRLite application is the direct reaction on topics generated by Czech physicians. For example the version of the MUDR record with applications in stomatology can work on stand-alone workstation without connection with a remote server, see *Fig. 3*. Moreover, proposed solutions can be also verified in two ambulatories of preventive cardiology supervised by both hospitals of the EuroMISE Centre. The project has been also focused on the design and evaluation of a proper infrastructure supporting management of electronic health care documentation and on the development of a health care system for continuous shared care about citizens among various hospitals and general practitioners. One of possible applications is the use of Internet in sharing data among physicians and patients at remote places. We have developed the e-Health portal (www.euromise.cz) where online advice can be given by physicians to patients on distance under valid Czech and European legislative rules of medical data security, safety and privacy. Further issues of continuous shared care are connected with partial automatic structuring of information coved by free text medical record and promoting medical guidelines. Automatic extraction of structured data from medical records written in free text has been done by methods based on analysis using regular rules. Application was tested on medical records from General Faculty Hospital and Municipal Hospital in Caslav [11] and embedded in the MUDR EHR, see *Fig. 4*.

Finally, some knowledge acquired in medicine is possible to represent by medical guidelines, which make decision process in concrete case more easy and transparent. For computer implementation and processing, it is necessary to have medical guidelines explicitly structured. The most important and nowadays mostly used is the GLIF (Guideline Interchange Format) model. The main goal of GLIF is to enable sharing of guidelines among institutions and across computer applications. The GLIF browser is designed as a general tool that can present any formalized medical guidelines in a user-friendly manner. It can be

***Figure 4*. Embedded analysis of free medical text in MUDR EHR**

used for education of students and as a decision support system in medical practice. Our method for automatic comparing of medical guidelines with EHR has the following advantages [12].

General applicability. System based on proposed method can work with arbitrary guidelines. Only at first the guidelines must be transformed into GLIF graph model. The transformation of free text guidelines into GLIF model or some similar structured and precisely defined formal model should be accomplish anyway, because only in this way one can be certain, that guidelines are unambiguous and non-contradictory.

Facilitation of changes in the system. When some part of guidelines is changed, it is not necessary to correct the set of rules used for checking of input data. What is sufficient to do is making corresponding change in guideline model.

Support of distributed computing. The guidelines models can be maintained on a server running at one site and EHR and the system for its comparing with GLIF model and GLIF browser on a user computer.

A method of medical guidelines modelling in GLIF and its implementation in XML have been developed and first version of Processing Medical Guidelines is available on the web pages (www.euromise.cz).

Conclusions

The ability to manage modern communication tools such as Internet services is one of the most important prerequisites for the development and use of e-education (especially in the area of lifelong education) and e-Health applications. The computer literacy and how to prove it, it is a very important task for today. We discussed the role of the ECDL concept for measuring of computer literacy in population. Nowadays, younger groups are more confident in Internet services than the older people. Therefore it is very difficult to implement some e-health application concerning home care, care for elderly or disabled people. However, links between computer literacy among health care professionals and quality of health care are obvious. Everyone understands that the main source of wealth of any nation is information management and the efficient transformation of information into knowledge. There appear completely new decisive factors for the economics of the near future based on circulation and exchange information. It is clear that modern health care cannot be build without information and communication technologies. Telemedicine is largely based on electronic health record and new methods and tools how to structure information, extract information from free medical texts and sharing knowledge is the main objective of many research projects.

Acknowledgement

The work was partly supported by the grant number 1ET200300413 of the Academy of Sciences of the Czech Republic and by the Institutional Research Plan AV0Z10300504.

References

1. Final Report of the project “Statistical Indicators Benchmarking the Information Society” (http://www.scac.cz/SIBIS/); 2004.
2. Stepankova O, Engova D. Continuing Education in e-Age. To be published in Methods of Information in Medicine; 2005.
3. Zvárová J, Svaćina Š. New Czech Postgraduate Doctoral Program in Biomedical Informatics. In: Surján G, Engelbrecht R, McNair P, editors. Health Data in the Information Society, Proceedings of MIE 2002, Amsterdam, IOS Press; 2002, p. 766-76.
4. Zvárová J. Basic Statistics for Biomedical Disciplines, Charles University, Prague, Carolinum; 2001, 2004.
5. Zvárová J, Mazura I. Stochastic Genetics Charles University, Prague, Carolinum; 2001.
6. Bencko V, Hrach K, Malý M, Pikhart H, Reissigová J, Svaćina Š, Tomečková M, Zvárová J. Statistical Methods in Epidemiology, Charles University, Prague, Carolinum; 2003.
7. Zvárová J, Hanzlíček P, Hejl J, Jirkovec Z, Pikhart H, Pribík V, Smitková V, Zvára K. Basic Informatics for Biomedicine and Healthcare Charles University, Prague, Carolinum; 2002.
8. Zvárová J, Zvára K. Evaluation of Knowledge using ExaMe program on the Internet. In: Iakovidis I, Maglaveras N, Trakatellis A, editors. User Acceptance of Health Telematics Applications, Amsterdam, IOS Press 2000; p. 145-51.
9. Martinková P, Zvára K jr, Zvárová J, Zvára K. The New Features of the ExaMe Evaluation System and Reliability of its Fixed Tests. Met Inform Med; 2005, in press.
10. Hanzlíček P, Špidlen J, Nagy M. Universal Electronic Health Record MUDR. In: Duplaga M, Zielinski K, Ingram D, editors. Transformation of Healthcare with Information Technologies, Amsterdam: IOS Press, 2004; p. 190-201.
11. Semecký J, Zvárová J. On Regular Analysis of Medical Reports. Proceedings of NLPBA 2002, Cyprus, 2002; p. 13-6.
12. Veselý A, Zvárová J, Peleška J, Buchtela D, Anger Z. Medical Guidelines Presentation and Comparing with Electronic Health Record. Int J Med Inform; 2005, in press.

Peroxisome proliferator-activated receptor γ ligand prevents the development of chronic pancreatitis through modulating NF-κB-dependent proinflammatory cytokine production and pancreatic stellate cell activation

Hisada S, Shimizu K*, Shiratori K, Kobayashi M

Department of Gastroenterology and Pathology, Tokyo Women's Medical University, School of Medicine, Tokyo, Japan

Abstract

Purpose: Thiazolidinedione derivatives (TZDs) are known to be ligands of peroxisome proliferator-activated receptor γ (PPARγ). In this study, we investigated the effect of a TZD, troglitazone, on inflammation and fibrogenesis in the pancreas of an experimental model of chronic pancreatitis.

Material and methods: Male WBN/Kob rats with spontaneous chronic pancreatitis were fed rat chow containing 0.2% troglitazone from 1 to 4 months of age. Immunohistochemical studies of rat pancreas were carried out with monoclonal mouse antibody against human alpha-smooth muscle actin (α-SMA) or rabbit polyclonal antibody against collagen type I, collagen type III, or fibronectin. Cytokine production was measured by enzyme-linked immunosorbent assay. The inhibitory action of troglitazone on nuclear factor-κB (NF-κB) binding activity in activated macrophages was also investigated.

Results: Long-term administration of troglitazone reduced inflammatory cell infiltration and fibrosis in the pancreas of WBN/Kob rats, and expression of α-SMA, procollagen I, III, and fibronectin was significantly reduced by troglitazone. The increase in TNF-α production by activated macrophages was significantly decreased by troglitazone. Peritoneal macrophages isolated from WBN/Kob rats produced a large amount of TNF-α, whereas those from troglitazone-treated WBN/Kob rats produced only a marginal amount of TNF-α. Lipopolysaccharide-induced NF-κB binding activity in peritoneal macrophages was also significantly reduced by troglitazone.

Conclusions: Troglitazone prevented the progression of chronic pancreatitis via inhibition of ECM synthesis and proinflammatory cytokine production mediated by the inhibition of NF-κB activity.

Key words: thiazolidinedione derivatives, chronic pancreatitis, PPARγ, TNF-α, NF-κB.

* CORRESPONDING AUTHOR:
Department of Gastroenterology
Tokyo Women's Medical University, School of Medicine
8-1 Kawada-cho, Shinjuku-ku
Tokyo 162-8666, Japan
Tel: 81-3-3353-8111; Fax: 81-3-5269-7435
e-mail: kyoko@ige.twmu.ac.jp (Kyoko Shimizu)

Received 20.06.2005 Accepted 23.06.2005

Introduction

Chronic pancreatitis is an irreversible, progressive disease characterized by pancreatic exocrine and endocrine dysfunction and by morphological damage, including inflammatory responses, subsequent fibrosis, and destruction of acinar and duct cells. Thiazolidinedione derivatives (TZDs) are known antidiabetic agents and ligands for peroxisome proliferator-activated receptor γ (PPARγ), a member of the nuclear receptor superfamily of transcription factors [1-5]. We previously reported that a TZD, troglitazone, improves exocrine pancreatic function in rats with streptozotocin-induced diabetes mellitus and inhibits the progression of pancreatic damages and pancreatic exocrine insufficiency in an animal model of chronic pancreatitis through morphological improvement of inflammation and fibrogenesis [6,7]. Pancreatic stellate cells (PSCs) have been characterized as the major source of extracellular matrix and cytokine production and as playing a pivotal role in the fibrogenesis in chronic pancreatitis [8]. We have reported finding that troglitazone prevents the progression of chronic pancreatitis by interfering with the fibrogenic action of PSCs in an *in vitro* study [9], and PPARγ ligands have been reported to inhibit inflammatory cytokine production [10,11]. Su et al. found that PPARγ ligands reduce colonic inflammation in a mouse model of inflammatory bowel disease by down-regulating proinflammatory cytokine production at the transcriptional level through inhibition of activation of a transcription factor, nuclear factor-κB (NF-κB) [12]. In the present study, we found that long-term

administration of a PPARγ ligand prevents the progression of chronic pancreatitis by modulating the pancreatic inflammatory and fibrogenic response through inhibition of NF-κB-dependent proinflammatory-cytokine production.

Material and methods

Animals and experimental protocol

One-month-old male WBN/Kob rats were fed 0.2% troglitazone (supplied by Sankyo Company Ltd, Tokyo, Japan) containing rat chow for 3 months, and male WBN/Kob rats and male Wistar rats were fed the same rat chow without troglitazone, as controls. For the morphological studies, pancreata were fixed overnight in freshly prepared 4% paraformaldehyde in PBS (pH 7.2) before embedding them in paraffin by the standard method.

Morphological examination of the pancreas

After deparaffinizing the sections and rehydrating them through a xylene and ethanol series, they were stained with hematoxylin-eosin or Masson's trichrome stain and evaluated histopathologically. To measure the area of the pancreas that was fibrotic, the whole pancreas was divided into a splenic half and a duodenal half, and each half was sliced at 3 mm intervals to provide cross-sections of tissue. The area of the fibrotic tissue identified by staining with Masson's trichrome was calculated in all specimens with image analysis software (SPICA II, Olympus Optical Co., Tokyo, Japan). The proportion (%) of the pancreas that was fibrtoric was calculated thus: (fibrotic area / total area of the specimen x 100).

Immunohistochemical study

Serial sections were used for the immunohistochemical study. The protocol basically consisted using the avidin-biotin complex method and reagents provided by Vector Laboratories Inc (Burlingame, CA). Briefly, sections were incubated overnight at 4°C with monoclonal mouse antibody directed against human alpha-smooth muscle actin (α-SMA), rabbit polyclonal antibody against collagen type I, collagen type III, or fibronectin (DakoCytomation, Kyoto, Japan), and then incubated with a biotinylated secondary antibody. Peroxidase staining was performed with 3,3'-diaminobenzidine (DAB). Immunoglobulin from non-immunized animals was substituted for the primary antibody to provide a negative control.

Protein extraction and western blot analysis

The pancreas was homogenized in lysis buffer containing 0.15M NaCl, 50 mM Tris-HCl (pH 7.2), 1% deoxycholic acid, 1% Trion X-100, 0.1% SDS, and 1 mM PMSF, plus 5 μg/ml of each of the following proteinase inhibitors: pepstatin, leupeptin, chymostatin, antipain, and aprotinin. After centrifuging the homogenates at 15,000 rpm for 10 min at 4°C, the protein concentrations were determined. A 25 μg sample of protein was separated by SDS-PAGE and transferred electrophoretically to the membrane, and nonspecific binding was blocked by 1-hour incubation of the membrane in 3% low-fat milk in PBS (pH 7.6). The blots were then incubated with monoclonal mouse antibody directed against α-SMA. Following incubation with peroxidase-conjugated secondary antibodies, signals were developed by a chemiluminescence system.

Isolation of peritoneal macrophages

The abdomen was opened and lavaged with 5 ml of cooled PBS. The peritoneal lavage fluid was then collected and centrifuged at 1500 rpm for 15 min. The cell pellet was washed once in PBS and resuspended at a concentration of 2×10^6 cells/ml in RPMI 1640 medium supplemented with 10% FCS, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were plated in chamber slides and cultured at 37°C under a 5% CO_2 atmosphere for 2 hours. Nonadherent cells and the medium were then removed, and the adherent cells were washed three times with PBS and used as peritoneal macrophages.

Measurement of the concentration of TNF-α released by peritoneal macrophages

To measure the TNF-α released by peritoneal macrophages, cells isolated from male WBN/Kob rats were resuspended at a concentration of 1×10^5 cells/200 ml/well in 10% FBS-RPMI 1640 medium containing 100 U/ml penicillin and 100 mg/ml streptomycin and cultured at 37°C under 95% O_2 and 5% CO_2 for 3 days. The culture supernatant was used to determine the TNF-α concentration. Whether troglitazone modulates TNF-α secretion by activated macrophages was also investigated. Freshly isolated peritoneal macrophages (1×10^6 cells/ml) from male Wistar rats were grown for 18 hours in standard medium, and then for 2 hours in fresh medium containing troglitazone, after which the cells were cultured in the presence of lipopolysaccharide (LPS) (10 ng/ml) for 3 days. TNF-α in the supernatant released by the peritoneal macrophages was measured with an enzyme-linked immunosorbent assay (ELISA) kit (Genzyme, USA).

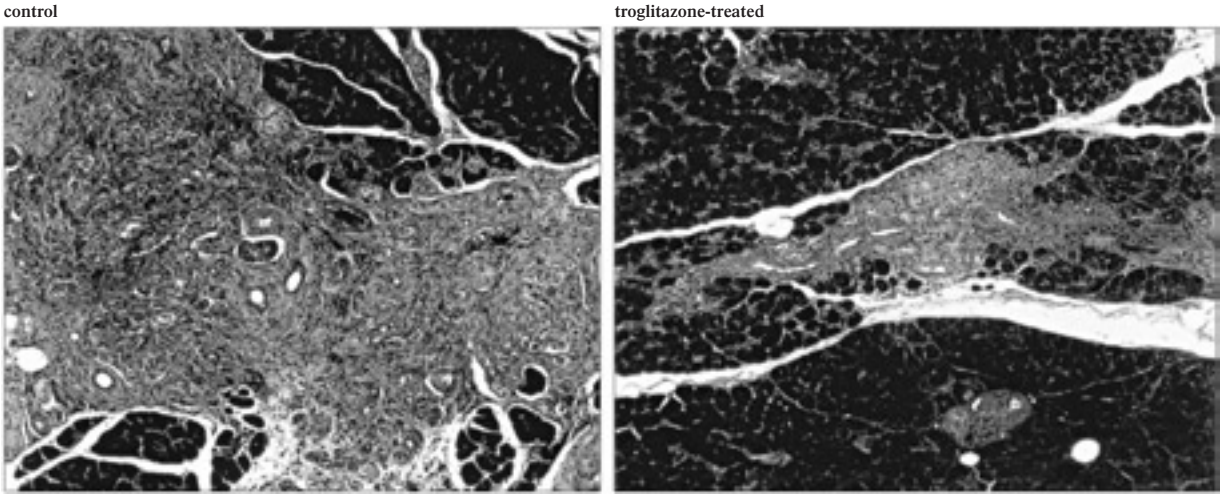
Nuclear protein extracts

Peritoneal macrophages isolated from male Wistar rats were exposed to LPS for 3 hours in the presence or absence of troglitazone, and the cells were harvested for nuclear extract preparation. Cells were washed twice in cold PBS and lysed in 1.2 ml of ice-cold hypotonic lysis buffer (10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, pH 8.0, 1 mM DTT, TritonX 0.5% containing protease inhibitor) for 30 min on ice, and centrifuged at 6500 rpm for 1 min. The pellets were resuspended in 50 μl of nuclear extraction buffer (20 mM HEPES, pH 7.9, 400 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, pH 8.0). The lysate was incubated for 20 min, and an aliquot of the culture supernatant was stored at -80°C.

Electrophoretic mobility shift assay (EMSA)

EMSA was performed by using a gel shift assay system kit (Promega, USA). Complementary oligonucleotides were ^{32}P-end-labeled with T4 polynucleotide kinase. Oligonucleotide probes were incubated with 10 μg of nuclear extracts in binding buffer with or without unlabeled competitor or noncompetitor oligonucleotide. DNA-binding protein complexes were separated with 6% DNA retardation gel (Novex, USA) at 250V and visualized by autoradiography.

Figure 1. **Histological appearance of the pancreas of WBN/Kob rats. Focal inflammatory cell infiltration, hemorrhage, and fibrosis were observed in 4-months-old WBN/Kob rats. These findings were markedly prevented by administration of troglitazone. Original magnification x100**



Statistical analysis

All data are expressed as the mean ± standard error. Statistical analysis was performed by the unpaired Student's t test or by one-way analysis of variance followed by Fisher's multiple comparison test. All differences with p values <0.05 were regarded as significant.

Results

Troglitazone prevents the progression of chronic pancreatitis in male WBN/Kob rats

Significant focal inflammatory cell infiltration, hemorrhage, and fibrosis were observed in all of the 4-month old WBN/Kob rats, and troglitazone administration for 3 months significantly limited the development of the pancreatic inflammation (*Fig. 1*). The proportion of the total area of the pancreatic specimen occupied by the fibrotic area, which stained positive with Masson's trichrome, was calculated to evaluate the effect of troglitazone on pancreatic fibrosis. The fibrotic area (10.5 ± 1.5 mm^2) accounted for only 2-3% of the total area (303.7 ± 19.3 mm^2) measured in the WBN/Kob rats fed troglitazone for 3 months, as opposed to approximately 7% in the control WBN/Kob rats (16.8 ± 0.5 mm^2/240.0 ± 11.1 mm^2). The immunohistochemical study revealed the presence of spindle-shaped α-SMA-positive cells, thought to be myofibroblasts, scattered throughout the fibrotic area in the control WBN/Kob rats. Administration of troglitazone significantly reduced the number of α-SMA positive cells, and the areas positive for collagen I, III, and fibronectin were also reduced by troglitazone (*Fig. 2*). Western blot analysis demonstrated strong expression of α-SMA in the control WBN/Kob rats, and its expression was decreased in the WBN/Kob rats fed troglitazone (*Fig. 3*). These findings suggest that PSCs may be an important target in the mechanism of the anti-fibrotic action of troglitazone.

TNF-α production by peritoneal macrophages is decreased by exposure to troglitazone

We investigated the mechanism of the inhibitory action of troglitazone on proinflammatory cytokine production. Peritoneal macrophages isolated from WBN/Kob rats produced a much greater amount of TNF-α (1061 ± 28 pg/ml/10^5 cells) during three days of culture than cells from normal Wistar rats (31 ± 14 pg/ml/10^5 cells). However, the cells from troglitazone-treated rats produced only a marginal amount of TNF-α. LPS-stimulated macrophages from normal Wistar rats released a larger amount of TNF-α than unstimulated macrophages, and exposure to troglitazone significantly decreased the LPS--induced TNF-α production (*Fig. 4*).

Troglitazone dose-dependently inhibits NF-κB/Rel activation in activated macrophages

We investigated whether troglitazone affects NF-κB/Rel binding activity in LPS-stimulated macrophages. No NF-κB/Rel binding activity was detected in nuclear extracts prepared from unstimulated cells, and the specificity of the NF-κB/Rel binding activity induced by LPS was confirmed by a competition study. Increasing doses of troglitazone suppressed the NF-κB/Rel binding activity in a dose-dependent manner (*Fig. 5*).

Discussion

In previous *in vivo* studies, we found that pancreatic exocrine dysfunction in rats with streptozotocin-induced diabetes mellitus and in an animal model of chronic pancreatitis was improved by administration of troglitazone [6,7], and the severity of the morphological pancreatic damage, including inflammatory cell infiltration and fibrosis, was suppressed by the inhibitory effect of troglitazone on intrapancreatic proinflammatory cytokine expression and PSC activation. We also found

Figure 2. **Immunohistochemical staining for α-SMA, collagen I, III, and fibronectin. The area of the pancreas that stained positive for α-SMA, collagen I, III, and fibronectin in the troglitazone (TRO)-treated rats was much smaller that in the control WBN/Kob rats**

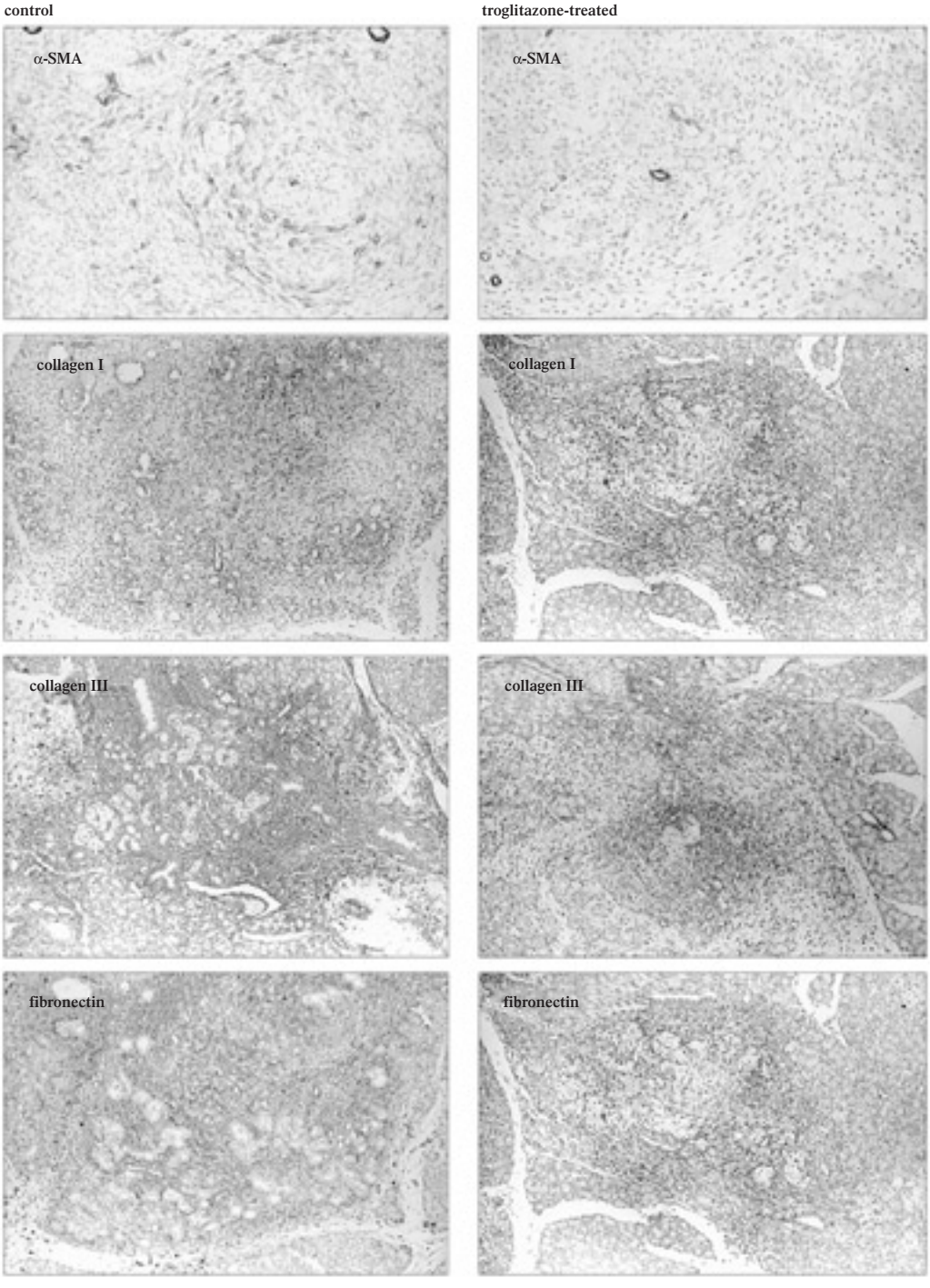


Figure 3. **Western blot analysis showed a significantly lower α-SMA protein level in the pancreas of troglitazone-treated WBN/Kob rats than in the control WBN/Kob rats**

that PSC proliferation was inhibited by troglitazone's blocking effect on cell-cycle progression beyond the G1 phase via PPARγ-dependent and independent mechanisms [9]. The present study demonstrated the area of the pancreas that was positive for collagen I, III, and fibronectin, and expression of α-SMA was markedly diminished by troglitazone in an animal model of chronic pancreatitis, suggesting that troglitazone contributes to the prevention of the progression of chronic pancreatitits by inhibiting ECM production.

Figure 4. **TNF-α production by peritoneal macrophages. Peritoneal macrophages were isolated from male Wistar rats (n=4) and exposed to LPS 10 ng/ml for 3 days. TNF-α released into the medium by the peritoneal macrophages was determined by ELISA. LPS exposure increased TNF-α secretion, and LPS-stimulated TNF-α secretion was significantly inhibited by troglitazone. Each value represents a mean ± SEM**

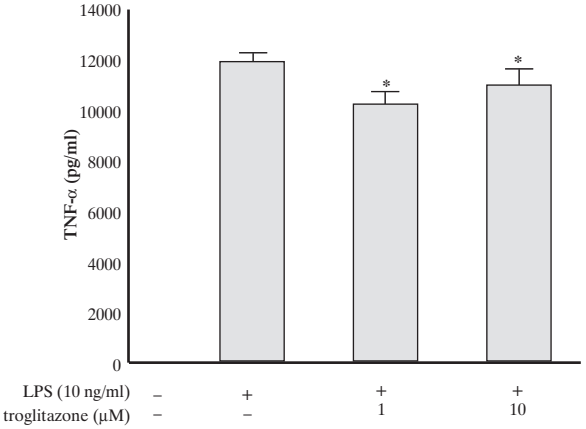
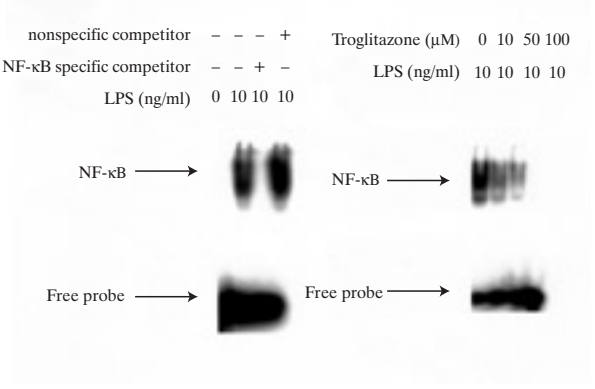


Figure 5. **Analysis of NFκB binding activity by the EMSA. Peritoneal macrophages were activated by LPS, and NFκB binding activity was analyzed by EMSA with a ^{32}P-end labeled NFκB oligomer. Specific binding activity was determined by an excess amount of unlabeled NF-κB oligonucleotide or a nonspecific oligomer containing the SP-1 DNA recognition motif. Peritoneal macrophages were incubated in medium at a concentration of 1, 50, or 100 μM of troglitazone for 2 hours and then exposed to LPS 10 ng/ml for 3 hours**



When PSCs are stimulated by various soluble mediators released by activated macrophages and aggregating platelets, the phenotype of quiescent fat-storing cells converts to a myofibroblast-like cell phenotype that produces collagen type I, III, and IV, and fibronectin [13,14], and transforming growth factor (TGF)-β and TNF-α accelerate the change in cell phenotype. Platelet-derived growth factor is a potent stimulator of cell proliferation, and TGF-β, PDGF, and basic fibroblast growth factor (bFGF) stimulate ECM synthesis [14,15]. Macrophage activation is closely associated with the fibrogenic action of PSCs. The present study revealed that the TNF-α release by peritoneal macrophages isolated from WBN/Kob rats was significantly suppressed by troglitazone, and similar results were obtained in LPS-stimulated peritoneal macrophages isolated from normal Wistar rats. PPARγ ligands have been reported to inhibit inflammatory cytokine gene expression and promotor activity by antagonizing the activity of the transcription factors of AP-1, STAT, and nuclear factor-κB (NF-κB) [10,11], and PPARγ ligands inhibit the nuclear translocation and subsequent DNA binding of NF-κB by inhibiting the degradation of IκB-α [11, 16-18]. NF-κB is a transcription factor that binds to enhancer elements involved in a variety of inflammatory cytokine genes, such as the genes encoding TNF-α, IL-6, IL-8, and inducible nitric oxide synthase. NF-κB/Rel is known to be activated in animal models of acute pancreatits, including cerulein-induced pancreatitis and taurocholate pancreatitis [19,20], and we found that LPS-induced NF-κB/Rel activation in peritoneal macrophages isolated from rats with chronic pancreatitis was inhibited by troglitazone, suggesting that one of the mechanisms by which troglitazone decreases inflammatory cytokine production is mediated by the inhibition of NF-κB/Rel activation.

The matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs) are crucial modulators of fibrogenesis and fibrolysis. PSCs express not only the ECM but membrane type-1 MMP, MMP-2, and MMP-9, which are known to degrade basement membrane collagen, and MMP-13, which degrades fibrillar collagen, and their inhibitors, TIMP-1 and TIMP-2 [14,21]. Activated PSCs also express activated TGF-β1, which up-regulates collagen I and down-regulates MMP-3 and MM-9 [14]. Recent reports suggest that pioglitazone and rosiglitazone inhibit pancreatic cancer cells invasiveness by suppressing their gelatinolytic and fibrinolytic activity though inhibition of MMP-2 activity [22], and a study on hepatic stellate cells reported that pioglitazone prevents the activation of hepatic stellate cells, thereby resulting in a reduction in expression of type I procollagen, MMP-2, TIMP-1, and TIMP-2 mRNA and acceleration of fibrolysis in the liver [23]. While the regulation of fibrogenic and fibrolytic action on ECM by the combination of MMPs and TIMPs is complicated, the balance between ECM synthesis and degradation by MMPs and TIMPs may be a candidates for the mechanism that regulates the anti-fibrogenic effect of TZDs in chronic pancreatitis.

Prevention of inflammation and fibrosis through inhibition of proinflammatory cytokine production and activation of PSCs is a major target of the treatment of chronic pancreatitis. Our recent study suggests that PSCs act as resident phagocytic cells and that scavenger receptor CD36 promotes troglitazone-induced phagocytic activity via activation of endogenous PPARγ [24]. TZD may be a useful drug for the treatment of chronic pancreatitis.

Acknowledgement

This work was supported by a Grant-in-Aid for Scientic Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan and a grant from the Yayoi Yoshioka award.

References

1. Fujiwara T, Yoshioka S, Yoshioka T, Ushiyama I, Horikoshi H. Characterization of new oral antidiabetic agent CS-045. Studies in KK and ob/ob mice and Zucker Fatty rats. Diabetes, 1988; 37: 1549-58.
2. Fujiwara T, Wada M, Fukuda K, Fukami M, Yoshioka S, Yoshioka T, Horikoshi H. Characterization of CR-045, a new oral antidiabetic agent: II. Effects on glycemic control and pancreatic islet structure at a late stage of the diabetic syndrome in C57BL/KsJ-db/db mice. Metabolism: Clinical & Experimental, 1991; 40: 1213-8.
3. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM. A prostaglandin J_2 metabolite binds peroxisome proliferators-activated receptor γ and promotes adipocyte differentiation. Cell, 1995; 83: 813-9.
4. Forman BM, Tontonoz P, Chen J, Brun RP, Spiegelman BM, Evans RM. 15-deoxy^{12,14}-prostaglandin J_2 is a ligand for the adipocyte determination factor PPARγ. Cell, 1995; 83: 803-12.
5. Tafuri SR. Troglitazone enhances differentiation, basal glucose uptake, and Glut1 protein levels in 3Y3-L1 adipocytes. Endocrinology, 1996; 137: 4706-12.
6. Shimizu K, Shiratori K, Hayashi N, Fujiwara T, Horikoshi H. Effect of troglitazone on exocrine pancreas in rats with streptozotocine-induced diabetes mellitus. Pancreas, 2000; 21: 421-6.
7. Shimizu K, Shiratori K, Hayashi N, Kobayashi M, Fujiwara T, Horikoshi H. Thiazolidinedione derivatives as novel therapeutic agents to prevent the development of chronic pancreatitis. Pancreas, 2002; 24: 184-90.
8. Apte MV, Haber PS, Applegate TL, Norton ID, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Periacinar stellate shaped cells in rat pancreas: identification, isolation, and culture. Gut, 1998; 43: 128-33.
9. Shimizu K, Shiratori K, Kobayashi M, Kawamata H. Troglitazone inhibits the progression of chronic pancreatitis and the profibrogenic acitivity of pancreatic stellate cells via a PPARγ-independent mechanism. Pancreas, 2004; 29: 67-74.
10. Ricote M, Li AC, Willson TM, Kelley C, Glass CK. The peroxisome proliferator activated receptor-γ is a negative regulator of macrophage activation. Nature, 1998; 391: 79-82.
11. Jiang C, Ting AT, Seed B. PPAR-γ agonists inhibit production of monocyte inflammatory cytokines. Nature, 1998; 391: 82-6.
12. Su CG, Wen X, Bailey ST, Jiang W, Rangwala SM, Keilbaugh SA, Flanigan A, Murthy S, Lazar MA, Wu GD. A novel therapy for colitis utilizing PPARγ ligands to inhibit the epithelial inflammatory response. J Clin Invest, 1999; 104: 383-9.
13. Apte MV, Haber PS, Darby SJ, Rodgers SC, McCaughan GW, Korsten MA, Pirola RC, Wilson JS. Pancreatic stellate cells are activated by proinflammatory cytokines: implication for pancreatic fibrogenesis. Gut, 1999; 44: 534-41.
14. Shek FWT, Benyon RC, Walker FM, McCrudden PR, Pender SLF, Williams EJ, Johnson PA, Johnson CD, Bateman AC, Fine DR, Iredale JP. Expression of transforming growth factor-β1 by pancreatic stellate cells and its implications for matrix secretion and turnover in chronic pancreatitis. Am J Pathol, 2002; 160: 1787-98.
15. Schneider E, Schmid-Kotsas A, Zhao J, Weidenbach H, Schmid RM, Menke A, Adler G, Waltemberger J, Grunert A, Bachem MG. Identification of mediators stimulating proliferation and matrix synthesis of rat pancreatic stellate cells. Am J Physiol Cell Physiol, 2001; 281: C532-43.
16. Li M, Pascual G, Glass CK. Peroxisome proliferators-activated receptor gamma-dependent repression of the inducible nitric oxide synthase gene. Mol Cell Biol, 2000; 20: 4699-707.
17. Delerive P, Martin-Nizard F, Chinetti G, Trottein F, Fruchart JC, Najib J, Duriez P, Staels B. Peroxisome proliferators-activated receptor activators inhibit thrombin-induced endothelin-1 production in human vascular thrombin-induced endothelin-1 production in human vascular endothelial cells by inhibiting the activator protein-1 signaling pathway. Circ Res, 1999; 85: 394-402.
18. Desreumaux P, Dubuquoy L, Nuten S, Peuchmaur M, Englaro W, Schoonjans K, Derijard B, Desvergne B, Wahli W, Chambon P, Leibowitz MD, Colombel JF, Auwerx J. Attenuation of colon inflammation through activators of the retinoid X receptor (RXR)/ peroxisome proliferator-activated receptor gamma (PPARgamma) heterodimer. A basis for new therapeutic strategies. J Exp Med, 2001; 193: 827-38.
19. Steinle AU, Weidenbach H, Wagner M, Adler G, Schmid RM. NF-κB/Rel activation in cerulein pancreatitis. Gastroenterology, 1999; 116: 420-30.
20. Satoh A, Shimosegawa T, Fujita M, Kimura K, Masamune A, Koizumi M, Toyota T. Inhibition of nuclear factor-κB activation improves the survival of rats with taurocholate pancreatitis. Gut, 1999; 44: 253-8.
21. Phillips PA, McCarroll JA, Park S, Wu M-J, Pirola R, Korsten M, Wilson JS, Apte MV. Rat pancreatic stellate cells secrete matrix metalloproteinases: implications for extracellular matrix. Gut, 2003; 52: 275-82.
22. Galli A, Ceni E, Crabb DW, Mello T, Salzano R, Grappone C, Milani S, Surrenti E, Surrenti C, Casino A. Antidiabetic thiazolidinediones inhibit invasiveness of pancreatic cancer cells via PPARγ independent mechanisms. Gut, 2004; 53: 1688-97.
23. Kawaguchi K, Sakaida I, Tsuchiya M, Omori K, Takami T, Okita K. Pioglitazone prevents hepatic steatosis, fibrosis, and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient L-amino acid-defined diet. Biochem Biophys Res Commun, 2004; 315: 187-95.
24. Shimizu K, Kobayashi M, Tahara J, Shiratori K. Cytokines and peroxisome proliferator-activated receptor γ ligand regulate phagocytosis by pancreatic stellate cells. Gastroenterology, 2005; 125: 2105-18.

Single Nucleotide Polymorphisms in exon 3 of the adiponectin gene in subjects with type 2 diabetes mellitus

Krętowski A[1]*, Gugała K[2], Okruszko A[1], Wawrusiewicz-Kurylonek N[1], Górska M[1]

[1] Department of Endocrinology, Diabetology and Internal Medicine, Medical University of Białystok, Poland
[2] Department of Invasive Cardiology, Medical University of Białystok, Poland

Abstract

Purpose: Adiponectin (APM1) – a newly discovered adipocytokine secreted by fat tissue – was recently suggested to play a role in the genetic predisposition to type 2 diabetes, obesity and insulin resistance. Adiponectin gene is localized on chromosome 3q27 within the region which was identified as susceptibility locus for type 2 diabetes and metabolic syndrome. Till now genetic associations of two SNP in exon 2 (+45T/G) and intron 2 (+276G/T) of adiponectin gene with type 2 diabetes and adiponectin level were reported in Japanese population and with insulin resistance in some Caucasian populations (Italy, Germany). Moreover, in the proximal promoter region of the APM1 gene: SNP-11426A/G and –11391A/-11377G haplotype predicted the associations with fasting plasma glucose, type 2 diabetes and adiponectin levels. On the other hand the role of mutations in exon 3 of the adiponectin gene is not so well studied.

Material and methods: The aim of our study was the screening for rare mutation in exon 3 of adiponectin gene in the Polish subjects with type 2 diabetes as there is no data available about the frequency and role of these mutations in our population. The study was performed in the group of 187 Polish origin patients with type 2 diabetes (32 female and 155 male, mean age 54.1±8.6 yrs) and 102 age and sex matched healthy controls.

Results: The frequency of adiponectin gene mutations in exon 3 was 3.9%, while in the control group 0.98% and this difference was not statistically significant. We also observed that adiponectin level is significantly lower in patients with c.331 T→C mutation (Y111H) in comparison to subjects without this mutation (5.0 ug/ml vs 14.4 ug/ml, p=0.0148).

Conclusions: To our knowledge the present study is the first which shows that in Polish populations.

Key words: adiponectin, polymorphism, exon 3, type 2 diabetes.

Introduction

Adiponectin (APM1) – a newly discovered adipocytokine secreted by fat tissue – was recently suggested to play a role in the genetic predisposition to type 2 diabetes, obesity and insulin resistance [1,2].

There is an increasing evidence that hypoadiponectinemia, observed in these different forms of metabolic syndrome, may have a role in the pathogenesis of insulin resistance [3-5]. It was shown that administration of adiponectin to animals with insulin resistance lowered free fatty acid by improving their oxidation in skeletal muscle and decreasing serum levels of glucose [6].

Adiponectin gene is localized on chromosome 3q27 within the region which was identified as susceptibility locus for type 2 diabetes and metabolic syndrome [7]. Till now genetic associations of two SNP in exon 2 (+45T/G) and intron 2 (+276G/T) of adiponectin gene with type 2 diabetes and adiponectin level were reported in Japanese population and with insulin resistance in some Caucasian populations (Italy, Germany) [8-11]. Moreover, in the proximal promoter region of the APM1 gene: SNP-11426A/G and -11391A/-11377G haplotype predicted the associations with fasting plasma glucose, type 2 diabetes and adiponectin levels [9,12].

On the other hand the role of mutations in exon 3 of the adiponectin gene is not so well studied. However, the association of these rare mutation with type 2 diabetes was recently shown in French Caucasians, but it was not confirmed in by the Swedish group [8,12].

* CORRESPONDING AUTHOR:
Department of Endocrinology, Diabetology and Internal Medicine
Medical University of Białystok
ul. M. Skłodowskiej-Curie 24a
15-276 Białystok, Poland
Tel: +48 85 746823; Fax: +48 85 7447611
e-mail: akretows@amb.edu.pl (Adam Krętowski)

Received 14.04.2005 Accepted 07.06.2005

Table 1. **Clinical chracteristics of the patients with the mutations detected in exon 3 of the adiponectin gene and subjects without these mutations**

Subject ID	Age	Sex	DM T2	CHD	Mutation in exon 3	Amino acid substitution	Adiponectin serum level	BMI	Hypertension	Hyperlipdemia
CASE 024	49	m	yes	yes	TAC→CAC	Tyr111His	5.7	26.3	1	1
CASE 037	49	m	yes	yes	TAC→CAC	Tyr111His	3.2	26.1	0	0
CASE 173	54	m	yes	yes	TAC→CAC	Tyr111His	9.4	30.1	1	0
CASE 067	62	m	yes	yes	ACC→ACT	Thr83Thr	26.0	25.1	1	0
CONT 186	51	m	no	yes	TAC→CAC	Tyr111His	1.5	25.9	0	1
CASEs without mutation	53.8	32f/ 150m	99/182	111/182	no	no	14.4	30.3	103/182	123/182

The aim of our study was the screening for rare mutation in exon 3 of adiponectin gene in the Polish subjects with type 2 diabetes as there is no data available about the frequency and role of these mutations in our population.

Material and methods

The study was performed in the group of 187 Polish origin patients with type 2 diabetes (32 female and 155 male, mean age 54.1±8.6 yrs) and 102 age and sex matched healthy controls.

For the study of the adiponectin gene mutations: c.250 G→C(GGA→CGA) G84R (Glycine→Arginine); c.268 G→A (GGT→ATG) G90S (Glycine→Serine); c.331 T→C (TAC→CAC) Y111H (Tyrosine→Histidine); c.491 TAC (ATC→ACC) I164T (Isoleucine→Threonine) 476 bp fragment of exon 3 was directly sequenced using forward: 5'-GTGAGTGGGAGCCACAGGGATG-3' and rewers: 5'-GCCGGAGGCCTGGTCCACATTA-3' primers.

Results

In the studied group with type 2 diabetes we have found four mutations in exon 3 of the adiponectin gene (three missence Y111H and one silent mutation at the position c.249 C→T, and only one mutation (Y111H) in subject without type 2 diabetes (but with the history of CHD and hyperlipidemia).

In our population we didn't find any mutation which were earlier observed in Japanese population (including c.491 T→C (I164T) and in the previous studies in French Caucasians: at the positions c.250 G→C (G84R) or c.268 G→A (G90S) [8,13].

The frequency of adiponectin gene mutations in exon 3 was 3.9%, while in the control group 0.98% and this difference was not statistically significant. We also observed that adiponectin level is significantly lower in patients with c.331 T→C mutation (Y111H) in comparison to subjects without this mutation (5.0 ug/ml vs 14.4 ug/ml, p=0.0148) (*Tab. 1*).

Discussion

To our knowledge the present study is the first which shows that in Polish populations. Tyr111His mutation in exon 3 of adiponectin gene could contribute to the lower level of adiponectin in the peripheral blood. Similar observation about the association of at least one of G84R, G90S or Y111H variants with adiponectin level and type 2 diabetes was previously described in the group of 1373 French Caucasian subjects with obesity and type 2 diabetes [8]. Contrary to our and Vasseur and col. observations these three SNPs were at a very low frequency in Swedish patients and were not associated with type 2 diabetes [12].

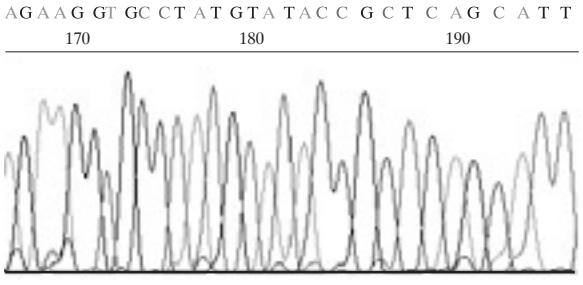
At present it is difficult to explain how the observed mutation could influence the adiponectin level or function. As Y111H missense mutation is located in the highly conserved region of the adiponectin gene – at the hinge between the collagen and globular domains, it was previously suggested that this mutation may partially hinder the complexation of collagenous homotrimers in boundles and alter the spatial organization of protein, interfere in post-translational modifications or influence the interactions of proteins with their receptor [8].

In our study the frequency of adiponectin gene mutations in exon 3 was three times higher than in the control group (3.9% vs 0.98%) but this difference didn't reach the statistical significance. We think that the main reason for the lack of statistical differences between the groups is that the frequency of the studied mutation is relatively low and to prove the difference one would have to study much larger group of subjects.

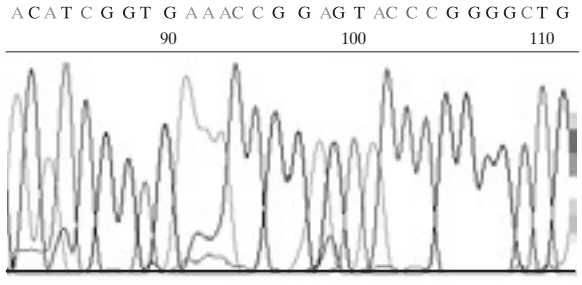
In summary our study suggests that Y111H missense mutation is associated with the lower levels of adiponectin in the peripheral blood and its potential association with type 2 diabe-

Figure 1. **Fragments of DNA sequences of exon 3 of adiponectin gene with or without studied mutations**

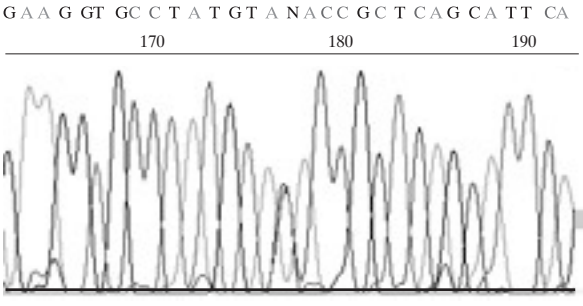
A) DNA sequence without c.331 T→C mutation (Y111H)



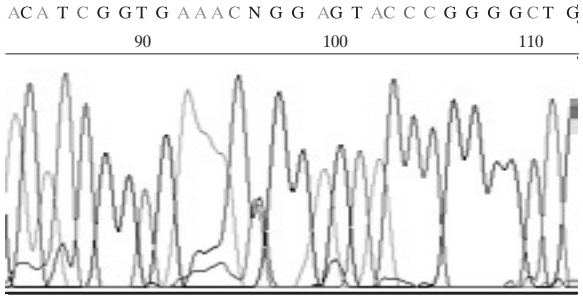
C) DNA sequence without c.249 C→T mutation (Thr83Thr)



B) DNA sequence with c.331 T→C mutation (Y111H)



D) DNA sequence with c.249 C→T mutation (Thr83Thr)



tes, coronary artery disease and maybe some other disorders of metabolic syndrome need to be studied in the larger population of subjects.

References

1. Ukkola O, Ravussin E, Jacobson P, Sjostrom L, Bouchard C. Mutations in the adiponectin gene in lean and obese subjects from the Swedish obese subjects cohort. Metabolism, 2003; 52(7): 881-4.

2. Yang WS, Tsou PL, Lee WJ, Tseng DL, Chen CL, Peng CC, Lee KC, Chen MJ, Huang CJ, Tai TY, Chuang LM. Allele-specific differential expression of a common adiponectin gene polymorphism related to obesity. J Mol Med, 2003; 81(7): 428-34.

3. Ohashi K, Ouchi N, Kihara S, Muramoto A, Niwa R. Possible roles of adiponectin gene mutation in the occurrence of the metabolic syndrome. 63rd Scientific Session of ADA, 2003.

4. Ouchi N, Kihara S, Arita Y, Maeda K, Kuriyama H, Okamoto Y, Hotta Y, Hotta K, Nishida M, Takahashi M, Nakamura T, Yamashita S, Funahashi T, Matsuzawa Y. Novel modulator for endothelial adhesion molecules; Adipocyte-derived plasma protein adiponectin. Circulation, 1999; 100: 2473-6.

5. Adamczak M, Wiecek A, Funahashi T, Chudek J, Kokot F, Matsuzawa Y. Decreased plasma adiponectin concentration in patients with essential hypertension. Am J Hypertens, 2003; 16(1): 72-5.

6. Fruebis J, Tsao TS, Javorschi S, Ebbets-Reed D, Erickson MR, Yen FT, Bihain BE, Lodish HF. Proteolytic cleavage product of 30-kDa adipocyte complement-related protein increases fatty acid oxidation in muscle and causes weight loss in mice. Proc Natl Acad Sci USA, 2001; 98(4): 2005-10.

7. Kissebah AH, Sonnenberg GE, Myklebust J, Goldstein M, Broman K, James RG, Marks JA, Krakower GR, Jacob HJ, Weber J, Martin L, Blangero J, Comuzzie AG. Quantitative trait loci on chromosomes 3 and 17 influence phenotypes of the metabolic syndrome. Proc Natl Acad Sci USA, 2000; 97(26): 14478-83.

8. Vasseur F, Helbecque N, Dina C, Lobbens S, Delannoy V, Gaget S, Boutin P, Vaxillaire M, Lepretre F, Dupont S, Hara K, Clement K, Bihain B, Kadowaki T, Froguel P. Single-nucleotide polymorphism haplotypes in the both proximal promoter and exon 3 of the APM1 gene modulate adipocyte-secreted adiponectin hormone levels and contribute to the genetic risk for type 2 diabetes in French Caucasians. Hum Mol Genet, 2002; 11: 2607-14.

9. Menzaghi C, Ercolino T, Di Paola R, Berg AH, Warram JH, Scherer PE, Trischitta V, Doria A. A haplotype at the adiponectin locus is associated with obesity and other features of the insulin resistance syndrome. Diabetes, 2002; 51: 2306-12.

10. Hara K, Boutin P, Mori Y, Tobe K, Dina C, Yasuda K, Yamauchi T, Otabe S, Okada T, Eto K, Kadowaki H, Hagura R, Akanuma Y, Yazaki Y, Nagai R, Taniyama M, Matsubara K, Yoda M, Nakano Y, Tomita M, Kimura S, Ito C, Froguel P, Kadowaki T. Genetic variation in the gene encoding adiponectin is associated with an increased risk of type 2 diabetes in the Japanese population. Diabetes, 2002; 51(2): 536-40.

11. Stumvoll M, Tschritter O, Fritsche A, Staiger H, Renn W, Weisser M, Machicao F, Haring H. Association of the T-G polymorphism in adiponectin (exon 2) with obesity and insulin sensitivity: interaction with family history of type 2 diabetes. Diabetes, 2002; 51: 37-41.

12. Gu HF, Abulaiti A, Ostenson CG, Humphreys K, Wahlestedt C, Brookes AJ, Efendic S. Single nucleotide polymorphisms in the proximal promoter region of the adiponectin (APM1) gene are associated with type 2 diabetes in Swedish caucasians. Diabetes, 2004; 53(Suppl. 1): S31-5.

13. Zietz B, Barth N, Scholmerich J, Schmitz G, Schaffler A. Gly15Gly polymorphism within the human adipocyte-specific apM-1gene but not Tyr111His polymorphism is associated with higher levels of cholesterol and LDL-cholesterol in caucasian patients with type 2 diabetes. Exp Clin Endocrinol Diabetes, 2001; 109(6): 320-5.

Interleukin 18 and sICAM-1 serum levels in families with type 1 diabetes mellitus

Mirończuk K, Okruszko A, Wawrusiewicz-Kurylonek N, Krętowski A*, Kinalska I, Górska M

Department of Endocrinology, Diabetology and Internal Medicine, Medical University of Białystok, Poland

Abstract

It is well known that subjects with type 1 diabetes are at an increased risk of death from coronary heart disease in comparison to non-diabetic age-matched individuals because hyperglycaemia is believed to be a key risk factor for the development of micro- and macrovascular complications. On the other hand there is increasing evidence about the role of inflammatory mediators in the pathogenesis of atherosclerosis and the development of acute coronary syndromes. It has been recently suggested that IL-18 and sICAM-1 have a strong predictive value for cardiovascular diseases and deaths in patients with coronary artery disease and/or in apparently healthy men.

The aim of our study was to estimate the serum levels of IL-18 and sICAM-1 in subjects with type 1 diabetes and their relatives, who share HLA diabetic susceptibility genes (with or without pancreatic autoantibodies), but still without glucose level disturbances, as an evaluation of the possible role of genetic predisposition to the presence of IL-18 in diabetic families. The study was carried out in 35 type 1 diabetic subjects, their 101 healthy first-degree relatives: 36 siblings and 65 parents and the control group consisted of 31 healthy volunteers.

We have found increased IL-18 and sICAM-1 levels in subjects with type 1 diabetes and their first degree relatives, who share diabetic HLA haplotypes: DRB1*03/DRB1*04 or DRB1*03/*04/DQB1*02 independently of their autoimmune status. There was a strong positive correlation between IL-18 and sICAM-1 levels in diabetic subjects and their first degree relatives without glucose level disturbances.

To our knowledge this is the first study, which suggests that sICAM-1 elevations could be a result of IL-18 overproduction in type 1 diabetic subjects and their first degree relatives. Since in previous studies IL-18 and sICAM-1 were found to be predictors of death in subjects with CHD, one could speculate that high levels of IL-18 observed in subjects with genetic predisposition, but still with normal glucose levels, are an in addition to hyperglycaemia, pathogenic factors responsible for a higher risk of acute coronary events in subjects with diabetes type 1.

Key words: coronary heart disease, diabetes type 1, interleukin 18, soluble Intercellular Adhesion Molecule 1.

Introduction

It is well known that subjects with type 1 diabetes have a two to four times increased risk of death from coronary heart disease in comparison to non-diabetic age-matched individuals and hyperglycaemia is believed to be a key risk factor for the development of micro- and macrovascular complications [1]. However, in contrast to the beneficial influence on microvascular complications, the role of good glycaemic control in the prevention of cardiovascular deaths isn't still well documented in diabetic patients [2,3].

On the other hand there is increasing evidence about the role of inflammatory mediators in the pathogenesis of atherosclerosis and development of acute coronary syndromes [4-6].

Interleukin 18 (IL-18) is a pleiotropic pro-inflammatory cytokine involved in the regulation of innate and acquired immune responses, playing a key role in autoimmune, inflammatory and infectious diseases [7]. IL-18 strongly stimulates INF-γ production by T cells and NK cells and in synergy with IL-12 promotes the development of Th1 responses [7,8].

The possible role of IL-18 in the pathogenesis of cardiovas-

* CORRESPONDING AUTHOR:
Department of Endocrinology, Diabetology and Internal Medicine
Medical University of Białystok
ul. M. Skłodowskiej-Curie 24a, 15-276 Białystok, Poland
Tel: +48 85 74 68 239; Fax: +48 85 74 47 611
e-mail: akretows@amb.edu.pl (Adam Krętowski)

Received 14.04.2005 Accepted 24.05.2005

Table 1. **Serum concentration of IL-18 and sICAM-1 in type 1 diabetic patients, their first degree relatives and healthy controls**

	Type 1 diabetes (n=35)	Siblings (n=36)	Parents (n=65)	Healthy controls (n=31)
IL-18 (pg/ml)	157.3±80.1**	170.3±66.9**	146.9±72.6*	115.1±30.4
sICAM-1 (ng/ml)	357.2±69.4###	311.2±75.1##	322.0±74.6#	257.3±46.7

Data are means ±SD, P values vs controls: ** p<0.01, * p<0.05, ### p<0.000001, ## p<0.00002, # p<0.00003

cular disease was previously suggested by Blankenberg et al., who have found IL-18 to be a strong predictor of cardiovascular death in stable and unstable angina [9]. An increased expression of IL-18 has been reported in human atherosclerotic plaque and proposed to be involved in the plaque destabilisation [10,11]. Increased plasma IL-18 concentrations also correlated with the severity of myocardial dysfunction in patients with acute coronary syndromes [12].

The soluble form of intercellular adhesion molecule 1 (ICAM-1) is another marker of inflammation recently found to be related with cardiovascular mortality and ischemic stroke independently from other traditional risk factors [13-15]. ICAM-1 plays a crucial role in leukocytes migration from circulation into inflamed tissues and the soluble form of ICAM-1 is supposed to be cleaved during this process [13-15]. High levels of circulating ICAM-1 are associated with the early stages of atherosclerosis development and their concentrations were shown to correlate with pro-inflammatory cytokines: TNF-α, IL-6 [16,17].

Moreover it was recently shown in *in vitro* studies that IL-18 in time- and concentration-dependent fashion up-regulates the expression of ICAM-1 in the monocytes population in human PBMC. On the other hand ICAM-1/LFA-1 interactions induced by IL-18 are important for the enhanced production of INF-γ, TNF-α, IL-12 [18,19].

The aim of our study was to estimate the serum levels of IL-18 in subjects with type 1 diabetes and their relatives sharing HLA diabetic susceptibility genes (with or without pancreatic autoantibodies), but as yet with no glucose level disturbances, as an evaluation of the possible role of genetic predisposition for the determination of IL-18 in diabetic families.

In addition we have investigated the relationship between IL-18 and sICAM-1 serum levels as their relationship has been suggested in *in vitro* studies and both markers were found to have a strong predictive value for cardiovascular diseases and deaths in the patients with coronary artery disease and/or in apparently healthy men [9,14,20].

Material and methods

The study was carried out in 35 type 1 diabetic subjects (mean age 15.5±7.6 yrs, F/M=11/24, with good metabolic control – HbA_1C<7%) and their 101 healthy first-degree relatives: 36 siblings (16.1±9.9, F/M=13/23) and 65 parents (40.9±9.5 yrs, F/M=33/32). Diagnosis of type 1 diabetes was made according to the criteria defined by WHO in 1985, the presence of ketosis, low body mass index and need for insulin therapy.

The control group consisted of 31 healthy volunteers from our staff families (mean age 16.4±3.1, F/M=13/18), who had no family history of type 1 diabetes and other autoimmune diseases.

The concentrations of IL-18 were measured by ELISA using two monoclonal antibodies against two different epitopes of human IL-18 (MBL, Ltd, Nagoya, Japan). The sensitivity of the assay is 12.5 pg/ml.

sICAM-1 levels in the serum were determined by solid phase ELISA assay (Parameter, R&D Systems, Abingdon, UK; the minimum detectable concentration was 0.35 ng/ml).

Antibodies against glutamic acid decarboxylase (GADA) and against tyrosine phosphatase (IA-2A) were measured by RIA (Euroimmun, GmbH, Lübeck, Germany). HbA_1C was quantified by high-performance liquid chromatography (Bio-Rad, München, Germany).

The genotyping of HLA alleles associated with type 1 diabetes mellitus in the Polish population: DRB1*03, DRB1*0401, DQB1*02 was performed using SSP-PCR method as described previously [21,22].

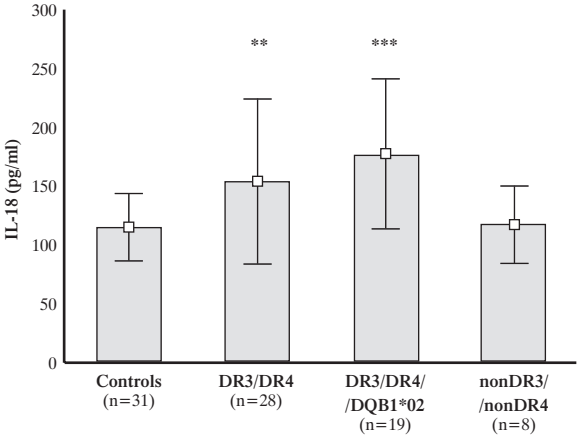
The results of IL-18 or sICAM-1 levels are presented as a mean ±SD. The statistical significance between the groups were evaluated by the Mann-Whitney U test, for regression analysis Spearman's correlation coefficient was used (Statistica 5.0, StatSoft, Tulsa, OK, USA) and the differences where considered significant at p<0.05.

Results

In subjects with type 1 diabetes fasting serum levels of IL-18 and sICAM-1 were increased in comparison to the healthy controls (*Tab. 1*) and the degree of these elevations were not dependent on the degree of glycaemic control. No correlation was observed between HbA_1C and IL-18 or sICAM-1 levels. We also didn't find any correlation between IL-18 levels and age or gender (data not shown).

Moreover our study has shown that IL-18 and sICAM-1 levels were higher in siblings and parents of diabetic subjects in comparison to the controls (*Tab. 1*). More detailed analysis revealed that increased IL-18 levels are observed in the first degree relatives, who share diabetic HLA haplotypes: DRB1*03/DRB1*04 or DRB1*03/*04/DQB1*02 (*Fig. 1*) independently of their autoimmune status (no difference in subjects with or without GADA, IA-2A autoantibodies). There was a strong positive correlation between IL-18 and sICAM-1 levels in diabetic subjects and their first degree relatives with no glucose level disturbances (r=0.4, p<0.0002) (*Fig. 2*).

Figure 1. **Serum concentration of IL-18 in association to HLA diabetic susceptibility alleles in siblings of type 1 diabetic patients**



** $p<0.03$ vs control group *** $p<0.006$ vs control group

Discussion

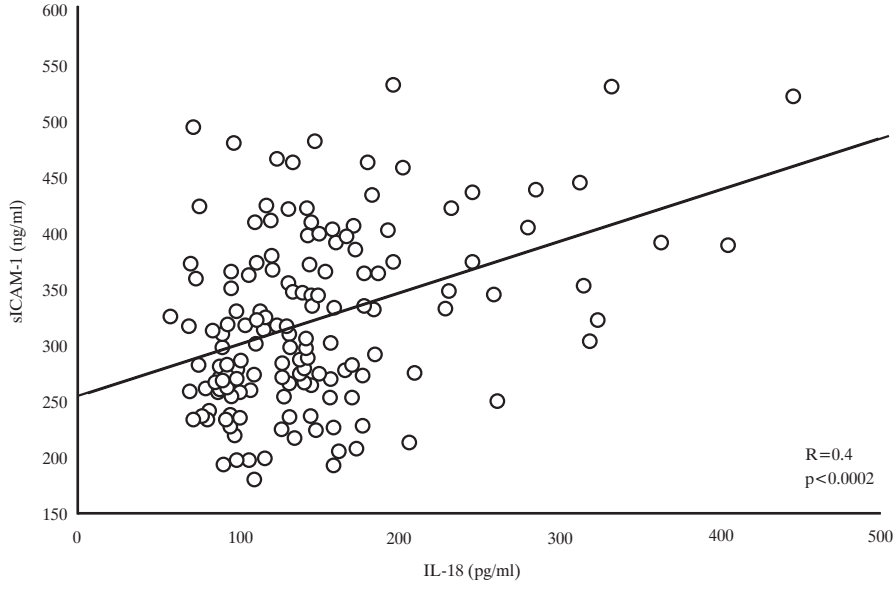
In our previous study we have shown an association between type 1 diabetes and G→C polymorphism at position -137 in the promoter of IL-18 gene [23]. These findings together with our present observations, that IL-18 levels are increased in type 1 diabetic patients and their healthy relatives, who share HLA diabetic susceptibility alleles, seems to suggest that IL-18 levels could be influenced by genetic predisposition in type 1 diabetic subjects and their relatives with alleles associated with the disease.

To our knowledge till now there has been only one paper published concerning the levels of IL-18 in type 1 diabetes [24]. Nicoletti et al have found higher levels of IL-18 in the pre-diabetic stage of type 1 diabetes, but in contrast to our results, not in the newly diagnosed subjects [24]. This difference could result from the methodological reasons, since their assay was able to measure detectable levels of IL-18 only in 3 out of 35 (8.6%) diabetic subjects. However It was recently reported that different ELISA assays can detect different subtypes of IL-18 in human serum [25]. In our assay two monoclonal antibodies were used against two different epitopes of human IL-18 (commercially available ELISA test, previously used in other published studies), IL-18 levels were detected in all the studied subjects and the mean levels of cytokine in the healthy controls were similar to the concentrations observed in other studies [9].

Moreover in the present study we found increased levels of sICAM-1 in type 1 diabetic patients and their first degree relatives and a strong positive correlation between IL-18 and sICAM-1 levels, which could suggest that high concentrations of sICAM-1 could be, at least partially, influenced by IL-18 overproduction in diabetic patients and their relatives.

In the present study we didn't observe any relationship between the both studied parameters and glycaemic control or immune status. No relation between HbA_1C or duration of disease and increased sICAM-1 in type 1 diabetes was previously reported by Fasching et al., who suggested that the high concentrations of sICAM-1 reflected endothelial cell stimulation and leukocyte activation [26]. Moreover Jude et al. observed that higher levels of ICAM-1 in the sera of patients with diabetes were of good predicture value of the development of macrovascular disease in diabetic patients independently of glycaemic control [14]. On the other hand Esposito et al. have shown that in subjects with IGT, as well as in healthy controls, acute hyperglycaemia increased IL-18 concentration during the clamp study [27]. In the light of their findings we believe that no correlation between HbA_1C and IL-18 levels in our study could

Figure 2. **Relationship between serum IL-18 and sICAM-1 levels in type 1 diabetic subjects and their first degree relatives**



result from good glycaemic control ($HbA_1C<7\%$) observed in the studied type 1 diabetic patients.

In summary, to our knowledge this is the first study which suggests that sICAM-1 elevations could be a result of IL-18 overproduction in type 1 diabetic subjects and their first degree relatives. Since IL-18 and sICAM-1 have been the predictors of death in subjects with CHD, one could speculate that higher levels of IL-18, observed in subjects with genetic predisposition to type 1 diabetes, but still with normal glucose levels, could be in addition to hyperglycaemia, considered a pathogenic factor responsible for a higher risk of acute coronary events in subjects with diabetes type 1 in comparison to non-diabetic age-matched individuals.

References

1. Krolewski AS, Kosinski EJ, Warram JH, Leland OS, Busick EJ, Asmal AC, Rand LI, Christlieb AR, Bradley RF, Kahn CR. Magnitude and determinants of coronary artery disease in juvenile-onset, insulin-dependent diabetes mellitus. Am J Cardiol, 1987; 59: 750-5.
2. The Diabetes Control and Complications Trial Research Group. Effect of intensive diabetes management on macrovascular events and risk factors in the Diabetes Control and Complications Trial. Am J Cardiol, 1995; 75: 894-903.
3. UK Prospective Diabetes Study Group. Intensive blood-glucose control with sulphonylureas or insulin compared with conventional treatment and risk of complications in patients with type 2 diabetes. Lancet, 1998; 352: 837-53.
4. Ross R. Atherosclerosis: an inflammatory disease. N Engl J Med, 1999; 340: 115-26.
5. Buffon A, Biasucci LM, Liuzzo G, D'Onforio G, Crea F, Maseri A. Widespread coronary inflammation in unstable angina. N Engl J Med, 2002; 347: 5-12.
6. Libby P. Current concepts of the pathogenesis of the acute coronary syndromes. Circulation, 2001; 104: 365-72.
7. Biet F, Locht C, Kremer L. Immunoregulatory functions of IL-18 and its role in defence against bacterial pathogens. J Mol Med, 2002; 80: 147-62.
8. McInnes IB, Gracie JA, Leung BO, Wei XO, Liew FY. Interleukin 18: a pleiotropic participant in chronic inflammation. Immunol Today, 2000; 21: 312-5.
9. Blankenberg S, Tiret L, Bickel C, Peetz D, Cambien F, Meyer J, Rupprecht HJ for the AtheroGene Investigators. Interleukin-18 is a strong predictor of cardiovascular death in stable and unstable angina. Circulation, 2002; 106: 24-30.
10. Mallat Z, Corbaz A, Scoazec A et al. Expression of IL-18 in human atherosclerotic plaques and relation to plaque instability. Circulation, 2001; 104: 1598-603.
11. Gerdes N, Sukhova GK, Libby P et al. Expression of IL-18 and functional IL-18 receptor on human vascular endothelial cells, smooth muscle cells and macrophages: implications for atherogenesis. J Exp Med, 2002; 195: 245-57.
12. Mallat Z, Henry P, Fressonnet R, Alouani S, Scoazec A, Beaufils P, Chvatchko Y, Tedgui A. Increased plasma concentration of IL-18 in acute coronary syndromes. Heart, 2002; 88: 467-9.
13. Ridker PM, Hennekens CH, Roitman-Johnson B, Stampfer MJ, Allen J. Plasma concentration of soluble adhesion molecule 1 and risks of future infarction in apparently healthy men. Lancet, 1998; 351: 88-92.
14. Jude EB, Douglas JT, Anderson SG, Young MJ, Boulton AJ. Circulating cellular ICAM-1, VACAM-1, P- and E-selectin in the prediction of cardiovascular disease in diabetes mellitus. Eur J Intern Med, 2002; 13: 185-9.
15. Tanne D, Haim M, Boyko V, Goldbourt U, Reshef T, Matetzky S, Adler Y, Mekori A, Behar S. Soluble ICAM-1 and risk of future ischemic stroke. A nested case-control study from the bezafibrate infarction prevention study cohort. Stroke, 2002; 33: 2182-6.
16. Hulthe J, Wikstrand J, Mattsson-Hulten L, Fagerberg B. Circulating ICAM-1 is associated with early stages of atherosclerosis development and with inflammatory cytokines in healthy 58-year-old men: the AIR study. Clin Sci, 2002; 103: 123-9.
17. Marfella R, Esposito K, Giunta R, Coppola G, De Angelis L, Farzati B, Paolisso G, Giugliano D. Circulating molecules in humans: role of hyperglycaemia and hyperinsulinaemia. Circulation, 2000; 101: 2247-51.
18. Yoshida A, Takahashi HK, Nishibori M, Iwagaki H, Yoshino T, Morichika T, Yokoyama M, Kondo E, Akagi T, Tanaka N. IL-18-induced expression of ICAM-1 in human monocytes: involvement in Il-12 and INF-γ production in PBMC. Cell Immunol, 2001; 210: 106-15.
19. Takahashi HK, Iwagaki H, Mori T, Morichika T, Itoh H, Yokoyama M, Kubo S, Kondo E, Akagi T, Tanaka N, Nishibori M. Prostaglandin E1 inhibits IL-18 induced ICAM-1 and B7.2 expression through EP2/EP4 receptors in human peripheral blood mononuclear cells. J Immunol, 2002; 168: 4446-54.
20. Ballantyne CM, Entman ML. Soluble adhesion molecules and the search for biomarkers for atherosclerosis. Criculation, 2002; 106: 766-7.
21. Krętowski A, Kinalska I. DQA1 and DQB1 HLA genes as markers of insulin-dependent diabetes mellitus in the Polish population. Pol Arch Med Wewn, 1999; 101: 205-11.
22. Krętowski A, Kinalska I. Evaluation of selected HLA DRB1 genes as genetic markers of type 1 diabetes in the population of north-eastern Poland. Pol Merkuriusz Lek, 1999; 41: 208-11.
23. Krętowski A, Mirończuk K, Karpińska A, Bojaryn U, Kinalski M, Puchalski Z, Kinalska I. Interleukin 18 promoter polymorphisms in type 1 diabetes. Diabetes, 2002; 51: 3347-9.
24. Nicoletti F, Conget I, DiMarco R, Speciale AM, Morinigo R, Bendzen K, Gomis R. Serum levels of interferon-gamma-inducing cytokine interleukin 18 are increased in individuals at high risk of developing type 1 diabetes. Diabetologia, 2001; 44: 309-11.
25. Shida K, Shiratori I, Matsumo M, Fukumori Y, Matsuhisa A, Kikkawa S, Tsuji S, Okamura H, Toyoshima K, Seya T. An alternative form of IL-18 in human blood plasma: complex formation with IgM defined by monoclonal antibodies. J Immunol, 2001; 166: 6671-9.
26. Fasching P, Veitl M, Rohac M, Streli C, Schneider B, Waldhausl W, Wagner OF. Elevated concentrations of circulating adhesion molecules and their associations with microvascular complications in IDDM. J Clin Endocrinol Metab, 1996; 81: 4413-7.
27. Esposito K, Nappo F, Marfella R, Giugliano G, Giugliano F, Ciotola M, Quagliaro L, Ceriello A, Giugliano D. Inflammatory cytokine concentrations are actuely increased by hyperglycaemia in humans. Role of oxidative stress. Circulation, 2002; 106: 2067-72.

Long-term outcome of percutaneous transhepatic drainage for benign bile duct stenoses

Eickhoff A[1]*, Schilling D[1], Jakobs R[1], Weickert U[1], Hartmann D[1], Eickhoff JC[2], Riemann JF[1]

[1] Medical Department C – Gastroenterology, Hepatology and Oncology – Klinikum Ludwigshafen, Academic Hospital of the University of Mainz, Germany
[2] Department of Biostatistics & Medical Informatics, University of Wisconsin, Madison, WI, USA

Abstract

Purpose: The occurrence of benign bile duct stenoses is mostly associated with prior biliary surgery, pancreatic diseases or sclerosing cholangitis. It remains a challenging problem for gastroenterologists and surgeons, especially in case the endoscopic approach is not possible. The exact role of percutaneous transhepatic stenting for these patients has not been clearly defined yet.

Material and methods: 36 patients with symptomatic benign bile duct stenoses or strictures after surgery underwent percutaneous transhepatic stenting and were studied prospectively. We were particularly interested in how many patients would achieve resolution of the stricture and tolerate removal of the drainage in the long-run.

Results: The primary success rate of percutaneous transhepatic biliary drainage (PTBD) was 92% (33/36 patients). All patients presented improvement of jaundice and cholestasis. Relief of the stricture and clinical improvement was achieved in 72% (26/36) of patients after a median stenting time of 14.5 (6-34) months. 5.5% (2/36) required further stenting due to a persistent stricture. A clinical recovery without radiological stricture regression after stenting demonstrated 22% (8/36) of patients. Long-term failures were noted in 27% (10/36) of patients after a median follow-up of 48 months.

Conclusions: Percutaneous transhepatic stenting of symptomatic benign biliary strictures is safe and highly effective in achieving adequate internal bile drainage. There seems to be a therapeutic benefit not only for short-term interventional treatment but also as a sufficient long-term therapeutic alternative to surgery with tolerable complication rates.

Key words: benign biliary stricture, percutaneous transhepatic drainage, long-term stenting.

Introduction

The etiology of benign bile duct stenoses is often associated with open or laparoscopic cholecystectomy, pancreatic diseases, sclerosing cholangitis and biliary anastomoses – e.g. after liver transplantation or hepaticojejunostomy [1,2]. Postoperative bile duct injuries occur in 0.2% to 0.5% of patients undergoing open cholecystectomy and in 0% to 2.7% after laparoscopic cholecystectomy. Biliary tract complications, ranging from 7% to 31% after orthotopic liver transplantation, have been reported in the literature [3-5]. Benign bile duct strictures (BBS) represent a significant clinical problem, despite technological developments that have facilitated diagnosis and management. Long-term complications may lead to recurrent chronic cholangitis in up to 9% and secondary biliary cirrhosis in about 7% of patients. Sepsis due to pyogenic liver abscess has also been described in a number of patients [6].

Two treatment modalities for management of BBS are available: surgical biliary drainage (mainly Roux-en Y hepaticojejunostomy) and the non-surgical approach with endoscopic stricture dilation (consisting of repeated balloon dilation and/or temporary insertion of a plastic stent) or percutaneous transhepatic biliary drainage (PTBD) for patients with inaccessible papilla or intrahepatic strictures. These two therapeutic modalities provide comparable results with stricture relapses demonstrated in 15% to 45% of cases after mean follow-up periods of 4 to 9 years [2,4-9]. However, surgical biliary drainage is associated with a mortality of more than 10% although a mortality of almost zero has been seen in more recent but smaller studies

* CORRESPONDING AUTHOR:
Medical Department C
Klinikum Ludwigshafen
Bremserstr. 79
D-67063 Ludwigshafen, Germany
Tel: (+49) (0)621-503-4101; Fax: (+49) (0)621-503-4114
e-mail: eickhofa@klilu.de (Axel Eickhoff)

Received 04.05.2005 Accepted 04.05.2005

Table 1. **Previous surgical interventions before PTBD**

Operation	N
Cholecystectomy	13 (8 laparoscopic)
Biliodigestive anastomosis	13
Whipple's procedure	4
Hepaticojejunostomy	2
No prior surgery	4

[10-16]. Therefore, over the last decade there have been many efforts to explore endoscopic management [17]. Endoscopic biliary stenting has been proved with good long-term results in more than 80% of cases, respectively [5,8,9,18,19].

On the other side, data on the efficacy of the percutaneous transhepatic approach for BBS is based only on small uncontrolled trials. Overall, PTBD has achieved success rates of approximately 70% to 80% in patients with medium-term follow-up periods only [18,20-23]. The data are more limited with regard to long-term results, and the patients described have been subject to greater selection.

To clarify the role of PTBD, we prospectively studied the efficacy and safety of this approach for BBS in patients with an inaccessible papilla, intrahepatic stricture or in whom surgical intervention was to be avoided. Overall, the main focus was to investigate the long-term outcome of this treatment.

Material and methods

Patients

A prospective analysis of the "'Ludwigshafen' benign biliary stricture register" revealed 127 cases of benign bile duct stenoses treated interventionally at the Endoscopy Unit, Department of Gastroenterology, Klinikum Ludwigshafen, Germany, from 01.01.1996 until 01.01.2004, respectively. Patients with malignant strictures or cholestasis due to distal common bile duct stricture on chronic pancreatitis were excluded. A total of 36 cases with symptomatic cholestasis were treated via the percutaneous transhepatic route. Endoscopic approach was not suitable in all 36 patients and they were referred for percutaneous transhepatic biliary drainage (PTBD) therefore.

The patients previous surgeries are listed in *Tab. 1*. Of 36 patients, 32 had undergone biliary operations while 4 patients had had no previous surgery in the biliary system overall. However, these patients had intrahepatic strictures or had undergone Billroth-II resection of the stomach and gastroduodenectomy though that might have indirectly affected the biliary system and they were included into the analysis. The median interval between surgery and our subsequent percutaneous biliary drainage was 36.5±64 (1-252) months.

Endoscopic Technique

Percutaneous access of the biliary system was achieved in case ERCP was not successful, because the papilla could not be reached due to anatomical difficulties, biliodigestive anastomoses or intrahepatic strictures. Depending on the anatomy and the site of cholestasis, a right and/or left peripheral bile duct was punctured and a guide wire was used to pass the stricture. The technique of percutaneous transhepatic cholangiography (PTC) was performed as described previously by other authors [24]. After insertion of a 5 Fr plastic sheath over the stricture a more rigid guide wire was placed into the intestine. The transhepatic tract was sequentially (usually within 7-10 days) dilated by exchange of transhepatic plastic tubes with an increasing diameter up to 16-18 Fr until a stable cutaneobiliary fistula is achieved. We performed percutaneous transhepatic cholangioscopy (PTCS; CHFX-P20, Olympus, Hamburg, GER) in every patient to clarify the macroscopic-endoscopic dignity of the stricture and to obtain histological specimen. In patients with concomitant bile duct stones adjuvant endoscopic techniques like laser lithotripsy and elctrohydraulic lithotripsy (EHL), was used and the stone fragments were pushed into the intestine or extracted with a basket afterwards.

Percutaneous transhepatic biliary drainage/stenting was carried out with transhepatic flexible tubes (Yamakawa® type, FA. Pflugbeil, Zorneding, GER). These polyethylene tubes are available with an outer diameter of 14-18 Fr and have sideholes for biliary drainage. The proximal tip of the tube was locked and positioned at the skin level. Technical success was defined as correct placement across the biliary stricture and a free flow of bile was noted through the stent into the intestine. Complications were defined immediate if related to the procedure itself or if they occurred within 1 week post-procedure. The patients were advised to flush the tube with sterile saline solution at least twice a week. Re-evaluation of the patients was scheduled every 4 months and the stents were exchanged electively to prevent complications of clogging in the follow-up. Patients who developed cholangitis received intravenous antibiotic treatment and stent exchange.

Follow-up and outcome

The charts of the patients were reviewed retrospectively for indication, effectiveness, success and complications of PTBD. Additionally, patients were contacted regularly to obtain current clinical status and to document any symptoms of stent related problems in the follow-up. Clinical and laboratory data of the patients were assessed using the patients' records and by personal interviews. Successful stenting was defined as radiological stricture regression with clinical improvement after stent removal in the long-run. Treatment was defined as not successful when patients presented no radiological and clinical stricture regression and required ongoing stenting or surgical approach in the follow-up. Prognostic factors were evaluated for a definitive successful outcome of biliary stenting. The results are specified as mean +/- standard deviation and range of values. Statistical analysis was performed using the χ^2 test to verify the independence of two variables, and the Fisher's exact test for case numbers <50, $p<0.05$ was defined as statistically significant.

Results

Percutaneous transhepatic biliodigestive stent therapy was carried out in 36 patients with symptomatic benign biliary stricture. There were 19 woman and 17 men with a mean age

Figure 2. **High-grade biliary stricture**

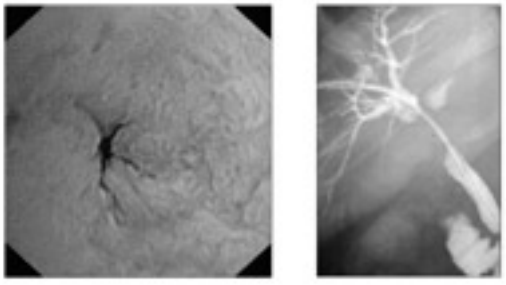
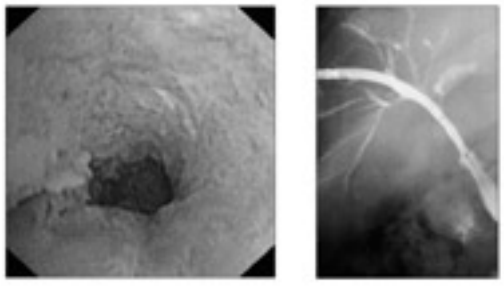


Figure 3. **Regression after 1-year stenting**



of 67.5 ±12.5 (38-87) years. All cases presented with signs of obstructive jaundice or cholangitis when the indication of PTBD was defined. The patients demonstrated the following laboratory data before biliary drainage: total bilirubine 3.3±2.8 mg/dl (0.2-14 mg/dl), alkaline phosphatase (AP) level 687.3±417 U/l (53-2412 U/l) and the median diameter of the largest pre-stenotic/ anastomotic bile duct segment measured by ultrasound was 9.52±3.1 mm (5-18 mm). The length of the stricture did not exceed 15mm in most cases. Concomitant bile duct stones were present in 21 patients and were successfully removed after PTCS-guided laser lithotripsy or EHL before placement of transhepatic tubes. Complete clearance of the bile ducts was achieved after 2.2±0.5 treatment sessions, respectively.

Successful internal biliary drainage was achieved primarily within the PTC-procedure in 33/36 (92%) of cases and a 9 Fr transhepatic polyethylene bougie was placed with the distal tip in the intestine. After sequential dilation of the cutaneobiliary tract and the stricture to a width of at least 14 Fr, a 14-18 Fr Yamakawa® prosthesis was successfully positioned into the right hepatic duct (n=22), into the left hepatic duct (n=10) or into both ducts (n=4). The primary failed cases underwent percutaneous cholangioscopy and the biliary stricture was passed with a guide-wire under endoscopic control in all 3 patients. Therefore, of the 36 primary attempts of PTBD, all were successful. Overall, adequate internal biliary drainage was noted in 100% (36/36) of patients.

Short-term results:

Adequate drainage with significantly lowered bilirubine and alkaline phosphatase (AP) levels was recognized in the short-term follow-up in all 36 patients. Total bilirubine decreased from 3.3 mg/dl to 1.3±1.4 mg/dl (0.3-10.8 mg/dl), alkaline phosphatase (AP) level from 687.3 U/l to 261.2±156 U/l (76-1200 U/l) and the diameter of the proximal common bile duct diminished remarkable to a width of 5.58±2.1 mm (3-9 mm), respectively. There was no problem of early clogging or perforation of the prosthesis. No procedure-related mortality was encountered. Therefore, the 30-day mortality rate was 0% while we recognized a morbidity rate of 13%, respectively.

The early complications related to the percutaneous transhepatic approach consisted of 2 cases of temporary cholangitis, 2 patients with minor bleeding episodes due to biliovenous fistulas and one patient with active arterial bleeding after dilation of the transhepatic tract. Patients with cholangitis received antibiotics via the i.v. route and this was successful in all cases. Hemostasis due to biliovenous fistula was achieved by placement of transhepatic polyethylene tubes with no sideholes at the site of the fistula. Management of the arterial bleeding after dilation included angiography with superselective coiling of a peripheral branch of the hepatic artery. The mean hospitalization of the patients for initial PTC and PTBD was 17.5±-4.8 (8-29) days.

Long-term results:

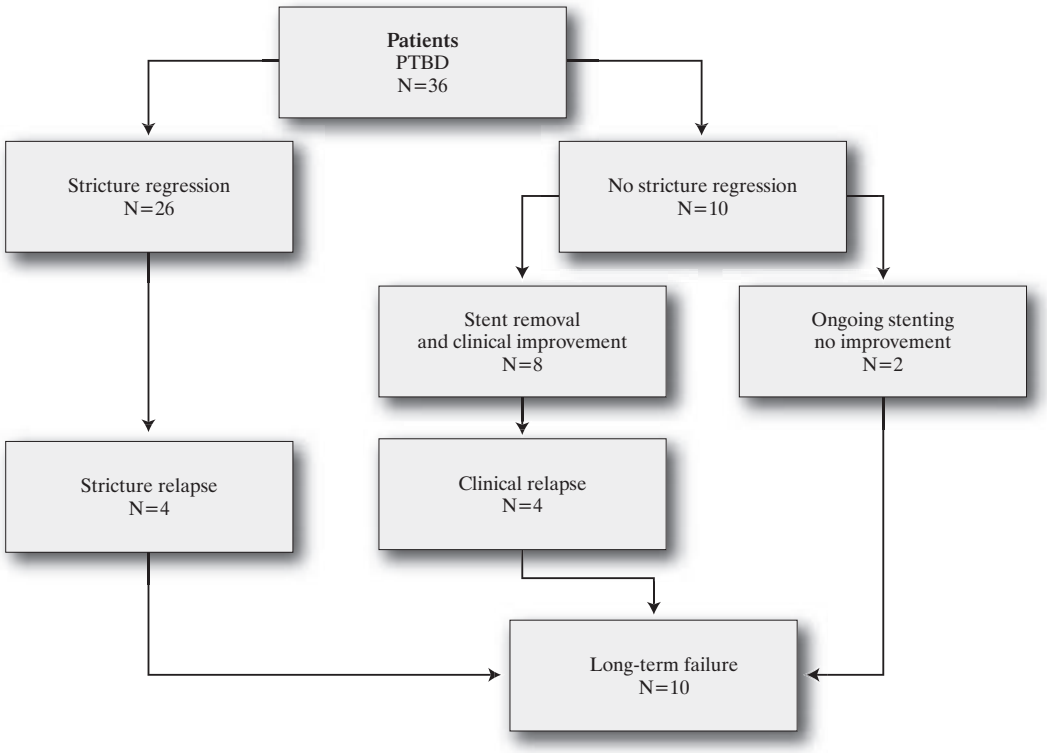
The median duration of drainage was 12±9.9 (1-52) months and the subsequent follow-up for all patients irrespective of treatment allocation was 48±14.2 (2-156) months. We recognized a radiological verified regression of the biliary stenosis in 26 patients after a stenting period of 14.4±6.1 (4-36) months. The radiographic measurement after removal of the stent demonstrated a mean dilation of the stenotic biliary segment from 1.4±0.6 (1-3) mm to 2.4±2.9 (1-10) mm. However, 10 of 36 patients (27%) presented a persistent biliary stricture after transhepatic stenting therapy.

Overall, in 22 patients, the transhepatic tubes could be removed permanently while objective stricture regression and clinical improvement was observed in the long-term follow-up. The median stenting time for these patients was 14.9±6.2 (6-34) months (*Fig. 2*,*3*). In 8 of the 10 patients without radiological stricture regression, we removed the drains after a median stenting time of 13.5±4.7 (8-22) months and the patients demonstrated clinical improvement (no jaundice, pain or cholangitis) without a stent in situ. However, in the long-term follow-up 4 of the 8 patients were re-admitted because of recurrent jaundice, bile duct stones or cholangitis after 23.5±4.6 (17-24) months.

Four patients (11%) with primary stricture regression developed relapse of cholestasis after 20.2±2.6 (18-24) months. The objective radiological long-term success rate (stricture regression + clinical improvement) with percutaneous treatment was 22 of 36 patients (62%), while the overall benefit from therapy was 26 of 36 patients (72%).

The remaining 10 cases, which had to be regarded as long-term failures, should be differentiated in primary failures (no stricture regression and/or no clinical improvement after attempted PTBD extraction) and secondary failures (recurrence/relapse of stricture and/or clinical improvement in the follow-up (*Fig. 1*).

Figure 1. **Long-term results and outcome of PTBD in biliary stenoses**



Persistent stricture without clinical improvement after attempted PTBD was noted in 2 of 36 (6%) patients. The median stenting time for these patients was 11.9±12 (2-52) months. The reason for ongoing stenting were contraindications for surgery in both patients. The percutaneous drainages were exchanged regularly every 4 months to prevent complications like clogging and cholangitis.

Eight patients (22%) developed relapse of the biliary stricture or recurrence of clinical symptoms (jaundice, cholangitis, cholestasis) after 21.8±4.4 (17-24) months. The median stenting period of these patients was 14.2±5.1 (4-36) months. Of these 8 patients, 2 were managed by insertion of a percutaneous self-expandable metal stent, one patient underwent biliary drainage surgery and 5 patients were treated with ongoing percutaneous drainage again. Four patients (11%) with primary PTBD could be switched to a biliodigestive stenting via the endoscopic route. All patients with continued percutaneous transhepatic stenting have remained well, with uneventful exchanges of the Yamakawa drains every 4 months. Currently, 4/36 patients with persistent stenosis or stricture relapse have stents in place at the time of writing.

Possible influencing factors for determining superior long-term outcome like age, gender, etiology of the stricture, stricture location, cholestatic serum parameters, width of the common bile duct and treatment time were investigated as well. Statistical analysis (Fisher's exact test) revealed minor significance only for proximal or intrahepatic location, anastomotic strictures, short strictures (p=0.07) and extended percutaneous stenting time (p=0.08). All other variables demonstrated no significant influence for successful long-term outcome.

Long-term complications

Stent occlusion was the predominant late complication and was found in 14 (38%) of 36 patients. In total, we observed 19 (9.5%) occlusions of 198 stents in 36 patients. There was no early or late perforation of the prosthesis in our patients. Diameter and the length of the prosthesis were irrespective parameters for stent occlusion. However, Yamakawa drains with sideholes beginning at 7.5 cm from the proximal tip seems to be superior to drains with sideholes beginning at 15 cm from the proximal tip. An occlusion occurred more frequently in patients with stent renewal "on demand" than in patients with elective replacements every 4 months.

Discussion

Benign bile duct stricture (BBS) caused by prior biliary surgery (open/laparoscopic cholecystectomy), pancreatic diseases, sclerosing cholangitis or biliary anastomoses represent a difficult clinical challenge for gastroenterologists and surgeons [6,11,15,25]. If untreated, there is a high risk of repeated cholangitis, biliary cirrhosis, hepatic failure, or death [10,13,19]. Many patients have undergone various treatment approaches before admission to specialized centers [3,5,9]. However, adequate management of these patients is based on a "skilled team

approach" between endoscopists and surgeons. Dependant factors for the "team approach" are patient's age, comorbidities, course and etiology of the stricture [6,7,11-13].

Today, emerging endoscopic and transhepatic treatment modalities are particularly attractive because of their low morbidity in comparison to surgical approaches [1,2,8,26]. The characteristics of BBS as well as the success of the non-surgical treatment are well documented in the literature [5]. Surgery and endoscopic approach have demonstrated similar long-term success rates [2,9,13,16,26]. Bergman et al. found a long-term success of biliary stenting in 80% of their patients while 20% suffered from stricture relapse within the first 2 years of stent therapy. They advise endoscopic treatment as the initial management of choice for postoperative bile duct stenosis [26]. For patients with tight benign strictures, an endoscopic dilation therapy for a minimum of one year is recommended [1,2,7,17,26].

Indications for surgery are complete transection, failed previous repairs and failure of initial interventional therapy. All other patients should be candidates for endoscopic stenting as initial and definitive treatment [3,5,9,11]. However, only a few direct comparisons between long-term results of surgery and interventional techniques have been made until today.

More favorable long-term results have been demonstrated in uncontrolled series for percutaneous transhepatic treatment, with a success rate between 70% to 93% at a mean follow-up of 24 to 36 months [18,20,27-29]. The goal of the percutaneous transhepatic approach, especially in patients with inaccessible papilla and intrahepatic stenoses, is to establish adequate internal biliary drainage to prevent cholestasis, cholangitis, and sludge or stone formation. Obviously, it is necessary that the new interventional and minimal invasive techniques like percutaneous transhepatic treatment should be compared with surgical results. However, it is difficult to assess the relative value of the new interventional and percutaneous approaches in comparison to surgery, and not only because of the limited patient numbers published in the literature until today. Moreover, it is problematical to give guidelines on when the percutaneous transhepatic tube can be removed and there is lack of confidence about the time of stenting as well.

This manuscript is the first published paper presenting long-term (>4 years) results of the treatment with percutaneous transhepatic drains (Yamakawa® prosthesis) in benign biliary strictures. The transhepatic prosthesis should not be left in place longer than 4 months. After this time the Yamakawa® drainage should be replaced in case of persistent stricture every 4 months up to 1 year. We defined successful treatment as stricture regression and/or ongoing clinical absence of cholestasis after removal of the prosthesis. Our results show that of 36 patients with BBS who were treated during an 8-year period, about 72% were able to benefit from the percutaneous treatment. In the long-term follow-up of 48 months, we noted an acceptable rate of stricture relapse (22%) and a reasonable rate of complications (13%).

Our short- and medium-term results are comparable with the results from previously published data. Despite of these promising outcome, we should take into consideration the shortcomings of our data. First, the patient number in our study was 36 and this makes a reliable statistical comparison more difficult. Second, the uncontrolled character of the study may lead to an inhomogenous patient group. Therefore a systemic bias concerning outcome variables and complication rate cannot be ruled out.

Table 2. **Stricture location and overall success rate of PTBD**

Stricture location	Success of treatment
Anastomotic	9/10
Distal common bile duct	1/7
Mid/proximal common bile duct	3/7
Intrahepatic	12/12
All locations	26/36 (72%)

On the other side, as our study involves unselective consecutive patients, we conclude that our results provide a realistic and reliable assessment of the outcome of percutaneous transhepatic stent therapy in the clinical reality.

In 21 patients (58%) concomitant intrahepatic stones were diagnosed due to intrahepatic or anastomotic stricture. The overall stone-free success rate after 2.2 treatment sessions was 100% without any complications using laser lithotripsy or electrohydraulic lithotripsy (EHL). Comparable high success rates of 83-100% were reported with the practice of different techniques of lithotripsy under cholangioscopic control [24,30,31].

Long-term success defined as no further stricture and no recurrence of cholestasis or cholangitis is the vital issue for patients with benign biliary strictures. The overall success rate in the present study is comparable with those demonstrated in most of the published reports [18,20,22,29]. In accordance to the findings of Citron et al., treatment was more successful in strictures located intrahepatic or in the upper parts of the common bile duct while their lowest success rates were in the distal common bile duct (*Tab. 2*) [32].

Direct comparison of results between surgery and interventional techniques is difficult because data have rarely been published. The success rate of primary operation is about 80% and after 6-8 years varies between 75% and 90% [9-11,16]. In comparison with these data we had a primary success rate of 94% (34 of 36 patients), and 26 of 36 (72%) of patients were asymptomatic after percutaneous therapy after a long-term follow-up of 48 months.

As shown in our results, over 70% of patients presented a radiological verified regression of the stricture in the follow-up. However, on the other hand, 22% of our patients demonstrated an unchanged radiological stricture but clinical improvement after stent removal in the long-run. We therefore presume that the bile duct diameter alone is not the only factor influencing biliary clearance function.

The success of treatment probably also depends on the period during which the stents were in place, as the present results also show. Until now, there are no data on how long a transhepatic tube should remain in place. From our experience we conclude that percutaneous transhepatic tubes should be placed for up to 1 year. A stricture remaining after this length of treatment should be operated, because it is unlikely that conservative treatment would be successful. However, ongoing percutaneous transhepatic stent therapy should become the therapy of choice in poor surgical candidates or those who deny surgery.

The decision on when to remove the transhepatic tube is difficult, since a new percutaneous tract into the small bowel has to be created if there is a recurrence, and this is sometimes problematic, especially after surgical interventions. Born et al. reported two different approaches to solve the problem [18]. They tried to leave a small-diameter (7 Fr) non-functioning catheter in place for 2 to 4 months in order to maintain the cutaneobiliary tract for follow-up radiographics. After this time the likelihood of stricture recurrence may be lower. Another option is to leave a shortened percutaneous tube in place, not bridging the stenosis but keeping the sinus tract open. However, we have to keep in mind the high rate of tube dislocation and cholangitis in the follow-up with this approach.

Conclusions

In conclusion, taking into account all the available data, however, the problem of what to recommend to the individual patient is difficult. Despite the improving success rates with surgical anastomosis [9,10,12,15,25] the minimal invasive approach with transhepatic stenting should be performed as first line therapy - and not only for patients with high surgical risk [14,28,33], since we have to keep in mind the substantial differences in the success rates and associated risks involved in primary and repeated interventions for surgical repair [17]. Moreover, we recommend that the transhepatic approach should be the definitive treatment of choice for patients with intrahepatic strictures, inoperable cases or after hepaticojejunostomy. Percutaneous transhepatic stent therapy for BBS is an effective and low-risk procedure and its associated long-term results promise a real alternative to surgery.

References

1. Riemann JF, Maier M, Schilling D, Kohler B. Benigne Gallengangsstenosen- konservativ so lange wie möglich? Schweiz Rundschau Med Praxis, 1994; 83: 883-5.
2. Dumonceau JM, Deviere J, Delhaye M, Baize M, Cremer M. Plastic and metal stents for postoperative benign bile duct strictures: the best and the worst. Gastrointest Endosc, 1998; 47: 8-17.
3. Schilling D, Zopf T, Adamek HE, Riemann JF. Possibilities and limits of invasive endoscopy in treatment of endoscopic surgical complications after operations of the biliary tract. Zentralbl Chir, 1998; 123(Suppl 2): 84-8.
4. Zajko AB, Sheng R, Zetti GM, Madariaga JR, Bron KM. Transhepatic balloon dilation of biliary strictures in liver transplant patients: a 10-year experience. J Vasc Interv Radiol, 1995; 6(1): 79-83.
5. Davids PH, Tanka AK, Rauws EA, van Gulik TM, van Leeuwen DJ, de Wit LT, Verbeek PC, Huibregtse K, van der Heyde MN, Tytgat GN. Benign biliary strictures. Surgery or endoscopy? Ann Surg, 1993; 217(3): 237-43.
6. Frattaroli FM, Reggio D, Guadalaxara A, Illomei G, Pappalardo G. Benign biliary strictures: a review of 21 years of experience. J Am Coll Surg, 1996; 183(5): 506-13.
7. Geenen DJ, Geenen JE, Hogan WJ, Schenck J, Venu RP, Johnson GK, Jackson A Jr . Endoscopic therapy for benign bile duct strictures. Gastrointest Endosc, 1989; 35(5): 367-71.
8. Hartmann D, Jakobs R, Schilling D, Riemann JF. Endoscopic and radiological interventional therapy of benign and malignant bile duct stenoses. Zentralbl Chir, 2003; 128(11): 936-43.
9. Pitt HA, Kaufman SL, Coleman J, White RI, Cameron JL. Benign postoperative biliary strictures. Operate or dilate? Ann Surg, 1989; 210(4): 417-25.
10. Csendes A, Diaz C, Burdiles P, Nava O, Yarmuch J, Maluenda F, Fernandez E. Indications and results of hepaticojejunostomy in benign strictures of the biliary tract. Hepatogastroenterology, 1992; 39(4): 333-6.
11. Lillemoe KD, Pitt HA, Cameron JL. Current management of benign bile duct strictures. Adv Surg, 1992; 25: 119-74.
12. Nealon WH, Urrutia F. Long-term follow-up after bilioenteric anastomosis for benign bile duct stricture. Ann Surg, 1996; 223(6): 639-45.
13. Pellegrini CA, Thomas MJ, Way LW. Recurrent biliary stricture. Patterns of recurrence and outcome of surgical therapy. Am J Surg, 1984; 147(1): 175-80.
14. Tocchi A, Costa G, Lepre L, Liotta G, Mazzoni G, Sita A. The long-term outcome of hepaticojejunostomy in the treatment of benign bile duct strictures. Ann Surg, 1996; 224(2): 162-7.
15. Abdel Wahab M, el-Ebiedy G, Sultan A, el-Ghawalby N, Fathy O, Gad el-Hak N, Abo Elenin A, Abo Zid M, Ezzat F. Postcholecystectomy bile duct injuries: experience with 49 cases managed by different therapeutic modalities. Hepatogastroenterology, 1996; 43(11): 1141-7.
16. Rothlin MA, Lopfe M, Schlumpf R, Largiader F. Long-term results of hepaticojejunostomy for benign lesions of the bile ducts. Am J Surg, 1998; 175(1): 22-6.
17. Smith MT, Sherman S, Lehman GA. Endoscopic management of benign strictures of the biliary tree. Endoscopy, 1995; 27(3): 253-66.
18. Born P, Rosch T, Bruhl K, Sandschin W, Allescher HD, Frimberger E, Classen M. Long-term results of endoscopic and percutaneous transhepatic treatment of benign biliary strictures. Endoscopy, 1999; 31(9): 725-31.
19. Warren KW, Mountain JC, Midell AI. Management of strictures of the biliary tract. Surg Clin North Am, 1971; 51(3): 711-31.
20. Schumacher B, Othman T, Jansen M, Preiss C, Neuhaus H. Long-term follow-up of percutaneous transhepatic therapy (PTT) in patients with definite benign anastomotic strictures after hepaticojejunostomy. Endoscopy, 2001; 33(5): 409-15.
21. Gallacher DJ, Kadir S, Kaufman SL, Mitchell SE, Kinnison ML, Chang R, Adams P, White RI Jr, Cameron JL. Nonoperative management of benign postoperative biliary strictures. Radiology, 1985; 156(3): 625-9.
22. Martin EC, Fankuchen EI, Laffey KJ, Sibley RE. Percutaneous management of benign biliary disease. Gastrointest Radiol, 1984; 9(3): 207-12.
23. Hausegger KA, Kugler C, Uggowitzer M, Lammer J, Karaic R, Klein GE, Maurer M. Benign biliary obstruction: is treatment with the Wallstent advisable? Radiology, 1996; 200(2): 437-41.
24. Neuhaus H, Hoffmann W, Classen M. The benefits and risks of percutaneous transhepatic cholangioscopy. Dtsch Med Wochenschr, 1993; 118(16): 574-81.
25. Millis JM, Tompkins RK, Zinner MJ, Longmire WP Jr, Roslyn JJ. Management of bile duct strictures. An evolving strategy. Arch Surg, 1992; 127(9): 1077-82.
26. Bergman JJ, Burgemeister L, Bruno MJ, Rauws EA, Gouma DJ, Tytgat GN, Huibregtse K. Long-term follow-up after biliary stent placement for postoperative bile duct stenosis. Gastrointest Endosc, 2001; 54: 154-61.
27. Lee MJ, Mueller PR, Saini S, Hahn PF, Dawson SL. Percutaneous dilatation of benign biliary strictures: single-session therapy with general anesthesia. AJR Am J Roentgenol, 1991; 157(6): 1263-6.
28. Vogel SB, Howard RJ, Caridi J, Hawkins IF Jr. Evaluation of percutaneous transhepatic balloon dilatation of benign biliary strictures in high-risk patients. Am J Surg, 1985; 149(1): 73-9.
29. Born P, Rosch T, Willkomm G, Sandschin W, Fitz N, Weigert N, Ott R, Frimberger E, Allescher H, Classen M. Initial experience with a new Yamakawa-type prosthesis for long-term percutaneous transhepatic drainage. Endoscopy, 1999; 31(9): 748-50.
30. Bonnel DH, Liguory CE, Cornud FE, Lefebvre JF. Common bile duct and intrahepatic stones: results of transhepatic electrohydraulic lithotripsy in 50 patients. Radiology, 1991; 180(2): 345-8.
31. Mo LR, Hwang MH, Yueh SK, Yang JC, Lin C. Percutaneous transhepatic choledochoscopic electrohydraulic lithotripsy (PTCS-EHL) of common bile duct stones. Gastrointest Endosc, 1988; 34(2): 122-5.
32. Citron SJ, Martin LG. Benign biliary strictures: treatment with percutaneous cholangioplasty. Radiology, 1991; 178(2): 339-41.
33. Pereira-Lima L. Biliary reconstruction in benign postoperative stricture with transhepatic tubes. Am J Surg, 1992; 164(2): 124-8.

Trends in the incidence of the free wall cardiac rupture in acute myocardial infarction. Observational study: experience of a single center

Sobkowicz B[1]*, Lenartowska L[2], Nowak M[2], Hirnle T[3], Borys D[4], Kosicki M[4], Prajs P[4], Wrabec K[2]

[1] Department of Cardiology, Medical University of Białystok, Poland
[2] Department of Cardiology, County Hospital, Wrocław, Poland
[3] Department of Cardiac Surgery, Medical University of Białystok, Poland
[4] Division of Pathology, County Hospital, Wrocław, Poland

Abstract

Purpose: Free wall cardiac rupture (CR) is one of the most common cause of in-hospital death in acute myocardial infarction (AMI). The early diagnosis of CR and selection of the patients predisposed to CR become an important clinical tool. Aim: assessing the occurrence of CR in patients with AMI, to determine the factors which could help to identify the patients threatened with CR.

Material and methods: 2320 consecutive patients with AMI. CR was proved by autopsy or by echocardiography performed during cardio-pulmonary resuscitation (CPR).

Results: In-hospital mortality was 11% (254 patients). 50 patients (2%) died from CR. CR was the cause of 20% of total in-hospital death. Patients with CR were older than survivors (72 vs 60 years, $p<0.0001$). Women prevailed in CR group: (62% in CR group vs 27% in the survivors, $p<0.01$). 29% of patients were treated with thrombolytics (Th+). Out of 58 patients from Th (+) group who died, 17 (29.3%) died because of CR. CR occurred in 33 (16.8%) patients out of 196 died in Th (-) group. In the logistic regression analysis only age and sex remained as predictors of CR. 16 patients died from CR during first 24 h from admission (ECR). In 34 patients CR occurred >24 h (LCR). In ECR group were no prevalence of women, while in LCR women constituted 68%. In ECR group all but one patient had no previous history of MI ($p=0.06$). Frequency of thrombolythic therapy was equal.

Conclusions: Advanced age patients, particularly women with first AMI are at risk of CR. Decision of thrombolytic treatment in this group of patients must be very cautious.

* CORRESPONDING AUTHOR:
Department of Cardiology
Medical University of Białystok
ul. M. Skłodowskiej-Curie 24a
15-276 Białystok, Poland
Tel: +48 85 7468638; Fax: +48 85 7468604
e-mail: sobkowic@wp.pl (Bożena Sobkowicz)

Received 08.04.2005 Accepted 24.05.2005

Key words: free wall cardiac rupture, acute myocardial infarction, thrombolytic therapy.

Introduction

Free wall cardiac rupture (CR) became a second most important cause of death in acute myocardial infarction (AMI). Almost inevitably CR leads to death and is considered to be a hopeless situation. Some data suggest, that CR occurs more often than it is clinically diagnosed and in case of subacute CR conservative or surgical treatment could be lifesaving [1,2]. On the other hand different kind of reperfusion therapy (trombolysis or primary PCI) seems to influence the occurrence of CR [3-5]. Therfore the early diagnosis of CR and selection of the patients predisposed to CR become an important clinical tool.

The aim of the study was to assess the trends of occurrence and frequency of CR in patients suffering from AMI admitted to our department and to determine the demographic and clinical factors which could help to identify the patients especially threated with CR.

Material and methods

2320 consecutive patients with AMI admitted to Cardiological Department of Wrocław County Hospital from 1985 to 2001 were retrospectively analysed.

Diagnosis of AMI was established according to the "old" WHO definition: retrosternal pain not responding to sublingual nitrates, ST elevation in at least 2 ECG leads and/or CPK elevation (>2 x normal level).

In respect of AMI localisation, we divided patients into 3 groups: group 1 – anterior Q wave AMI, group 2 – inferior Q wave AMI, group 3 – patients with isolated lateral MI, MI of uncertain localisation, non-Q AMI.

Until 1987 AMI treatment consisted of administration of unfractionated Heparin and intravenous Nitroglycerin. Since

Table 1. **Baseline characteristics of study population by sex**

	All (%) n=2320	Women (%) n=672	Men (%) n=1648	P value
Age	60.9±12	66.0±11	58.6±12	<0.001
AMI localisation				
anterior	910 (*39.2*)	269 (*40.0*)	641 (*38.9*)	
inferior	1083 (*46.7*)	293(*43.6*)	790 (*48.0*)	NS
other	325 (*14.0*)	110 (*16.4*)	215 (*13.1*)	
Thrombolysis	661 (*28.5*)	186 (*27.7*)	475 (*28.9*)	NS
Total mortality	254 (*11.0*)	111 (*16.5*)	143 (*8.7*)	<0.001
CR	50 (*2.2*)	31 (*4.6*)	19 (*1.2*)	<0.00001
OD	204 (*8.8*)	80 (*11.9*)	124 (*7.5*)	<0.001

CR – cardiac rupture; OD – other death

Figure 1. **Bar graphs demonstrating percentage men/women over analysed period**

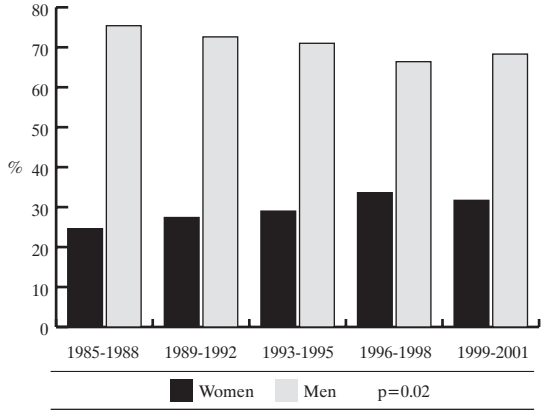
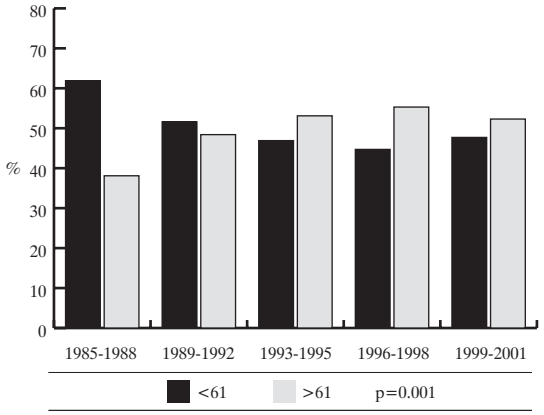


Figure 2. **Bar graphs demonstrating aging of study population (median = 61 years)**



1987 fibrinolytic treatment with streptokinase was introduced, since 1990 – new generations of thrombolytics (t-PA, rt-PA, TNK-tPA) were applied as well as a routine administration of aspirin. Up to 2001 primary percutaeous interventions (PCI) were not performed.

CR was diagnosed in 50 patients. In 39 of them on autopsy. The other 11 patients were included in the CR group on the basis of the signs of electromechanical dissociation (EMD) during cardiopulmonary resuscitation, with CR confirmed by echocardiography. Echocardiographic diagnose of CR was made when liquid layer (more than 1 cm) was present in the pericardial sac, distributed regularly around the heart. Sometimes hyperechogenic structures (clots) were visible.

CR population was divided into 2 subgroups: early CR (ECR) subgroup consisted of 16 patients who died within 24 hours after hospital admission. The remaining 34 patients were included to the late CR (LCR) subgroup.

We analysed demographic and clinical data, localisation of the AMI as well as influence of fibrinolytic treatment. In the CR group we assessed also the anamnestic data like previous myocardial infarction, preceding angina and hypertension, thrombolytic treatment.

Statistical analysis

Results are presented as a mean ± standard deviation (SD) for continuous and normally distributed variables, as median and as percentage for categorical data. Analysis of normality was performed with Kolmogorov-Smirnov test. For continuous variables comparisons between two groups were performed with unpaired two-tailed t-test. Categorical data and proportion were analysed using Chi-square test. Stepwise logistic regression model was developed to analyse the effect of baseline characteristics for the prediction of CR. A p value < 0.05 was considered statistically significant.

Results

Characteristic of the study group is shown in *Tab. 1*.

There were 29% women in study population. Women were significantly older than men. During observation time, the percentage of women with AMI was growing up significantly, AMI population was growing old (*Fig. 1*,*2*).

254 patients (11%) died. During study period hospital mortality ranged from 15.9% to 5.7% and significantly decreased (p=0.01). CR constituted 19.7% of whole deaths. The frequency of CR fluctuated, but increased (*Fig. 3*). CR as a cause of death was noted significantly more often in women than in men. The mean age of AMI survivors (S) was nearly 7 years lower, than those who died, and more than 11 years lower than those who died of CR (*Tab. 2*).

In whole AMI group, inferior wall MI occurred slightly more often, in CR group anterior wall MI was in prevalence.

Cardiac rupture and thrombolysis

661 patients (28.5%) received thrombolytic treatment. Frequency of thrombolytic therapy rose rapidly, with maximum in 1999, when 53% of patients received this kind of treatment. There was difference in mortality between groups in favour of those who received thrombolytic treatment Th (+): (8.8% vs 11.8% respectively, p=0.033).

Figure 3. **Free wall cardiac rupture ploted against thrombolytic treatment**

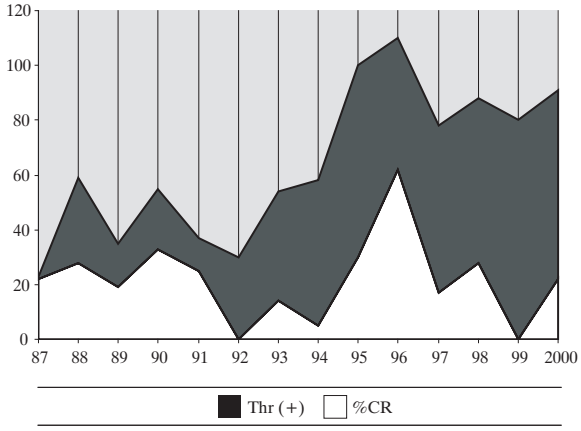


Table 2. **Subgroup analysis: hospital outcomes**

	S (%) n=2066	CR (%) n=50	OD (%) n=204	P value
Age	60.0±12	71.7±9	68.1±12	<0.001
Sex				
women	561 (*27.2*)	31 (*62*)	80 (*39.2*)	<0.01
men	1505 (*72.8*)	19 (*38*)	124 (*60.8*)	
AMI localisation				
anterior	805 (*39.0*)	25 (*50.0*)	80 (*39.2*)	
inferior	991 (*48.0*)	20 (*40.0*)	72 (*35.3*)	NS
other	268 (*13.0*)	5 (*10.0*)	52 (*25.5*)	
Thrombolysis	603 (*29*)	17 (*34.0*)	41 (*20.1*)	p=0.034
Time to death (day) Median		4.1±3.6 4	5.6±10 2	NS

CR – cardiac rupture; OD – other death; S – survivors

Table 3. **Demographic and clinical data in patients with (CR) divided into early and late rupture (ECR and LCR)**

	CR (%) n=50	ECR (%) n=16	LCR (%) n=34
Age	71.7±9	69±9	73±8
sex			
women	31 (*62*)	8 (*50*)	23 (*68*)
men	19 (*38*)	8 (*50*)	11 (*32*)
Previous MI	11 (*24*)	1 (*7*)	10 (*32*)
Previous angina	14 (*32*)	3 (*23*)	11 (*36*)
Hypertension	23 (*46*)	6 (*38*)	17 (*50*)
AMI localisation			
anterior	25 (*50*)	9 (*56*)	16 (*47*)
inferior	22 (*44*)	6 (*38*)	16 (*47*)
other	3 (*6*)	1 (*6*)	2 (*6*)
Thrombolysis	17 (*34*)	6 (*38*)	11 (*32*)

CR – cardiac rupture; ECR – early cardiac rupture; LCR – late cardiac rupture

Out of 58 patients from Th (+) group who died, 17 (29.3%) died because of CR. Out of 196 patients who died and did not received thrombolytic treatment (Th (-) group) frequency of CR was lower: 33 patients (16.8%) p=0.036. Only 2 patients (12%) who died from CR received thrombolytic treatment later than 6 h from the onset of chest pain. In first case time interval was 7 h, in second one 12 h after beginning of chest pain.

32% of CR occurred during first 24 hours after admission (ECR group). The differences between ECR and LCR groups were not signifficant. However, in ECR group were no prevalence of women, while in LCR women constituted 68% (*Tab. 3*). In ECR group only one patient had previous history of MI while in LCR 11 (p=0.06). Frequency of thrombolythic therapy was equal in both groups.

All investigated variables were included in a logistic regression analysis for the prediction of CR. In the forward stepwise procedure the variables that remained as predictors of CR were age (OR 1.072 CI 1.042 – 1.103) and sex (OR 0.349 CI 0.187 – 0.652) both for p<0.001.

Discussion

Our study had an observational character and consisted of unselected consecutive patients with AMI treated in one cardiac centre during consecutive 17 years. Thrombolysis was the only one reperfusion therapy administered.

We noticed significant improvement of survival in patients with AMI during analysed period in spite of growing old population and increasing percentage of women resulted in well known fact of greater mortality in this subgroups of patients [6-10].

The occurrence of CR was consistent with previously published papers [11,12]. Probably the percentage could be greater, if as in most studies, all patients died with signs of EMD were qualified as CR. Some authors consider appearance of EMD as diagnostic for CR [13,14]. In our study we considered only incontestable cases of CR confirmed on autopsy and/or by echocardiography.

In our material, as in previous studies CR occurred in significantly older patients, with prevalence of women [15-17]. The prevalence of CR in women population is not obvious. Significantly higher mean age of women is of great importance. The different course of CAD in women plays also an important role. The differences concern anatomical properties of coronary circulation and atherosclerotic plaque [19,20].

Cardiac rupture in respect of thrombolysis

There are three mechanisms possibly responsible for an increased occurrence of CR in patients received thrombolysis: 1) hemorrhage to the ischemic zone resulting loose of strength of muscular tissue [20,21]; 2) thrombolytic agents increase degradation of collagen and restrain its synthesis [22]; 3) lymphocyte migration to the infarct zone initiate absorbtion of collagen and proteolysis [21].

The results of GISSI-1 trial and Honan investigation showed, that percentage of CR positively correlates with the delay of thrombolytic treatment [23,24]. LATE trial did not confirm this results, suggesting that the late thrombolysis (>12

hours), did not increase the risk of CR, but accelerated occurrence of CR as compared to placebo group [13]. Other authors suggest, that thrombolysis decrease the rate of CR and the lack of reperfusion of the culprit vessel is responsible for increased risk of CR [25]. In fundamental anlysis concerning CR based on U.S. registry of 35000 patients with AMI, Becker found, that in the thrombolytic era, percentage of CR as a cause of death increased. Moreover, thrombolysis accelerates occurrance of CR as compared to patients who did not received thrombolysis [12].

We found higher percentage of CR in patients treated with thrombolytics but in logistic regression analysis this difference did not reach statistical significance perhaps because of small sample size. We cannot confirm an observation concerning acceleration of CR by late administration of thrombolytics as well as an observation concerning shorter time interval from the onset of chest pain to CR in patients treated with thrombolythics.

There are no clear classification of CR. Generally 2 classification are proposed: acute CR (death within 30 min) and subacute CR (30% of cases) or early CR (up to 72 h) and late CR [1,2,26]. We were searching for the predisposing factors for CR during first 24 hours hospitalisation when the choice of reperfusion therapy takes place. We can not explain why the significant prevalence of women in CR group did not concern the ECR subgroup, where the proportion between men and women were equal. Some authors had similar observation [1,2]. In contrary to ECR group, in LCR group 50% of patients suffered from hypertension which is regarded by some authors as a factor increasing the risk of CR [11]. All but one patient from the ECR group had previously MI, only a few had history of angina. More severe course of MI and greater tendency to CR was described in patients suffered from the first MI with no preinfarction angina [27]. This observation may be explained by lack of the effect of ischemic preconditioning. According to some authors CR occurres frequently in patient with one vessel disease, when the collateral circulation is not developed. Mentioned above differences between the ECR and LCR group suggest not homogenous mechanisms leading to CR. Cardiomyocyte apoptosis, neutrophylic infiltration and the importance of defective metalloproteinases (MMPs) activation during LV remodeling after AMI could lead to CR [5,28]. On a mouse model of AMI it has been demonstrated that MMPs inhibition may prevent CR [29]. Genetic susceptibility for CR (i.e. polymorphism in MPPs promoters) is also taken into consideration [5].

Some investigators call in question the advantage of thrombolytic therapy in patients >75 years, because of high risk of such treatment [30]. The optimal reperfusion therapy in this group of patients is now widely discussed. Primary PCI reduces the risk of CR, although its influence on frequency of CR occurrence in AMI is now the matter of investigation [31,32].

Conclusions

Selection of patients with AMI particulary threaten with CR may be possible. This are the patients older than 70 years with first AMI, particulary women. In this group of patients, thrombolytic therapy if given should be followed by careful (probably echocardiographic) observation.

Limitations: The material presented was analysed in most cases retrospectively, so it was impossible to focus on same important problems like eventual analysis of subacute CR. The obduction was not performed in all patients died because of AMI, even in patients died within signs of EMD, which with no doubt influenced on lowering the percentage of CR in our material.

References

1. Figueras J, Cortadellas J, Evangelista A. Medical management of selected patients with left ventricular free wall rupture during acute myocardial infarction. J Am Coll Cardiol, 1997; 29: 512-8.
2. Purcaro A, Costantini C, Ciampani N. Diagnostic criteria and management of subacute ventricular free wall rupture complicating acute myocardial infarction. Am J Cardiol, 1997; 80: 397-405.
3. Oka RK, Fortman SP, Varady AN. Differences in treatment of acute myocardial infarction by sex, age, and other factors (the Stanford Five-City Project). Am J Cardiol, 1996; 78: 861-5.
4. Bartoletti A, Fantini A, Meucci F, Idini R, Abbondanti A, Mangialavori G, Margheri M, Vergasola R. Primary coronary angioplasty in acute myocardial infarction: is it possible to prevent postinfaction cardiac rupture? Ital Heart J, 2000; 1: 400-6.
5. Wehrens XHT, Doevendans PA. Cardiac rupture complicating myocardial infarction. Int J Cardiol, 2004; 95: 285-92.
6. Widdershoven J, Gorgeles A, Vermeer F. Changing characteristics and in-hospital outcome in patients admitted with acute myocardial infarction. Observations from 1982 to 1994. Eur Heart J, 1997; 18, 1073-80.
7. Naylor CD, Chen E. Population-wide mortality trends among patients hospitalysed for acute myocardial infarction: The Ontario experience, 1981 to 1991. J Am Coll Cardiol, 1994; 24: 1431-8.
8. Vaccarino V, Krumholz HM, Berkman L, Horowitz R. Sex difference in mortality after myocardial infarction. Is there evidence for an increased risk for women? Circulation, 1995; 91: 1861-71.
9. Marrugat J, Sala J, Masia R, Pavesi M. Mortality differences between men and women following first myocardial infarction. JAMA, 1998; 280: 1405-9.
10. Mahon NG, Mc Kenna CJ, Codd MB, O'Rorke C, McCann HA, Sugrue DD. Gender differences in the menagement and outcome of acute myocardial infarction in unselected patients in the thrombolytic era. Am J Cardiol, 2000; 85: 921-6.
11. Pollak H, Diez W, Spiel R. Early diagnosis of subacute free wall rupture complicating acute myocardial infarction. Eur Heart J, 1993: 14: 640-8.
12. Becker R, Gore J, Labrew C. A composite view of cardiac rupture in United States National Registry of myocardial infarction. JACC, 1996; 27: 1321-6.
13. Becker RC, Charlesworth A, Hampton J. Cardiac rupture associated with thrombolytic therapy: impact of the time to treatment in the Late Assessment of Thrombolytic Efficacy Study. J Am Coll Cardiol, 1995; 25(5): 1063-8.
14. Figueras J, Curos A, Cortadellas J. Reliability of electromechanica dissociation in the diagnosis of left ventricular free wall rupture in acute myocardial infarction. Am Heart J, 1996; 131: 861-4.
15. Salem B, Lagos J, Haikal M, Gowda S. The potential impact of the thrombolytic era on cardiac rupture complicating acute myocardial infarction. Angiol, 1994; 45: 931-6.
16. Sobkowicz B, Lenartowska L, Borys D, Hirnle T, Nowak M, Zawada J, Wrabec K. Pęknięcia serca w świeżym zawale. Ocena kliniczno-patologiczna chorych leczonych w dużym Oddziale Internistycznym. Kardiol Pol, 2000; 52: 85-92.
17. Hutchins K, Skurnick J, Lavenhar M, Natarajan M. Cardiac rupture in acute myocardial infarction. Am J Forensic Med Pathol, 2002; 23: 78-82.
18. Chyczewski L, Musiatowicz B, Kurłowicz-Sidun B. Analiza pęknięć serca w materiale sekcyjnym Katedry i Zakładu Anatomii Patologicznej Akademii Medycznej w Białymstoku. Pol Arch Med. Wewn, 1972; 49: 587-90.
19. Syska-Stumińska J, Cybulska K, Dłużniewski M. Coronary artery disease in women – is it different than in men? Medipress Karidologia, 1999; 1: 3-8.

20. Becker R, Charlesworth A, Wilcox R, Hampton J, Skene A, Gore JM, Topol EJ. Cardiac rupture associated with thrombolytic therapy. Impact of time to treatment in the late assessment of thrombolytic efficacy. J Am Coll Cardiol, 1995; 25: 1063-8.

21. Kawano H, Miyauchi K, Okada R, Daida H, Yokoi H, Miyano H, Takaya J, Satoh H, Yamaguchi H, Suda K. Histopathological study of cardiac rupture following myocardial infarction with and without thrombolytic therapy. J Cardiol, 1994; 24: 249-55.

22. Peuhkurinen K, Risteli L, Jounela A, Risteli J. Changes in interstitial collagen metabolism during acute myocardial infarction treated with streptokinase or tissue plasminogen activator. Am Heart J, 1996; 131: 7-13.

23. Gruppo Italiano per lo Studio della Streptochinasi nell'Infarcto Miocardico (GISSI). Effectiveness of intravenous thrombolytic treatment of acute myocardial infarction. Lancet, 1986; 1: 397-402.

24. Honan MB, Harrel FE, Reimer KA. Cardiac rupture, mortality once the timing of thrombolytic therapy: A meta-analysis. J Am Coll Cardiol, 1990; 16: 359-67.

25. Cherix E, De Sward H, Dijkman L, Havenith MG, Maessen JG, Engelen DJ, Wellens HJ. Myocardial rupture after myocardial infarction is related to the perfusion status of the infarct-related coronary artery. Am Heart J, 1995; 129: 644-50.

26. Nakatsuchi Y, Minamino T, Fuji K, Negoro S. Clinicopathological characterisation of cardiac free wall rupture in patients with acute myocardial infarction: difference between early and late phase rupture. Int J Cardiol, 1994; 47: S33.

27. Kobayashi Y, Miyazaki S, Itoh A, Daikoku S, Morii I, Matsumoto T, Goto Y, Nonogi H. Previous angina reduces in-hospital death in patients with acute myocardial infarction. Am J Cardiol, 1998; 81: 117-22.

28. Anzai T, Yoshikawa T, Shiraki H. C-reactive protein as a predictor of infarct expansion and cardiac rupture after a first Q-wave acute myocardial infarction. Circulation, 1997; 96: 778-84.

29. Heymans S, Luttun A, Nuyens D, Theilmeier G. Inhibition of plasminogen activator or matrix metalloproteinase prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nature Med, 1999; 5: 1135-42.

30. Thiemann DR, Coresh J, Schulman SP, Gerstenblith G, Oetgen WJ, Powe NR. Lack of benefit for intravenous thrombolysis in patients with myocardial infarction who are older than 75 years. Circulation, 2000; 101: 2239-46.

31. Holmes D, White H, Pieper K, Ellis S, Califf R, Topol E. Effect of age on outcome with primary angioplasty versus thrombolysis. J Am Coll Cardiol, 1999; 33: 412-9.

32. Nishiyama K, Nakagawa Y, Okino S, Shirai S, Ando K, Nobuyoshi M. Angiographical predictors of free wall rupture after primary coronary angioplasty for acute myocardial infarction. Eur Heart J, 2003; 24(Suppl.): 294.

Thrombin activatable fibrinolysis inhibitor (TAFI) in stable angina pectoris patients undergoing coronary artery bypass grafting (CABG)

Lisowski P[1]*, Małyszko J[2], Hirnle T[1], Lisowska A[3], Jackowski R[1], Małyszko JS[2], Buzun L[1], Myśliwiec M[2]

[1] Department of Cardiac Surgery, Medical University of Białystok, Poland,
[2] Department of Nephrology and Transplantology, Medical University of Białystok, Poland,
[3] Department of Cardiology, Medical University of Białystok, Poland

Abstract

Purpose: Thrombin activatable fibrinolysis inhibitor (TAFI) seems to be a potential haemostatic risk factor of coronary artery disease (CAD). Taking into account interactions between TAFI and haemostasis, especially during cardiopulmonary bypass, we decided to determine concentration of TAFI and activated TAFI (TAFIa) and other haemostasis markers in CABG patients.

Material and methods: 45 CAD patients (11 women, 34 men) undergoing elective CABG were included in the study. Blood samples were taken before the operation, on the 3rd, 7th day and 3 months after CABG. A value of $p<0.05$ was considered statistically significant.

Results: We found a significant decrease in TAFIa concentration on 3rd postoperative day: 6 μg/ml (0.3-43.2) vs 8.9 μg/ml (0.5-37) before CABG ($p<0.05$), a significant increase in TAFI concentration on the 7th postoperative day: 127.7%±36.8 vs 112.18%±30.34 of standard plasma concentration before CABG ($p<0.05$), significant increase in plasmin-antyplasmin (PAP) complexes concentration on 3rd and 7th day, respectively: 645 μg/l (323-1237) vs 406 μg/l (197-1840) before CABG ($p<0.001$); and 1030 μg/l (640-2149) vs 406 μg/l (197-1840) before CABG ($p<0.0001$). Before operation we found a significant negative correlation between PAP complexes concentration before CABG and EuroSCORE risk scale value ($p<0.01$).

Conclusions: In CABG patients, there is a significant increase in fibrinolytic activity due to decrease in TAFIa concentration, with simultaneous increase in PAP complexes. A significant negative correlation between PAP complexes concentration before CABG and EuroSCORE risk scale value stressed a potentially higher operation risk in patients with lower fibrinolytic activity.

Key words: thrombin activatable fibrinolysis inhibitor (TAFI), coronary artery bypass grafting (CABG), coronary artery disease (CAD), haemostasis.

Intoduction

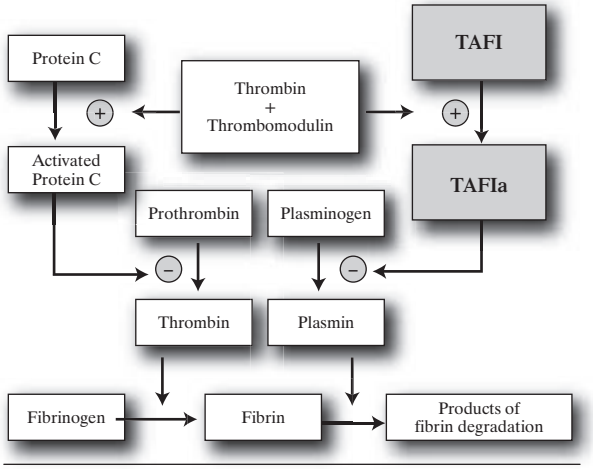
Coronary artery disease (CAD) is a leading cause of mortality in well developed societies. This occurs in spite of growing knowledge of atherosclerosis pathogenesis. Atherosclerosis begins as a functional or/and structural changes in endothelium, which in turn causes its injury and impairs humoral and secreting function. Haemostasis plays an important role in the progression of atherosclerosis, development of cardiac complications (acute coronary syndromes) – especially after cardiac surgery with cardiopulmonary bypass. Increased risk of cardiovascular diseases is combined with high activity of coagulation system and lower activity of fibrinolytic system, enhanced platelets activation, and dysfunction of the endothelium. In spite of that, research still continues to determine the precise role of each of haemostatic factors in increased risk of coronary artery disease and its complications.

Decrease of fibrinolytic activity is considered to be a risk factor for arterial thrombosis. Thrombin activatable fibrinolysis inhibitor (TAFI), a glikoprotein identified by Bajzar [1] in 1995, seems to play special role as a potential risk factor of CAD. Mosnier et al. [2] proved that plasma TAFI concentration in normal individuals correlates with clot lysis time. Activated TAFI (TAFIa) inhibits the conversion of Glu-plasminogen to Lys-plasminogen [3]. Thrombin-thrombomodulin complex is a physiological activator not only for TAFI, but also for

* CORRESPONDING AUTHOR:
Department of Cardiac Surgery
Medical University of Białystok
ul. M. Skłodowskiej-Curie 24a
15-276 Białystok, Poland
Tel. +48 85 7468464; Fax: +48 85 7468630
e-mail: lispiotr@poczta.onet.pl (Piotr Lisowski)

Received 09.05.2005 Accepted 22.07.2005

Figure 1. **Physiological role of TAFI in haemostasis**



TAFI – thrombin activatable fibrinolysis inhibitor
TAFIa – activated thrombin activatable fibrinolysis inhibitor

Table 1. **Clinical characteristics of the study group**

Number of patients (n)	45
Females	11
Males	34
CCS class (n)	
II	25
III	20
Age (years)	60.4±9.38 (35-75)
Body mass index (kg/m^2)	29±3.5
Ejection fraction of the left ventricle (%)	48.18±9.85
Ejection fraction between 50% and 30% (n)	13
Ejection fraction <30% (n)	3
EuroSCORE (points)	2.2±1.5
Aspirin withdraw before CABG (days)	12.24±7.81 (5-30); median – 9
MI (n)	
Q-wave	18
Non-Q	10
Segmental wall motion abnormalities of the left ventricle (n)	36
Previous coronary angioplasty (n)	4
Hypertension (n)	32
Cigarette smoking (n)	17
Obesity (n)	17
Peripheral atherosclerosis (n)	4
Obstructive lung disease (n)	1
Mild renal failure (n)	1

MI – myocardial infarction; EuroSCORE – cardiac surgery operative risk scale; CCS class – Canadian Cardiovascular Society Grading Scale for Angina Pectoris

protein C – that is why TAFI could be a kind of link between coagulation and fibrinolysis. Activation of TAFI is mediated by thrombin – generated by the coagulation cascade [4]. TAFIa is a substrate for transglutaminases – e.g. factor XIIIa builds TAFIa into fibrin net – it ensures localisation activity and may protect against its inhibitors [4]. The physiological role of TAFI in haemostasis is shown on *Fig. 1*.

In an animal arterial and vein thrombolysis models inhibition of TAFI by specific inhibitors enhanced tPA induced lysis of a thrombus [5]. Van Tilburg et al. [6] showed that TAFI plasma concentration above the 90th percentile of the controls increased the risk for thrombosis nearly 2-fold compared with TAFI plasma concentration below the 90th percentile. Schroeder et al. [7] revealed in intracoronary plasma samples of CAD patients a significant increase of TAFI concentration compared to the control group. They showed also a positive correlation between TAFI concentration and fibrinogen and total cholesterol concentration, but they showed no correlation neither between TAFI and others cardiovascular risk factors nor between TAFI and extent of coronary atherosclerosis. They suggested that TAFI might be a risk factor for development of CAD. Silveira et al. [8] found a significant increase in TAFI plasma concentration in pre-CABG patients with stable angina pectoris (110 men) compared to the control group. In a subset of 31 men, they observed a decrease in TAFI concentration on 3rd postoperative day and its increase on 6th postoperative day compared with preoperative concentration. Silveira et al. [9] suggested that increased TAFI concentration could enhance early occlusion of venous bypass grafts as well as acceleration of thrombosis in native atherosclerotic coronary arteries by fibrinolysis inhibition. Lau et al. [9] have proposed a novel potential predictor of angiographic coronary restenosis after percutaneous transluminal coronary angioplasty (PTCA) – increased concentration of TAFI together with decreased concentration of PAI-1.

However, the final role of plasma TAFI concentration in arterial thrombotic events is not clear yet. Brouwers et al. [10] investigated 209 unstable angina pectoris (UAP) patients of which 76 were refractory to medical treatment. Patients with more severe form of UAP had significantly lower plasma TAFI concentration. Moreover, Juhan-Vague et al. [11] observed that low plasma TAFI concentration was associated with significantly higher risk of myocardial infarction (MI).

Taking all these data into consideration, we designed a prospective study to determine the effects of CABG on TAFI concentration and activated TAFI (TAFIa) concentration, as well as on markers of coagulation: prothrombin fragments F 1+2, thrombin-antythrombin (TAT) complexes, fibrinolysis: plasmin-antyplasmin (PAP) complexes and endothelial dysfunction: von Willebrand factor (vWF), thrombomodulin (TM). We assessed also correlations between TAFI, other biochemical and perioperative parameters and clinical outcome of CABG patients.

Material and methods

Patients

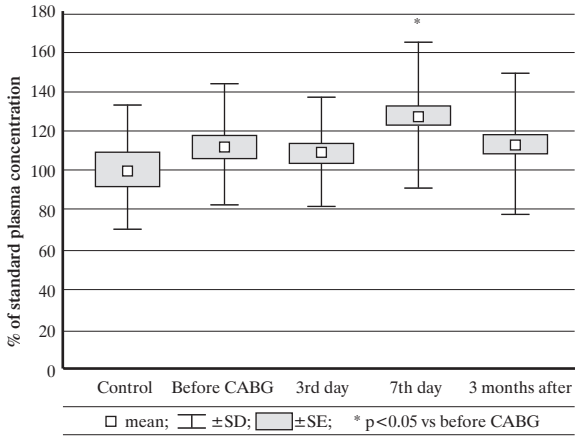
Forty-five stabile angina pectoris patients (11 women, 34 men) with CAD confirmed by angiography, udergoing elective CABG were included in the study. Patients were qualified for the operation according to ACC/AHA Guidelines for CABG – class I and IIa. *Tab. 1* shows characteristics of the study group. Exclusion criteria were: diabetes mellitus, liver dysfunction, treating with oral anticoagulants, unstable angina pectoris, and

Table 2. **Procedural data and clinical perioperative parameters**

Procedural data		
Duration of procedure (hours)	6.19±1.16	
Duration of cardiopulmonary bypass (min)		106.02±36
Cross-clamping time (min)		59.8±28.03
Number of grafts (n)		2.73±0.81
Postoperative course		
Myocardial ischaemia events (n)		12
Perioperative MI (n)	Non-Q	3
	Q-Wave	1
Intra-aortic balloon pump (n)		2
Blood transfusion (n)		34
Haemofiltration (n)		4

MI – myocardial infarction

Figure 2. **Plasma TAFI concentration**



absence of lipid disorders. Aspirin therapy was withdrawn to all the patients about two weeks before the operation (average 12 days, minimum 5 days) and restarted in the evening of the day of CABG. There were no significant differences in pharmacology treatment in the study after the surgery. That way its potential disturbing influence on investigated parameters was minimized. The Ethics Committee of the Medical University of Białystok approved the study protocol.

Methods

CABG was performed typically – via median sternotomy. Standard anesthetic and cardiopulmonary bypass (CPB) techniques with normotermia were used. *Tab. 2* shows procedural data and clinical perioperative parameters. Blood samples were taken the day before the operation, on the 3rd, 7th day and 3 months after CABG. The first post-CABG measurements were performed three days after the procedure to minimize the effects of surgical stress, blood transfusion or agents used perioperatively on the concentration of examined haemostatic parameters.

Blood samples were taken from antecubital vein without the stasis, in the morning and were collected on citrate (3,8% trisodium citrate – 1 ml of citrate, 9 ml of blood). Blood samples were centrifuged within one hour at 3000 rpm for 10 minutes. Half of the supernatant was taken from the middle layer of plasma. Plasma samples were stored at -70°C until assayed.

The control group consisted of 33 age- and sex-matched healthy volunteers to obtain the normal ranges of the haemostatic parameters studied.

TAFI antigen concentration (TAFI Ag) was determined by commercially available immunoassay (TAFI-EIA, Affinity Biologicals Inc, Canada). TAFI concentration was expressed as percentage of standard plasma concentration. TAFIa concentration was determined by commercial chromogenic assay (ACTICHROME® Plasma TAFI Activity Kit, American Diagnostica, USA). Other haemostatic markers were also determined by using commercial immunoassays: prothrombin fragments 1 +2 (F 1+2) – Enzygost® F 1+2 micro, Dade – Behring, Germany; thrombin-antythrombin (TAT) complexes – Enzygost® TAT micro, Dade – Behring, Germany; plasmin-antyplasmin (PAP) complexes – Enzygost® PAP micro Dade – Behring; Germany; thrombomodulin (TM) – IMUBIND® Thrombomodulin ELISA Kit, American Diagnostica, USA; von Willebrand factor (vWF) – IMUBIND® vWF ACTIVITY Elisa, American Diagnostica Stago USA. All assays were performed according to manufacturer's instructions by the same person.

Other coagulation and biochemical parameters were determined using standard laboratory methods: prothrombin time (PT), INR, activated partial thromboplastin time (APTT), fibrinogen, complete blood count, platelets, creatinine, urea, bilirubin, sodium, potassium, total protein, alanine aminotransferase (AlAT), aspartate aminotransferase (AspAT), creatinine kinase (CK) and its cardiac isoenzyme (CK-MB), glucose, total cholesterol and its fractions.

We estimated the operative risk for each patient from the study group, according to EuroSCORE (cardiac surgery operative risk scale). The higher EuroSCORE value indicated higher operative risk.

Statistical analysis

Statistical analyses were performed using Statistica 6.0 PL software for Windows (Tulsa, OK, USA). Shapiro-Wilk test was used to check the data distribution. Whenever possible in skewed distribution, logarithmic transformation was performed before the analysis. In normally distributed variables statistical analysis was performed using one way ANOVA test with following post hoc Tukey's test. In a case on non-normal distribution, Kruskall-Wallis analysis of variance with post hoc Mann-Whitney test was used. All the normally distributed parameters were presented as means ±SD. Others are given as medians and minimal-maximal values. A value of $p<0.05$ was considered statistically significant. Correlations between parameters were analyzed with Pearson's or Spearman's correlation coefficient, as appropriate.

Results

We found a significant increase in TAFI concentration on the 7th day after CABG: 127.7%±36.8 vs 112.18%±30.34 of standard plasma concentration before CABG, $p<0.05$ (*Fig. 2*).

Figure 3. **Plasma TAFIa concentration**

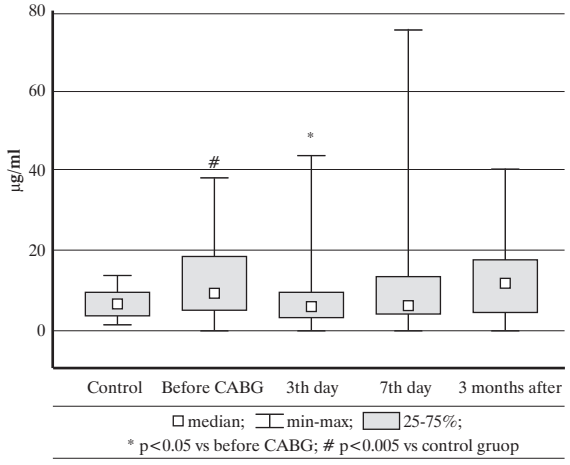


Figure 4. **Plasma plasmin-antiplasmin (PAP) complexes concentration**

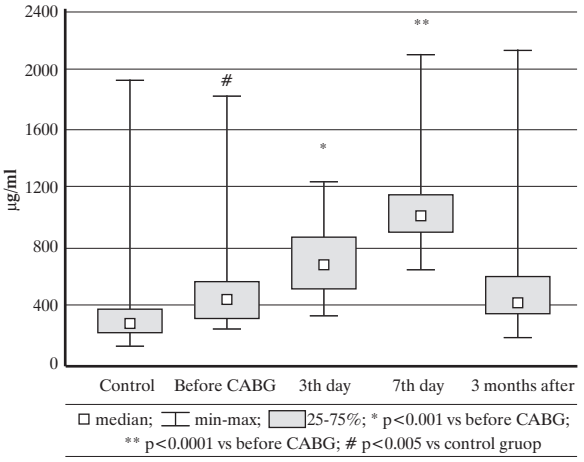


Figure 5. **Plasma von Willebrand factor (vWF) activity**

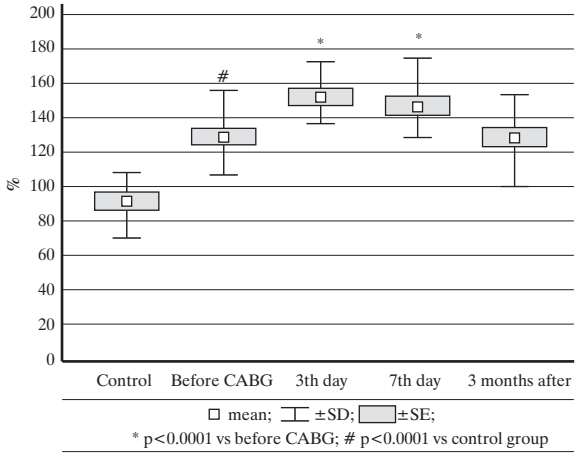
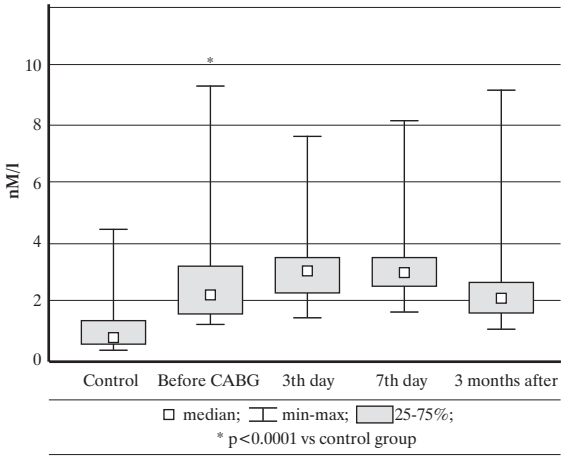


Figure 6. **Plasma prothrombin fragments 1+2 (F 1+2) concentration**



There was a significant decrease in TAFIa concentration on the 3rd postoperative day: 6 µg/ml (0.3-43.2) vs 8,9 µg/ml (0.5-37) before CABG, p<0.05 (*Fig.* 3).

Significant increase in PAP complexes on the 3rd and 7th day was found, respectively: 645 µg/l (323-1237) vs 406 µg/l (197-1840) before CABG, p<0.001; and 1030 µg/l (640--2149) vs 406 µg/l (197-1840) before CABG, p<0.0001 (*Fig. 4*). Significant increase in vWF activity on the 3rd and 7th day, respectively: 154.3%±17.8 vs 129.6%±24.4 before CABG, p<0.0001 and 150.95%±21.1 vs 129.6%±24.4 before CABG, p<0.0001 (*Fig. 5*). No significant F 1+2 fragments (*Fig. 6*) and TAT complexes (*Fig. 7*) concentration alteration, before vs after CABG were found. On the 3rd postoperative day a tendency to decrease in TAT complexes was observed when compared to the pretreatment values. However, it did not reach statistical significance (p=0.06) (*Fig. 7*). There was no significant TM concentration alteration, before vs after CABG. However on the 3rd postoperative day we found statistical margin increase in TM concentration compared to the values from before the procedure: 4 ng/ml (0.64-15,88) vs 2.52 ng/ml (0.12-25,8) before CABG, p=0.059) (*Fig. 8*).

TAFIa concentration, PAP complexes concentration, F 1+2 fragments concentration, TAT complexes concentration, vWF activity were significant higher in studied patients before an operation then in control group. There were respectively: TAFIa concentration 8.9 µg/ml (0.5-37) vs 4.4 µg/ml (1.1-10.6) p<0.005 (*Fig. 3*); PAP complexes 406 µg/l (197-1840) vs 285 µg/ml (126-1917) p<0.005 – *Fig. 4*; F 1+2 fragments 2.07 nM/l (1.11--9.28) vs 0.63 nM/l (0.26-4.52) p<0.0001 (*Fig. 6*); TAT complexes 7.95 µg/l (0.4-124.4) vs 1 µg/l (1-4) p<0.0001 (*Fig. 7*); vWF 129.6%±24.4 vs 88.2%±15.2 p<0.0001 (*Fig. 5*). TAFI (*Fig. 2*) and TM (*Fig. 8*) concentrations did not differ significantly between control group and patients before the CABG.

In the study group we also found significant decrease in total cholesterol, LDL-cholesterol and triglycerides concentrations 3 months after CABG compared to before an operation, respectively: 4.77 mmol/l±1.38 vs 5.71 mmol/l±1.11 p<0.05; 2.91 mmol/l±1.15 vs 3.59 mmol/l±1.14 p<0.05; 1.43 mmol/l±0.59 vs 1.9 mmol/l±0.79 p<0.05. A significant decrease in haemoglobin, erythrocytes concentrations and haematocrit value on 7-th postoperative day compared to before an operation was found, respectively: 7.01 mmol/l±1.09 vs 8.53 mmol/l±0.72

Figure 7. **Plasma thrombin-antithrombin (TAT) complexes concentration**

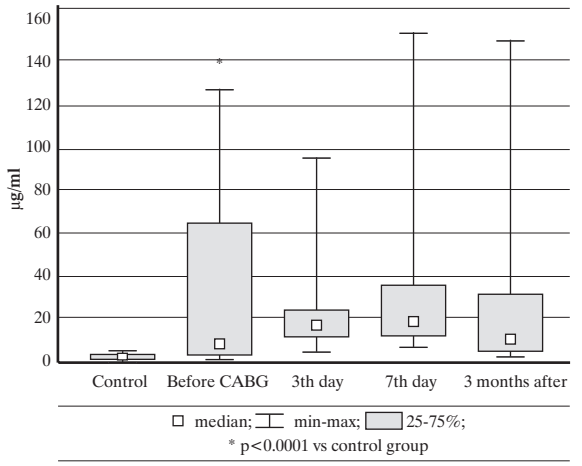
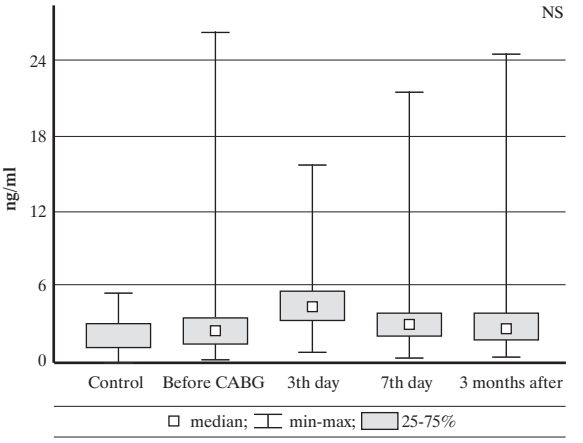


Figure 8. **Plasma thrombomodulin (TM) concentration**



p<0.05; 3.86 x10^9/mm^3±0.78 vs 4.52 x10^9/mm^3±0.34 p<0.05; 34.98%±6.03 vs 41.18%±3.42 p<0.05. On 7th postoperative day compared to before an operation a significant increase in platelets concentration was found: 331.33 x10^3/mm^3±108.32 vs 215.84 x10^3/mm^3±45.52.

Correlations

– significant positive correlation between TAFIa concentration before CABG and total cholesterol concentration (p<0.05; r=0.41);

– significant positive correlation between TAFIa concentration on the 3rd postoperative day and concentration of fibrinogen (p<0.05; r=0.49);

– significant negative correlation between PAP complexes concentration before CABG and EuroSCORE risk scale value (p<0.01; r=-0.44);

– significant positive correlation between F 1+2 fragments concentration on the 3rd postoperative and aorta clamping time (p<0.05; r=0.31);

– significant positive correlation between vWF activity on the 7th postoperative day and duration time of the procedure (p<0.05; r=0.33).

Discussion

Since TAFI has been identified and its role in haemostasis and thrombosis has been confirmed, a possibility of important role of TAFI in CAD, has been considering. There was few data on TAFI concentration, and/or TAFIa concentration and their correlations with independent risk factors for CAD and other haemostasis parameters in CAD patients [7,9-11].

In the studied group, there was significant increase of TAFI concentration on the 7th postoperative day compared to the values before the procedure. Taking into account simultaneous increase of the markers of plasmin formation (PAP-complexes), increase of TAFI concentration could be a response to increased fibrinolytic activity. That is even more prominent because increase of TAFI concentration took place despite hemodilution after CABG.

Silveira et al. [8] also revealed increase of TAFI concentration on the 6th day after CABG in CAD patients, compared to the values obtained before the procedure and to the control group. Simultaneously in a subset of 31 men, they observed decrease of TAFI concentration on the 3rd postoperative day and its increase on the 6th postoperative day compared with preoperative values [8]. In our study on the 3rd postoperative day we observed statistical decrease of TAFIa concentration, however we did not observe statistical decrease in TAFI concentration. In our study TAFIa concentration before CABG was higher than in healthy volunteers. So, decreased TAFIa concentration on the 3rd postoperative day could rather reflect an increased fibrinolytic activity in our studied group. Moreover, PAP complexes concentration statistically increased on the 3rd and 7th postoperative day when compared to the pretreatment values. Concentrations of thrombin generation markers – (F 1+2 fragments and TAT complexes) were no statistically different before and after CABG. These preliminary results on the effects of CABG on markers of ongoing coagulation and vWF were reported previously [12]. On the other hand, TAFIa concentration as well as F 1+2 and TAT complexes were significanlty higher in patients undergoing CABG when compared to the healthy volunteers. All haemostatic parameters studied returned to baseline values after 3 months following CABG. An observed decrease of TAFIa concentration after CABG could be a result of used CPB. There was a hypercoagulability state before the operation followed by hyperfibrinolysis in early postoperative period (3rd to 7th day). CPB could decrease TAFIa concentration by reduction of TAFI activator-thrombin concentration [13]. The significant negative correlation between TAFIa concentration on the 3rd postoperative day and cross clamping time of aorta during the operation may speak in favour that CPB could reduce TAFIa concentration.

Silveira et al. [8] reported also a significant decrease in PAI-1 concentration on the 3rd postoperative day and a little higher, but still lower than before the CABG on the 6th post-

operative day. It may suggest an increased plasma fibrinolytic activity. In our study we found an increase in PAP complexes on the 3rd and 7th day after CABG, which reflected an increased plasma fibrinolytic activity. We did not find an increase in TAFIa concentration on the 7th day after CABG, but a tendency to a decrease in TAFIa concentration. Silveira et al. [8] suggested that a mechanism of impaired fibrinolysis resulted in more stable fibrin deposits and increased the risk of precocious CAD as well as early occlusion of venous bypass grafts. They proposed that high plasma TAFI concentration might be a potential risk factor for CAD and for early vein graft occlusion [8].

In our study we found that TAFIa concentration was higher in stable CAD patients before CABG when compared to the healthy volunteers, therefore our data might support the results reported by Silveira et al. [8]. In fact, Juhan-Vague et al. [11] observed that TAFI concentration above the 90th percentile significantly correlated with lower risk of MI experienced between 3 to 6 months before the study. They even suggested that TAFI increase could protect against MI. It was a multicentre study, which comprised of patients both from the North and South Europe. However, non-prosepctive design and wide TAFI polymorphism are the main limitations of this study [11]. We should also stress that in Juhan-Vague et al. [11] study the healthy volunteers were on Mediterranean diet. Moreover, secondary prevention of CAD in post MI patients was different in countries studied. Brouwers et al. [10] investigated UAP patients with refractory and non-refractory form to medical treatment. Plasma TAFI concentration was significantly higher in non-refractory patients compared to refractory patients. They determined also the association between plasma TAFI concentration, TAFI gene polymorphism, other biochemical parameters and clinical outcome of UAP patients. Patients with more severe form of UAP had significantly lower plasma TAFI concentration [10]. But as showed in our study – increase of TAFI concentration might have been a consequence of the increase of fibrinolytic activity. However, the Brouwers et al. [10] did not study fibrinolytic activity or markers of ongoing fibrinolysis in their patients. On the other hand, according to Lau et al. [9] reported that patients with serious restenosis (>50%) after PTCA exhibited a significantly higher concentration of TAFI and lower concentration of PAI-1. Therefore, it seems that the role of TAFI in arterial thrombotic events remains unclear.

In the literature TAFI was also considered as an acute phase protein. In our study we found a positive correlation between TAFIa concentration and fibrinogen on the 3rd postoperative day. Similar data were reported previously [7,8], however, other did not agree with this suggestion [14]. In patients undergoing CABG, it is very difficult to assess whether TAFI is an acute phase protein, because CPB always caused huge general inflammatory response [15-17], and we did not observe a significant increase in TAFI concentration on the 3rd postoperative day. A negative correlation between TAFI concentration and fibrinogen after 3 months following CABG did not support this suggestion.

We observed a positive significant correlation between TAFIa concentration and total cholesterol concentration only on the 7th postoperative day. A significant positive correlation between TAFI concentration and total cholesterol, LDL-cholesterol and VLDL-cholesterol concentrations were observed by Silveira et al. [8] only in the study group, but not in the control group. Schroeder et al. [7] showed a similar correlation between TAFI concentration and total cholesterol concentration. However, Juhan-Vague et al. [11] did not observe such a correlation it in nearly 600 CAD patients after MI. These discrepances may be due to the fact that all patients studied were taking statins before and 3 months after CABG. Statins were withdrawn at least one week before the CABG because of temporary increase in aminotransferases activity caused by CPB. All the patients studied obligatory continued statin therapy since 7-14th postoperative day. Therefore, a significant decrease in total cholesterol, LDL-cholesterol and triglycerides, was found 3 months after CABG. According to accepted standards, all patients should receive statins after CABG, as a secondary prevention, unless contraindicated.

We found a significant negative correlation between PAP complexes concentration before CABG and EuroSCORE value. EuroSCORE is universally administered scale which estimates risk of cardiac surgery operation in European population [18]. Patients which have more points in EuroSCORE (have higher operative risk) had lower PAP complexes concentration. It means that they had lower plasma fibrinolytic activity. These patients could have higher risk of acute coronary syndrome occurrence during/after CABG, especially because they also revealed a hypercoagulable state [19] before the procedure.

In studied group, on the 3rd and 7th postoperative day there was significant increase of vWF activity (a marker of disturbed endothelium function), compared to the baseline values. After 3 months – vWF activity returned to the baseline values. Simultaneously vWF activity in studied group before CABG was significantly higher than in healthy volunteers as described by others [20,21]. A rise in vWF may suggest a further endothelium injury after operations with CPB. In our study we found a statistically positive correlation between vWF activity on the 7th postoperative day and duration time of the procedure.

We found a significant increase in platelet count occurring 7 days after CABG. Several morphotic blood elements, including platelets, undergo destruction during CPB [22]. An increase in the number of platelets 7 days after CABG may be a compensatory reaction to peri-operative injury. In studied group we also found a significant decrease in haematocrit value, erythrocyte count and haemoglobin concentration on 7th day after CABG. These findings may be due to haemodilution – a process caused by CPB, or due to both peri- and postoperative blood loss. All these parameters returned to the baseline values 3 months after CABG.

Due to the fact that haemostatic disturbances after CPB are still not fully understood, further studies are needed to recognize the pathogenetic mechanisms of haemostatic abnormalities in coronary heart diease and the effect of CABG, as well as the role of TAFI in these processes.

Conclusions

1. In patients with stable angina pectoris undergoing CABG, an increase in fibrinolytic activity may be due to the

fall in TAFIa concentration with simultaneous rise in PAP complexes.

2. Positive significant correlation between TAFIa concentration and total cholesterol concentration before CABG may suggest the role for TAFI as a potential risk factor for CAD.

3. Significantly negative correlation between PAP complexes and EuroSCORE value stressed a potentially higher operation risk in patients with lower fibrinolytic activity.

4. Positive significant correlation between F 1+2 fragments concentration on the 3rd postoperative day and aorta clamping time could prove that F 1+2 fragments may contribute to postoperative acute coronary syndromes.

5. Postoperative rise in vWF may suggest an endothelium injury after operations with CPB.

References

1. Bajzar L, Manuel R, Nesheim ME. Purification and characterization of TAFI, a thrombin-activable fibrinolysis inhibitor. J Biol Chem, 1995; 270: 14477-84.
2. Mosnier LO, von dem Borne PA, Meijers JC, Bouma BN. Plasma TAFI levels influence the clot lysis time in healthy individuals in the presence of an intact intrinsic pathway of coagulation. Thromb Haemost, 1998; 80: 829-35.
3. Nesheim M, Wang W, Boffa M, Nagashima M, Morser J, Bajzar L. Thrombin, thrombomodulin and TAFI in the molecular link between coagulation and fibrinolysis. Thromb Haemostas, 1997; 78: 386-91.
4. Valnickova Z, Enghild JJ. Human procarboxypeptidase U, or thrombin-activatable fibrinolysis inhibitor, is a substrate for transglutaminases. Evidence for transglutaminase-catalyzed cross-linking to fibrin. J Biol Chem, 1998; 273: 27220-4.
5. Nagashima M, Werner M, Wang M, Zhao L, Light DR, Pagils R, Morser J, Verhallen P. An inhibitor of activated thrombin-activatable fibrinolysis inhibitor potentiates tissue-type plasminogen activator-induced thrombolysis in a rabbit jungular vein thrombolysis model. Thromb Res, 2000; 98: 333-42.
6. van Tilburg NH, Rosendaal FR, Bertina RM. Thrombin activatable fibrinolysis inhibitor and the risk for deep vein thrombosis. Blood, 2000; 95: 2855-9.
7. Schroeder V, Chatterjee T, Mehta H, Windecker S, Pham T, Devantay N, Meier B, Kohler HP. Thrombin activatable fibrinolysis inhibitor (TAFI) levels in patients with coronary disease investigated by angiography. Thromb Haemost, 2002; 88: 1020-5.
8. Silveira A, Schatteman K, Goossens F, Moor E, Scharpe S, Stromqvist M, Hendriks D, Hamsten A. Plasma procarboxypeptidase U in men with symptomatic coronary artery disease. Thromb Haemost, 2000; 84: 364-8.
9. Lau H K, Segev A, Hegele R A, Sparkes JD, Teitel JM, Chisholm RJ, Strauss BH. Thrombin-activatable fibrinolysis inhibitor (TAFI): a novel predictor of angiographic coronary restenosis. Thromb Haemost, 2003; 90: 1-5.
10. Brouwers GJ, Leebeek FW, Tanck MW, Jukema JW, Kluft C, de Maat MP. Association between thrombin-activatable fibrinolysis inhibitor (TAFI) and clinical outcome in patients with unstabile angina pectoris. Thromb Haemost, 2003; 90: 92-100.
11. Juhan-Vague I, Morange PE, Aubert H, Henry M, Aillaud MF, Alessi MC, Samnegard A, Hawe E, Yudkin J, Margaglione M, Di Minno G, Hamsten A, Humphries SE. Plasma Thrombin-Activatable Fibrinolysis Inhibitor antigen concentration and genotype in relation to myocardial infarction in the north and south of Europe. Arterioscler Thromb Vasc Biol, 2002; 22: 867-73.
12. Lisowski P, Małyszko J, Jackowski R, Myśliwiec M, Lisowska A, Łapiński T, Pawlak K, Hirnle T. Effects of coronary artery by-pass grafting on haemostatic factors. Kardiol Pol, 2004; 60: 112-7.
13. Davidson SJ, Burman JF, Philips SM, Onis SJ, Kelleher AA, De Souza AC, Pepper JR. Correlation between thrombin potential and bleeding after cardiac surgery in adults. Blood Coagul Fibrinol, 2003; 14: 175-9.
14. Boffa MB, Brown D, Nesheim ME, Koschinsky ML. Characterization of the response of the human TAFI promoter to acute phase mediators. Supplement to the journal Thrombosis and Haemostasis 2001. Abstract: OC1757.
15. Holmes JH, Connolly NC, Paull DL, Hill ME, Guyton SW, Ziegler SF, Hall RA. Magnitude of the inflammatory response to cardiopulmonary bypass and its relation to adverse clinical outcomes. Inflamm Ress, 2002; 51: 579-86.
16. Kirklin JK, Westaby S, Blackstone EH, Kirklin JW, Chenoweth DE, Pacifico AD. Complement and the damaging effects of cardiopulmonary bypass. J Thorac Cardiovasc Surg, 1983; 86: 845-57.
17. Struber M, Cremer JT, Gohrbandt B, Hagl C, Jankowski M, Volker B, Ruckoldt H, Martin M, Haverich A. Human cytokine responses to coronary artery bypass grafting with and without cardiopulmonary bypass. Ann Thorac Surg, 1999; 68: 1330-5.
18. Roques F, Nashef SA, Michel P, Gauducheau E, de Vincentiis C, Baudet E, Cortina J, David M, Faichney A, Gabrielle F, Gams E, Harjula A, Jones MT, Pinna Pintot P, Salamon R, Thulin L. Risk factors and outcome in European cardiac surgery: analysis of the EuroSCORE multinational database of 19030 patients. Eur J Cardiothoracic Surg, 1999; 15: 816-23.
19. De Lorenzo F, Xiao H, Scully M, Kadziola Z, Kakkar VV. Prethrombotic state and impared fibrinolytic potential in coronary heart disease patients with left ventricular dysfunction. Blood Coagul Fibrinol, 2003; 14: 67-75.
20. Tsang GM, Allen S, Pagano D, Wong C, Graham TR, Bonser RS. von Willebrand factor and urinary albumin excertion are possible indicators of endothelial dysfunction in cardiopulmonary bypass. Eur J Cardiothorac Surg, 1998; 13: 385-91.
21. Valen G, Blomback M, Sellei P, Lindblom D, Vaage J. Release of von Willebrand factor by cardiopulmonary bypass, but not by cardioplegia in open heart surgery. Thromb Res, 1994; 73: 21-9.
22. Wahba A, Rothe G, Lodes H, Barlage S, Schmitz G. The influence of the duration of cardiopulmonary bypass on coagulation, fibrinolysis and platelet function. Thorac Cardiovasc Surg, 2001; 49: 153-6.

Concentration of interferon-inducible T cell chemoattractant and monocyte chemotactic protein-1 in serum and cerebrospinal fluid of patients with Lyme borreliosis

Grygorczuk S*, Zajkowska J, Świerzbińska R, Pancewicz S, Kondrusik M, Hermanowska-Szpakowicz T

Department of Infectious Diseases and Neuroinfections, Medical University of Białystok, Poland

Abstract

Purpose: Chronic inflammation in Lyme borreliosis may be sustained by aberrant inflammatory response, characterized by Th1 lymphocyte predominance, which in turn may be determined by chemokines synthesized in inflammatory focus. The aim of the study was to evaluate synthesis of chemokines: interferon-induced T cell chemoattractant (I-TAC – chemoattractant for Th1 lymphocytes), and monocyte chemotactic protein (MCP-1) in Lyme borreliosis.

Material and methods: Study group consisted of 13 patients with erythema migrans, 10 with Lyme arthritis and 6 with neuroborreliosis. Serum, as well as cerebrospinal fluid (CSF) in neuroborreliosis, was obtained before (examination 1) and during (examination 2) antibiotic treatment. Control serum was obtained from 8 healthy volunteers and control csf from 8 patients in whom meningitis and neuroborreliosis was excluded after diagnostic lumbar puncture. The samples were assayed for MCP-1 and I-TAC by ELISA.

Results: Serum mean I-TAC concentration in examination 1 was 73.0 pg/ml in erythema migrans, 78.9 pg/ml in Lyme arthritis and 87.3 pg/ml in neuroborreliosis (29.9 pg/ml in controls, difference significant for neuroborreliosis) and did not change significantly in examination 2. MCP-1 serum concentration was significantly increased to 497.5 pg/ml in neuroborreliosis in examination 2. I-TAC concentration in csf remained low, while MCP-1 concentration in examination 1 was increased to 589.1 pg/ml, significantly higher than simultaneously in serum.

Conclusions: I-TAC synthesis is increased in Lyme borreliosis and may be a factor favoring predominance of Th1 lymphocyte subset. MCP-1 creates chemotactic gradient towards central nervous system and may contribute to csf pleocytosis in neuroborreliosis.

Key words: Lyme borreliosis, meningitis, chemokines.

* CORRESPONDING AUTHOR:
Department of Infectious Diseases and Neuroinfections
Medical University of Białystok
ul. Żurawia 14
15-540 Białystok, Poland
Tel. +48 85 7409519; Fax. +48 85 7409515
e-mail: neuroin@amb.edu.pl (Sambor Grygorczuk)

Received 22.11.2004 Accepted 26.01.2005

Introduction

Lyme borreliosis is an infectious disease whose etiologic agent, Borrelia burgdorferi spirochete, is transmitted from animal reservoir to humans by Ixodes ticks. Three stages can be distinguished in course of the disease: localized infection, early disseminated infection and chronic infection (lasting for >12 months) [1]. Typically affected sites are skin (primary lesion – erythema migrans, EM), musculoskeletal system (Lyme arthritis, LA) and central (CNS) and peripheral nervous system (neuroborreliosis) [1]. Spirochetes found in affected tissues are innumerous related to the intensity of inflammatory and destructive processes, which suggests that functional disorders of the immune system and autoimmune processes take important part in the pathogenesis of this disease, especially in its chronic stage [2-4]. Consistently with that, clinical course of Lyme borreliosis is often prolonged and antibiotic treatment tends to be inefficient in chronic disease. The more profound understanding of the pathogenesis of inflammation in patients with chronic Lyme borreliosis could possibly lead to development of new therapeutic approaches, aimed at the aberrant and prolonged inflammatory response.

Chemokines are family of cytokines characterized by a potent chemotactic activity towards leukocytes and playing a vital role in the development of inflammatory reactions. According to amino acid sequence and spectrum of activity they are divided into CXC-chemokine family (including, among the others, interleukine 8; Il-8) and CC-chemokine family [5]. Synthesis

of chemokines is induced by proinflammatory cytokines (interleukine 1β – Il-1β, tumor necrosis factor alpha – TNF-α) and viral and bacterial antigens including surface lipoproteins of Borrelia burgdorferi, and takes place in leukocytes, fibroblasts, epithelial and endothelial cells [5-7]. The enhanced synthesis of chemokines has been observed in the inflammation site in course of various infectious diseases. In meningitides of viral and bacterial etiology increased concentrations of chemokines in cerebrospinal fluid (CSF) are observed [8-12]. A dominant role of endogenous chemotactic factors, including chemokines, in driving leukocytes into inflammatory infiltrates in Lyme borreliosis is very likely, especially that membrane components of Borrelia burgdorferi spirochetes themselves do not show chemotactic activity [13].

Monocyte chemoattractant protein 1 (MCP-1) is a CC chemokine chemotactic for most of mononuclear cell populations [5,14]. Its receptor, CC-chemokine receptor 2 (CCR2), is expressed on Il-2-activated T lymphocytes, as well as on monocytes and basophils [5]. Borrelia burgdorferi and its surface lipoproteins induce MCP-1 synthesis in endothelial cells, fibroblasts and monocytes [6,7,15]. The capability of astrocytes and microglia to synthesize MCP-1 and its presence in CSF of patients with both viral and bacterial meningitis suggest that MCP-1 may be also synthesized within CNS in course of neuroborreliosis [11,16,17].

Interferon-induced T cell chemoattractant (I-TAC) is a CXC chemokine acting via a CXC-chemokine receptor 3 (CXCR3) (18,19). CXCR3 is expressed on T lymphocytes under stimulation with Il-2 and is present on active T lymphocytes and memory cells as well as, to a lesser extent, on NK cells and B lymphocytes [20-24]. I-TAC synthesis by monocytes and neutrophils may lead to recruitment of activated T lymphocytes to the inflammation site. I-TAC is also produced by astrocytes, which suggests its role in the inflammatory processes within CNS [20,21]. I-TAC synthesis is induced by IFN-γ and further enhanced by tumor necrosis factor alpha (TNF-α), Il-1 and lipopolisacharide (LPS), which, however, do not induce I-TAC expression on their own [18,20,21]. Il-4 and Il-10 suppress I-TAC synthesis, which may contribute to the regulation of the inflammatory response in vivo [20,21]. As far as its amino acid sequence and activity, I-TAC shows much similarity to IP-10 and Mig chemokines, which are also induced by IFN-γ and bind CXCR3, but with less affinity [19,20].

The purpose of our study was to evaluate the role of MCP-1 and I-TAC as factors possibly responsible for migration of mononuclear cells, especially activated T lymphocytes, into the inflammatory focus in Lyme borreliosis. To check for their increased synthesis in Lyme borreliosis in vivo and their role in creating chemotactic gradient, we measured MCP-1 and I-TAC concentration in serum of patients with different clinical forms of Lyme borreliosis, as well as in CSF of patients with neuroborreliosis.

Material and methods

The study included 29 patients with Lyme borreliosis (14 females and 15 males, age 21-68 years, mean 44.6) hospitalized in the Department of Infectious Diseases and Neuroinfections of the Medical University of Białystok or treated in the Outpatient Department of the Dłuski Regional Specialist Hospital in Białystok. Diagnosis was based on epidemiological data, anamnesis, physical examination and presence of anti-Borrelia burgdorferi antibodies in serum, and in neuroborreliosis also in CSF. Generally, diagnosis made by treating physician and recorded in medical documentation was a rationale for patients' inclusion into the study group. All Lyme borreliosis patients reported either tick bites or frequent exposure to ticks in endemic areas. The clinical forms of Lyme borreliosis were distinguished according to Åsbrink [1]. Diagnosis of EM was based on the presence of typical skin lesion at least 5 cm in diameter, developing within several days or weeks around the place of tick bite. Neuroborreliosis (NB) was diagnosed in patients either with lymphocytic meningitis or symptoms of chronic CNS involvement, antibodies against Borrelia burgdorferi detectable in serum and CSF and no indication of other probable etiology. Lyme arthritis (LA) was diagnosed in patients complaining of chronic or recurrent musculoskeletal pain, affecting mainly large joints of upper and lower extremities, who had IgG antibodies against Borrelia burgdorferi detectable in serum and no features of acute inflammation in basic laboratory tests (ESR, leukocyte and platelet count). Depending on clinical picture, other examinations were carried out (detection of rheumatoid factor by standard laboratory test, radiological examination of affected joints), which excluded other probable causes of musculoskeletal symptoms.

Patients were divided into three groups: with EM – 13 patients, 7 females and 6 males (21-68 years, mean – 45.5); with LA – 10 patients, 4 females and 6 males (24-64 years, mean – 44.5) and with NB – 6 patients, 3 females and 3 males (27-51 years, mean – 42.8). Typically, patients from different study groups represented consecutive stages of Lyme borreliosis. EM patients had short history of the disease, with symptoms lasting for days or weeks (early localized infection), NB patients presented with duration of illness from a few weeks (early disseminated infection) to 1-2 years while LA patients complained of chronic and/or recurrent symptoms (chronic infection). Control group (C) consisted of 16 people: serum samples were obtained from 8 healthy persons (blood donors from the Regional Blood Donation Center in Białystok) and CSF samples from 8 patients in whom meningitis and neuroborreliosis were excluded after performing diagnostic lumbar taps. Informed consent was obtained from the patients and the study design was approved by the Ethics Committee of the Medical Academy in Białystok.

For the purpose of the study we used serum samples obtained together with venous blood for routine laboratory examinations, before treatment (examination 1) and during or after antibiotic therapy with doxycycline or III generation cephalosporine (examination 2). In NB group, CSF obtained by spinal puncture performed for diagnostic purpose was also examined. All samples were stored at -80°C and tested simultaneously. In EM group the period between examination 1 and 2 was from 23 to 40 days (mean ± SD – 30.1 days ± 5.5), in LA group – from 5 to 52 days (14.6 days ± 13.6) and in NB group the period between taking serum samples was from 8 to 66 days (36.2 days ± 24.1); treatment-phase CSF samples were available

Table 1. **I-TAC concentrations in serum of patients with erythema migrans, Lyme arthritis and neuroborreliosis before treatment (examination 1) and during antibiotic therapy (examination 2) in comparison with the values observed in serum of controls (pg/ml)**

Group	Examination 1			Examination 2		
	min-max	$\bar{x}$	SD	min-max	$\bar{x}$	SD
EM (n=13)	2.79-173.16	73.00 ¶	54.18	2.03-379.48	88.66	112.16
LA (n=10)	5.14-440.31	78.87	132.51	3.93-630.63	90.26 ¥	191.43
NB (n=6)	46.00-149.73	87.25 **	38.58	70.56-137.93	96.76 ** ¥	28.82
C (n=8)	0.57-50.17	29.86	15.49	-	-	-

EM – erythema migrans; LA – Lyme arthritis; NB – neuroborreliosis; C – control group; min-max – the range of concentrations observed; $\bar{x}$ – mean; SD – standard deviation; ¶ – difference on the border of statistical significance in comparison with controls (p=0.0579); ** – statistically significant difference in comparison with controls (p< 0.01); ¥ – statistically significant difference between groups of patients with NB and with LA in examination 2 (p<0.05)

Table 2. **MCP-1 concentrations in serum of patients with erythema migrans, Lyme arthritis and neuroborreliosis before treatment (examination 1) and during antibiotic therapy (examination 2) in comparison with the values observed in serum of controls (pg/ml)**

Group	Examination 1			Examination 2		
	min-max	$\bar{x}$	SD	min-max	$\bar{x}$	SD
EM (n=13)	136.72-453.66	277.17	86.35	175.10-276.12	323.50	140.55
LA (n=10)	100.80-699.24	388.97	208.00	180.64-832.34	429.06¶	216.64
NB (n=6)	159.34-226.00	245.78 #§	78.24	226.00-775.74	497.47 * §	215.87
C (n=8)	152.32-314.60	253.71	55.25	-	-	-

EM – erythema migrans; LA – Lyme arthritis; NB – neuroborreliosis; C – control group; min-max – the range of concentrations observed; $\bar{x}$ – mean; SD – standard deviation; ¶ – difference on the border of statistical significance in comparison with controls (p=0.0506); * – statistically significant difference in comparison with controls (p<0.05); § – difference on the border of statistical significance between examination 1 and 2 in NB group (p=0.0796)

in 5 out of 6 NB patients and were obtained from 6 to 66 days after the first sample (32 days ±23.9).

IgM and IgG antibodies against Borrelia burgdorferi were detected with ELISA assay from Biomedica (Viena, Austria), according to manufacturer's instructions. Following Borrelia burgdorferi recombinant antigens were included in the assay: p21 (OspC), Borrelia garinii p41 and Borrelia afzelii p41 for IgM detection and p21, B. garinii p41, B. afzelii p41, p18 and p100 for IgG detection. Results were expressed as BBU/ml (Biomedica Borrelia units/ml) and >11 BBU/ml was considered positive. In case of borderline results (9-11 BBU/ml) sera were re-evaluated with Western blot assays Milenia Blot Borrelia IgM and IgG (DPC Bierman GmbH, Germany). Patients with symptoms of meningitis were examined for presence of antibodies against tick-borne encephalitis virus in class IgM and IgG in serum and CSF with ELISA assay from Virion/Serion (Würzburg, Germany). The CSF protein concentration and pleocytosis were measured on the day of CSF collection, with standard laboratory techniques. MCP-1 and I-TAC concentrations were measured in serum and CSF samples by ELISA assay with reagents from R&D Systems (USA), following the manufacturer's instructions.

Statistical analysis was performed by means of SSST software. The Mann-Whitney test was used for comparisons of the chemokine concentrations between groups and between serum and CSF. The levels in examination 1 and 2 were compared by means of Wilcoxon's paired test. Pearson's linear correlation coefficient was used to estimate correlations between variables. The value of $p < 0.05$ was considered statistically significant.

Results

I-TAC concentration in patients' serum together with statistical interpretation is shown in *Tab. 1*. Mean I-TAC concentration was significantly increased in NB group both before and after treatment, while in EM in examination 1 it was increased with borderline significance.

MCP-1 concentrations are presented in *Tab. 2*. Significant increase of MCP-1 concentration in comparison with control serum was observed in NB group in examination 2 and borderline increase – in LA in examination 2. In NB group there was also increase of MCP-1 concentration between examination 1 and 2.

The mean chemokine concentrations in CSF are shown in *Tab. 3*. I-TAC concentration was significantly increased in comparison with control CSF both before and after treatment, but it remained lower than simultaneous concentration of I-TAC in serum. Concentration of MCP-1 before treatment showed only a borderline tendency to increase when compared with control CSF, but was significantly higher than concentration observed at the same time in serum; this difference was no longer present in examination 2.

CSF of all patients with NB presented with changes characteristic of lymphocytic meningitis, except for one patient whose CFS parameters were within the normal range (<5 cells/mm^3 and protein concentration <45 mg/dl). The mean CSF pleocytosis was $\bar{x}$=153/mm^3 (from 1 to 343) in examination 1, whereas, in examination 2, it improved to $\bar{x}$=17/mm^3 (from 12 to 23), with percentage of mononuclear cells in both examinations

Table 3. **I-TAC and MCP-1 concentrations in cerebrospinal fluid of patients with neuroborreliosis before treatment (examination 1) and during antibiotic therapy (examination 2) in comparison with the values observed in serum of these patients and in control cerebrospinal fluid (pg/ml)**

Chemokine	Examination 1			Examination 2			C		
	min-max	$\bar{x}$	SD	min-max	$\bar{x}$	SD	min-max	$\bar{x}$	SD
I-TAC	25.63-55.42	36.69 ** †	13.12	28.72-58.41	44.84 ** †	11.70	16.79-26.75	23.23	3.38
MCP-1	306.36-872.16	589.11 ¶ ‡	235.11	214.40-940.82	510.95	253.17	306.13-400.50	356.01	36.94

C – control group; min-max – the range of concentrations observed; $\bar{x}$ – mean; SD – standard deviation; ¶ – difference on the border of statistical significance in comparison with controls ($p=0.0528$); ** – statistically significant difference in comparison with controls with $p<0.01$; † – significantly lower levels than observed simultaneously in serum ($p<0.05$); ‡ – significantly higher levels than observed simultaneously in serum ($p<0.05$)

ranging from 80 to 100%. The mean protein concentration was $\bar{x}=76.5$ mg/dl (44.3-145.4 mg/dl) in examination 1 and $\bar{x}=59.25$ mg/dl in examination 2.

The serum and CSF concentrations of chemokines were not significantly correlated with CSF inflammatory parameters (data not shown). In case of MCP-1 there was a tendency for positive correlation of its CSF concentration with CSF parameters (protein level, and total, mononuclear and polynuclear cell count), which, however, did not reach statistical significance.

Discussion

Our study revealed a consistent tendency for increased concentrations of I-TAC and MCP-1 in serum of patients with Lyme borreliosis. The role of I-TAC in Lyme borreliosis has not been evaluated so far. In our study we found mean concentration of I-TAC to be from 2.4 to 3.2 – fold higher in patients with different forms of Lyme borreliosis in comparison with controls, which, however, was statistically significant only in neuroborreliosis. The important role of MCP-1 in Lyme borreliosis was already suggested by results of in vitro studies by Sprenger's et al., who found that MCP-1 synthesis was most effectively stimulated by a small number of Borrelia burgdorferi cells (1 bacterial cell per 10 monocytes) [6]. In the same setting, Il-8 was produced most effectively at the spirochete to monocytes ratio of 1:1 and MIP-1α at 10:1 [6]. This suggests that production of MCP-1 in vivo may be initiated by a relatively small number of invading spirochetes [6]. In the study of Gergel et al. MCP-1 appeared to be a factor responsible for T lymphocyte migration across cultured human endothelium incubated with B. burgdorferi [25]. However, in contrast with in vitro data, in the study of Pashenkov et al. no elevated levels of MCP-1 were detected in serum and CSF of patients with Lyme meningitis [26]. In our study, MCP-1 levels were not significantly increased in serum of patients before treatment, but reached significantly increased values in examination 2 in neuroborreliosis (and of borderline significance in LA group).

I-TAC concentration remained significantly lower in CSF of patients with neuroborreliosis than in serum, arguing against its role in causing CSF pleocytosis. In contrast with that, the mean MCP-1 concentration before treatment, as well as individual concentrations in all studied patients, were higher in CSF than in serum, which suggests that MCP-1 may participate in stimulating migration of mononuclear cells to CSF in neuroborreliosis. So far, similar chemotactic gradient towards CSF in neuroborreliosis was observed for Il-8, however, it was present only in 10 out of 20 patients examined and in the whole studied group there was no difference between mean Il-8 concentration in CSF and serum [27]. MCP-1 is present in CSF of patients with viral and purulent meningitis, where it seems to be the main factor responsible for the migration of monocytes/macrophages to CSF and contributing to the migration of T lymphocytes [8,11,17]. Concentrations of MCP-1 in CSF were generally higher (3-6 ng/ml) and concentration gradient between CSF and serum was more evident in patients with viral and purulent meningitides than in patients with neuroborreliosis included in our study [8,11]. More similar to values we noted in neuroborreliosis was mean concentration of MCP-1 observed by Mastroianni et al. in patients with tuberculous meningitis (808 pg/ml), a condition which differs from neuroborreliosis in terms of severity but resembles it with its prolonged and subacute clinical course [8]. Lack of significant correlation between CSF pleocytosis and MCP-1 concentration does not exclude pathogenetic significance of this chemokine, as any correlation of this kind must be weakened by multiplicity of factors contributing to the inflammatory state within CSF. Other studies in which chemokine concentrations were measured in CSF of patients with meningitis showed either no correlation at all or only limited to certain forms of meningitis and certain leukocyte populations [6,8,9].

There was no decline in chemokine concentrations between examination 1 and 2, and even a weak tendency for increase could be observed, which reached the borderline significance in case of MCP-1 in patients with neuroborreliosis. Of note, the examination 2 was often carried out before completion of treatment, which typically lasted four weeks. However, this result is in contrast with our previous study, in which concentrations of Il-8 and β-chemokines: macrophage inflammatory protein-1α and 1β (MIP-1α and MIP-1β) in serum and CSF of patients with Lyme borreliosis fell several-fold during two weeks of treatment [27]. It may suggest the long-term maintenance of the synthesis of MCP-1 and I-TAC in patients with Lyme borreliosis and possibly their involvement in some form of protracted inflammatory reaction, which is sustained in spite of antibiotic therapy and spirochete elimination. Conversely, it could be hypothesized that the increased synthesis of I-TAC and/or MCP-1 at this time point is somehow related to the resolution of the inflammatory

condition. Further studies serially measuring concentrations of MCP-1 and I-TAC in larger groups of patients and relating them to other markers of inflammation and clinical course and outcome of the disease could possibly clarify that issue.

The tendency to predominance of inflammatory response dependent on lymphocytes of either Th1 (with increased synthesis of IFN-γ) or Th2 (synthesis of Il-4, Il-5 and Il-10) subset in different infections can vary individually and condition the host's capability to eliminate various pathogens [28]. According to Gergel et al., human endothelium incubated with B. burgdorferi promotes a selective migration of IFN-γ secreting T lymphocytes and may recruit them into inflammatory lesion, which suggests that Th1 response to B. burgdorferi typically dominates [25]. However, in Lyme borreliosis predominance of cytokines released by Th2 lymphocytes over the cytokines produced by Th1 cells may be associated with milder inflammatory reaction and better prognosis [29,30]. Th1 predominance was suggested to be associated with the prevailing cellular response, which may not be fully effective in Borrelia burgdorferi elimination and lead to prolonged infection, tissue damage and even autoimmunity [2,29]. The experiments on mice suggest an unfavorable effect of IFN-γ and favorable of Il-4 on symptoms of Lyme arthritis [30]. An increased IFN-γ and a decreased Il-4 expression was observed in the joints of patients with chronic LA, which may indicate that this type of response promotes a clinical manifestation of the disease in humans [29]. Also in neuroborreliosis IFN-γ synthesis in peripheral blood and CSF lymphocytes dominates over Il-4 synthesis [31].

According to the study of Sallusto et al., the predominance of Th1 or Th2 cells in inflammatory focus may depend on a spectrum of produced chemokines, which determine the selective migration and accumulation of different populations of T lymphocytes at the site of an inflammation [24]. Studies on I-TAC synthesis indicate its dependence on IFN-γ and inhibition by Il-4 and Il-10 [19-21]. According to Burns et al. synthesis of MPC-1 by the endothelium incubated with Borrelia burgdorferi was inhibited by Il-10 [15]. This may suggest the connection of both chemokines with the Th1 type response and suppression of their synthesis in the case of the Th2 response predominance. The receptor for I-TAC (CXCR3) is exposed on Th1 in several-fold higher quantities than on Th2 lymphocytes, which is reflected by stronger chemotactic effect of I-TAC on Th1 than on Th2 cells [22-24]. According to Quin et al., CXCR3 is found on practically all T cells forming inflammatory infiltrates in rheumatoid arthritis – a chronic inflammatory condition with a marked predominance of Th1 response [23]. Moreover, I-TAC and, to a lesser extent, related chemokines Mig and IP-10, act antagonistically on the chemokine receptor CCR3, expressed on eosinophils and Th2 lymphocytes, but not on Th1 cells [23,24,32]. This may allow I-TAC to inhibit Th2 cell migration stimulated through CCR3 and their accumulation in an inflammatory focus [32]. I-TAC seems to be connected by a positive feedback with Th1 cell activity, as it favors Th1 lymphocyte selective accumulation in inflammation site, while its synthesis is stimulated by Th1-related cytokines. As I-TAC demonstrates so evident association with the Th1 cytokine profile, the increase of its concentration in Lyme borreliosis seems consistent with the present knowledge about the cytokine patterns in this disease and may be considered another element of inflammatory processes unfavorable for its clinical course.

As for MCP-1, its relation to the specific cytokine profile in not explicit. The MCP-1 receptor is found on both main subsets of Th lymphocytes, but its expression is higher on Th1 cells; in vitro this chemokine attracts both Th1 and Th2 lymphocytes [23]. In the study of Gergel et al. migration of T lymphocytes across B. burgdorferi-stimulated endothelium driven by MCP-1 lead to selective enrichment in IFN-γ secreting cells [25]. However, some data from animal models suggest the connection between MCP-1 and Th2 type response [33,34]. In murine endotoxemia MCP-1 plays a protective role, enhancing the expression of Il-10 [33]. The differentiation of T lymphocytes towards Th2 phenotype due to MCP-1 was observed in mice, probably through the stimulation of Il-4 synthesis by MCP-1 [34]. Interestingly in this context, our study demonstrated significant increase of MCP-1 levels in serum, but not in CSF, of patients with Lyme borreliosis during the antibiotic treatment, suggesting its systemic synthesis being increased during the resolution phase of the inflammation caused by Borrelia burgdorferi.

Our study has shown the increased synthesis of chemokines acting on mononuclear cells, including activated T lymphocytes, MCP-1 and I-TAC, in patients with Lyme borreliosis. The increase in serum concentrations was more pronounced for I-TAC, and this chemokine, which is connected with Th1 profile of cytokine expression, may be one of the important pro-inflammatory factors in Lyme borreliosis and possibly promote its unfavorable clinical course. MCP-1 is present in CSF in neuroborreliosis and creates a chemotactic gradient between CSF and serum. It is likely that MCP-1 plays, next to Il-8, certain role in stimulating the inflow of leukocytes to CSF in course of neuroborreliosis.

References:

1. Åsbrink E, Hovmark A. Comments on the course and classification of Lyme borreliosis. Scand J Infect Dis, 1991; 77(Suppl): 41-3.
2. Sigal LH. Lyme disease: a review of aspects of its immunology and immunopathogenesis. Ann Rev Immunol, 1997; 15: 63-91.
3. Steere AC, Duray PH, Butcher EC. Spirochetal antigens and lymphoid cell surface markers in Lyme synovitis. Comparison with rheumatoid synovium and tonsillar lymphoid tissue. Arthritis Rheum, 1988; 31: 487-95.
4. Steere AC, Dwyer E, Winchester R. Association of chronic Lyme arthritis with HLA-DR4 and HLA-DR2 alleles. N Eng J Med, 1990; 323: 219-23.
5. Baggiolini M. Chemokines and leukocyte traffic. Nature, 1998; 392: 565-8.
6. Sprenger H, Krause A, Kauffman A, Priem S, Fabian D, Burmester GR, Gemsa D, Rittig MG. Borrelia burgdorferi induces chemokines in human monocytes. Infect Immun, 1997; 65: 4384-8.
7. Ebnet K, Brown KD, Siebenlist UK, Simon MM, Shaw S. Borrelia burgdorferi activates nuclear factor-κB and is a potent inducer of chemokine and adhesion molecule gene expression in endothelial cells and fibroblasts. J Immunol, 1997; 158: 3285-92.
8. Mastroianni CM, Lancella L, Mengoni F, Lichtner M, Santopadre P, D'Agostino CD, Ticca F, Vullo V. Chemokine profiles in the cerebrospinal fluid (CSF) during the course of pyogenic and tuberculous meningitis. Clin Exp Immunol, 1998; 114: 210-4.
9. Inaba Y, Ishiguro A, Shimbo T. The production of macrophage inflammatory protein-1α in the cerebrospinal fluid at the initial stage of meningitis in children. Ped Res, 1997; 42: 788-93.
10. Spanaus K-S, Nadal D, Pfister HW, Seebach J, Widmer U, Frei K, Gloor S, Fontana A. C-X-C and C-C chemokines are expressed in the cerebrospinal fluid in bacterial meningitis and mediate chemotactic activ-

ity on peripheral blood-derived polymorfonuclear and mononuclear cells in vitro. J Immunol, 1997; 158: 1956-64.

11. Sprenger H, Rosler A, Tonn P, Braune HJ, Huffmann G, Gemsa D. Chemokines in the cerebrospinal fluid of patients with meningitis. Clin Immunol Immunopathol, 1996; 80: 155-61.

12. Halstensen A, Ceska M, Brandtzaeg P, Redl H, Naess A, Waage A. Interleukin-8 in serum and cerebrospinal fluid from patients with meningococcal disease. J Infect Dis, 1993; 167: 471-5.

13. Sellati TJ, Abrescia LD, Radolf JD, Furie MB. Outer surface lipoproteins of Borrelia burgdorferi activate vascular endothelium in vitro. Infect Immun, 1996; 64: 3180-7.

14. Jiang Y, Beller DI, Frendl G, Graves DT. Monocyte chemoatractant protein-1 regulates adhesion molecule expression and cytokine production in human monocytes. J Immunol, 1992; 148: 2423-8.

15. Burns MJ, Furie MB. Borrelia burgdorferi and interleukin-1 promote the transendothelial migration of monocytes in vitro by different mechanisms. Infect Immun, 1998; 66: 4875-3.

16. Peterson PK, Hu S, Salak-Johnson J, Molitor TW, Chao CC. Differential production of and migratory response to β chemokines by human microglia and astrocytes. J Infect Dis, 1997; 175: 478-81.

17. Lahrtz F, Piali L, Spanaus KS, Seebach J, Fontana A. Chemokines and chemotaxis of leukocytes in infectious meningitis. J Neuroimmunol, 1998; 85: 33-43.

18. Tensen CP, Flier J, Rampersad SS, Sampat-Sardjoepersad S, Scheper RJ, Boorsma DM, Willemze R. Genomic organization, sequence and transcriptional regulation of the human CXCL 11 gene. Biochim Biophys Acta, 1999; 1446: 167-72.

19. Cox MA, Jenh Ch-H, Gonsiorek W, Fine J, Narula SK, Zavodny PJ, Hipkin RW. Human interferon-inducible 10 kDa protein and human interferon-inducible T cell a chemoattractant are allotopic ligands for human CXCR3: differential binding to receptor states. Mol Pharmacol, 2001; 59: 707-15.

20. Cole KE, Strick ChA, Paradis TJ, Ogborne KT, Loetscher M, Gladue RP, Lin W, Boyd JG, Moser B, Wood DE, Sahagan BG, Neote K. Interferon-inducible T cell alpha chemoattractant (I-TAC): a novel non-ELR CXC chemokine with potent activity on activated T cells through selective high affinity binding to CXCR3. J Exp Med, 1998; 187: 2009-21.

21. Gasperini S, Marchi M, Celzetti F, Laudanna C, Vicentini L, Ossen H, Murphy M, Liao F, Farber J, Casatella MA. Gene expression and production of the monokine induced by IFN-γ (MIG), IFN-inducible T cell a chemoattractant (I-TAC), and IFN-γ-inducible protein-10 (IP-10) chemokines by human neutrophils. J Immunol, 1999; 162: 4928-37.

22. Bonecchi R, Bianchi G, Bordignon PP, D'Ambrosio D, Lang R, Borsatti A, Sozzani S, Allavena P, Gray PA, Mantovani A, Sinigaglia F. Differential expression of chemokine receptors and chemotactic responsiveness of type 1 T helper cells (Th1s) and Th2s. J Exp Med, 1998; 187: 129-34.

23. Qin S, Rottman JB, Myers P, Kassam N, Weinblatt M, Loetscher M, Koch AE, Moser B, Mackay CR. The chemokine receptors CXCR3 and CCR5 mark subsets of T cells associated with certain inflammatory reactions. J Clin Invest, 1998; 101: 746-54.

24. Sallusto F, Lenig D, Mackay CR, Lanzavecchia A. Flexible programs of chemokine receptor expression on human polarized T helper 1 and 2 lymphocytes. J Exp Med, 1998; 187: 875-83.

25. Gergel EI, Furie MB. Populations of human T lymphocytes that traverse the vascular endothelium stimulated by Borrelia burgdorferi are enriched with cells that secrete gamma interferon. Infect Immun, 2004; 72: 1530-6.

26. Pashenkov M, Teleshowa N, Kouwenhoven M, Smirnova T, Jin Y-P, Kostulas V, Huang Y-M, Pinegin B, Boiko A, Link H. Recruitment of dendritic cells to the cerebrospinal fluid in bacterial neuroinfections. J Neuroimmunol, 2002; 122: 106-16.

27. Grygorczuk S, Pancewicz S, Kondrusik M, Świerzbińska R, Zajkowska J, Hermanowska-Szpakowicz T. Serum- and cerebrospinal fluid concentration of inflammatory proteins MIP-1α and MIP-1β and of interleukine 8 (Il-8) in the course of neuroborreliosis. Neurol Neurochir Pol, 2003; 37: 73-87.

28. Mosmann TR, Sad S. The expanding universe of T-cell subsets: Th1, Th2 and more. Immunol Today, 1996; 17: 138-46.

29. Yin Z, Braun J, Neure P, Wu P, Eggens U, Krause A, Kamradt T, Sieper J. T cell cytokine pattern in the joints of patients with Lyme arthritis and its regulation by cytokines and anticytokines. Arthritis Rheum, 1997; 40: 69-79.

30. Keane-Myers A, Nickell SP. Role of Il-4 and IFN-γ in modulation of immunity to Borrelia burgdorferi in mice. J Immunol, 1995; 155: 2020-28.

31. Ekerfelt C, Ernerudh J, Bunikis J, Vrethem M, Aagsen J, Roberg M, Bergström S, Forsberg P. Compartmentalization of antigen specific cytokine responses to the central nervous system in CNS borreliosis: secretion of IFN-gamma predominates over Il-4 secretion in response to outer proteins of Lyme disease Borrelia spirochetes. J Neuroimmunol, 1997; 79: 155-62.

32. Loetscher P, Pellegrino A, Gong J-H, Mattioli I, Loetscher M, Bardi G, Bagiollini M, Clark-Lewis I. The ligands of CXC chemokine receptor 3, I-TAC, Mig, and IP-10, are natural antagonists for CCR3. J Biol Chem, 2001; 276: 2986-91.

33. Zisman DA, Kunkel SL, Strieter RM, Tsai WC, Bucknell K, Witkowski J. MCP-1 protects mice in lethal endotoxemia. J Clin Invest, 1997; 99: 2832-6.

34. Karpus WJ, Lukacs NW, Kennedy KJ, Smith WS, Hurst SD, Barrett TA. Differential CC chemokine-induced enhancememt of T helper cell cytokine production. J Immunol, 1997; 158: 4129-36.

HBV-DNA and sFas, sFasL concentrations in serum of healthy HBsAg carriers

Łapiński TW[1]*, Kowalczuk O[2], Prokopowicz D[1], Chyczewski L[2], Jaroszewicz J[1]

[1] Department of Infectious Diseases, Medical University of Białystok, Poland
[2] Laboratory of Molecular Biology, Medical University of Białystok, Poland

Abstract

Purpose: Increased HBV-DNA concentration is a prognostic factor of disease progression in chronic hepatitis B patients. Moreover, active hepatic inflammation during HBV replication influences apoptosis intensification. The aim of this study was to estimate occurrence of HBV replication among carriers of HBsAg. Furthermore, we analysed the correlation between HBV replication and HBeAg or anti-HBe presence as well as known apoptosis indicators – sFas and sFasL concentration.

Material and methods: The study included 34 HBV infected patients, aged 20-43 yrs defined as HBsAg healthy carriers. HBV-DNA was extracted from patients' serum using two different DNA isolation kits: the QIAamp DNA Mini Kit (QIAGEN Ltd, USA) and the Gene Elute Mammalian Genomic DNA Miniprep Kit (Sigma, USA).

HBV-DNA concentration in serum was measured by RT-PCR based on TaqMan Universal Master Mix (Applied Biosystems). The detection limit of this system was as few as 10 HBV-DNA copies/mL of serum. HBV-DNA concentration was calculated from a linear standard curve obtained between 10 and 10^8 DNA copies/reaction.

HBeAg and anti-HBe in serum were detected by MEIA method (ABBOTT, Germany). The concentration of sFas and sFasL in serum was – estimated by ELISA method (Bender MedSystems, Austria).

Results: HBV active replication was detected in 79% HBsAg carriers. The HBV-DNA levels exceeding 10^5 copies/mL were observed in 64% patients. Among HBsAg carriers presenting HBeAg, HBV replication occurred more often and was more intensify than in HBsAg carriers presenting anti-HBe antibodies. The sFasL occurrence in serum of 56% HBsAg carriers shows an active apoptosis, independent from ALT and AST activity within normal ranges.

Key words: HBsAg carriers, HBV-DNA levels, apoptosis.

Introduction

Healthy HBsAg carriers show correct liver function. Some of those persons eliminate HBV. However, Hou et al. [1] showed, progressing liver inflammation in approximately 30% of chronic HBsAg carriers. Exact prognostic factors defining a direction of an HBV infection evolution in such persons are not known. It seems that high HBV replication may is suggest the developments of chronic liver inflammation.

HBeAg presence in serum of persons with high HBV replication indicates the infection of "wild" type HBV. Among patients that show anti-HBe antibodies existence, replication of HBV is frequent and is mainly caused by mutant HBV infection, namely YMDD [2,3]. The HBsAg carriers with high levels of HBV-DNA are a group of increased risk of chronic liver inflammation development. Antiviral therapy initiation should be considered in such patients.

Patients with active hepatitis B present escalation of apoptosis and cytotoxic reactions. One of the indicators of this process is high Fas and FasL serum concentration. Simultaneous confirmation of a high HBV-DNA replication and the Fas, FasL serum concentration in HBV infected patient should indicate a need of an antiviral therapy consideration [4].

The aim of this study was to define the frequency of HBV replication among HBsAg carriers as well as to investigate the HBV-DNA levels in reference to HBeAg, anti-HBe presence and Fas, FasL serum concentrations.

* CORRESPONDING AUTHOR:
Department of Infectious Diseases
Medical University of Białystok
ul. Żurawia 14
15-540 Białystok, Poland
Tel/Fax: +48 85 7416921
e-mail: twlapinski@poczta.onet.pl (Tadeusz Wojciech Łapiński)

Received 07.03.2005 Accepted 23.05.2005

Material and methods

The study included 34 HBV infected patients, 15 women (aged 20-43 yrs) and 19 men (aged 21-38 yrs) defined as HBsAg healthy carriers.

Inclusion criteria were:

1. age 18 and over
2. chronic HBV infection evidenced by HBsAg presence for at least 1 year
3. ALT, AST, bilirubin, albumin and prothrombin time within normal range in period of yearly observation (triple investigation)
4. no changes in USG liver examination.

Patients presenting one or more of following criteria were excluded:

1. significant laboratory abnormalities in ALT or AST activity
2. past or present history of drug or alcohol abuse
3. autoimmune disorders (ANA in serum), past or present
4. HIV, delta virus or hepatitis C co-infection
5. patients undergoing dialysis because of chronic renal injury
6. history of malignant diseases.

Informed consent was obtained from each patient and the Bioethics Committee at the Medical University of Białystok approved the study protocol.

Extraction of HBV-DNA from patient sera

HBV-DNA was extracted from 200 μl of patient sera using two different kits for DNA isolation: the QIAamp DNA Mini Kit (QIAGEN Ltd, USA) and the "Gene Elute Mammalian Genomic DNA Miniprep Kit" (Sigma, USA). In the first method (QIAamp DNA Mini Kit) 200 μl of serum was added to 200 μl lyses solution (AL) supplemented with 5 μg of oligo $(A)_{25}$ carrier DNA and 400 μg of QIAGEN protease. After 10 minutes at 56°C incubation, 230 μl of 96% ethanol was added and the mixture was transferred onto a QIAamp spin column combined attached to a collective tube. The microcolumn was centrifuged for 1 minute at 6000 x g in a standard tabletop centrifuge at room temperature. After being washed once with 500 μl of washing buffer (AW1) and once with washing buffer (AW2), DNA was eluted with 50 μl of elution buffer AE (10 mM Tris-HCl, 0.5 mM EDTA-Na_2, pH 8.0). This resulted in obtaining fourfold concentration of the original input material.

In the second method (Genomic DNA Miniprep Kit) 200 μl of serum was added to 200 μl lyses solution with 400 μg of proteinase K. After 10 minutes at 70°C incubation for, 200 μl of 96% ethanol was added and the mixture was transferred onto a spin column attached to a collective tube. The microcolumn was centrifuged for 1 minute at 12000 x g in a standard tabletop centrifuge at room temperature. After being washed twice with 500 μl of washing buffer DNA was eluted with 200 μl of elution buffer AE (10 mM Tris-HCl, 0.5 mM EDTA-Na_2, pH 8.0).

Obtained DNA solutions were stored at -20°C until further processing.

All serum samples were processed at the same time with both negative (water) and positive control (HBV-positive serum) samples. To minimize the risk of samples cross-contamination, only one tube of serum was opened at a time during the aliquoting and lyses steps.

HBV-DNA quantification

In order to detect HBV-DNA sequences in sera of the patients the conventional PCR method with conserved pre-S/S region primers was used. Thermal cycling was performed using the following conditions: initial incubation at 96°C for 120 s, and then 40 cycles in 94°C for 30 s, 50°C for 30 s and 72°C for 60 s.

We detected HBV-DNA concentration in sera by real-time detection PCR based on TaqMan chemistry. Amplification was performed in 25 μl reaction mixture containing 2 x TaqMan Universal Master Mix (Applied Biosystems) with uracil N'-glycosylase, 30 pmol of forward primer, 30 pmol of reverse primer, 30 pmol TaqMan probe (5'-FAM) and 5 μl of isolated DNA. The primers and probe were selected in the pre-S region of the HBV genome and generated a product of 89 bp. After incubation for 2 minutes at 50°C, which enables uracil N'-glycosylase to inactivate possible contaminating amplicons, incubation for 10 min at 95°C allowed AmpliTaq Gold polymerase to activate and inactivate the uracil N'-glycosylase. The PCR cycling program consisted of 45 two-step cycles of 15 w at 95°C and 60 s at 60°C. Analysis of raw data was done with the Sequence Detector V1.6.3 software (PEBiosystems). Data were collected at the annealing step (60°C) of every cycle, and the threshold cycle (Ct) for each sample was calculated by determining the point at which the fluorescence exceeded the threshold limit, which was set at 0.04 U. The standard curve was calculated automatically by plotting the Ct values against each standard of known concentration. For preparation of the external standards an international reference VQC plasma preparation panel (CLB) containing well-characterized HBV-DNA levels was used. Sample copy numbers were calculated by interpolation of the experimentally determined standard curve. The detection limit of this system was as few as 10 HBV-DNA copies/ml of serum. A linear standard curve was obtained between 10 and 10^8 DNA template copies/reaction.

Serological analyses

HBV infection was diagnosed on the basis of HBsAg, HBeAg and anti-HBe the presence. The HBsAg, HBeAg and anti-HBe were detected by microenzyme immunological method (MEIA, ABBOTT, Germany).

sFas and sFasL

The sFas and sFasL concentration in the serum was measured twice by immunology – enzymatic assay test (ELISA, Bender MedSystems, Austria) [5].

Statistical analyses

Statistical analysis was performed with use of Logistic Regression test and Fisher Exact test by program of SS for Windows. Values of $p<0.05$ were considered to be significant.

Table 1. **The HBV-DNA and sFas, sFasL serum concentration in healthy HBsAg carriers presenting HBeAg or anti-HBe antibodies**

No	sex	HBeAg	anti-HBe	HBV-DNA			HBV-DNA	sFas** pg/ml	SFasL*** pg/ml
				QIAGEN*	NESTED*	SIGMA*	QIAGEN* real-time		
1	♂	-	+	-	++	-	$8.4x10^5$	18	0.13
2	♀	-	+	+	-	-	$1.06x10^5$	16.5	0.1
3	♀	+	-	-	-	-	0	13.5	0
4	♀	-	+	+	-	+	$2.08x10^5$	8	2.57
5	♂	-	+	++	+	-	$9.09x10^4$	14	11.5
6	♀	-	+	+	-	-	$4.1x10^4$	8	2.53
7	♂	-	+	++	-	-	$3.04x10^5$	31	0
8	♂	-	+	++	-	-	$9.18x10^5$	8.5	0
9	♂	-	+	+++	+++	++	$6.27x10^5$	19	0
10	♂	-	+	++	+	-	$4.43x10^5$	7.4	0.13
11	♂	+	-	+	+	-	$2.72x10^5$	14	0.08
12	♀	-	+	++	++	-	$9.82x10^6$	7.5	0.08
13	♀	-	+	++	++	++	0	3.5	0.16
14	♂	-	+	-	-	-	$1.39x10^5$	43	0.83
15	♂	-	+	-	++	-	$1x10^4$	8	0
16	♀	+	-	+	+	-	$1.33x10^5$	19	0
17	♀	+	-	+	-	-	0	16	0
18	♂	+	-	+	-	+	$2.95x10^5$	6.5	0
19	♂	+	-	+++	++	+	$2.45x10^5$	14	9.89
20	♀	-	+	-	-	-	0	11	0
21	♀	-	+	+	++	-	$5.77x10^4$	22	0.08
22	♀	-	+	+	-	-	$4.15x10^4$	12.5	0.12
23	♀	-	+	+	++	+	$1.45x10^5$	13	0
24	♂	-	+	++	++	+	$1.43x10^6$	12.5	0
25	♀	-	+	-	-	+	0	17	0.81
26	♂	+	-	++++	++	+++	$1.93x10^9$	13	0
27	♀	+	-	++	-	-	$1.01x10^5$	23	0
28	♂	+	-	++++	++	+++	$8.03x10^9$	27	0.08
29	♂	-	+	++	++	+	$9.85x10^5$	25.5	0
30	♂	-	+	++	++	+	$2.09x10^5$	13	4.73
31	♂	-	+	+	++	-	0	31	0
32	♂	+	-	+++	+++	-	$5.57x10^6$	11.5	2.53
33	♂	-	+	-	-	-	0	7	0.07
34	♀	-	+	+	+	+	$1.1x10^5$	7.5	0.1

* – DNA isolation method; ** – normal range = 20.3 pg/ml, S.D. = ±4.9; *** – normal range = 0

Results

Our investigations showed that the QIAGEN test is a more sensitive in HBV-DNA detection in comparison to SIGMA test; however, we did not observed statistical differences between used tests.

HBV replication was confirmed in 24 (71%) of 34 HBsAg carriers. 22 (65%) of them presented HBV-DNA level exceeding 10^5 copies/mL. Moreover, 8 (80%) of HBsAg and HBeAg positive carriers showed HBV-DNA replication over 10^5/mL. Among HBsAg carriers presenting anti-HBe antibodies, only 14 (54%) had HBV-DNA concentration exceeding 10^5/mL. We did not observe significant differences level in HBV-DNA between HBsAg carriers in respect to HBeAg and anti-HBe antibodies occurrence. The HBV viral load below 10^4 copies/mL was observed only in HBsAg carriers with anti-HBe antibodies, (5/24 persons =21%).

The difference in sFas serum concentration between HBsAg carriers and control group (15.4 pg/ml vel 18.4 pg/ml; $p>0.05$) was not significant. Concentration of sFas in serum did not depend on HBeAg or anti-HBe antibodies presence (15.75 pg/ml vel 15.18 pg/ml; $p>0.05$).

None of individuals in control group expressed sFasL in serum. Conversely sFasL was detected in sera of 19 (56%) HBsAg carriers, which may indicate increased apoptosis activity in these patients.

Discussion

The HBsAg carriers are defined as healthy persons, thus do not require antiviral treatment. Furthermore, according to the National Institutes of Health, patients with serum HBV-DNA levels exceeding 10^5 copies/mL of HBV-DNA in serum should

be considered as a individuals with an active HBV replication and ongoing liver damage, thus should be classified as chronic hepatitis B [6]. Investigations oindicating correlation between histological changes in liver and HBV-DNA serum concentration may confirm this opinion. It is particularly apparent in patients whose HBV viral load in serum exceeds 10^6 copies/mL [7].

Several evidences suggest that advancement of liver inflammation is correlated with HBV viral load [1]. This is coherent with studies showing the relationship between the amount of liver inflammation and HBV-DNA in HBsAg carriers with suppression of immunological response [8]. Langer et al. [9] found active HBV-DNA replication in 49% of HBsAg carriers. Those persons didn't have any symptoms and biochemical markers of liver function were within normal ranges.

For a long-time HBeAg to anti-HBe seroconversion was recognised as a profitable indicator of HBV elimination. Tedder et al. [10] describedd a relationship between HBV-DNA viral load and the presence of HBeAg. On the other hand, Chu et al. [11] and Langer et al. [9], found higher proportion of anti-HBe antibodies in HBsAG carriers with active viral replication than in patients presenting HBeAg. The interesting observation was that in some individuals after HBeAg to anti-HBe seroconversion HBV-DNA concentration decreased [10,12].

Karasawa et al. [13], in 93% HBsAg carriers with anti-HBe antibodies detected HBV mutant "pre-core" existence. Moreover, investigations of Shan's et al. [14], showed a higher rate of HBV mutations among anti-HBe presenting carriers than in patients with HBeAg. It is possible that mutation process contributes to low level of HBV-DNA in persons presenting anti-HBe antibodies.

HBV active replication among HBsAg carriers leads to chronic hepatitis development. Furthermore, long lasting stimulation of hepatocytes to Fas synthesis may induce inflammatory – necrotic changes and fibrosis intensification in the liver [15].

Fas and FasL are proteins that regulate processes of apoptosis in patients with chronic liver injury [16-18]. The Kupffer' cells stimulate the synthesis of cytokines influencing biosynthesis of Fas in hepatocytes. In their study of Xin et al. [19] and Luo et al. [20] confirmed relationship between Fas and FasL serum or liver's tissue concentration and apoptosis activity in patients with chronic liver injury. Cytotoxic reaction and apoptosis run simultaneously in chronic hepatitis B patients and both are controlled by Fas and FasL synthesis [21]. Results of our investigations suggest that sFasL incidence in HBsAg carriers may reflect apoptosis activation. Regardless of correct biochemical markers of the liver function sFasL detection in the serum of HBsAg carriers may demonstrate activation of non-specific immunological processes against HBV infection.

Conclusions

High HBV replication is common within HBsAg carriers. It concerns healthy HBsAg carriers presenting HBeAg as well as anti-HBe antibodies in serum. However, the HBV replication is slightly decreased in carriers with anti-HBe antibodies incidence. The sFasL occurrence in over a half of studied HBsAg carriers may demonstrate activation of non-specific immunological processes against HBV infection.

References

1. Hou TI, Wu JC, Lee PC, Chau GY, Lui WY, Tsay SH, Ting LT, Chang FY, Lee SD. Sero-clearance of hepatitis B surface antigen in chronic carriers does not necessarily imply a good prognosis. Hepatology, 1998; 28: 231-8.
2. Fujiwara K, Yokosuka O, Ehata T, Chuang WL, Imazeki F, Saisho H, Omata M. The two different states of hepatitis B virus DNA in asymptomatic carriers: HBe-antigen-positive versus anti-HBe-positive asymptomatic carriers. Dig Dis Sci, 1998; 43: 368-76.
3. Rossi G, Pelizzari A, Motta M, Puoti M. Primary prophylaxis with lamivudine of hepatitis B virus reactivation in chronic HBsAg carriers with lymphoid malignancies treated with chemotherapy. Br J Haematol, 2001; 115: 58-62.
4. Qin B, Zhang D, Ma Y, Ren H. Role of tumor necrosis factor receptor 1 and Fas antigen in hepatcellular apoptosis of viral hepatitis B patients. Zhonghua Gan Zang Bing Za Zhi, 2001; 6: 337-9.
5. Hori Y, Wada H. Plasma sFas and sFas ligand in the joints of patients with rheumatoid arthritis and osteoarthritis. Arthritis Rheum, 1998; 4: 657-62.
6. Lok ASF, McMahon BJ. Chronic hepatitis B. AASLD practice guidelines. Hepatology, 2001; 34: 1225-41.
7. Mommeja-Marin H, Mondou E, Blum MR, Rousseau F. Serum HBV DNA as a marker of efficacy during therapy for chronic HBV infection: analysis and review of the literature. Heptology, 2003; 37: 1309-19.
8. Shibolet O, Ilan Y, Gillis S, Hubert A, Shouval D, Safadi R. Lamivudine therapy for prevention of immunosuppressive-induced hepatitis B virus reactivation in hepatitis B surface antigen carriers. Blood, 2002; 100: 391-6.
9. Langer BCA, Frosner GG, von Brunn A. Epidemiological study of viral hepatitis A, B, C, D and E among Inuits in West Greenland. J Viral Hep, 1997; 4: 339-49.
10. Tedder RS, Ijaz S, Gilbert N, Barbara JA, Corden SA, Gilson RJ, Boxall EH. Evidence for a dynamic host-parasite relationship in e-negative hepatitis B carriers. J Med Virol, 2002; 68: 505-12.
11. Chu CJ, Hussain M, Lok AS. Quantitative serum HBV DNA levels during different stages of chronic hepatitis B infection. Hepatology, 2002; 36: 1408-15.
12. Sugauchi F, Chutaputti A, Orito E, Kato H, Suzuki S, Ueda R, Mizokami M. Hepatitis B virus genotypes and clinical manifestation among hepatitis B carriers in Thailand. J Gastroenterol Hepatol, 2002; 17: 671-6.
13. Karasawa T, Shirasawa T, Okawa Y, Kuramoto A, Shimada N, Aizawa Y, Zeniya M, Toda G. Association between frequency of amino acid changed in core region of hepatitis B virus (HBV) and the presence of precore mutation in Japanese HBV carriers. J Gastroenterol, 1998; 32: 611-22.
14. Shan H, Niitsuma H, Nagasaki F, Cervantes J, Ojima T, Ueno Y, Kobayashi K, Ishii M, Shimosegawa T. Analysis of the entire nucleotide sequence of hepatitis B virus: characteristics of HBeAg-positive asymptomatic carriers, HBeAb positive asymptomatic carriers and patients with liver cirrhosis. Hepatol Res, 2002; 23: 251-5.
15. Yonehara S. Death receptor Fas and autoimmune disease: from the original generation to therapeutic application of agonistic anti-Fas monoclonal antibody. Cytokine Growth Factor Rev, 2002; 13: 393-9.
16. Canbay A, Higuchi H, Bronk SF, Taniai M, Sebo TJ, Gores GJ. Fas enhances fibrogenesis in the bile duct legated mouse: a link between apoptosis and fibrosis. Gastroenterology, 2002; 123: 1323-30.
17. Ehrmann J, Galuszkova D, Ehrmann J, Krc I, Jezdinska V, Vojtesek B, Murray PG, Kolao Z. Apoptosis-related proteins, BCL-2, BAX, FAS, FAS-L and PCNA in liver biopsies of patients with chronic hepatitis B virus infection. Pathol Oncol Res, 2000; 6: 130-5.
18. Lee MO, Kang HJ, Cho H, Shin EC, Park JH, Kim SJ. Hepatitis B virus X protein induced expression of the Nur77 gene. Biochem Biophys Res Commun, 2001; 5: 1162-8.
19. Xin SJ, Zhao JM, Wang LJ, Wang SS. Study on relationship between the apoptosis of hepatocytes and liver fibrosis of chronic viral hepatitis. Zhonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 2000; 1: 31-3.
20. Liaw YF, Tsai SL, Chien RN, Yeh CT, Chu CM. Prednisolone priming enhances Th1 response and efficacy of subsequent lamivudine therapy in patients with chronic hepatitis B. Hepatology, 2000; 3: 604-9.
21. Hayashi N, Mita E. Fas system and apoptosis in viral hepatitis. J Gastroenterol Hepatol, 1997; 12: S223-6.

Morpho-functional comparisons in *Helicobacter pylori* – associated chronic atrophic gastritis

Pasechnikov VD[1]*, Chukov SZ[2], Kotelevets SM[1], Chabannaya TA[1]

[1] Department of Therapy, Medical Academy, Stavropol, Russian Federation
[2] Department of Pathology, Medical Academy, Stavropol, Russian Federation

Abstract

Purpose: To evaluate serum pepsinogen I (PG I) and gastrin-17 (G-17) levels in patients with *Helicobacter pylori* (*H. pylori*) – associated chronic atrophic gastritis, with reference to endoscopical Kimura-Takemoto's staging, chromoendoscopical and histological features.

Material and methods: 267 dyspeptic *H. pylori*-infected patients were examined by chromoendoscopy with biopsy sampling according to the Sydney System and according to Kimura-Takemoto's scale. Simultaneous assessment of serum pepsinogen I (PG I) and gastrin-17 (G-17) levels by enzyme immunoassay was performed. The serologic and morphologic results were compared with correlation analysis.

Results: There was strong reverse correlation between the stomach mucosal atrophy (antral part or corpus) and the proper serologic markers (respectively, G-17 or PG I) in *H. pylori*-associated chronic gastritis when gastric biopsies taken according to the Sydney System were assessed. The use of Kimura-Takemoto's scale has revealed the decrease of serum PG I levels only at O-2 and O-3 grades of the corpus mucosa atrophy. Probably, these results reflects the development of functional failure of the stomach corpus mucosa at late stages of atrophy when its compensatory capacity becomes insufficient. There were not any advantages in sampling biopsies for the detecting of intestinal metaplasia (IM) by the Sydney System, or by Kimura-Takemoto's scheme. The obvious concordance between histologically proven extent of IM and the number of IM foci detected by chromoendoscopy has been revealed.

Conclusions: The biopsy sampling for the diagnosis of precancerous changes of the stomach mucosa after non-invasive screening of atrophic gastritis (e.g., by means of EIA) should be based preferably on the visual signs acquired via chromoendoscopy than through routine endoscopy, independently of the scheme of examination of stomach mucosa, either according to the Sydney System, or to the Kimura-Takemoto's scale.

Key words: *Helicobacter pylori*, pepsinogen I, gastrin-17, atrophy, intestinal metaplasia, chromoendoscopy.

* CORRESPONDING AUTHOR:
21 Aviatsionnaya Street
355017 Stavropol
Russian Federation
e-mail: passetchnikov@mail.ru (Victor D. Pasechnikov)

Received 05.06.2005 Accepted 12.06.2005

Introduction

Helicobacter pylori (*H. pylori*) is a major causative agent in the pathogenesis of chronic active gastritis, duodenal and gastric ulcer [1]. There is a strong evidence that *H. pylori* infection may be associated also with gastric carcinoma and low-grade MALT lymphoma [2]. Our knowledge of the pathogenesis of gastric neoplasms is therefore increasing, and new approaches to the prevention of gastric cancer by eradication treatment of gastric precancerous diseases, mainly *H. pylori* gastritis, can be considered. *H. pylori* infection causes the development of infiltration of gastric mucosa by mononuclear cells and neutrophils, the latter can serve as a marker for the activity of gastritis. Chronic active inflammation is ultimately accompanied by a replacement of the gastric foveolae by a regenerative type of epithelium and a decrease in the production of mucus [3]. Besides, this damage leads to a persistent state of proliferation and regeneration, and thus increases the risk of malignant alterations of the gastric stem cells at the neck region of the gastric tubes [4]. As a consequence, the multifocal features such as atrophy, intestinal metaplasia, and epithelial dysplasia may be found in association with *H. pylori* infection. In turn, atrophic gastritis and intestinal metaplasia have been considered precursor lesions of

intestinal type of gastric cancer [5]. The effect of eradication of *Helicobacter pylori* on the prevention or regression of atrophy and IM has not been fully elucidated. The point of no return at which such a regression is possible is not known [6]. This subject is very important, because it has a great influence on a strategy of gastric cancer prevention. It has been shown that the extent and topography of *H. pylori*-associated gastritis differs when compared with age- and sex-matched patients having peptic ulcer or merely gastritis. It appears that patients with gastric cancer have much more severe gastritis in the corpus of the stomach in contrast to the antrum-predominating gastritis in duodenal ulcer patients [7]. That pronounced gastritis in the corpus may be involved in the development of gastric cancer can be explained by the finding of a decrease in acid production associated with a shift in the distribution of gastritis toward the corpus [8]. This reduction in local acid production might then result in the suppression of a defense mechanism against dedifferentiated epithelium – since atypical cells are very acid sensitive – and might thus lead to persistence and progression of atypical cells [9].

The gastric mucosa can be evaluated by gastrointestinal endoscopic examination with biopsy [10], but this is an invasive and expensive technique. Easier objective methods for evaluating gastritis and *H. pylori* infection are needed. Of course, *H. pylori* infection can be diagnosed by the assay of anti-*H. pylori* antibodies, the urease test, culture or Giemsa stain of gastric mucosa, or the urea breath test. However, measurement of serum pepsinogen is easier and does not require special equipment or techniques. The concentration of serum pepsinogen, which is an ordinary zymogen of pepsin in gastric mucosa, is known to be a marker of atrophic gastritis [11], as well as being a marker of *H. pylori* infection and eradication [12]. Moreover, gastric mass screening can be performed by assays of serum pepsinogen concentrations [13]. The same is true about measurement of serum gastrin for the non-invasive detection of gastric antral atrophy [14]. Previously, we have found that non-invasive detection of atrophic gastritis requires further endoscopy with histological examination for the recognition of possible progression of atrophy into IM, dysplasia or carcinoma [15], so it is critically important to be sure that the method of endoscopy would allow the adequate biopsy sampling from the irregularly distributed foci of above mentioned preneoplastic changes of stomach mucosa.

The aim of the present study was to evaluate serum pepsinogen I (PG I) and gastrin-17 (G-17) levels in patients with *Helicobacter pylori*-associated chronic atrophic gastritis, with reference to endoscopical Kimura-Takemoto's staging, chromoendoscopical and histological features.

Material and methods

The study was carried out according to updated Declaration of Helsinki in a group of 267 dyspeptic *H. pylori*-infected patients (175 female, 92 male, aged from 15 to 89 years, in average 61.7±13.0 years), after informed consent for examinations. Any eventual medication with proton pomp inhibitors, H2 antagonists and NSAIDs were excluded for at least one month before examinations. The diagnostic test was histology in 267 cases; 158 cases were diagnosed by chromoendoscopy using the methylene blue dye scattering method and 109 – by endoscopy using the Kimura-Takemoto endoscopic classification [16]. The assessment of the type of mucosal atrophy according to Kimura-Takemoto's grading was as follows: C-0 – absence of atrophy, C-1 – pyloric mucosal atrophy, C-2 – atrophy on a lesser curvature of a lower third of the stomach, C-3 – the atrophy on a lesser curvature of a middle third of stomach, O-1 – a border of an atrophy is between lesser curvature and anterior wall; O-2 – atrophy within the limits of an anterior wall of a stomach; O-3 – the area of atrophy is distributed from anterior wall to the major curvature of the stomach. Biopsy specimens were fixed in 10% formalin, embedded in paraffin, cut in sequential 5 μm sections, and stained with hematoxylin and eosin, PAS/alcian blue (pH 2.5), and Giemsa stain. The grade of the stomach mucosal atrophy was estimated from 0 to 3 according to Houston visual analogous scale [10]. Fasting serum *H. pylori* antibodies (Hp-Ab), serum levels of PG I and G-17 were assayed by enzyme immunoassay (EIA) with Biohit GastroPanel® (Biohit Plc, Helsinki, Finland). According to the instruction of manufacturer, serum levels of PG I <25 μg/l were accepted as markers of gastric corpus atrophy; serum levels of G-17 <5 pmol/l were estimated as markers of gastric antral atrophy; serum levels of G-17 <10 pmol/l in a combination with serum levels of PG I <50 μg/l were estimated as markers of mild gastric corpus atrophy. Hp-Ab IgG titers were estimated as follows: <32 EIU (EIU – enzyme immunoassay unit) – negative result; 32-44 EIU – doubtful result; >44 EIU – positive result. The numerical meanings of assayed parameters were analyzed by the program GastroSoft® (Biohit Plc, Helsinki, Finland) enclosed to test-system Biohit GastroPanel®. On the basis of inserted data, the program composed the diagnosis in a view of the presence or absence of *H. pylori*-infection and mucosal atrophy, with estimating of gastric cancer or peptic ulcer risk and with recommendations on the treatment according to Maastricht-2 consensus.

The statistical analysis was used to estimate the mean values of investigated parameters and to calculate statistical significance of received data by Mann-Whitney criterion, and by the Spearman's correlation coefficient (r_s).

Results

According to EIA data, the absence of the corpus mucosal atrophy was detected in 155 patients, mild corpus atrophy – in 33 patients, moderate corpus atrophy – in 48 patients, and severe corpus atrophy – in 31 patients. The corresponding values of serum PG I are presented in *Tab. 1*.

The absence of antral mucosal atrophy was detected in 14 patients, mild antral atrophy – in 40 patients, moderate antral atrophy – in 94 patients, and severe antral atrophy – in 119 patients. The corresponding values of serum G-17 are presented in *Tab. 2*.

The correlation analysis has revealed strong reverse correlation between the presence and the degree of stomach corpus atrophy and the serum levels of PG I (r_s = -0.63; $P<0.05$). Simi-

***Table 1*. The mean values of serum PG I at different degrees of the stomach corpus musocal atrophy (μg/l) in chronic gastritis**

	Mean	95% confidence interval	σ	Number of patients
No atrophy	133.30	-10.63 – 277.23	71.96	155
Mild atrophy	42.61*	-21.10 – 106.31	31.85	33
Moderate atrophy	18.66*	-19.31 – 56.63	18.98	48
Severe atrophy	7.79*	-1.21 – 16.78	4.49	31
Total				267

*P<0.05 compared to non-atrophic state and to previous degree of atrophy

***Table 2*. The mean values of serum G-17 at different degrees of the stomach antral musocal atrophy (pmol/l) in chronic gastritis**

	Mean	95% confidence interval	σ	Number of patients
No atrophy	15.46	3.15 – 27.78	6.16	14
Mild atrophy	8.71*	3.49 – 13.94	2.61	40
Moderate atrophy	6.15*	2.61 – 9.70	1.77	94
Severe atrophy	1.31*	-2.34 – 4.95	1.82	119
Total				267

*P<0.05 compared to non-atrophic state and to previous degree of atrophy

***Table 3*. The mean number of foci of chromoendoscopically detected intestinal metaplasia (IM) in relation to histological detection of IM in *Helicobacter pylori* – associated atrophic gastritis**

	Mean	95% confidence interval	σ	Number of patients
No IM	1.05	0.5 – 1.6	0.27	51
Mild IM	5.04*	-17.24 – 27.32	11.14	50
Moderate IM	8.85*	1.09 – 16.60	3.88	38
Severe IM	23.89*	-11.50 – 59.29	17.70	19
Total				158

*P<0.05 compared to non-metaplastic state and to previous degree of IM

larly, we have observed the strong reverse correlation between the presence and the degree of stomach antral atrophy and the serum levels of G-17 (r_s = -0.73; P<0.05). There were no any marked correlations between serum Hp-Ab IgG titers and the presence and the degree of stomach corpus or antral atrophy (the values of r_s, respectively, 0.12 and 0.14; P>0.05 for both corpus or antral mucosa).

Among 158 patients, which were undergone chromoendoscopy, there were 107 histologically proven cases of intestinal metaplasia (IM), 50 patients of them had mild IM, 38 – moderate IM, and 19 – severe IM. Fifty one patients had no histological features of IM (*Fig. 1*).

We have compared the histologically detected extent of IM and the number of foci of IM detected by chromoendoscopy (*Tab. 3*). The results have confirmed the high accuracy of chromoendoscopy in distinguishing IM in atrophic gastritis (r_s = 0.92; P<0.05).

***Figure 1*. The prevalence of intestinal metaplasia (IM) in the patients with *Helicobacter pylori* – associated chronic atrophic gastritis**

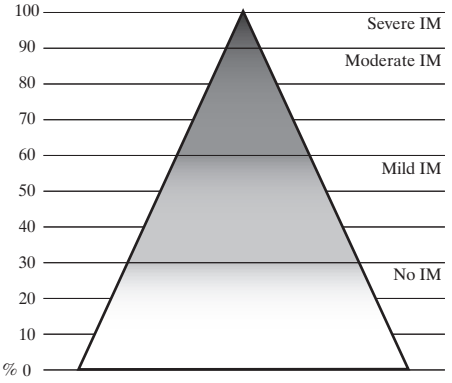
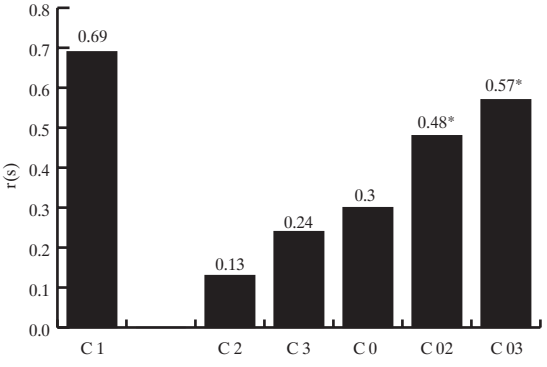


***Figure 2*. Correlations (r_s) between the results of EIA (serum G-17 levels for C 1, serum PG I levels for C 2 – O 3) with the type of stomach mucosal atrophy according to Kimura-Takemoto's grading** (*P<0.05 compared to C2 – O1 stages)



Further, we have compared the results of EIA with the type of stomach mucosal atrophy according to Kimura-Takemoto's grading (*Fig. 2*). There was strong reverse correlation between serum G-17 level and the presence of C-1 grade of stomach mucosal atrophy (antral). On the contrary, the correlations in the corpus of the stomach were not so obvious, and reached the significant values beginning from O-2 grade of corpus atrophy. So, the progression of corpus mucosal atrophy was accompanied with functional disorders only when the vast majority of gastric glands disappeared.

Discussion

Prior to the identification of *H. pylori* as the major cause of gastritis, the decline in the ability to secrete gastric acid due to atrophic change of gastric mucosa was considered a consequence of aging. The discovery of *H. pylori* has led to a reassessment of the importance of aging and has focused on the long-term effects of *H. pylori* infection and its role in the development of atrophic gastritis [17]. The updated Sydney

System allows precise histologic evaluation of gastritis [10], but requires gastrointestinal endoscopy and biopsy, which are invasive, expensive, and uncomfortable methods. Of course, as gastrointestinal endoscopic examination is the most accurate method of examination for gastric diseases, especially gastric malignancy, it should be used for patients exhibiting any relevant symptoms. However, in follow-up studies and screening of asymptomatic subjects, an easier and less expensive method is required.

The serum pepsinogen level reflects the secretory function of the gastric glands. Its levels decreased significantly in the patients with chronic atrophic gastritis, an important precursor of gastric carcinoma [11]. It has been suggested that the measurement of serum pepsinogens could identify people at high risk for gastric cancer. Chronic atrophic gastritis is believed to be an important premalignant condition for the development of gastric carcinoma, particularly the intestinal type [18]. The serum PG I/II ratio has been found to be reduced in gastric carcinoma, as pepsinogen I decreases proportionally more than pepsinogen II. In contrast, the serum level of gastrin was increased in atrophic corpus gastritis [19]. In Japan, where studies have shown a high prevalence of chronic gastritis, the serum pepsinogen level has been studied as a mass screening tool for the detection of gastric cancer. The Kimura-Takemoto's scale for the evaluation of gastric mucosa morphology was used in these studies [20].

Screening by using serum pepsinogen has advantages over other methods such as endoscopy and barium studies. It is simple and inexpensive, and there is no radiation hazard. In our study, there was strong reverse correlation between the presence and the degree of stomach mucosal atrophy (antral part or corpus) and the proper serologic markers (respectively, G-17 or PG I) in *H. pylori*-associated chronic gastritis when we assessed gastric biopsies taken according the Sydney System. However, the use of grading the stomach mucosal atrophy according to Kimura-Takemoto's scale has brought us some different results concerning the serum levels of PG I at various extent of corpus atrophy. We observed the decreasing of serum PG I levels only at O-2 and O-3 grades of corpus atrophy. In our opinion, these results reflects the development of functional failure of the stomach corpus mucosa at late stages of atrophy when its compensatory capacity becomes insufficient. In general, we could not find any advantages in sampling biopsies for detecting IM by the Sydney System recommendations, or by Kimura-Takemoto's scheme. Similar results are reported by Asaka et al. [21]. We can not agree with the interpretation of So et al. [22] results, showing an increase in serum PG I and II levels and a lower PG I/II ratio in gastric cancer patients. In their study, the gastric atrophy was present in a small proportion of patients with gastric cancer and the authors conclude that atrophic gastritis may not be an essential stage in gastric carcinogenesis. Hence, in their meaning, the serum pepsinogen measurement is not useful for the screening of gastric cancer in the investigated population. In contrast, many other studies have shown the correlation between the serum PG levels and the extent of atrophic gastritis.

Kreuning et al. [23] reported that serologic parameters in healthy volunteers were related to specific histologic features of the gastric body. Kawaguchi et al. [24] also confirmed that PG correlated well with the grade of atrophic gastritis regardless of the age or sex of patient. Kiyohira et al. [25] investigated the utility of serum PG concentrations for the diagnosis of *H. pylori* infection and the objective evaluation of histologic gastritis. The authors also investigated whether *H. pylori* infection could be diagnosed via serum PG concentrations alone. Kuipers et al. [26], Asaka et al. [27], and Knight et al. [28] have shown, that the serum concentrations of PG I and PG II increased and the I/II ratio decreased in *H. pylori* infection. Cave et al. [29] hypothesized that *H. pylori* leads to increased PG secretion. In patients with no or mild atrophy, both serum PG I and PG II concentrations were increased. The mass of chief cells decreases with the progression of atrophy, with chief cells gradually being replaced by pyloric gland cells. Serum PG I concentrations then decrease, but serum PG II concentrations continue to increase. Consequently, the PG I/II ratio decreases. In marked atrophy, both serum PG I and PG II concentrations decrease and the PG I/II ratio shows a marked fall.

Recently, several studies have demonstrated that successful treatment of *H. pylori* infection significantly reduces serum PG concentrations, significantly increases the PG I/II ratio, and clearly improves histological findings within approximately 1 month [12,30]. Therefore, these parameters may be useful for monitoring of anti-*H. pylori* treatment efficacy. Gastric mucosal evaluation by serum PG concentrations is an inexpensive, noninvasive, simple, and objective method.

Another important issue is revealing the intestinal metaplasia in *H. pylori*-associated atrophic gastritis. As the serum pepsinogen I/II ratio was known to be a good marker for gastric atrophy [31], Asaka et al. [21] hypothesized that the development of atrophic gastritis and intestinal metaplasia in the gastric mucosa was strongly associated with *H. pylori* infection. Recently the authors performed a case-control study of 85 asymptomatic healthy adults recruited from a health screening center in Sapporo [32]. All subjects underwent endoscopy and gastric biopsy. The prevalence of atrophic gastritis and intestinal metaplasia as assessed by pathological findings was significantly greater in those with *H. pylori* infection compared with those without *H. pylori* infection. The later study [21] was a large scale multicenter study involving different regions in Japan using three different methods to assess the prevalence of atrophic gastritis as well as evaluation of the presence of intestinal metaplasia by endoscopic biopsy. The authors have found that both atrophic gastritis and intestinal metaplasia were strongly associated with *H. pylori* infection and not with aging per se, and that the tight link is present between *H. pylori* infection, atrophic gastritis and intestinal metaplasia. The high prevalence of the precursor lesion, atrophic gastritis with intestinal metaplasia among those with *H. pylori* infection suggests that the risk of development of early gastric cancer will continue to remain high until *H. pylori* is eliminated either naturally or by therapy. Nomura et al. [33] have reported that persons with both *H. pylori* or CagA seropositivity and a low PG I level or PG I/II ratio are highly susceptible to development of noncardia gastric cancer. Hartleb et al. [34] have recently stated that the test panel composed of pepsinogen I and protein stimulated gastrin-17 may be used as the "serological gastric biopsy" detecting multifocal atrophic gastritis. The diagnostic sensitivity of this test panel is not

increased by knowledge of *H. pylori* status. Previously, we have shown similar results, with strong correlation between detection of IM by chromoendoscopy and by histology [15]. Our present study has confirmed these results in another group of patients, and there has been the obvious concordance between histologically detected extent of IM and the number of foci of IM detected by chromoendoscopy.

Conclusions

The natural history of *Helicobacter pylori*-associated atrophic gastritis is a permanent progression of the extent and severity of atrophy with ultimate imbalance between proliferation and differentiation leading to the development of intestinal metaplasia and dysplasia. The latter have patchy distribution through the stomach mucosa and, thus, the biopsy sampling for the diagnosis of precancerous changes of stomach mucosa after non-invasive screening of atrophic gastritis (e.g., by means of EIA) should be based preferably on the visual signs acquired via chromoendoscopy rather than through routine endoscopy. It holds true independently of the scheme of examination of the stomach mucosa, would it be based either on the Sydney System, or the Kimura-Takemoto's scale.

References

1. Fontham ET, Ruiz B, Perez A, Hunter F, Correa P. Determinants of Helicobacter pylori infection and chronic gastritis. Am J Gastroenterol, 1995; 90: 1094-101.
2. Parsonnet J, Friedman GD, Vandersteen DP, Chang Y, Vogelman JH, Orentreich N, Sibley RK. Helicobacter pylori infection and the risk of gastric carcinoma. N Engl J Med, 1991; 325: 1127-31.
3. Stolte M, Stadelmann O, Bethke B, Burkard G. Relationship between the degree of Helicobacter pylori colonization and the degree and activity of gastritis, surface epithelial degeneration and mucus secretion. Z Gastroenterol, 1995; 33: 89-93.
4. Bechi P, Balzi M, Becciolini A, Maugeri A, Raggi CC, Amorosi A, Dei R. Helicobacter pylori and cell proliferation of the gastric mucosa: possible implications for gastric carcinogenesis. Am J Gastroenterol, 1996; 91: 271-6.
5. Correa P. A human model of gastric carcinogenesis. Cancer Res, 1988; 48: 3554-60.
6. Tytgat GNJ. Remaining questions with respect to Helicobacter pylori. Gastroint Endosc, 1998; 47: 198-9.
7. Stolte M, Meining A. Helicobacter pylori and gastric cancer. Oncologist, 1998; 3: 124-8.
8. Stolte M, Bethke B. Elimination of Helicobacter pylori under treatment with omeprazole. Z Gastroenterol, 1990; 28: 271-4.
9. Axon ATR, Lynch DAF. Helicobacter pylori, gastric physiology and cancer. Eur J Gastroenterol Hepatol, 1993; 5: 109-13.
10. Dixon MF, Genta RM, Yardley J, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston, 1994. Am J Surg Pathol, 1996; 20: 1161-81.
11. Samloff IM, Varis K, Ihamaki T, Siurala M, Rotter JI. Relationships among serum pepsinogen I, serum pepsinogen II, and gastric mucosal histology: a study in relatives of patients with pernicious anemia. Gastroenterology, 1982; 83: 204-9.
12. Wagner S, Haruma K, Gladziwa U, Soudah B, Gebel M, Bleck J, Schmidt H, Manns M. Helicobacter pylori infection and serum pepsinogen A, pepsinogen C, and gastrin in gastritis and peptic ulcer: significance of inflammation and effect of bacterial eradication. Am J Gastroenterol, 1994; 89: 1211-8.
13. Yoshihara M, Sumii K, Haruma K, Kiyohira K, Hattori N, Kitadai Y, Komoto K, Tanaka S, Kajiyama G. Correlation of ratio of serum pepsinogen I and II with prevalence of gastric cancer and adenoma in Japanese subjects. Am J Gastroenterol, 1998; 93: 1090-6.
14. Suovaniemi O, Harkonen M, Paloheimo L, Sipponen P. GastroPanel: Diagnosing atrophic gastritis from serum – providing a tool for evidence-based medicine. Business Briefing: Global Health Care, 2003: 1-4.
15. Pasechnikov VD, Chukov SZ, Kotelevets SM, Mostovov AN, Polyakova MB, Mernova VP. The non-invasive diagnosis of precancerous changes of stomach mucosa. Rocz Akad Med Bial, 2004; 49: 66-71.
16. Kimura K, Takemoto T. An endoscopic recognition of the atrophic border and its significance in chronic gastritis. Endoscopy, 1969; 3: 87-97.
17. Katelaris PH, Seow F, Lin BP, Napoli J, Ngu MC, Jones DB. Effect of age, Helicobacter pylori infection, and gastritis with atrophy on serum gastrin and gastric acid secretion in healthy men. Gut, 1993; 34: 1032-7.
18. Correa P. Human gastric carcinogenesis: a multistep and multifactorial process. Cancer Res, 1992; 52: 6735-40.
19. Westerveld BD, Pals G, Lamers CB, Defize J, Pronk JC, Frants RR, Ooms EC, Kreuning J, Kostense PJ, Eriksson AW, et al. Clinical significance of pepsinogen A isozymogens, serum pepsinogen A and C levels, and serum gastrin levels. Cancer, 1987; 59: 952-8.
20. Kitahara F, Kobayashi K, Sato T, Kojima Y, Araki T, Fujino MA. Accuracy of screening for gastric cancer using serum pepsinogen concentrations. Gut, 1999; 44: 693-7.
21. Asaka M, Sugiyama T, Nobuta A, Kato M, Takeda H, Graham DY. Atrophic gastritis and intestinal metaplasia in Japan: results of a large multicenter study. Helicobacter, 2001; 6: 294-9.
22. So JB, Yeoh KG, Moochala S, Chachlani N, Ho J, Wong WK, Mack P, Goh PM. Serum pepsinogen levels in gastric cancer patients and their relationship with Helicobacter pylori infection: a prospective study. Gastric Cancer, 2002; 5: 228-32.
23. Kreuning J, Lindeman J, Biemond I, Lamers CB. Serological parameters in assessment of degree of gastritis in healthy volunteers. Dig Dis Sci, 1995; 40: 609-14.
24. Kawaguchi H, Haruma K, Komoto K, Yoshihara M, Sumii K, Kajiyama G. Helicobacter pylori infection is the major risk factor for atrophic gastritis. Am J Gastroenterol, 1996; 91: 959-62.
25. Kiyohira K, Yoshihara M, Ito M, Haruma K, Tanaka S, Chayama K. Serum pepsinogen concentration as a marker of Helicobacter pylori infection and the histologic grade of gastritis; evaluation of gastric mucosa by serum pepsinogen levels. J Gastroenterol, 2003; 38: 332-8.
26. Kuipers EJ, Uyterlinde AM, Pena AS, Roosendaal R, Pals G, Nelis GF, Festen HP, Meuwissen SG. Long-term sequelae of Helicobacter pylori gastritis. Lancet, 1995; 345: 1525-8.
27. Asaka M, Kimura T, Kudo M, Takeda H, Mitani S, Miyazaki T, Miki K, Graham DY. Relationship of Helicobactor pylori to serum pepsinogens in an asymptomatic Japanese population. Gastroenterology, 1998; 102: 760-6.
28. Knight T, Greaves S, Wilson A, Hengels K, Newell D, Corlett M, Webb P, Forman D, Elder J. Variability in serum pepsinogen levels in an asymptomatic population. Eur J Gastroentrol Hepatol, 1995; 7: 647-54.
29. Cave TR, Cave DR. Helicobacter pylori stimulating pepsin secretion from isolated rabbit gastric glands. Scand J Gastroenterol, 1991; 26(Suppl. 181): 9-14.
30. Furuta T, Kaneko E, Baba S, Arai H, Futami H. Percentage changes in serum pepsinogens are useful as indices of eradication of Helicobacter pylori. Am J Gastroenterol, 1997; 92: 84-8.
31. Miki K, Ichinose M, Shimizu A, Huang SC, Oka H, Furihata C, Matsushima T, Takahashi K. Serum pepsinogens as a screening test of extensive chronic gastritis. Gastroenterol Jpn, 1987; 22: 133-41.
32. Asaka M, Kato M, Kudo M, Katagiri M, Nishikawa K, Koshiyama H, Takeda H, Yoshida J, Graham DY. Atrophic changes of gastric mucosa are caused by Helicobacter pylori infection rather than aging: studies in asymptomatic Japanese adults. Helicobacter, 1996; 1: 52-6.
33. Nomura AM, Kolonel LN, Miki K, Stemmermann GN, Wilkens LR, Goodman MT, Perez-Perez GI, Blaser MJ. Helicobacter pylori, pepsinogen, and gastric adenocarcinoma in Hawaii. J Infect Dis, 2005; 191: 2075-81.
34. Hartleb M, Wandzel P, Waluga M, Matyszczyk B, Boldys H, Romanczyk T. Non-endoscopic diagnosis of multifocal atrophic gastritis; efficacy of serum gastrin-17, pepsinogens and Helicobacter pylori antibodies. Acta Gastroenterol Belg, 2004; 67: 320-6.

Gastric juice ammonia and urea concentrations and their relation to gastric mucosa injury in patients maintained on chronic hemodialysis

Blusiewicz K[1], **Rydzewska G**[2,4], **Rydzewski A**[3,4]*

[1] Dialysis Unit, Regional Hospital, Grajewo, Poland
[2] Department of Internal Medicine and Gastroenterology, Central Clinical Hospital of Ministry of Internal Affairs and Administration, Warsaw, Poland
[3] Department of Internal Medicine and Nephrology, Central Clinical Hospital of Ministry of Internal Affairs and Administration, Warsaw, Poland
[4] Institute of Medical Education, Świętokrzyska Academy, Kielce, Poland

Abstract

Purpose: **This study was undertaken to test the hypothesis that high concentrations of urea in gastric juice would have an influence on *Helicobacter pylori* infection in patients maintained on chronic hemodialysis (HD).**

Material and methods: **We investigated 30 patients (17 males, 13 females; mean age 50.8 ± 2.9 years) with end-stage renal disease (ESRD) undergoing hemodialysis treatment (HD) for at least 6 months, who were compared to 31 patients (16 males, 15 females; mean age 61.3 ± 2.2 years) with dyspeptic symptoms. Biopsies from the gastric antrum and body were taken for histological investigation. Urea and ammonia were measured in gastric juice, and the severity of gastritis was evaluated according to Sydney criteria.**

Results: ***H. pylori*** **infection was found in 19 (63%) HD patients and in 22 (71%) control subjects. Gastric juice urea concentration was significantly higher in HD patients than in controls and *H. pylori* infection caused a significant decrease in urea concentration in both groups. There was an inverse correlation between urea and ammonia concentration in gastric juice in both groups. Ammonia concentration in both groups was higher in *H. pylori* infected patients. In *H. pylori* negative subjects ammonia/urea ratio was lower in HD patients in comparison to controls. Ammonia/urea ratio was raised by *H. pylori* infection in both groups, and the difference between HD and control groups persisted. *H. pylori* infection was associated with polymorphonuclear infiltration of gastric mucosa. There was a significant correlation between gastric ammonia and mucosal polymorphonuclear leukocytes infiltration and gastritis score.**

Conclusions: **Higher urea levels in the gastric juice of chronically hemodialyzed patients do not seem to be a risk factor for infection with *Helicobacter pylori*.**

Key words: *Helicobacter pylori*, gastritis, urea, ammonia, hemodialysis.

Introduction

Dyspeptic complaints are present in up to 80% of patients with uremia [1-3]. The reasons for this are not entirely clear. *Helicobacter pylori* is an important causative factor of peptic ulcer disease and chronic gastritis. It is estimated, that in Poland about 70% of population may be infected [4,5]. Data concerning frequency of *H. pylori* infection in chronic hemodialysis patients are conflicting [3,6-11]. *H. pylori* has several adaptations for an acid milieu of the stomach. One of them is urease, which converts urea into ammonia and bicarbonate [12]. The ammonium hydroxide formed raises pH of gastric juice and enables *H. pylori* colonization of gastric mucosa. It has been argued that urease is essential for gastric colonization [13], although, urease negative strains have been isolated from patients [14].

In patients with chronic renal failure high gastric juice urea concentrations, by providing substrate, might lead to elevated concentrations of ammonia, and ammonia has been incriminated as a main factor injuring gastric mucosa by several groups [15-20]. It has also been found that in patients maintained on hemodialysis there is a lack of correlation between endoscopic findings and histopathology of gastric mucosa [2,3].

Therefore this study was undertaken to test the hypothesis that high concentrations of urea in the gastric juice would have an influence on *Helicobacter pylori* infection by investigating relationship between concentrations of urea and ammonia in gastric juice and histopathologic changes of gastric mucosa in

* CORRESPONDING AUTHOR:
Department of Internal Medicine and Nephrology
Central Clinical Hospital of Ministry
of Internal Affairs and Administration
ul. Wołoska 137
02-507 Warsaw, Poland
Tel: +48 22 5081207; Fax: +48 22 5081218
e-mail: Andrzej.Rydzewski@ckmswia.pl (Andrzej Rydzewski)

Received 27.06.2005 Accepted 25.07.2005

hemodialyzed patients in comparison to subjects with normal kidney function.

Material and methods

Patients

30 patients (17 males, 13 females; mean age 50.8±2.9 years) with end-stage renal disease (ESRD) undergoing hemodialysis treatment (HD) for at least 6 months, were compared to 31 patients (16 males, 15 females; mean age 61.3±2.2 years) with dyspeptic symptoms and normal levels of blood urea nitrogen and creatinine. ESRD patients were hemodialyzed 3 times a week for 3.5-5.5 hours with Fresenius 4008B machines. Bicarbonate dialysate was used and polysulphone dialysis membranes (Fresenius F5 and F6 dialyzers).

Study protocol

Patients from both groups were recruited for the study based on the presence of dyspeptic complaints and abstinence from alcohol. Exclusion criteria from both groups were as follows: administration of histamine$_2$-receptor antagonists, proton-pump inhibitors, bismuth, sucralfate, non-steroidal anti--inflammatory drugs, or antibiotics within 4 weeks before the study, prior gastrectomy or eradication therapy for *H. pylori* and abnormal liver function tests. The study protocol was approved by Ethics Committee of the Medical University of Białystok.

All the endoscopic procedures were performed by experienced endoscopist (KB). Patients underwent endoscopy before scheduled hemodialysis, after an overnight fast using Olympus GIF E10 endoscope. First 5 ml of gastric juice was withdrawn using sterile cannula and syringe and immediately transferred to a sterile tightly capped tube, and after cooling to 4°C centrifugated at 3000 g for 15 minutes. Five mucosa specimens were obtained using sterile biopsy forceps from the standard sites in antral region and stomach body for urease test and histopathology.

Histopathology

The formalin-fixed, paraffin-embedded tissue sections were stained with hematoxylin and eosin, as well as modified Giemsa stain, to detect *H. pylori*. All sections were assessed "blindly" by the same histopathologist according to the Sydney System [21]. *H. pylori* colonization, polymorphonuclear infiltration, mononuclear cells number, atrophy and intestinal metaplasia were scored as absent, mild, moderate or severe.

The scores for each evaluated factor from both antrum and body were added to give a total "gastritis score" for each patient. We also calculated polymorphonuclear score, mononuclear score, atrophy score, and intestinal metaplasia score by adding individual scores for antrum and corpus.

Patients were considered to be infected with *H. pylori* if either histological examination or urease test were positive.

Laboratory methods

Urease test was performed on a biopsy specimen using commercial kit (Institute of Food and Nutrition, Warsaw, Poland).

Urea concentration in gastric juice was measured with urease colorimetric method, based on hydrolysis of urea to ammonia and carbon dioxide in the presence of urease. Ammonia then reacts with oxoglutarate in the presence of GLDH and NADH, which is oxidised and measured at 340 nm, using reagents from Abbot (Urea Nitrogen List No 8D34-01) and Alcyon TM 300/300i analyzer (Abbot Laboratories). Correction was made for ammonia present in gastric juice according to Lieber [22]. Ammonia was determined with a commercial reagents (Randox Laboratories) using enzymatic UV method and Cobas Bio automatic analyzer (Roche Diagnostics). Urease activity index was calculated as ammonia/urea ratio in gastric juice.

Table 1. **Endoscopic findings in each investigated group. HD (hemodialyzed) patients**

Mucosa	HD – n (%)	Control – n (%)	P (Fisher's exact test)
Normal	10 (33)	10 (33)	NS
Chronic gastritis	10 (33)	12 (39)	NS
Chronic erosive gastritis	6 (20)	6 (19)	NS
Chronic atrophic gastritis	2 (7)	3 (10)	NS
Duodenitis	3 (10)	2 (6)	NS
Gastric ulcer	0	2 (6)	NS
Duodenal ulcer	1 (3)	4 (13)	NS

Statistical analysis

Results are expressed as mean ±SD. Chi-square test, Student's t-test, Mann-Whitney test, MANOVA, Spearman and Pearson correlations were used as appropriate to test the statistical significance of the differences between groups. A P value less than 0.2 was introduced into a backward stepwise logistic regression model. $P<0.05$ was considered statistically significant. The STATA software, version 8.2 (Stata Corporation, College Station, TX, USA) was used for statistical computations.

Results

Endoscopic findings in each investigated group are shown in *Tab. 1*.

Positive urease test was found in 19 (63%) HD patients and in 22 (71%) control subjects ($P=0.525$; NS). The presence of *H. pylori* in tissue sections was found in 7 (23%) HD patients and in 15 (48%) control subjects ($P=0.042$).

Serum urea concentration in HD patients infected with *H. pylori* was similar to that in HD subjects without *H. pylori* infection ($P=0.322$). There was a strong correlation between serum and gastric juice correlations of urea ($r=0.795$; $P<0.0001$).

As seen from *Fig. 1* and *Tab. 2* gastric juice urea concentration was significantly higher in HD patients than in controls (*Tab. 2*) and *H. pylori* infection caused statistically significant decrease in urea concentration in both groups (*Tab. 2*).

The mean gastric juice ammonia concentration in the *H. pylori* infected HD patients was 8.9±7.6 mmol/L compared with 7.4±3.1 mmol/L in the *H. pylori* positive patients with nor-

Figure 1. **Gastric juice urea concentration in *H. pylori* negative and positive control subjects and HD (hemodialyzed) patients** (for statistical significances see *Tab. 2*)

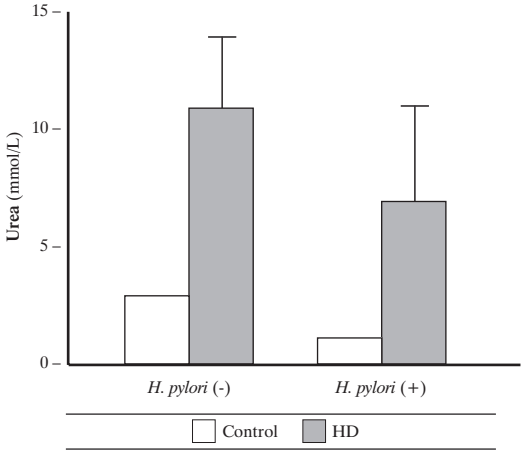
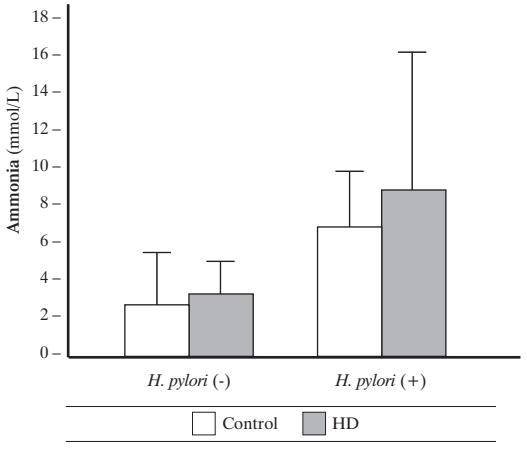


Table 2. **Influence of renal failure and *H. pylori* infection on ammonia and urea concentrations and urease index in gastric juice (MANOVA)**

	P value for		
Factor	**Ammonia**	**Urea**	**Urease index**
Renal failure	NS	<0.00001	<0.00001
H. pylori infection	<0.00001	=0.0004	<0.00001
Interaction between factors	NS	NS	0.0075

Figure 2. **Gastric juice ammonia concentration in *H. pylori* negative and positive control subjects and HD (hemodialyzed) patients** (for statistical significances see *Tab. 2*)



mal renal function (*Fig. 2*). In the *H. pylori* negative patients the values were 3.1±2.0 mmol/L and 1.5±1.0 mmol/L respectively in the HD patients and control subjects and this difference was statistically significant (*Tab. 2*).

There was an overlap of the urea and ammonia concentrations in gastric juice from both *H. pylori* positive and negative subjects (*Fig. 3*).

In *H. pylori* negative subjects ammonia/urea ratio was lower in HD patients in comparison to controls (*Fig. 4* and *Tab. 2*).

Figure 3. **Individual ammonia and urea concentrations in gastric juice from both *H. pylori* positive and negative subjects. HD (hemodialyzed) patients**

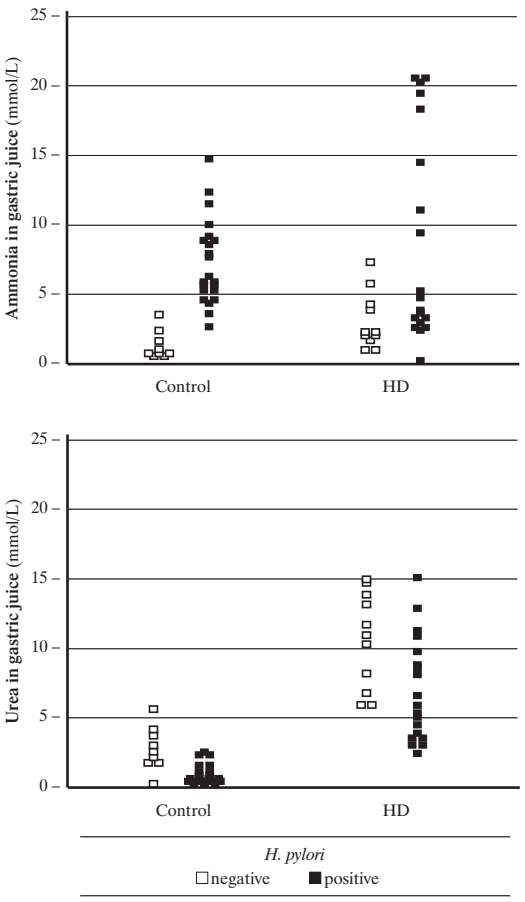
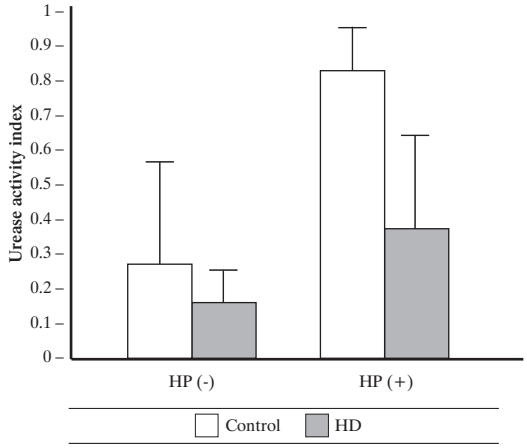


Figure 4. **Ammonia/urea ratio in gastric juice in *Helicobcter pylori* (HP) negative and positive control subjects and HD (hemodialyzed) patients** (for statistical significances see *Tab. 2*)



Ammonia/urea ratio was augmented in the presence of *H. pylori* infection in both groups, and the difference between HD and control groups persisted. Increase, however, was more pronounced in controls (*Tab. 2*).

There was an inverse correlation between urea and ammonia concentration in gastric juice in both groups (*Fig. 5*).

Figure 5. **Correlation between urea and ammonia in gastric juice. Regression for HD (hemodialyzed) patients (solid line) and controls (dashed line)**

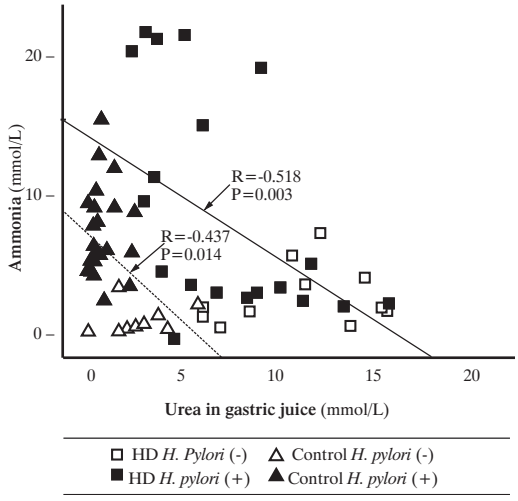


Table 3. **Results of backward stepwise logistic-regression analysis with *H. pylori* infection status as an outcome variable**

Variable	Odds Ratio	95% CI	P value
Polymorphonuclear infiltration score for antrum	4.656	1.852-11.707	0.001
Polymorphonuclear infiltration score for corpus	3.814	1.592-9.136	0.003

* Other variables that were examined but they did not have statistically significant associations with a *H. pylori* infection included patients being on HD treatment (lymphoplasmocytic infiltration score, mucosal atrophy score and intestinal metaplasia score)

The number of polymorphonuclears in antral and stomach corpus mucosa was proportional to ammonia concentration in gastric juice (Spearman's rho =0.339; P=0.008 and Spearman's rho =0.344; P=0.007 respectively).

When all the subjects were analyzed the total gastritis score was directly correlated with ammonia concentration (Spearman's rho =0.295; P=0.022), urease activity index (Spearman's rho =0.302; P=0.019) and *H. pylori* positive status (Spearman's rho =0.446; P=0.004), and inversely to urea in gastric juice (Spearman's rho =-0.284; P=0.028).

We also calculated polymorphonuclear score, mononuclear score, atrophy score, and intestinal metaplasia score by adding individual scores for antrum and corpus. Only polymorphonuclear score was significantly higher in HD in comparison to control group (P=0.045; Mann-Whitney test).

The results of backward stepwise logistic-regression analysis have shown that only polymorphonuclear infiltration in both corpus and antrum were predictive for *H. pylori* infection (*Tab. 3*).

Discussion

Since its discovery in 1982 [23], *Helicobacter pylori* has been linked to many gastroduodenal diseases, including gastritis, peptic ulcer and gastric malignancies. However, its role in the pathogenesis of gastrointestinal complications of renal failure has not been definitely proved. Uremic patients have very often dyspeptic symptoms, but their etiology is multifactorial, and most probably not related to *H. pylori* infection [2,3].

It has been argued that high gastric juice urea concentrations might create favourable environement for *H. pylori*, and therefore a higher frequency of infection in uremic patients should be expected. That has not been proved to be the case [24]. To the contrary, several studies reported lower prevalence of *H. pylori* infection in renal failure patients [3,6-8]. Frequent use of antibiotics in patients maintained on chronic HD might possibly be one of the reasons for the lower prevalence of *H. pylori* infection. We observed somewhat higher prevalence of *H. pylori* than in other studies, although not different from control subjects – fact perhaps related to the selection of patients presenting with dyspeptic complaints, as well as to rather high prevalence of *H. pylori* infection in Poland. In unselected populations it was reported to be about 70% [4,5].

Similar serum urea concentrations in both *H. pylori* positive and negative subjects indicate that differences in gastric juice urea concentration between these groups were in fact caused by infection with microorganism. This is in agreement with Neithercut et al. [25] and opposite to Ala-Kaila et al. [2]. Inverse relationship between urea and ammonia in gastric juice concentrations suggests that uremic state is an important factor causing raised ammonia concentration in gastric juice. Such effect can be mimicked by an infusion of urea into the stomach [26], and should accentuate any ammonia induced effects. Ammonia is transformed to $NH4^+$ ion (ammonium). The relative concentration of these two forms is pH dependent. Ammonia concentration raises with an increase of pH. Ammonia has been incriminated as a factor contributing to gastric mucosal injury [16]. It has been shown that ammonia accelerates apoptosis in gastric mucosa [18], inhibits proliferation and cell cycle progression at S-phase [19], cell migration and proliferation of gastric mucosa [20].

There was an overlap between urea and ammonia concentrations in gastric juice from *H. pylori* positive and negative subjects in both groups which precludes possible use of these parameters as indicators of *H. pylori* infection. It is interesting to note that ammonia/urea ratio was a good predictor of *H. pylori* infection in both groups of patients.

Total gastritis score was directly correlated with ammonia concentration and urease activity index and inversely to urea in gastric juice. It does not necessarily prove a direct causal link. It may also be an indirect marker of *H. pylori* presence which harms mucosa, as suggested by correlation with positive infection status.

Both in HD patients and control group polymorphonuclear infiltration in both corpus and antrum were predictive for *H. pylori* infection. It is well known that *Helicobacter pylori* infection causes gastric mucosa inflammation which is associated with neutrophilic activation [27,28]. Leukocytes are located

in the lamina propria of the mucosa, within the epithelium. The intensity of neutrophilic infiltration within the epithelium is correlated with the extent of mucosa damage and the intensity of *Helicobacter pylori* infection.

In conclusion higher urea levels in blood and gastric juice of chronically hemodialyzed patients do not seem to be a risk factor for *Helicobacter pylori* infection.

References

1. Hammer J, Oesterreicher C, Hammer K, Koch U, Traindl O, Kovarik J. Chronic gastrointestinal symptoms in hemodialysis patients. Wien Klin Wochenschr, 1998; 110: 287-91.
2. Ala-Kaila K, Vaajalahti P, Karvonen AL, Kokki M. Gastric Helicobacter and upper gastrointestinal symptoms in chronic renal failure. Ann Med, 1991; 23: 403-6.
3. Pietrzak-Zakrzewska M, Rydzewska G, Piaścik M, Blusiewicz K, Kosidło S, Rydzewski A. Objawy dyspeptyczne a infekcja Helicobacter pylori u chorych przewlekle hemodializowanych. Nefrol Dial Pol, 2001; 5: 227-31.
4. Bielański W. Epidemiological study on Helicobacter pylori infection and extragastroduodenal disorders in Polish population. J Physiol Pharmacol, 1999; 50: 723-33.
5. Matysiak-Budnik T, Megraud F. Epidemiology of Helicobacter pylori infection with special reference to professional risk. J Physiol Pharmacol, 1997; 48(Suppl. 4): 3-17.
6. Hruby Z, Myszka-Bijak K, Gościniak G, Blaszczuk J, Czyż W, Kowalski P, Falkiewicz K, Szymańska G, Przondo-Mordarska A. Helicobacter pylori in kidney allograft recipients: high prevalence of colonization and low incidence of active inflammatory lesions. Nephron, 1997; 75: 25-9.
7. Shousha S, Arnaout AH, Abbas SH, Parkins RA. Antral Helicobacter pylori in patients with chronic renal failure. J Clin Pathol, 1990; 43: 397-9.
8. Gladziwa U, Haase G, Handt S, Riehl J, Wietholtz H, Dakshinamurty KV, Glockner WM, Sieberth HG. Prevalence of Helicobacter pylori in patients with chronic renal failure. Nephrol Dial Transplant, 1993; 8: 301-6.
9. Giachino G, Sallio-Bruno F, Chiappero F, Saltarelli M, Rosati C, Mazzucco D, Pallante C, Forneris G, Suriani R. L'Helicobacter pylori in pazienti sottoposti ad emodialisi periodica. Minerva Urol Nefrol, 1994; 46: 213-5.
10. Davenport A, Shallcross TM, Crabtree JE, Davison AM, Will EJ, Heatley RV. Prevalence of Helicobacter pylori in patients with end-stage renal failure and renal transplant recipients. Nephron, 1991; 59: 597-601.
11. Kang JY. The gastrointestinal tract in uremia. Dig Dis Sci, 1993; 38: 257-68.
12. Montecucco C, Rappuoli R. Living dangerously: how Helicobacter pylori survives in the human stomach. Nat Rev Mol Cell Biol, 2001; 2: 457-66.
13. Tsuda M, Karita M, Mizote T, Morshed MG, Okita K, Nakazawa T. Essential role of Helicobacter pylori urease in gastric colonization: definite proof using a urease-negative mutant constructed by gene replacement. Eur J Gastroenterol Hepatol, 1994; 6(Suppl 1): S49-52.
14. Muraoka H, Kobayashi I, Hasegawa M, Saika T, Toda H, Nishida M, Suzuki J, Mine T, Fujita T. Urease-negative Helicobacter pylori isolates from gastrointestinal mucosa of patients with peptic ulcer. Kansenshogaku Zasshi, 1997; 71: 1216-20.
15. Murakami M, Yoo JK, Teramura S, Yamamoto K, Saita H, Matuo K, Asada T, Kita T. Generation of ammonia and mucosal lesion formation following hydrolysis of urea by urease in the rat stomach. J Clin Gastroenterol, 1990; 12(Suppl. 1): S104-9.
16. Triebling AT, Korsten MA, Długosz JW, Paronetto F, Lieber CS. Severity of Helicobacter-induced gastric injury correlates with gastric juice ammonia. Dig Dis Sci, 1991; 36: 1089-96.
17. Kim H, Park C, Jang WI, Lee KH, Kwon SO, Robey-Cafferty SS, Ro JY, Lee YB. The gastric juice urea and ammonia levels in patients with Campylobacter pylori. Am J Clin Pathol, 1990; 94: 187-91.
18. Igarashi M, Kitada Y, Yoshiyama H, Takagi A, Miwa T, Koga Y. Ammonia as an accelerator of tumor necrosis factor alpha-induced apoptosis of gastric epithelial cells in Helicobacter pylori infection. Infect Immun, 2001; 69: 816-21.
19. Matsui T, Matsukawa Y, Sakai T, Nakamura K, Aoike A, Kawai K. Ammonia inhibits proliferation and cell cycle progression at S-phase in human gastric cells. Dig Dis Sci, 1997; 42: 1394-9.
20. Sato K, Watanabe S, Yoshizawa T, Hirose M, Murai T, Sato N. Ammonia, hydrogen peroxide, and monochloramine retard gastric epithelial restoration in rabbit cultured cell model. Dig Dis Sci, 1999; 44: 2429-34.
21. Dixon MF, Genta RM, Yardley JH, Correa P. Classification and grading of gastritis. The updated Sydney System. International Workshop on the Histopathology of Gastritis, Houston 1994. Am J Surg Pathol, 1996; 20: 1161-81.
22. Lieber CS, Lefevre A. Ammonia as a source of gastric hypoacidity in patients with uremia. J Clin Invest, 1959; 38: 1271-7.
23. Warren JR, Marshall B. Unidentified curved bacilli on gastric epithelium in active chronic gastritis. Lancet, 1983; 1: 1273-5.
24. Di Giorgio P, Rivellini G, D'Alessio L, Di Benedetto A, De Luca B. The influence of high blood levels of urea on the presence of Campylobacter pylori in the stomach: a clinical study. Ital J Gastroenterol, 1990; 22: 64-5.
25. Neithercut WD, Rowe PA, el Nujumi AM, Dahill S, McColl KE. Effect of Helicobacter pylori infection on intragastric urea and ammonium concentrations in patients with chronic renal failure. J Clin Pathol, 1993; 46: 544-7.
26. Chittajallu RS, Neithercut WD, Macdonald AM, McColl KE. Effect of increasing Helicobacter pylori ammonia production by urea infusion on plasma gastrin concentrations. Gut, 1991; 32: 21-4.
27. Tennenberg SD, Dekhne N, Gordon D, Weller J, McCurdy B, Lange P, Kozol RA. Helicobacter pylori products upregulate neutrophil superoxide anion production. Int J Surg Investig, 1999; 1: 301-6.
28. Hatz RA, Meimarakis G, Bayerdorffer E, Stolte M, Kirchner T, Enders G. Characterization of lymphocytic infiltrates in Helicobacter pylori-associated gastritis. Scand J Gastroenterol, 1996; 31: 222-8.

Laser resection of lung parenchyma – a new technical and clinical approach

Rolle A[1]*, Kozłowski M[2]

[1] Department of Thoracic and Vascular Surgery, Coswig Specialised Hospital, Center for Pneumology and Thoracic Surgery, University Medical Center, Coswig/Dresden, Germany
[2] Department of Thoracic Surgery, Medical University of Białystok, Poland

Abstract

The introduction of a new 1318 nm wavelength Nd: YAG laser has created new possibilities in lung parenchyma surgery. The potentially curative surgical resection of pulmonary metastases in suitably selected cases had been recognized slowly. Using the new laser technology a greater number of patients can now offered salvage surgery. This paper reviews the history of surgical management of pulmonary metastases, development of new laser technology, conventional and extended indications for pulmonary metastasectomy and use of laser in thoracic surgery.

Key words: lung metastasectomy, laser surgery, thoracic surgery.

A long period of time was necessary to establish intended curative resection of metastases to the lung [1]. After chest wall resection for rib sarcoma Weinlechner in 1882 [2] performed the first resection of an isolated metastasis found in the underlying lung. Pulmonary metastasectomy as an independent operation was described in 1926 by Divis [3]. In 1939 Barney and Churchill [4] reported the first successful intentional lobectomy for metastatic kidney cancer, after failure of radiation therapy. Several years later elective surgery of solitary metastasis has been offered to patients selected to long disease free interval to their primaries [4,5]. In 1953 Mannix [6] performed simultaneous resection of multiple synchronous metastases. He removed a total of 6 lesions through lingulectomy and basal segmentectomy. In the beginning with metastasectomies 80% of patients underwent lobectomy or pneumonectomy and mortality was in the range of 10% [7,8]. Due to improvement of surgical techniques and progress to limited resections, a greater number of patients are now candidates for resection.

The effect of the Nd:YAG laser on lung tissue and small pulmonary tumours first was experimentally demonstrated by Minton et al. [9] in 1967. They excised and vaporised metastases implanted to rabbit lungs and successfully resected normal parenchyma of primates. Therefore Minton was the first to recognise this special laser indication that becomes more and more important in lung surgery today. But a lot of technical difficulties prevented the use of lasers in thoracic surgery for nearly 20 years.

In 1985 LoCicero et al. [10] reopend the discussion of clinical application on lung tissue by describing the mechanism of the laser's sealant effect using a CO_2 laser. Laser sealing works by means of a progressive collapse and shrinking of alveols producing a thick, multilayer and air-proof membrane. This result has been tried to achieve either by CO_2 or Neodymium-yttrium-aluminium-garnet (Nd:YAG) laser using low power density and defocussed laser beam. Because of his predominant absorption and low scattering quality in lung tissue the CO_2 laser, however, proved inadequate for lung surgery. Nd:YAG laser beam on the other hand is absorbed and scattered by water, black (anthracotic) structures and hemoglobin pigment thus allowing more coagulating activity up to 4 mm below the surface of the lung. Therefore a number of medical centres in the world and LoCicero as well started experimenting with 1064 nm Nd:YAG lasers using bare fibres and sapphire tips to deliver the radiation to lung parenchyma.

In 1988 Rolle et al. [11] first described the advantages of a new 1318 nm wavelength Nd:YAG laser due to experimental and clinical wedge and segmental resections in 47 patients. The

* CORRESPONDING AUTHOR:
Department of Thoracic and Vascular Surgery
Coswig Specialised Hospital
Center for Pneumology and Thoracic Surgery
University Medical Center
Neucoswiger Straße 21
D-01640 Coswig/Dresden, Germany
Tel: +49 3523 65102; Fax: +49 3523 65103
e-mail: dr.rolle@fachkrankenhaus-coswig.de (Axel Rolle)

Received 26.04.2005 Accepted 27.04.2005

standard 1064 nm Nd:YAG laser was employed parallel for peripheral resection of coin lesions by Moghissi et al. [12]. In 1991 Kodama et al. [13] described resection of lung metastases using 1064 nm Nd:YAG laser in 25 patients. Combination of metastasectomy with lobectomy in 51 patients using this laser was described by Branscheid et al. in 1992 [14]. In the same year Kodama et al. [15] reported of 1064 nm laser segmental resections in combination with bioadhesives in 25 patients with poor lung function and bronchial carcinoma. In 1994 Mineo et al. [16] once more emphasised the importance of laser resection of solitary and multiple metastases. In 1998 they reported that laser metastasectomies demonstrate statistically significant influence on tissue loss, postoperative air leakage and hospital stay and they continued to compare the impact of lobectomy versus minimal and versus laser resections to survival after surgery for lung metastases. The results reported in 2001 indicated that patients after limited resections have better survival than having anatomical resections but this comparison did not reach statistically significance [17,18].

In the majority of peripheral 1064 nm Nd:YAG laser resections bleeding or air leakage can be controlled by defocusing the laser beam, but deeper resections going to the centre of the lobe regularly require supplemental hand suturing or bioadhesives. Therefore Rolle et al. [11] started in 1988 to test the second wavelength of the Nd:YAG laser (1318 nm) in animal experiments based on the knowledge that the 1318 nm wavelength significantly differs from the standard wavelength (1064 nm) by its ten times higher absorption in water. On the other hand the 1318 nm laser offers sufficient scattering of laser light due to its proximity to the beginning near infra-red spectrum to satisfy coagulation requirement as well [19].

After a few tests it became clear that the 1318 nm wavelength provided the combination effect needed – cutting and coagulation qualities which could not be achieved by the 1064 nm wavelength. They also found strong lung tissue shrinkage providing two additional advantages: mechanical reinforcement of the coagulation area and closure of air leaks far into the central region of every lobe [20]. Areas coagulated and sealed by defocused 1318 nm laser irradiation were found to withstand artificial ventilation up to pressures of 25 cm H_2O.

To achieve higher laser power output for this 1318 nm wavelength a complete new laser system has been developed. The average energy efficiency of the Nd:YAG laser was almost doubled from 3% to 5%. The beam quality was improved to such an extend that the laser light of this special wavelength could easily be coupled into fibres of diameters less than 0.6 mm without any heat build up or energy losses [21].

The focusing handpiece featuring a four lens system was developed to allow a near one to one projection of fibre diameter relative to the working focus and enabling power densities up to 24 kw/cm^2. To be complete this laser system includes a high performance smog evacuator, which is needed to eliminate great deal of smog, produced by large-scale parenchyma resection [22].

Pulmonary metastasectomy is one of the main indications of potentially curative and palliative thoracic surgery and has been established now for more than 20 years [23]. Outside of centres of thoracic surgery even today it is widely believed that occurrence of lung metastases marks the final stage of the underlying disease so that only short survival times are expected and chemotherapy represents the only feasible treatment option, if any. However, this view has become obsolete. At the time of potential palliative surgical intervention modern patients are usually well informed about the state of their disease and have clear subjective about the state of things. This allows and requires a partnership approach with transparent patient guidance and care [24].

Conventional indication for resection of pulmonary metastases based on the conditions that the primary tumour should be removed completely (RO resection) and extrapulmonary foci can be ruled out by a preceeding examination of abdomen and skeletal system. In the past only patients with single side and long disease free interval to the primary were accepted for surgical treatment. In 2002 Rolle et al. [25] demonstrated that patients with multiple and synchronous metastases (i.e. metastases that are detected together with the primary tumour) are also eligible for surgical treatment. In 100 consecutive patients they showed that in 95% of all cases parenchyma sparing "precision" resection could be performed. Lobectomy was only necessary in the remaining 5% of patients (against an international lobectomy rate of 20% to 30%) [1], even though 6.3 metastases on average and a maximum of 124 metastases were removed per patient.

Due to a careful and comprehensive palpation of the collapsed lung, 25% more nodules could be resected than were indicated in spiral CT scan examination. Classical resection of pulmonary metastases is limited by surgical technique and functional conditions of the patient. Despite the fact that pulmonary metastases tend to grow locally and therefore can be resected with small margin of safety the number of clamp or stapler wedge resections is limited to three or five for one side. The development of this new 1318 nm laser system enables the thoracic surgeon to resect a much greater number of metastases with minimal loss of parenchyma furthermore to remove metastases located deep inside a lobe in an oncologically adequate manner while preserving the function of the lobe [22,25].

The comparison between wedge resection and laser resection was shown in the *Fig. 1*. One metastasis of 2.5 cm diameter located centrally within the lower lobe. Laser precision resection with 0.5 cm distance to the tumor has been demonstrated to be a secure oncological resection followed by a very low local recurrence rate. The parenchymal lost was calculated: 3 cm x 3 cm x 3 cm = 27 cm^3 with good ventilation and function of the rest lobe. The parenchymal lost for wedge resection with 1 cm stapler line distance to the tumor was calculated: 4.5cm x 5.5 cm x 7.0 cm = 173 cm^3 with no edges left which give problems due to atelectasis. The ratio in parenchymal lost wedge resection to laser resection was 173 cm^3 : 27 cm^3 = 7 : 1. We note a 7 times more parenchymal lost in classical resection of metastasis, which is highly significant.

Fig. 2a, *2b* and *2c* shows five metastases distributed in one lobe (peripheral and central location). In such case lobectomy is unavoidable in conventional resection technique. Laser precision resection is easy to perform by saving the lobe even in a higher number of metastases, due to simultaneous sealing and coagulation. The lung architecture and orientation was reconstructed following each nodular resection by reapproximating

Figure 1. **Sketch of the patient's metastasis. This is located centrally, within the lower lobe, where the lobe is on its thickest. Comparison between wedge resection and laser resection**

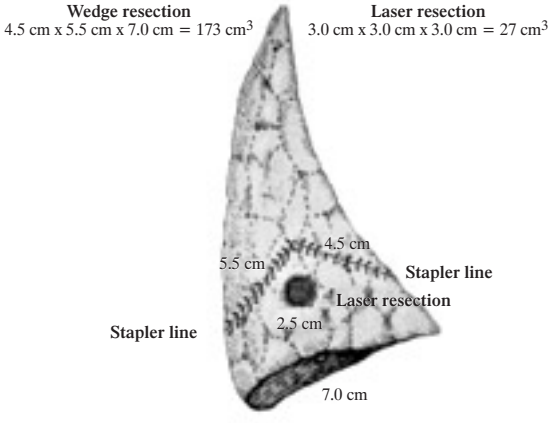


Figure 2a. **Sketch of five metastases distributed in one lobe**

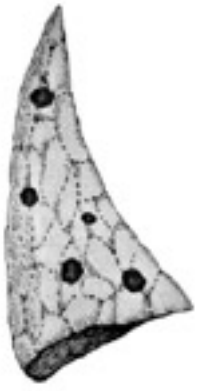


Figure 2b. **Sketch of the laser resection zone after removel of metastases**

Figure 2c. **Sketch of suturing the pleura visceralis**

the visceral pleura with a running absorbable suture. The rest of the lobe remains in function without atelectasis.

On condition that all metastases are complete resected there is no evidence of worse prognosis for patients with bilateral metastases. In cases of multiple and bilateral resections two staged operation via anterolateral, muscle sparing thoracotomy should be planned. The authors suggest to start resection at the side with the higher number or the more centrally located metastases. The other side is operated in the same manner in an average of four weeks later. This two staged approach ensures healing of the lung treated first under physiotherapy so that it can reliably take over the function of the other collapsed lung during the second operation. Overall this treatment significantly reduces the complication rate [26].

Metastases detected more than three years after removal of the primary tumour probably indicate an oncologically retarded disease process with more favourable prognosis. On the other hand metastases that develop within the first 12 months or are even diagnosed together with the primary tumour (so-called synchronous) are said to be followed by shorter survival. Rolle et al. [25] found complete resection to be the most important independent prognostic factor and suggested that patients with synchronous metastases should not be excluded from resection. Due to the parenchyma sparing and lobe saving effect of this laser operations it is possible to perform repeated resections in patients with recurrent metastases. If resection is complete once again there is no evidence of poor outcome after multiple thoracotomies. The indication of pulmonary metastasectomy can be extended to patients with previous complete resection of extrapulmonary metastases (brain, liver) [26].

This new 1318 nm laser system enables the thoracic surgeon to seal persistent air leakage from primary or secondary pneu-

mothorax, to cut adhesions, to resect bullae and perform photothermal pleurectomy. This technique can now be used as well in open thoracic as in videothoracic assisted surgery [26,27].

Compared to traditional resection techniques this laser system offers notable advantages. Blood loss and air leaks are remarkably lower in patients undergoing laser resection of deep located tumours or in patients with underlying severe emphysema. Due to more rapid reexpansion of residual lung parenchyma laser resection allows reduction of chest drainage time and thus leads to lower morbidity rate and shorter hospitalisation time. The use of this laser is the method of choice for the resection of multiple metastases and can successfully be applicated in all kinds of parenchymal resections.

References

1. Pastorino U, Friedel G, Buyse M, Ginsberg RJ, Goldstraw P, Johnston M, McCormack P, Pass H, Puznam JB, Zoomes H. Resection of lung metastases: long-term results and prognostic analysis based on 5206 cases. The International Registry of Lung Metastases. J Thorac Cardiovasc Surg, 1997; 113: 37-49.
2. Weinlechner JW. Tumoren an der Brustwand und deren Behandlung (Resektion der Rippen). Eröffnung der Brusthöhle und partielle Entfernung der Lunge. Wien Med Wschr, 1892; 32: 590.
3. Divis G. Ein Beitrag zur operativen Behandlung der Lungengeschwülste. Acta Chir Scand, 1927; 62: 329-34.
4. Barney JD, Churchill ED. Adenocarcinoma of the kidney with metastases to the lung cured by nephrectomy and lobectomy. J Urol, 1939; 42: 269-76.
5. Alexander J, Haighat C. Pulmonary resection for solitary metastatic sarcomas and carcinomas. Surg Gynec Obstet, 1947; 85: 129-46.
6. Manix EP Jr. Resection of multiple pulmonary metastases fourteen years after amputation for osteochondrogenic sarcoma of the tibia: Apparent freedom from recurrence two years later. J Thorac Surg, 1953; 26: 544-9.
7. Seiler HH, Clagett OT, McDonald JR. Pulmonary resection for metastatic malignant lesions. J Thorac Surg, 1950; 19: 655-79.
8. Gliedman NIL, Horowitz S, Lewis FJ. Lung resection for metastatic cancer: 29 cases from the University of Minesota and a collected review of 264 cases. Surgery, 1957; 42: 521-32.
9. Minton JP, Andrews NC, Jesseph JE. Pulsed laser energy in the management of multiple metastases. J Thorac Cardiovasc Surg, 1967; 54: 707-13.
10. LoCicero J, Hartz RS, Frederiksen JW, Michaelis LL. New applications of the laser in pulmonary surgery. Hemostasis and sealing of air leaks. Ann Thorac Surg, 1985; 40: 546-50.
11. Rolle A, Unsöld E, Ruprecht L, Permanetter W, Frank F. Morphologic aspects of Nd:YAG laser application (wavelengths 1064 nm and 1318 nm) on lung tissue. Laser Med Surg, 1988; 4: 10-4.
12. Moghissi K. Local excision of pulmonary nodular (coin) lesions with noncontact yttrium-aluminium-garnet laser. J Thorac Cardiovasc Surg, 1989; 97: 147-51.
13. Kodama K, Doi O, Higashiyama M, Massayuki T, Iwanaga T. Usefulness of resection with the neodymium: yttrium-aluminium-garnet laser with median stemotomy. J Thoracic Cardiovasc Surg, 1991; 51: 973-8.
14. Branscheid D, Krysa S, Wollkopf G, Bulzebruck H, Probst G, Horn M, Schirren J, Vogt-Moykopf I. Does Nd:YAG laser extend the indications for resection of pulmonary metastases? Eur J Cardiothorac Surg, 1992; 6: 590-6.
15. Kodama K, Doi O, Higashiyama M, Yokoouchi H. Usefulness of Nd: YAG laser for the excision of multiple lung metastases and segmentecatomy for lung cancer. Kyobu Geka, 1992; 45: 51-5.
16. Mineo TC, Cristino B, Ambrogi V, Natali GL, Casciani CU. Usefulness of the Nd:YAG laser in parenchyma-sparing resection of pulmonary nodular lesions. Tumori, 1994; 80: 365-9.
17. Mineo TC, Ambrogi V, Pompeo E, Nofroni L. The value of the Nd:YAG laser for the surgery of lung metastases in a randomized trial. Chest, 1998; 113: 1402-7.
18. Mineo TC, Ambrogi V, Tonini G, Nofroni L. Pulmonary metastasectomy: might the type of resection affect survival? J Surg Oncol, 2001; 76: 47-52.
19. Bayly IG, Kartha VB, Stevens WH. The absorption spectra of liquid phase H20, HDO and D20 from 0.7 micron to 10 micron. Infrared Physics, 1963; 3: 211-23.
20. Rolle A, Thetter O, Haussinger K, Hallfeldt KKJ, Schmölder A. Einsatz des Neodym YAG Lasers in der Thoraxchirurgie. Herz Gefäss Thorax Chir, 1989; 3: 88-91.
21. Stokes LF, Auth DC, Tanaka D, Gray JL, Gulasik C. Biomedical utility of 1320 nm Nd:YAG laser radiation. IEEE Trans Biomed Eng, 1981; 28: 297-9.
22. Rolle A, Eulerich E. Extensive multiple and lobe-sparing pulmonary resections with the ND:YAG laser and a new wavelength of 1318 nm. Acta Chir Hung, 1999; 38: 115-7.
23. Pastorino U. History of the surgical management of pulmonary metastases and development of the international registry. Semin Thorac Cardiovascul Surg, 2002; 14: 18-28.
24. Inderbitzi R, Rolle A. Palliative surgery for primary and secondary thoracic malignancies. Ther Umsch, 2001; 58: 435-41.
25. Rolle A, Koch R, Alpard SK, Zwischenberger JB. Lobe-sparing resection of multiple pulmonary metastases with a new 1318-nm Nd:YAG laser – first 100 patients. Ann Thorac Surg, 2002; 74: 865-9.
26. Rolle A. Laser applications in lung parenchyma surgery. Med Laser Appl, 2003; 18: 1-10.
27. Divisi D, Battagalia C, Di Francescantonio W, Toresini G, Crisci R. Giant bullous emphysema resection by VATS. Analysis of laser and stapler techniques. Eur J Cardiothorac Surg, 2002; 22: 990-4.

Serum endostatin levels in patients with lung carcinoma

Świdzińska E[1], Ossolińska M[1], Naumnik W[1], Trojan S[2], Chyczewska E[1]*

[1] Department of Lung Diseases and Tuberculosis, Medical University of Białystok, Poland
[2] Department of Clinical Molecular Biology, Medical University of Białystok, Poland

Abstract

The purpose of our study was to evaluate the clinical usefulness of serum endostatin levels during chemotherapy of lung cancer in relation to the histopathological type of the tumor, clinical stage and response to therapy

Material and methods: Serum concentrations of endostatin were determined in 37 patiens (24 with non-small cell lung cancer and 13 with small cell lung cancer), 10 healthy subjects constituted controls. To determine endostatin levels (ELISA), venous blood samples were collected from each patient before treatment and after 4-6 courses of chemotherapy.

Results: The serum concentrations of endostatin were found significantly higher in patients in comparison with controls (p=0.003). No statistically significant differences were established between the concentrations of endostatin with regard to such clinical features, as: performance status, clinical stage (III and IV) and histopathological type (non-small cell lung cancer and small cell lung cancer). The concentrations of endostatin did not change after chemotherapy. There was no change of endostatin concentration caused by the response to treatment.

Conclusions: The serum endostatin concentrations were elevated in lung cancer patients.

Key words: endostatin, lung carcinoma.

* CORRESPONDING AUTHOR:
Department of Lung Diseases and Tuberculosis,
Medical University of Białystok
ul. Żurawia 14
15-540 Białystok, Poland
Tel/Fax: +48 85 732 41 49
e-mail: klpluc@amb.edu.pl (Elżbieta Chyczewska)

Received 11.04.2005 Accepted 14.07.2005

Introduction

Malignant tumor growth and metastasis formation depend on the development of new vessels [1]. This process, called neoangiogenesis is regulated, among the others, by the factors derived from the tumor and caused by the shift of balance between stimulating and inhibiting factors, to the formers' advantage [2-4]. Neovascularization results in the progression through providing oxygen and nutrients necessary to the tumor's growth and facilitates the penetration of tumor cells and their transportation to distant organs [2]. Endostatin is one of natural antiangiogenic factors. It was discovered in the medium of the cell line of mice hemangioblastoma in 1997 year. Endostatin is generated due to the enzymatic digestion of collagen XVIII and it inhibits endothelial cell proliferation and induces the 15-30 – fold increase in their apoptosis coefficient. It prevents from VEGF – induced phosphorylation of VEGFR–1 and VEGFR–2 receptors and inhibits VEGF – induced endothelial cell migration. In animal studies, it exhibited an antiangiogenic effect and inhibited the tumor's growth and metastasis [5,6]. Endostatin has also been found in the sera of healthy volunteers. Its increased levels have been reported in various malignant tumors [6-10]. However, a significance and a role of endostatin in patients with lung cancer are still unknown.

The purpose of our study was to evaluate serum endostatin levels in patients with lung cancer (NSCLC and SCLC) treated with chemotherapy, and their relation to response to therapy.

Material and methods

The study was carried out on 37 patients (31 men and 6 women) aged from 45 to 78 (mean age 60 years), diagnosed with lung cancer and treated by chemotherapy (*Tab. 1*). There were 24 patients with diagnosed non-small cell lung cancer (NSCLC) and 13 patients with small cell lung cancer (SCLC).

In the group examined, the clinical stage was assessed according to TNM classification modified in 1997. Among the

Table 1. **Description of patients**

	Clinical stage	n	Performance status according to Zubrod			F	M	Treatment response			
			0	1	2 and more			CR	PR	NC	P
			n	n	n			n	n	n	n
NSCLC	III A	2	2	0	0	0	2	1	1	0	0
	III B	6	3	2	1	0	6	1	5	1	0
	IV	16	4	4	8	2	6	0	1	10	3
SCLC	III	11	3	1	7	4	3	0	5	4	2
	IV	2	0	1	1	0	2	0	0	2	0

NSCLC – non-small cell lung cancer, SCLC – small cell lung cancer, F – females, M – males, CR – complete response, PR – partial response, NC – stabilization, P – progression of the disease

Table 2. **Comparison of serum endostatin concentration (ng/ml) in patients and controls**

	Patients (n=37)			Controls (n=10)			
	Q_1	Me	Q_3	Q_1	Me	Q_3	p-value*
Endostatin	113.5	147.1	185.1	75.6	102.8	118.1	0.003 #

p-value for Wilcoxon's test; # – statistically significant differences at the significance level of 0.05; Me – median, Q1, Q3 – quartile 1 and 3

examined, there were 19 stage III patients (2 patients – stage III a and 17 – stage III b) the rest of patients – 18 individuals had stage IV. In case of SCLC, stage III corresponded to the limited stage of the disease and stage IV – to the extensive disease (*Tab. 1*). Additionally, performance status of patients was determined according to Zubord's scale. The total of 12 patients had the performance status classified as – 0; 8 patients – 1; and 17 patients – 2 or 3. All patients analysed in the study were treated with chemotherapy. In case of SCLC, they were given 4 – 6 courses of cisplatin + etoposid, and in case of NSCLC, they received 4 – 6 courses of cisplatin + vinorelbin or cisplatin + gemcytabin. In the whole group of patients, 2 patients (with NSCLC) had complete remission, 12 (7 with NSCLC and 5 with SCLC) – partial remission, 17 (11 with NSCLC and 6 with SCLC) – stable disease and 5 (3 with NSCLC and 2 with SCLC) – progressive disease.

The control group included 10 healthy volunteers (3 women and 7 men; mean age 58 years). Blood samples were collected before begining and four weeks after the treatment. Peripheral blood serum was examined. Blood samples of 5 ml each were drawn on clot on empty stomach. After blood clotting and centrifugation at the rotation of 2000 rpm for 10 minutes, the serum was separated and frozen at the temperature of -70°C to determine endostatin.

Endostatin levels were measured in the serum by the use of immunoenzymatic method ELISA with the Quantikine kits of R&D Systems (Minneapolis, USA) according to the producer's instruction. The reader of microplatelets ELx800 of BIOTEK Instruments Inc. was used in our examinations.

Statistical analysis

The correlations between the histopathological tumor type, performance status, clinical stage, tumor size and the concentrations of endostatin were analysed before treatment using non-parametric tests of Wilcoxon and Kruskal-Wallis. The differences between the pairs of groups were examined by means of Dunn's test for multiple comparisons. The hypothesis of normal distribution in particular groups was rejected (Shapiro-Wilk test).

New parameters of x_endostatin were calculated to check the correlation between the response and the therapy:

$$X\_\,\text{endostatin} = \frac{(\text{endostatin after treatment} - \text{endostatin before treatment})}{\text{endostatin before treatment}}$$

It represents relative changes of endostatin values.

The response influence on x_ endostatin was analysed using a variation analysis test with a single classification. The assumptions of variation analysis were checked by means of Shapiro-Wilk's and Bartlett's tests. The differences between the pairs of groups were examined using Bonferroni's test for multiple comparisons. Most of the tests performed were bilateral. Unilateral tests were notified in the description. The significance level was 0.05. The analysis was performed basing on the kit of SAS STAT Release 8.2.

Results

Endostatin was determined in the serum of patients and controls. Endostatin concentration differed significantly between patients and controls (*Tab. 2*). Additionally, the serum concentrations of endostatin obtained in patients were significantly higher (unilateral test) than in controls (p=0.0014).

No statistically significant differences were found between the concentrations of endostatin, and the clinical parameters, such as performance status, clinical stage and histopathologic form defined as small cell and non-small cell lung cancer (*Tab. 3*).

Endostatin concentrations measured after treatment showed no statistically significant changes in comparison with the preliminary levels and did not depend on the response obtained, and remained higher in patients than in controls.

Discussion

Lung cancer still remains the leading cause of mortality in cancer patients in the world. A high percentage of relapses after surgical resection is due to the presence of micrometastases at diagnosis. This high metastatic potential of lung cancer may be enhanced by intensive angiogenesis within the tumor.

Table 3. **Serum endostatin concentration with regard to histopathological pattern and performance status and clinical staging**

	n	Q_1	Me	Q_3	p-value
NSCLC	24	121	146	187	0.75[a]
SCLC	13	114	147	18	
Performance status					0.13[b]
0	12	85	111	166	
1	8	137	149	195	
2 and more	17	133	149	191	
Clinical stage					0.79[a]
III	19	102	147	185	
IV	18	122	145	190	

NSCLC – non-small cell lung cancer; SCLC – small cell lung cancer; a – p-value for Wilcoxon's test; b – p-value for Kruskal-Wallis's test; Me – median, Q1,Q3 – quartile 1 and 3

Figure 1. **Endostatin concentration (ng/ml) in patients and controls**

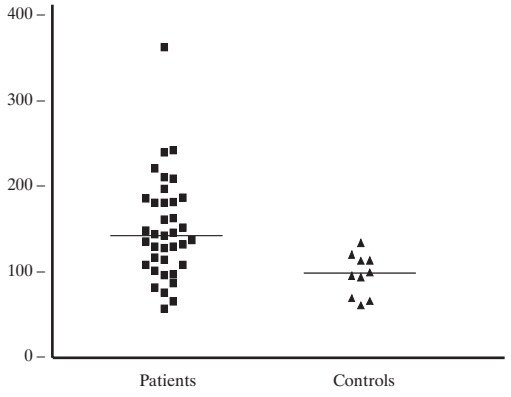


Table 4. **Effect of after – treatment response on relative changes of endostatin concentrations in serum of lung cancer patients**

	Complete and partial remission (n=14) mean +/- of standard deviation	Disease stabilization (n=17) mean +/- of standard deviation	Disease progression (n=5) mean +/- of standard deviation	p-value*
x_endostatin	0.27±0.96	0.21±0.5	0.2±0.72	0.98

* – p – value for the test of variation analysis

In the present study, the concentration of endostatin – a natural angiogenesis inhibitor – was determined in the serum of patients with lung cancer. The concentration of endostatin was significantly higher in patients with lung cancer in comparison with controls. So far, only two studies [8,9] evaluating serum endostatin concentrations in patients with NSCLC, have been published. In these studies the serum endostatin levels were also significantly higher in patients than in healthy controls.

Our study showed no differences of endostatin concentrations between patients with NSCLC and SCLC. To our knowledge, the current study is the first to report serum endostatin levels in SCLC patients.

In our study there were no differences in relation to clinical stage (III vs IV) of lung cancer. Conversely, M. Suzuki et al. [8] found significantly higher endostatin concentrations in patients with the clinical stage higher than Ib disease and in patients with their tumor classified as more than T2 compared to other patients. There were no patients in our study in I and II clinical stage. In our opinion, that is the reason for differences, in the results.

No differences of this inhibitor concentrations were found before and after treatment in the present study. There was no change of endostatin concentration as a response to treatment. After treatment it remained higher when compared to controls. To our knowledge, there were no other published studies evaluating serum endostatin levels in lung cancer patients after chemotherapy.

In a few studies, serum endostatin concentrations were determined in various malignant tumors, such as non-Hodgkin lymphoma, carcinoma of the bladder, acute leukemia, colorectal cancer, soft tissue sarcoma, and they were found higher in the serum of patients than controls [10-14]. In one study, a high concentration of endostatin correlated with the extent stage of disease (10), in another, a high concentration of endostatin was associated with poor prognosis [8,10,14-16]. In most studies, regardless of surgery treatment or chemotherapy, endostatin concentrations remained enhanced in patients with malignant tumors in comparison with controls [12,15]. However, Dhar et al. [17] proved a significantly lower concentration of endostatin in the serum of patients after radical resection of the liver cancer. In Miyashita's et al. [18] and Homer's et al. [19] studies, no differences of endostatin concentrations were found between patients with cancer and healthy volunteers.

These results indicated that elevated levels of endostatin were present in the serum of lung cancer patients, however, the reason for this remains unknown. Further studies are necessary to clarify the source of endostatin production, site of interaction, and mechanism of the activity.

Basing on the results of our study, it may be concluded that the serum endostatin concentrations were elevated in lung cancer patients.

References

1. Folkman J. Clinical applications of research on angiogenesis. N Engl J Med, 1995; 333: 1757-63.
2. Cox G. Angiogenesis and non-small cell lung cancer. Lung Cancer, 2000; 27: 81-100.
3. Denekamp J. The tumor microcirculation as a target in cancer therapy: a clearer perspective. Eur J Clin Invest, 1999; 29: 733-6.
4. Ferrara N. Role of vascular endothelial growth factor in the regulation of angiogenesis. Kidney Int, 1999; 56: 794-814.
5. O'Reilly MS, Boehm T, Shing Y, Fukai N, Vasios G, Lane WS, Flynn E, Birkhead JR, Olsen BR, Folkman J. Endostatin: an endogenous inhibitor of angiogenesis and tumor growth. Cell, 1997; 88: 277-85.
6. Sim BKL, MacDonald NJ, Gubish ER. Angiostatin and Endostatin: Endogenous inhibitors of tumor growth. Cancer Metastasis Rev, 2000; 19: 181-90.

7. Shepherd FA, Sridhar SS. Angiogenesis inhibitors under study for the treatment of lung cancer. Lung Cancer, 2003; 41: 563-72.

8. Suzuki M, Izasa T, Ko E, Baba M, Saitoh Y, Shibuya K, Sekine Y, Yoshida S, Hiroshima K, Fujisawa T. Serum endostatin correlates with progression and prognosis of non-small cell lung cancer. Lung Cancer, 2002; 35: 29-34.

9. Iizasa T, Chang H, Suzuki M, Otsuji M, Yokoi S, Chiyo M, Motohashi S, Yasufuku K, Sekine Y, Iyoda A, Shibuya K, Hiroshima K, Fujisawa T. Overexpression of collagen XVIII is associated with poor outcome and elevated levels of circulating serum endostatin in non-small cell lung cancer. Clin Cancer Res, 2004; 10: 5361-6.

10. Bono P, Teerenhovi L, Joensuu H. Elevated serum endostatin is associated with poor outcome in patients with non-Hodgkin lymphoma. Cancer, 2003; 97: 2767-75.

11. Guan KP, Ye HY, Yan Z, Wang Y, Hou SK. Serum levels of endostatin and matrix metalloproteinase-9 associated with high stage and grade primary transitional cell carcinoma of the bladder. Urology, 2003; 61: 719-23.

12. Glenjen N, Mosevoll KA, Bruserud O. Serum levels of angiogenin, basic fibroblast growth factor and endostatin in patients receiving intensive chemotherapy for acute myelogenous leukemia. Int J Cancer, 2002; 101: 86-94.

13. Feldman AL, Alexander HR Jr, Bartlet DL, Kranda KC, Miller MS, Costouros NG, Choyke PL, Libutti SK. A prospective analysis of plasma endostatin levels in colorectal cancer patients with liver metastases. Ann Surg Oncol, 2001; 1: 741-5.

14. Feldman AL, Pak H, Yang JC, Alexander HR Jr, Libutti SK. Serum endostatin levels are elevated in patients with soft tissue sarcoma. Cancer, 2001; 15: 1525-9.

15. Feldman AL, Alexander HR Jr, Yang JC, Linehan WM, Eyler RA, Miller MS, Steinberg SM, Libutti SK. Prospective analysis of circulating endostatin levels in patients with renal cell carcinoma. Cancer, 2002; 95: 1637-43.

16. Hata K, Dhar DK, Kanasaki H, Nakayama K, Fujiwaki R, Katabuchi H, Okamura H, Nagasue N, Miyazaki K. Serum endostatin levels in patients with epithelial ovarin cancer. Anticancer Res, 2003; 23: 1907-12.

17. Dhar DK, Ono T, Yamanoi A, Soda Y, Yamagouchi E, Rahman MA, Kohno H, Nagasue N. Serum endostatin predicts tumor vascularity in hepatocellular carcinoma. Cancer, 2002; 95: 2188-95.

18. Miyashita M, Tajiri T, Yanagi K, Shimizu T, Futami R, Sasajima K. Serum levels of vascular endothelial growth factor, basic fibroblast growth factor and endostatin in human metastatic liver tumors. Hepatogastroenterology, 2003; 50: 308-9.

19. Homer JJ, Greenman J, Stafford ND. Circulating angiogenic cytokines as tumour markers and prognostic factor in head and neck squamous cell carcinoma. Clin Otolaryngol, 2002; 27: 32-7.

An assessment of telemedicine possibilities in massive casualties situations

Paunksnis A[1,3]*, Barzdžiukas V[1,3], Kurapkienė S[1,3,4], Vaičaitienė R[2], Sereika V[2]

[1] Telemedicine Center of Kaunas University of Medicine, Kaunas, Lithuania
[2] Military Medical Service of Lithuanian Armed Forces, Lithuania
[3] Department of Ophthalmology of Institute for Biomedical Research of Kaunas University of Medicine, Kaunas, Lithuania
[4] Kaunas University of Technology, Kaunas, Lithuania

Abstract

The use of existing possibilities of Telemedicine Center of Kaunas University of Medicine allows the live distant consultations from high-level medical specialised centers to rural areas. On July 2004 the Telemedicine Center took part in the RESCUER/MEDCEUR project exercise. A special objective was the use of telemedicine facilities for distant consultations and sorting of victims directly at the event place. Telemedicine Center used appropriate telecommunication devices for joint activities of civil and multinational military services in critical situations such as mass casualty events. There were used ISDN lines and IP radio-connection.

On the final and most intensive day of the anti-terrorism drills, the multinational force of medics at the Kairiu Training Range in Lithuania reacted to a large mass casualty event – treating hundreds of victims from a simulated train crash. Using on-line telemedicine facilities from Kaunas Medical University Hospital there were corrected the tactics of giving the first help and sorting of casualties. The most complicated initiated cases of eye trauma, neurosurgical trauma, maxilloface trauma and traumatic amputation of limbs evaluated and selected for emergent evacuation to the third level hospitals. All those cases transported to Kaunas and Vilnius Universities Hospitals by helicopters (200 and 300 km from the event place).

The common use of existing military and civil telemedicine infrastructure showed the possibilities of interaction in management, giving the first help and sorting of casualties between military and civil medical services during the rescue operations.

* CORRESPONDING AUTHOR:
Telemedicine Center of Kaunas University of Medicine
Eiveniu 4, LT-50009 Kaunas, Lithuania
Tel: +370 37 338049; Fax: +370 37 327095
e-mail: apaun@medi.lt (Alvydas Paunksnis)
http://tmc.kmu.lt

Received 25.05.2005 Accepted 30.05.2005

Key words: telemedicine, civil and military medicine, teleconsultations, medical information.

Introduction

The use of existing possibilities of Telemedicine Center of Kaunas University of Medicine (http://tmc.kmu.lt) allows the live (on-line) distant consultations from high-level medical specialised centers to rural areas [1-3]. In order to expand the use of distant consultations facilities is essential to apply its possibilities in large mass casualty events, decreasing geographical isolation of the event place.

On 23 and 28 of July 2004 the Telemedicine Center took part in the RESCUER/MEDCEUR project exercise. MEDCEUR – Medical Central Europe Exercises. RESCUER/MEDCEUR 2004 is a USAREUR led "In the Spirit of Partnership for Peace" (ISO PfP) exercise designed to train US, NATO and Partner nations, to respond to a disaster relief/mass casualty situation. 393 participants from 16 countries, namely Lithuania, Armenia, Azerbaijan, Bulgaria, Estonia, Georgia, United States of America, Croatia, Latvia, Moldova, Romania and Ukraine took part in the RESCUER/MEDCEUR 2004 exercises, alongside the 6 observers from the Netherlands, Poland, Luxembourg, and Germany [4].

The main objectives of exercises were:

- To train medical subdivisions with interaction between international medical forces;
- Solution of massive casualties events;
- The actions of the first reaction group;
- To assess collaboration between military medical service and civil health care services;
- A special project (telemedicine) was the use of telemedicine facilities for distant consultations, management of interaction between military and civil medical services and sorting of casualties directly at the event place.

Materials and methods

The main scenario of exercises was:

- The terrorists groups trying to complicate the joining NATO of Estonia, Latvia and Lithuania provoced fires in the woods using high explosives.
- Demonstrating help and solidarity USEUCOM together with NATO and ISO PfP countries dislocated special military groups for the fight againt the fires and for the humanitarian aid.

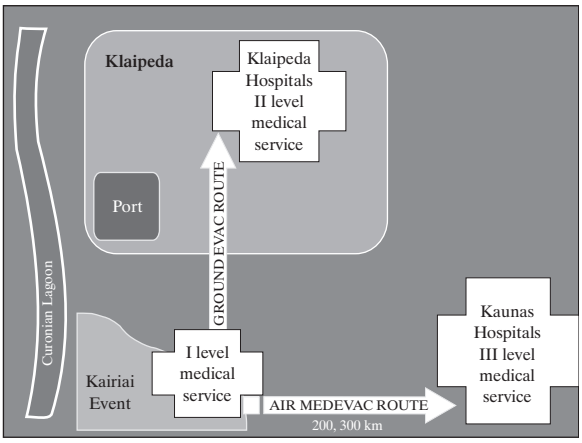
Exercises held at the Kairiu training range in Lithuania near Klaipeda (*Fig. 1*). Participants involved from medical side were:

- Emergency Center of Klaipeda city;
- Hospital of Klaipeda city;
- Klaipeda District Hospital;
- Klaipeda Marine Hospital;
- Emergency Hospital of Vilnius University;
- Kaunas Medical University Hospital;
- Telemedicine Center of Kaunas University of Medicine.

On the place of event the Telemedicine Center arranged live, direct high-level medical multispecialists teleconsultations from Kaunas Medical University Hospital. Military and civil networks were involved in this project (*Fig. 2*).

Telemedicine Center used appropriate telecommunication devices (Satellite, ISDN, IP) for joint activities of civil and multinational military services in critical situations such as mass casualty events. There were used ISDN lines and IP radio-connection [5].

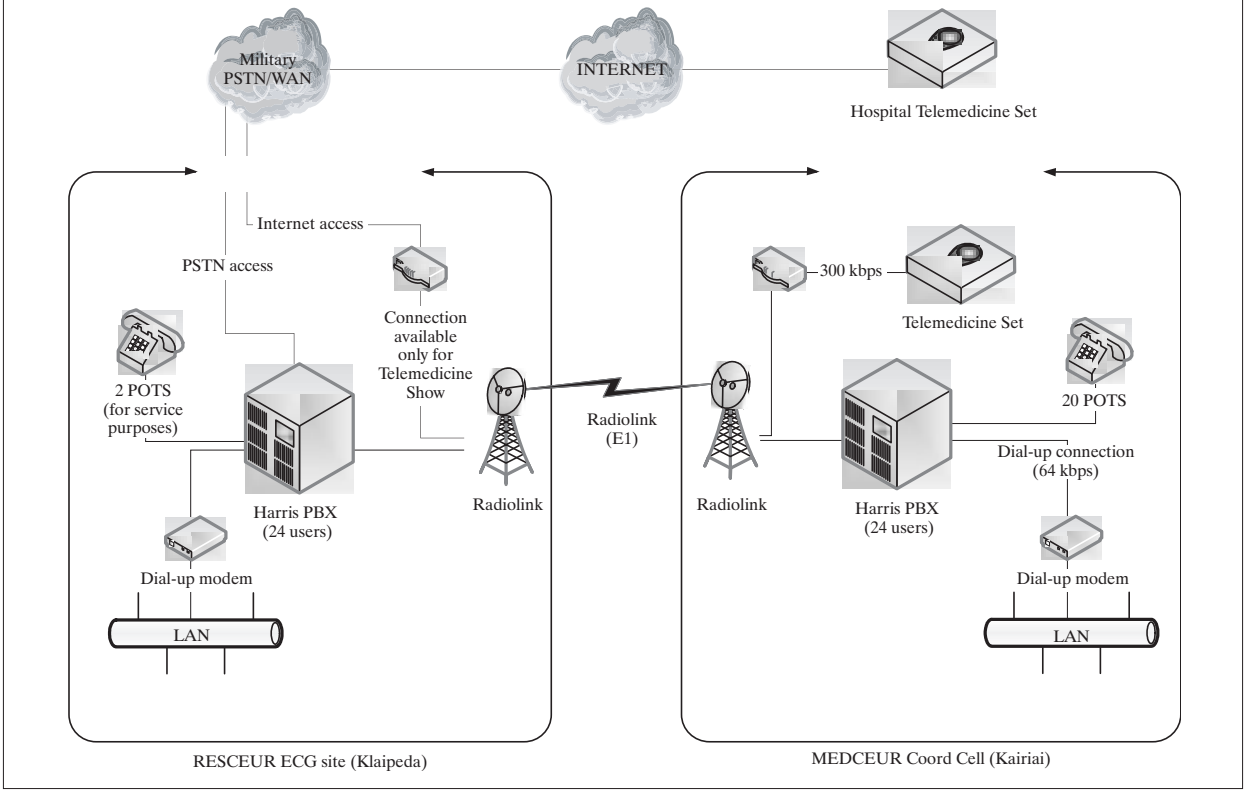
Figure 1. **Dislocation of involved medical services**



Results

On 28 of July, the final and most intensive day of the anti-terrorism drills, the multinational force of medics at the Kairiu Training Range in Lithuania reacted to a large mass casualty event – treating hundreds of victims from a simulated train crash. Using on-line telemedicine facilities from Kaunas Medical University Hospital there were corrected the tactics of giving the first help and sorting of casualties. The most complicated initiated cases of eye trauma, neurosurgical trauma, maxillo-

Figure 2. **Radio link connectivity**



face trauma and traumatic amputation of limbs evaluated and selected for emergent evacuation to the third level hospitals. All those cases transported to Kaunas and Vilnius Universities Hospitals by helicopters (200 and 300 km from the event place).

Conclusions

The common use of existing military and civil telemedicine infrastructure showed the possibilities of interaction in management, giving the first help and sorting of casualties between military and civil medical services during the rescue operations.

These results show the facilities of existing telemedicine infrastructure and needs for further development of existing system into International Integrated eHealth Network for very fast international exchange of medical information, remote consultations of high skilled specialists in emergent or a large mass casualty events from the best civil and military medical centers.

References

1. Paunksnis A, Barzdžiukas V, Kurapkienė S, Severgardh P. Trends of telemedicine development in Lithuania. Abstracts in International eHealth exhibition and conference "Med-e-Tel", Luxembourg, 21-23 April 2004: 71-2.

2. Paunksnis A. Integrated ehealth network in Lithuania for clinical practise, education and research. Ministerial Conference and Exhibition eHealth "The contribution of ICT to Health", 22-23 May, 2003, Brussels, 10-1.

3. Barzdžiukas V, Paunksnis A, Lukoševičius M, Kurapkienė S, Almquist L-O, Severgardh P. Teleophthalmology for second opinion and clinical decision support. Proceedings in the 2nd international conference "ehealth in Common Europe", 11-12 March, 2003, Krakow (Poland), p. 61.

4. Kurapkienė S, Paunksnis A, Barzdžiukas V, Vaičaitienė R, Sereika V. An assessment of telemedicine possibilities in massive casualties situations. Euroregional Conference on Building Information Society in the Healthcare in Euroregion Niemen, Book of abstracts. 17-19 Feb 2005, p. 62.

5. Marozas V, Jurkonis R, Kazla A, Lukoševičius M, Lukosevič ius A, Gelžinis A, Jegelevičius D. Development of Teleconsulatations Systems for eHealth. In a book "Transformation of Healthcare with Information Technologies" (editors M. Duplaga, K.Zielinsky, D.Ingram), series Studies in Health Technology, IOS Press. 2004, Vol. 105: 337-48.

Soluble form of TRAIL, Fas and FasL in the serum of patients with B-CLL

Jabłońska E[1]*, Kiersnowska-Rogowska B[2], Rogowski F[3], Parfieńczyk A[3], Pużewska W[1], Bukin M[1]

[1] Department of Immunology, Medical University of Bialystok, Poland
[2] Department of Hematology, Medical University of Bialystok, Poland
[3] Department of Nuclear Medicine, Medical University of Bialystok, Poland

Abstract

Purpose: Although many studies demonstrated expression of TNF family members in the course of B-CLL, there is a little known about relationships between soluble forms of these proteins. Furthermore, there is no study reported on effects of used therapy on this relation. The present study was designed to asses the relationships between the serum concentrations of sFas, sFasL and sTRAIL in patients with B-CLL regarding their correlation with clinical stage and used therapy.

Material and methods: We studied 40 patients with B-cell chronic lymphocytic leukemia (B-CLL) at diagnosis, before treatment and four weeks after therapy. To measure sFas, sFasL and sTRAIL levels in serum commercially available ELISA kits were used.

Results: We found increased concentrations of sFas in sera of all patients with B-CLL before treatment in comparison to the control group. There were no significant differences in concentrations of sFasL and sTRAIL between patients and control group. Increased sFasL concentrations after FC and CC therapy as well as decreased concentrations after 2CdA therapy in comparison to values before treatment were found. The concentrations of sTRAIL after FC and CC therapy were higher than those in patients before treatment.

Conclusions: Results obtained suggest that relationship between sFas, sFasL and sTRAIL in sera of patients with B--CLL before treatment may facilitate the growth B leukemic cells. Changes in these relations after therapy with FC and CC can make a contribution to inhibit B cells growth on the apoptosis way in this patient group.

Key words: B-cell chronic lymphocytic leukemia, sFas, sFasL, soluble TNF-related apoptosis-inducing ligand.

Introduction

TNF superfamily proteins such as TNF-related apoptosis-inducing ligand (TRAIL)/TRAIL-R and Fas ligand (FasL)/Fas play a role in cancer development of the lymphoid system [1]. B-cell chronic lymphocytic leukemia (B-CLL) is a clinically heterogenous disease characterized by the accumulation of a clonal population of B lymphocytes. This accumulation is considered to result from the prolonged survival of B-CLL cells arrested in the G_0 stage of the cell cycle and by resistance toward apoptosis-inducing agents [2,3]. TRAIL and FasL cooperate in limiting lymphocyte proliferation following activation and use common pathways leading to apoptotic cell death [1,4].

TRAIL is present as a type II membrane protein and a soluble protein in culture supernatants and interacts with five distinct receptors: TRAIL-R1 (DR4), TRAIL-R2 (DR5), TRAIL-R3 (DcR1), TRAIL-R4 (DcR2) and osteoprotegerin (OPG) [5,6]. The ability to transmit apoptosis is restricted to TRAIL-R1 and TRAIL-R2 which contain "death domain" responsible for transducing the death signal [5-7]. The other receptors, TRAIL-R3, TRAIL-R4 and OPG, lack a functional death domain, are decoy receptors unable to transduce a death signal [5,8]. These receptors compete with TRAIL-R1 and TRAIL-R2 for TRAIL binding [7]. It has been observed that B cells from B-CLL expressed levels of the TRAIL-R1 and TRAIL-R2 [9,10]. Despite TRAIL death receptor expression B-CLL cells were relatively resistant to induction of apoptosis by rhTRAIL [10].

Soluble TRAIL is generated by deletion of the transmem-

* CORRESPONDING AUTHOR:
Department of Immunology
Medical University of Białystok
ul. Waszyngtona 15A
15-274 Białystok, Poland
e-mail: ewaj@amb.edu.pl (Ewa Jabłońska)

Received 31.03.2005 Accepted 07.06.2005

***Table1*. Serum levels of TNF molecules in patients with B-CLL before treatment**

	Control	Patients before treatment			
		Stage I n=8	Stage II n=4	Stage III n=7	Stage IV n=5
sFas pg/ml	179±84.36	293.6*±129	297.5*±42.7	345*±197	378*±168
sFasL ng/ml	60±18.0	87.7±44.79	75±37.4	46.6±25.3	44.7±21.7
sTRAIL pg/ml	79.6±25.20	55.6±28.65	54.5±15.2	44.1±21.8	45.6±33.19

* – statistical difference with control ($p<0.05$)

brane and intracellular domains of mTRAIL by immune cells including lymphocytes, NK cells, monocytes, dendritic cells, macrophages and recently by neutrophils [6,8,11].

Although TRAIL signaling appears similar to Fas signaling, TRAIL induces apoptosis more efficiently than FasL. In contrast to TRAIL expressed on many tissues, the expression of FasL is restricted to activated lymphocytes and sites of immune privilege [12]. FasL (APO-1, CD95L) is a type II integral membrane protein and Fas (APO-1, CD95) is a type I membrane protein [1].

FasL and Fas expression were found on the surface of neoplastic plasma cells [13,14]. Binding FasL to Fas induces apoptosis of the Fas positive cells [1,13,14]. Both Fas and FasL exist as a soluble forms. Soluble Fas is generated by differential splicing via deletion of an exon encoding the transmembrane domain of Fas. In contrast, sFasL, a 26-kDa glycoprotein, is generated by proteolytic cleavage of the membrane bound form [13,15].

Although many studies demonstrated expression of TNF family members in the course of B-CLL, there is a little known about relationships between soluble forms of these proteins that may influence the B cells growth [13-16]. Furthermore, there is no study reported on effects of used therapy on the concentrations of these proteins.

The present study was designed to asses the relationships between the serum concentrations of sFas, as inhibitor of apoptosis, and sFasL and sTRAIL, as inducers of apoptosis, in patients with B-CLL regarding their correlation with clinical stage and used therapy.

Material and methods

Patient group

We studied 40 patients with B-cell chronic lymphocytic leukemia (B-CLL) at diagnosis, before treatment and four weeks after therapy. The average of these patients was 62.1 years, and the male to female ratio was 22/18. The diagnosis of leukemia was established on the basis of clinical observation, morphological composition of peripheral blood, marrow puncture and cytochemical tests, such as the reaction of alpha-naphtyl and black Sudan-B for peroxidases (POX), PAS and esterases. Leukemic B-CLL, according to generally accepted criteria was confirmed by flow cytometry immunophenotypic analysis of CD5, CD19, CD23, CD3, CD4 and CD8 using a EPIX XL analyzer (Coulter, USA). Patients were graded according to Rais' staging system as follows: stage I [8], stage II [10], stage III [14], stage IV [8]. Patients with active infection or allergic reaction were excluded from the study.

All patients with clinical stage III and IV disease were eligible for treatment. The treatment course consisted of 2CdA (Biodrybin) given at dose of 0.12 mg/kg/d in a 2-h intravenous infusion (i.v.) for 5 days; 2CdA 0.12 mg/kg/d in a 2-h i.v. for 3 days and mitoxantrone 10 mg/m^2 on day 1st and cyclophosphamide 650 mg/m^2 i.v. on day 1st (CMC); cyclophosphamide 650 mg/m^2 i.v. on 1st day (CC), fludarabine 25 mg/m2 i.v. for 3rd day and cyclophosphamide 250 mg/m^2 i.v. for 3rd day (FC).

The study was approved by the Local Ethical Committee and all patients gave written informed consent.

Control group

Control subjects (n=15) were normal healthy volunteers of the same age (workers and students of the Medical University of Bialystok).

Peripheral venous blood was drawn from into pyrogen-free blood collection tubes without additives. The serum was collected after centrifugation at 2500 rpm for 10 min and then was stored at -70°C until analyzed.

To measure sFas, sFasL and sTRAIL levels in serum commercially available ELISA kits were used (R&D Systems, Minneapolis, USA), according to the manufacturer's instructions. Recombinant sFas, sFasL and sTRAIL were used as respective standards.

The results are expressed as mean ± standard deviation. Data was analyzed according to the nonparametric U Mann-Whitney test. Correlations were calculated using the Pearson's test. A p value less than 0.05 was considered statistically significant.

Results

In sera of all patients with B-CLL before treatment we found increased concentrations of sFas in comparison to the control group of healthy person ($p<0.05$) (*Tab. 1*). There were no significant differences in concentrations of sFasL and sTRAIL between sera of these patients and the control group (*Tab. 1*). The concentrations of all parameters were analyzed according to Rais' stages. However, there was no significant differences between patients in different stages (*Tab. 1*)

To answer the question of which kind of therapy used in patients with B-CLL leads to specific changes in concentrations

Table 2. **The mean concentrations of parameters examined in the serum of patients with B-CLL before and after treatment**

	Patients with B-CLL			
	Before treatment	After treatment		
	$\bar{x}$±SD		*n*	$\bar{x}$±SD
sFas pg/ml	328.2±136.8	2CdA	13	368±15.3
		CMC	10	309±10.4
		FC	9	335.0±39.5
		CC	8	325±78.2
sFasL ng/ml	63±31	2CdA	13	37.6[a]±1.42
		CMC	10	58.3±1.60
		FC	9	87.5[a]±1.31
		CC	8	126[a]±0.84
sTRAIL pg/ml	49.5±16.1	2CdA	13	46.8±9.42
		CMC	10	60±8.65
		FC	9	84.7[a]±6.40
		CC	8	81[a]±8.91

[a] – statistical difference between patients before and after treatment ($p<0.05$)

of mediators examined their values we analyzed in detail due to 2CdA, CMC, FC or CC therapy.

We didn't find significant differences between concentrations of sFas in sera of patients after 2CdA, CMC, FC and CC therapy in comparison to the values obtained before treatment (*Tab. 2*). In contrast, the sera concentrations of sFasL in patients after used therapy were changed. Patients treated with FC and CC showed an increase in sFasL concentrations as compared to examinations before treatment ($p<0.05$) (*Tab. 2*). We also found that concentrations of sFasL in patients after 2CdA therapy were lower in comparison to examinations before treatment ($p<0.05$) (*Tab. 2*). In contrast to 2CdA therapy, CMC treatment didn't significantly influence on the sFasL concentrations in patients with B-CLL. In the present study we also observed absence of changes in sTRAIL concentrations in sera of patients after 2CdA and CMC treatment. In contrast, FC and CC therapy led to increased sTRAIL concentrations in patients with B-CLL (*Tab. 2*).

In this study we analyzed relationship between the concentrations of parameters examined in sera of patients before and after treatment. However, there were no correlation between these molecules.

Discussion

Results of the present study revealed an unfavorable relations between the soluble form of TNF superfamily molecules in sera of patients with B-CLL before treatment. Increased sFas and simultaneous decreased sFasL and sTRAIL concentrations can make a contribution to B leukemic cells growth and progression of B-CLL.

High concentrations of sFas, observed in the present study, can block death of B leukemic cells by inhibiting the interaction between Fas and FasL and can help B-CLL cells to escape from induction of apoptosis by FasL positive cells, such as autologous T cells [13,15].

Decreased concentrations of sFasL and sTRAIL as inducers of apoptosis may also be responsible for progression of B-CLL. Membrane – bound (mTRAILl) and soluble TRAIL (sTRAIL) were shown to rapidly induce apoptosis in susceptible tumor cells upon trimerization of its receptor and subsequent activation of the caspase cascade, leading to fragmentation of DNA [8].

It has been demonstrated that FasL and TRAIL synergistically induce apoptosis of chronic lymphocytic leukemia B cells [16]. Dicker et al. indicated that two or more death receptors are more effectively than one of these. This is similar to the interactions to achieve optimal immune co-stimulation during the immune response to antigen. Similarly, a requirement for co-ligation of multiple death receptors via FasL and TRAIL, could be an important mechanism for enhancing the specificity of killing by immune mechanisms [16]. Since sFasL and sTRAIL are generated by proteolytic cleavage of the membrane bound FasL and TRAIL, decreased sFasL and sTRAIL in the sera of these patients may be associated with high expression of their membrane forms. In fact, Tinhofer et al. demonstrated that the CD19+ B cells fraction from PBMC B-CLL patients were found to significantly express FasL [14].

Changes in sFasL and sTRAIL concentrations, besides a direct effect on B leukemic cells growth, may have different implications for the immune response in patient with B-CLL. For example, it has been demonstrated that Fas/FasL and TRAIL-R/TRAIL regulate myelopoiesis and dendritic cell functions [1]. Furthermore, sFasL has been shown to induce activation of NF-kB and its low concentration can be responsible for impaired synthesis of different molecules [6].

Elevated concentrations of sFas according to progression disease appear to confirm its role in pathogenesis of B-CLL and to indicate that sFas in patients' sera is, at least partly, derived from B leukemic cells. This observation is in agreement with data presented by Osorio et al. who found sFas in supernatants from *in vitro* cultured B-CLL cells. They also demonstrated a correlation between sFas serum levels and clinical progression [13].

The key question of this study is whether therapy used may change the relations between sFas, sFasL and sTRAIL in sera of patients with B-CLL. Results obtained revealed that any kind of therapy didn't significantly influence on the sFas concentrations in sera of patients. In contrast, increased sFasL and sTRAIL concentrations in sera of patients after FC and CC therapy were observed. Additionally, in sera of patients treated with CMC increased concentrations of sTRAIL without effect on sFasL levels were observed. Absence of changes in high concentrations of sFas after FC and CC therapy appear to be balanced by simultaneous increased concentrations of sFasL and sTRAIL. Alterations above can make a contribution to enhance B leukemic cells apoptosis and lead to inhibit progression of B-CLL in patients after treatment.

Since sTRAIL-induced apoptosis is more effectively than sFasL, soluble recombinant derivatives of TRAIL are considered as novel tumors therapeutics because of their selective apoptosis inducing activity in a variety of human tumors but not in normal

cells [17]. On the other hand, elevated sTRAIL concentrations may have unfavorable influence on the T cell function. Soluble TRAIL has been shown to inhibit T cell activation and proliferation without inducing T cell death [8].

Summarizing, our results indicate that relationship between sFas, sFasL and sTRAIL in sera of patients before treatment may facilitate the growth B leukemic cells in patients with B-CLL. Therapy with FC and CC lead to favorable changes in these relations. However, larger prospective studies, involving more molecules of TNF superfamily, are needed to explain a diagnostic value of these observations and to design appropriate therapeutic strategies for B-CLL.

References

1. Greil R, Anether G, Johrer K, Tinhofer I. Tracking death dealing by Fas and TRAIL in lymphatic neoplastic disorders: pathways, targets, and therapeutic tools. J Leukocyte Biol, 2003; 74: 311-30.

2. Jewell AP. Role of apoptosis in the pathogenesis of B-cell chronic lymphocytic leukemia. B J Med Science, 2002; 59(4): 235-6.

3. Munzert G, Kirchner D, Stobbe H, Bergman L, Schmid RM, Döhner H, Heimpel H. Tumor necrosis factor receptor – associated factor 1 gene over expression in B-cell chronic lymphocytic leukemia: analysis of NF-κB/Rel-regulated inhibitors of apoptosis. Blood, 2002; 100: 3749-56.

4. Phillips TA, Ni J, Pan G, Ruben SM, Wei YF, Pace JL, Hunt JS. TRAIL(Apo-2L) receptors in human placentas: implications for immune privilege. J Immunol, 1999; 162: 6053-9.

5. Griffith TS, Rauch CT, Smolak PJ, Waugh JY, Boiani N, Lynch DH, Smith CA, Goodwin RG, Kubin MZ. Functional analysis of TRAIL receptors using monoclonal antibodies. J Immunol, 1999; 162: 2597-605.

6. Herbeuval JP, Lambert C, Sabido O, Cottier M, Fournel P, Dy M, Genin C. Macrophages from cancer patients: analysis of TRAIL, TRAIL receptors, and colon tumor cell apoptosis. J Natl Cancer I, 2003; 958: 611-21.

7. Uno K, Inukai T, Kayagaki N, Goi K, Sato H, Nemoto A, Takahashi T, Kagami K,Yamaguchi N,Yagita H, Okumura K, Koyama-Okazaki T, Suzuki T, Sugita K, Nakazawa S. TNF-related apoptosis-inducing ligand (TRAIL) frequently induces apoptosis in Philadelphia chromosome-positive leukemia cells. Blood, 2003; 101: 3658-67.

8. Ehrlich S, Infante-Duante C, Seeger B, Zipp F. Regulation of soluble and surface-bound TRAIL in human T cells, B cells, and monocytes. Cytokine, 2003; 24: 244-53.

9. MacFarlane M, Harper N, Snowden RT, Dyer MJ, Barnett GA, Pringle JH, Cohen GM. Mechanisms of resistance to TRAIL-induced apoptosis in primary B-cell chronic lymphocytic leukemia. Oncogene, 2002; 21(44): 6809-18.

10. Olsson A, Diaz T, Aqullar-Santelises M, Osterborg A, Celsing F, Jondal M, Osorio LM. Sensitization to TRAIL-induced apoptosis and modulation of FLICE-inhibitory protein in B-cell chronic lymphocytic leukemia by actinomycin D. Leukemia, 2001; 15(12): 1868-77.

11. Tecchio C, Huber W, Scapini P, Calzetti F, Margotto D, Todeschini G, Pilla L, Martinelli G, Pizzolo G, Rivoltini L, Cassatella MA. INF-α-stimulated neutrophils and monocytes release a soluble form of TNF-related apoptosis-inducing ligand (TRAIL/Apo-2 ligand) displaying apoptotic activity on leukemic cells. Blood, 2004; 103: 3837-44.

12. Mongkolsapaya J, Cowper AE, Xu XN, Morris G, McMichael AJ, Bell JI, and Screaton GR. Cutting Edge: Lymphocyte inhibitor of TRAIL(TNF-related apoptosis-ligand): A new receptor protecting lymphocytes from the death ligand TRAIL. J Immunol, 1998; 160: 3-6.

13. Osorio LM, Aqular-Santelises M, DeSantiago A, Hachiya T, Mellstedt H, Jondal M. Increased serum levels of soluble Fas in progressive BCLL. Eur J Haematol, 2001; 66: 342-6.

14. Tinhofer I, Marschitz I, Kos M, Henn T, Egle A, Villunger A, Greil R. Differential sensitivity of CD4+ and CD8+ lymphocytes to the killing efficacy of Fas(Apo-1/CD95) ligand+tumor cells in B-cell chronic lymphocytic leukemia. Blood, 1998; 91: 4273-81.

15. Felderhoff-Mueser U, Herold R, Hochhaus F, Kohene P, Ring-Mrozik E, Obladen M, Bührer C. Increased cerebrospinal fluid concentrations of soluble Fas(CD95/Apo-1) in hydrocephalus. Arch Dis Child, 2001; 84: 369-72.

16. Dicker F, Kater AP, Fukuda T, Kipps TJ. Fas-ligand (CD178) and TRAIL synergistically induce apoptosis of CD40-activated chronic lymphocytic leukemia B cells. Blood, 2004; 10: 1182.

17. Wajant H, Moosmayer D, Wüest TH, Bartke T, Gerlach E, Schönherr U, Peters N, Scheurich P, Pfizenmaier K. Differential activation of TRAIL-R1 by soluble and membrane TRAIL allows selective surface antigen-directed activation of TRAIL-R2 by soluble TRAIL derivative. Oncogene, 2001; 20: 4101-6.

Relationship between insulin-like growth factors (IGF-I and IGF-II), IGF-binding proteins (IGFBP-3, IGFBP-2), leptin and anthropometric parameters (height, body mass index) during antileukaemic treatment in children

Krawczuk-Rybak M[1]*, Muszyńska-Rosłan K[1], Kitszel A[1], Sawicka-Żukowska M[1], Wołczyński S[2]

[1] Department of Pediatric Oncology, Medical University of Białystok, Poland
[2] Department of Gynecological Endocrinology, Medical University of Białystok, Poland

Abstract

Purpose: **The aim of the study was to estimate the anthropometric parameters and their relationship to serum levels of IGF-I, IGF-II, IGFBP-3, IGFBP-2 and leptin before and during intensive antineoplastic treatment for acute lymphoblastic leukaemia in children.**

Material and methods: **In 46 children in median age 6.6 years (range from 1.6 to 16) we evaluated at the time of diagnosis, after protocol I and after intensive treatment, height, body mass index (BMI) and IGF-I, IGF-II, IGFBP-3, IGFBP-2 and leptin.**

Results: **Height SDS lowered in successive points of analysis whereas BMI SDS rose after protocol II. IGF-I SDS was low and similar at each point, IGF-II SDS and IGFBP-3 SDS values augmented progressively and IGFBP-2 SDS was significantly elevated before treatment and lowered (but not normalized) during the therapy. Leptin SDS was elevated, especially after protocol I.**

Conclusion: **leukaemia and its treatment affect directly growth factors, its binding proteins and leptin production leading to growth retardation and overweight.**

Key words: growth, body mass index, growth factors, leptin, acute lymphoblastic leukaemia, children.

* CORRESPONDING AUTHOR:
Department of Pediatric Oncology
Medical University of Białystok
ul. Waszyngtona 17
15-274 Białystok, Poland
Tel/Fax: +48 85 7450846
e-mail: rybak@amb.edu.pl (Maryna Krawczuk-Rybak)

Received 08.06.2005 Accepted 08.07.2005

Introduction

Leukaemia and its composed treatment have severe catabolic effect especially on children, their development, growth and body composition. Corticosteroids and cytotoxic chemotherapy impair normal organ and tissues function such as liver, endocrine glands or cartillage growth plates [1]. Nutritional state and growth rest under influence of hormonal factors such as leptin and growth hormone (GH) which action is mediated by insulin-like growth factors (IGFs) and binding proteins (IGFBPs). Leptin, produced by adipose tissue, plays an important role in regulation of energy intake, expenditure and consequently – in regulation of body composition [2,3]. The importance of hormonal factors (mentioned above) is well known during normal development, growth and puberty in healthy children. In the present study we evaluated the relation between IGF-I, IGF-II, their binding proteins, leptin values and anthropometric parameters in children with newly diagnosed acute lymphoblastic leukaemia (ALL) and during intensive anticancer therapy.

Material and methods

Forty-six children (32 boys) in median age 6.58 years (range from 1.56 to 16.0) with newly diagnosed ALL (42-preB ALL, 4-T-ALL) were included in our study. All patients were treated according to ALLIC/BFM 2002 protocol.

The analysis was made:

1. at the time of diagnosis,
2. after protocol I (induction remission consisting prednisolone 60 mg/m^2 for 28 days, vincristine, asparaginase, cyclophosphamide, daunorubicine, cytarabine, adriamycine, 6-mercaptopurine and methotrexate – ith.), together 64 days,
3. after the end of intensive treatment; that is after protocol M including 6-mercaptopurine, methotrexate (iv. and ith.) and protocol II – with dexamethasone 10 mg/m^2 for 22 days, vincristine, doxorubicine, asparaginase, cyclophosphamide, cytarabine, 6-thioguanine).

Table 1. **Mean (±SEM) SDScore values for auxological and biochemical parameters in analysed points of ALL treatment**

	Height (SDS) N=46	BMI (SDS) N=46	IGF-I (SDS) N=46	IGF-II (SDS) N=46	IGFBP-3 (SDS) N=46	IGFBP-2 (SDS) N=46	Leptin (SDS) N=46	IGFBP-2: IGF-I N=46	Leptin: BMI N= 46
At diagnosis	0.54±1.7	-0.42±2.0	-1.71±0.9	-0.01±1.5	-0.27±2.3	10.96±8.5	2.12±1.5	28.39±21.0	0.30±0.3
After Protocol I	0.15±1.5	-0.39±2.4	-1.86±1.3	0.64±1.4	0.92±1.9	10.74±7.5	3.13±3.3	17.27±11.8	0.28±0.2
After Protocol II	-0.36±1.0	0.2±2.1.0	-1.8±0.9	0.83±2.0	1.75±2.4	8.03±7.3	2.88±3.1	12.49±11.7	0.52±1.3

All intensive treatment (protocols I, M, II) lasted on six months.

We measured at each time-point: height, weight, body mass index (BMI) – calculated as weight (kg) divided by the square of height (m^2) and biochemical parameters.

The samples were obtained after overnight fast and the serum was stored at -70°C until the analysis. Using radioimmunoassay methods, we determined the serum concentration of IGF-I (Source kit), IGF-II (DSL kit), IGF BP-3 (DSL kit), IGF BP-2 (OBRA-Polatom kit) and leptin (Linco HL-81 kit).

All data were presented as SDScore according to age and sex using Polish reference values for height and BMI [4]. The values of IGF-I, IGF-II, IGFBP-2, IGFBP-3 and leptin were compared to those obtained by Blum et al. and Juul et al. [2,3].

The study was approved by the Local Ethics Commitee.

Statistical analysis was performed using Statistica 5.0 for Windows. Results are showed as mean and standard deviation for normally distributed values and median significance levels were calculated according to the nonparametric Wilcoxon test. The Spearman correlation coefficient was also used. A level of $p<0.05$ was regarded as significant.

Results

All data of auxological values and biochemical parameters (as SDScore) are presented in *Tab.1*.

1. Auxological values: The mean height SDS at diagnosis (H1SDS) was 0.54±1.73 and lowered after protocol I (H2SDS) to 0.15±1.5 and to -0.36±1.04 after the end of intensive treatment (H3SDS) – $p<0.008$ between H1SDS and H3SDS and $p<0.005$ between H2SDS and H3SDS.

The mean BMI SDS did not differ at the time of diagnosis (-0.42±1.99) and after protocol I (-0.39±2.42) but rose after protocol II (0.20±2.10 ($p<0.05$ between BMI 2 SDS and BMI 3 SDS).

2. Biochemical parameters: IGF-I SDS was similar at diagnosis (-1.71±0.86), after protocol I (-1.86±1.35) and after protocol II (-1.8±0.89). IGF-II SDS augmented after each point of analysis: it was at the beginning -0.01±1.5, after protocol I – 0.64±1.39 ($p<0.001$), after protocol II – 0.83±2.01 ($p<0.0006$ between first and third point of analysis). IGFBP-3 SDS rose from -0.27±2.27 at diagnosis to 0.92±1.95 after protocol I ($p<0.006$) and to 1.75±2.42 after protocol II ($p<0.00007$ between first and third point of analysis and $p<0.03$ between second and third point). IGFBP-2 SDS was significantly elevated in all moments of analysis but lowered gradually from 10.96±8.46 (at diagnosis) to 10.74±7.47 (after protocol I) and to 8.03±7.32 (after protocol II); $p<0.05$ between values at diagnosis and the end of treatment.

The values of leptin SDS was higher after protocol I (3.13±3.3) comparing with the time of diagnosis (2.12±1.54) ($p<0.01$) and – with the end of intensive treatment (2.88±3.08).

The ratio of IGFBP-2: IGF-I was highest at diagnosis (28.39±21.05), lowered after protocol I (17.27±11.84, $p<0.006$) and after protocol II (12.49±11.75, $p<0.007$). The ratio of leptin: BMI did not change during the time of observation – at diagnosis it was 0.30±0.28, after protocol I – 0.28±0.25 and after protocol II – 0.52±1.29.

3. At the time of diagnosis we found a positive correlations between: a) height and: IGF-I ($r=0.75$, $p<0.001$) and IGFBP-3 ($r=0.485$, $p<0.01$) b) BMI and: leptin ($r=0.44$, $p<0.003$), IGF-I ($r=0.53$, $p<0.0003$), IGFBP-3 ($r= 0.68$, $p< 0.001$).

After protocol I we observed a positive correlation between a) height and IGF-I ($r=0.79$, $p< 0.0001$), IGFBP-3 ($r=0.45$, $p<0.01$) and leptin ($r=0.40$, $p<0.03$), b) BMI and: leptin ($r=0.59$, $p<0.001$) and IGFBP-3 ($r=0.49$, $p<0.02$).

After the end of intensive treatment we found the positive correlations between a) height and: IGF-I ($r=0.57$, $p<0.001$), IGFBP-3 ($r=0.53$, $p<0.002$), IGF-II ($r=0.48$, $p<0.008$), leptin ($r=0.32$, $p<0.05$) b) BMI and IGF-I ($r=0.55$, $p<0.008$), IGFBP-3 ($r=0.69$, $p<0.0001$), IGF-II ($r=0.53$, $p<0.02$) and – c) negative correlation between BMI and IGF BP-2 ($r=-0.32$, $p<0.05$).

We also observed the following correlations: at the time of diagnosis – a positive correlation between IGF-I and IGFBP-3 ($r=0.60$, $p<0.002$), IGF-I and leptin ($r=0.67$, $p<0.001$), IGF-I and IGF-II ($r=0.44$, $p<0.004$), IGF-II and IGFBP-3 ($r=0.57$, $p<0.0001$) and negative correlation between IGF BP-2 and: IGF-II ($r=-0.40$, $p<0.02$) and IGF-I ($r=-0.65$, $p<0.001$). After protocol I we found a positive correlation between IGF-I and leptin ($r= 0.73$, $p<0.0001$), IGF-I and IGFBP-3 ($r=0.63$, $p<0.002$), IGF-II and IGFBP-3 ($r=0.37$, $p<0,03$), IGFBP-3 and leptin ($r=0.53$, $p<0.001$) and after protocol II: a positive correlation between IGF-I and IGFBP-3 ($r=0.75$, $p<0.0001$), IGF-II and IGFBP-3 ($r=0.82$, $p<0.0001$), IGF-I and IGF-II ($r=0.52$, $p<0.02$) (*Tab. 2*).

Discussion

We made the simultaneous analysis of growth, body mass index and serum insulin-like growth factors, its binding proteins

Table 2. **Correlations between auxological and biochemical parameters expressed as absolute values: I – at diagnosis, II – after protocol I, III – after protocol II**

I

IGF-II	r=0.44 p=0.004					
IGFBP-3	r=0.60 p<0.002	r=0.57 p<0.001				
IGFBP-2	r=-0.65 p<0.001	R=-0.4 P<0.02	r=-0.24 p=0.13			
Leptin	r=0.67 p<0.001	r=0.23 p=0.3	r=0.22 p=0.16	r=-0.28 p=0.08		
Height	r=0.75 p<0.001	r=0.41 p=0.05	r=0.49 p<0.01	r=-0.36 p=0.02	r=0.13 p=0.39	
BMI	r=0.53 p=0.0003	r=0.22 p=0.32	r=0.68 p=0.001	r=-0.11 p=0.63	r=0.44 p<0.003	r=0.49 p=0.02
	IGF-I	**IGF-II**	**IGFBP-3**	**IGFBP-2**	**Leptin**	**height**

II

IGF-II	r=0.35 p=0.12					
IGFBP-3	r=0.63 p<0.002	r=0.37 p=0.003				
IGFBP-2	r=-0.9 p=0.67	r=0.04 p=0.82	r=0.14 p=0.44			
Leptin	r=0.73 p=0.0001	r=-0.0002 p=0.98	r=0.53 p=0.001	r=-0.12 p=0.6		
Height	r=0.79 p=0.0001	r=0.31 p=0.09	r=0.45 p<0.01	r=-0.01 p=0.93	r=0.4 p=0.03	
BMI	r=0.43 p=0.053	r=0,09 p=0.63	r=0,49 p<0.02	r=0.12 p=0.53	r=0.59 p=0.001	r=0.04 p=0.8
	IGF-I	**IGF-II**	**IGFBP-3**	**IGFBP-2**	**Leptin**	**height**

III

IGF-II	r=0.52 p=0.02					
IGFBP-3	r=0.75 p=0.0001	r=0.82 p=0.0001				
IGFBP-2	r=-0.24 p=0.15	r=0.003 p=0.99	r=-0.20 p=0.38			
Leptin	r=0.17 p=0.45	r=0.19 p=0.39	r=0.8 p=0.12	r=-0.18 p=0.42		
Height	r=0.57 p=0.001	r=0.48 p<0.008	r=0.53 p<0.002	r=-0.27 p=0.15	r=0.32 p=0.05	
BMI	r=0.55 p=0.008	r=0.53 p=0.02	r=0.69 p=0.0001	r=-0.32 p=0.05	r=0.29 p=0.19	r=0.39 p=0.03
	IGF-I	**IGF-II**	**IGFBP-3**	**IGFBP-2**	**Leptin**	**height**

and leptin during intensive treatment for ALL. In our previous study we demonstrated normal height and weight values in children and adolescent treated with chemotherapy alone or additionally received 12Gy for central nervous system [5]. The actual study made during the intensive treatment showed the tendency to decline growth velocity at the following point of analysis. BMI SDS values did not change after protocol I (with prednisolone) but increased after protocol II (with dexamethasone). This confirms the observations made by Wallace et al. [6] who proved that dexamethasone leads to greater change in BMI than prednisolone during induction chemotherapy for ALL.

The prospective study of Ahmed et al. [7] during intensive treatment for ALL showed a decline of height SDS at the beginning of treatment with a nadir at 6 month of chemotherapy with the increase of weight SDS and BMI SDS.

The longitudinal studies made during and after chemotherapy suggest that linear growth is most affected during intensive and maintenance therapy and followed by "catch-up" after the end of treatment. The tendency to overweight was affirmed by many investigators not only during the treatment, but also after the termination of therapy [1,8,9]. This weight gain is due to excessive fatness. Increased whole body percent of fat was observed even in patients with normal body mass index, what it was confirmed by Dalton et al. and in our previous study [8,10].

The patogenesis of developmental disturbances is multifactorial including malignancy per se, intensive chemotherapy and especially – corticotherapy and radiotherapy for CNS, reduced physical activity. The disturbances in the GH-IGF axis, impaired production of IGFs and its binding proteins by the liver are responsible for those problems [8,9]. Our observations indicated lowered values of IGF-I SDS accompanied by the increase of IGF-II SDS (from –0.01 to 0.83) and IGFBP-3 SDS (from -0.27 to 1.75).

IGFBP-2 production was most affected. We noted significantly elevated IGFBP-2 SDS values at the time of diagnosis (10.96±8.5) which lowered (but did not normalise) at the termination of intensive treatment (8.03±7.3). Similar tendency was observed by Mohnike et al. [11] who suggested the involvement of IGF system, especially IGFBP-2, in the proliferation of leukaemic cell clones. On the other hand IGFBP-2 levels are higher in other pathological situations such as growth hormone deficiency, hyponutrition which often accompany leukaemia and its treatment [12]. We found considerably elevated values of IGFBP-2: IGF-I ratio, especially at diagnosis, which lowered but rested high during all observation. Similar situation was recorded 6 months after diagnosis by Arguelles et al. [13] and Barrios et al. [12] and in their opinion it is a sign of catabolic state. The autors suggest partial and transient GH insensitivity provoked by leukaemia per se and its aggressive treatment what may explain the growth retardation in those patients. According to Brennan et al. [14], reduced IGF-I SDS values in children with malignancies are a sign of nutritional status, whereas normal IGFBP-3 values result from increased activity of IGFBP-3 protease.

Attard-Montallo et al. [15] in the study concerning different malignancies showed normal IGF-I and IGFBP-3 levels before and after intensive chemotherapy with the decrease at the time of febrile neutropenia. In their opinion these catabolic episodes provoke GH resistance.

We observed at diagnosis a positive correlation between IGF-I, IGF-II and IGFBP-3 and negative – between IGFBP-2 and IGF-I and IGF-II. During and after intensive treatment the similar correlations between IGF-I, IGF-II and IGFBP-3 and lack of correlations with IGFBP-2 were found. The similar results obtained by Mohnike et al. [11] suggest the different

regulation of IGFBP-2 and IGFBP-3 and the importance of IGFBP-2 in lymphoblasts proliferation.

In our group the leptin SDScore was increased at each point of observation, with its highest level after protocol I. Hyperleptinaemia was observed by Davies et al. [16] and Arguelles et al. [9] not only during intensive treatment for ALL but also increased after its cessation, suggesting a leptin resistance. We did not observe differences in leptin: BMI ratio although this values progressively augmented. Wallace et al. [6] found the increase of leptin: BMI ratio between fourth and sixth week of treatment with the decrease in following weeks. They suggest that increase in fat mass with leptin resistance is due to corticotherapy and the direct effect of glucocorticoids on adipocytes.

We noted a positive correlations between leptin and IGF-I before treatment and after protocol I and between BMI and IGF-I, IGFBP-3 and leptin which affirm the role of growth factors and its binding proteins in weight gain.

Conclusions

Our study demonstrates that leukaemia and its intensive chemotherapy affect the production of growth factors, its binding proteins and leptin, which may be responsible for growth retardation and overweight.

Acknowledgement

We thank Magdalena Topczewska from Faculty of Computer Science, Białystok Technical University for performing the statistical analysis.

References

1. Birkebaek NH, Fisker S, Clausen N, Tuovinen V, Sindet-Pedersen S, Christiansen JS. Growth and endocrinological disorders up to 21 years after treatment for acute lymphoblastic leukemia in childhood. Med Ped Oncol, 1998; 30: 351-6.

2. Blum WF, Englaro P, Hanitsch S, Juul A, Hertel NT, Muller J, Skakkebaek NE, Heiman ML, Birkett M, Attanasio AM, Kiess W, Rascher W. Plasma leptin levels in healthy children and adolescents: dependence on body mass index, body fat mass, gender, pubertal stage, and testosterone. J Clin Endocrinol Metab, 1997; 82: 2904-10.

3. Juul A, Dalgaard P, Blum WF, Bang P, Hall K, Michaelsen KF, Muller J, Skakkebaek NE. Serum levels of insulin-like growth factor (IGF)-binding protein-3 (IGFBP-3) in healthy infants, children, and adolescents: the relation to IGF-I, IGF-II, IGFBP-1, IGFBP-2, age, sex, body mass index, and pubertal maturation. J Clin Endocrinol Metab, 1995; 80: 2534-42.

4. Palczewska I, Szilagyi-Pagowska I. Ocena rozwoju somatycznego dzieci i młodzieży. Med Prakt, 2002; 3: 1-30.

5. Krawczuk-Rybak M, Kaliszewski J, Kitszel A. The influence of age, gender and modifications of therapeutic protocols on physical development of children and adolescents after acute lymphoblastic leukemia therapy completed. Endokrynol Diabetol Chor Przemiany Materii Wieku Rozw, 2003; 9: 41-7.

6. Wallace AM, Tucker P, Williams DM, Hughes IA, Ahmed SF. Short-term effects of prednisolone and dexamethasone on circulating concentrations of leptin and sex hormone-binding globulin in children being treated for acute lymphoblastic leukaemia. Clin Endocrinol, 2003; 58; 770-6.

7. Ahmed SF, Wallace WH, Kelnar CJ. An anthropometric study of children during intensive chemotherapy for acute lymphoblastic leukaemia. Horm Res, 1997; 48: 178-83.

8. Dalton VK, Rue M, Silverman LB, Gelber RD, Asselin BL, Barr RD, Clavell LA, Hurvitz CA, Moghrabi A, Samson Y, Schorin M, Tarbell NJ, Sallan SE, Cohen LE. Height and weight in children treated for acute lymphoblastic leukemia: relationship to CNS treatment. J Clin Oncol, 2003; 21: 2953-60.

9. Argüelles B, Barrios V, Buno M, Madero L, Argente J. Anthropometric parameters and their relationship to serum growth hormone-binding protein and leptin levels in children with acute lymphoblastic leukemia: a prospective study. Eur J Endocrinol, 2000; 143: 243-50.

10. Krawczuk-Rybak M, Muszyńska-Rosłan K, Konstantynowicz J, Solarz E, Wolczynski S, Protas P. Relationship of leptin values to fat body mass (FMB) and lean body mass (LBM) after antineoplastic treatment. Med. Wieku Rozwojowego, 2004; 8: 297-307.

11. Mohnike KL, Kluba U, Mittler U, Aumann V, Vorwerk P, Blum WF. Serum levels of insulin-like growth factor-I, -II and insulin-like growth factor binding proteins-2 and -3 in children with acute lymphoblastic leukemia. Eur J Pediatr, 1996; 155: 81-6.

12. Barrios V, Buno M, Pozo J, Munoz MT, Argente J. Insulin-like growth factor-binding protein-2 levels in pediatric patients with growth hormone deficiency, eating disorders and acute lymphoblastic leukemia. Horm Res, 2000; 53: 221-7.

13. Arguelles B, Barrios V, Pozo J, Munoz MT, Argente J. Modifications of growth velocity and the insulin-like growth factor system in children with acute lymphoblastic leukemia: a longitudinal study. J Clin Endocrinol Metab, 2000; 85: 4087-92.

14. Brennan BM, Gill M, Pennells L, Eden OB, Thomas AG, Clayton PE. Insulin-like growth factor I, IGF binding protein 3 and IGFBP protease activity: relation to anthropometric indices in solid tumours or leukaemia. Arch Dis Child, 1999; 80: 226-30.

15. Attard-Montalto SP, Camacho-Hubner C, Cotterill AM, D'Souza-Li L, Daley S, Bartlett K, Halliday D, Eden OB. Changes in protein turnover, IGF-I and IGF-binding proteins in children with cancer. Acta Pediatr, 1998; 87: 54-60.

16. Davies JH, Evans BA, Jones E, Evans WD, Jenney ME, Gregory JW. Osteopenia, excess adiposity and hyperleptinaemia during 2 years of treatment for childhood acute lymphoblastic leukaemia without cranial irradiation. Clin Endocrinol, 2004; 60: 358-65.

Patient acceptance of diagnostic laparoscopy

Weickert U[1]*, Bühl A[1], Jakobs R[1], Eickhoff A[1], Löffler W[2], Riemann JF[1]

[1] Department of Internal Medicine C, Klinikum Ludwigshafen, Academic Hospital of the University of Mainz, Germany
[2] Department of Biostatistics, Klinikum Ludwigshafen, Academic Hospital of the University of Mainz, Germany

Abstract

Purpose: **To assess patient acceptance of diagnostic conventional laparoscopy and minilaparoscopy under sedoanalgesia.**

Materials and methods: **120 consecutive patients undergoing diagnostic laparoscopy were enrolled prospectively in this study. Within the first week after diagnostic laparoscopy the patients were asked to answer a total of eight questions with regard to the acceptance of the procedure.**

Results: **The inconvenience of laparoscopy was assessed with a mean of 1.6 on a scale from 0 to 10 (0= no inconvenience, 10= very unpleasant). The discomfort in the two days following laparoscopy were graded with a mean of 2.1 on a scale from 0 to 10 (0= no inconvenience, 10= very unpleasant). There was no difference between conventional laparoscopy and minilaparoscopy. Only 10% of the patients described laparoscopy more inconvenient in comparison to diagnostic gastroscopy, whereas 29% of the patients assessed diagnostic gastroscopy more inconvenient.**

Conclusions: **Diagnostic laparoscopy under sedoanalgesia is a very well tolerated procedure. There is no difference between conventional laparoscopy and minilaparoscopy.**

Key words: acceptance, diagnostic laparoscopy, questionnaire.

* CORRESPONDING AUTHOR:
Department of Internal Medicine C
Klinikum Ludwigshafen
Academic Hospital of the University of Mainz
Bremserstr 79
67063 Ludwigshafen am Rhein, Germany
Tel: +49-621-503-4100; Fax: +49-621-503-4114
e-mail: weickeru@klilu.de (Uwe Weickert)

Received 17.03.2005 Accepted 25.04.2005

Introduction

Despite the availability of modern imaging methods diagnostic laparoscopy still is an important diagnostic tool of gastroenterologists [1-3]. The main indication for laparoscopy is liver disease and staging of gastrointestinal tumors [4-7].

There are several large studies available comparing the laparoscopic findings with the histological results of biopsies taken during laparoscopy in patients with various liver diseases. These studies consistently demonstrate that histology alone may miss the diagnosis of cirrhosis in up to a quarter of patients [8-11]. The diagnosis of cirrhosis is not made by histology alone especially in the case of macronodular disease and early stages of cirrhosis [12,13].

Small metastases to the liver surface and/or peritoneum missed by ultrasound, computed tomography (CT) and magnetic resonance tomography (MRT) can be easily diagnosed by laparoscopy [14]. With the recent development of small diameter laparoscopes the method has gained more widespread acceptance [15,16]. Its diagnostic capacity in liver disease and as a staging procedure has been proven in recent studies [13,14].

However, as there are no data published with respect to patient acceptance of minilaparoscopy compared with conventional laparoscopy, we addressed this topic in a prospective study.

Material and methods

Patients

120 consecutive patients undergoing diagnostic laparoscopy (conventional laparoscopy n=64, minilaparoscopy n=56) were enrolled prospectively in this study.

Laparoscopy

Diagnostic laparoscopy was performed as a standard procedure with the patient under conscious sedation with midazolam and pethidine. For conventional laparoscopy the Veres needle was advanced at the point of Monroe (lower left abdominal

quadrant). After insufflation of N_2O the laparoscope was introduced into the abdominal cavity through a trocar which was inserted at the point of Kalk (periumbilical). In the patients undergoing minilaparoscopy a small trocar for the Veres needle was advanced at the point of Kalk and after insufflation of N_2O the laparoscope was introduced through the same trocar. Liver biopsies and/or peritoneal biopsies were taken dependent on the underlying disease.

Questionnaire

Within the first week after diagnostic laparoscopy the patients were asked to answer a total of eight questions concerning the acceptance of the procedure. Questions 4 and 8 were constructed as a visual analog scale [17,18].

Questionnaire

1. Did you undergo gastroscopy in the past:
○ no
○ yes, without sedation
○ yes, with sedation

2. How did you experience laparoscopy in comparison with former gastroscopy:
○ I have not undergone gastroscopy
○ I don't know
○ more unpleasant
○ comparable
○ less unpleasant

3. How did you experience laparoscopy in comparison with former ultrasound guided liver biopsy:
○ I have not undergone ultrasound guided liver biopsy
○ I don't know
○ more unpleasant
○ comparable
○ less unpleasant

4. How was your overall experience of laparoscopy (left: no inconvenience, right: very unpleasant):
○ I can't remember

|_____|
no inconvenience very unpleasant

5. Would you retrospectively give your consent for laparoscopy:
○ yes
○ perhaps
○ no

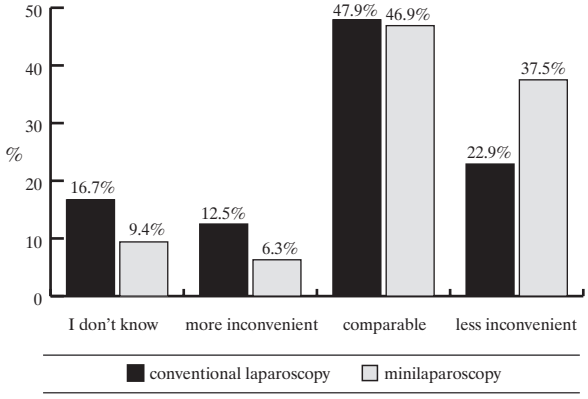
6. Would you undergo laparoscopy again, if indicated:
○ yes
○ yes, but only under deeper sedoanalgesia
○ perhaps
○ no

7. If you had to undergo another laparoscopy, what was your preference with respect to sedoanalgesia:
○ more sedoanalgesics
○ less sedoanalgesics
○ same medication

8. What was the grade of discomfort in the two days following laparoscopy (left = no discomfort, right = severe discomfort):

|_____|
no discomfort severe discomfort

Figure 1. **"How did you experience laparoscopy in comparison with former gastroscopy?"**



Statistics

The frequency of answers was described for each group and the associations in the one-way and two-way tables were measured with Fisher exact test. The Mann-Whitney-U-Test was used for testing the hypothesis that two independent samples are from populations with the same distribution (rank-sum test, questions 4 and 8).

Results

No patient declined to answer the questionnaire. The mean age (71 men, 49 women) was 54 years. In all patients sedoanalgesia was performed with a combination of midazolam and pethidin. The mean dose of midazolam was 5.2 mg. One hundred seventeen patients received 50 mg pethidin, one patient 75 mg and two patients 100 mg. There was no statistical significant difference between the groups of patients investigated by conventional laparoscopy and minilaparoscopy with respect to the dosage of sedoanalgesics, sex distribution, indication for laparoscopy or body-mass-index.

Eighty of 120 patients (67%) had undergone gastroscopy in the past, 23 patients (29%) without and 57 patients (71%) after administration of sedatives. Thirty eight patients (48%) judged the inconvenience of gastroscopy and laparoscopy as comparable, 8 patients (10%) described laparoscopy more inconvenient and 23 patients (29%) less inconvenient. Eleven patients (14%) could not decide on the topic. There was no difference between the group of patients who had undergone conventional laparoscopy and minilaparoscopy (p=0.42) (*Fig. 1*).

Figure 2. **"How was your overall experience of laparoscopy (0=no inconvenience, 10=very unpleasant)?"**

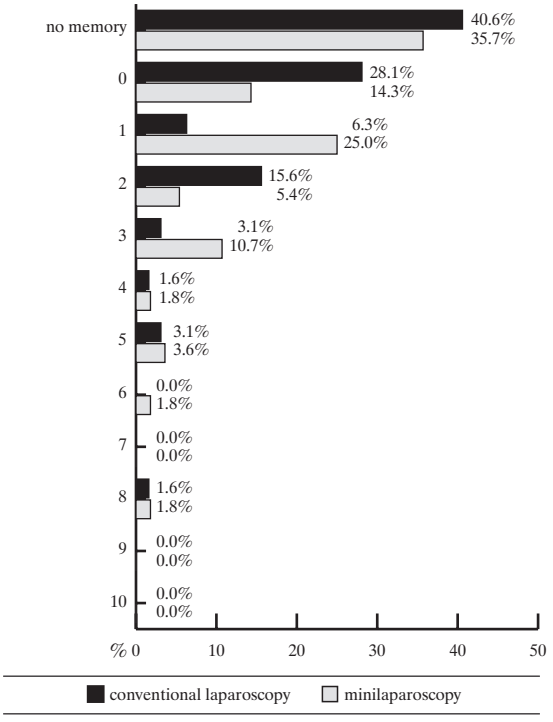


Figure 3. **"Would you retrospectively give your consent for laparoscopy?"**

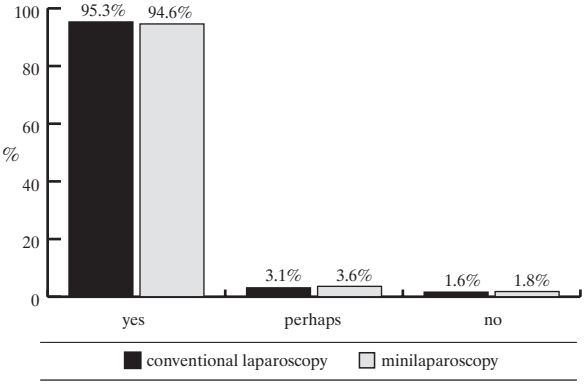
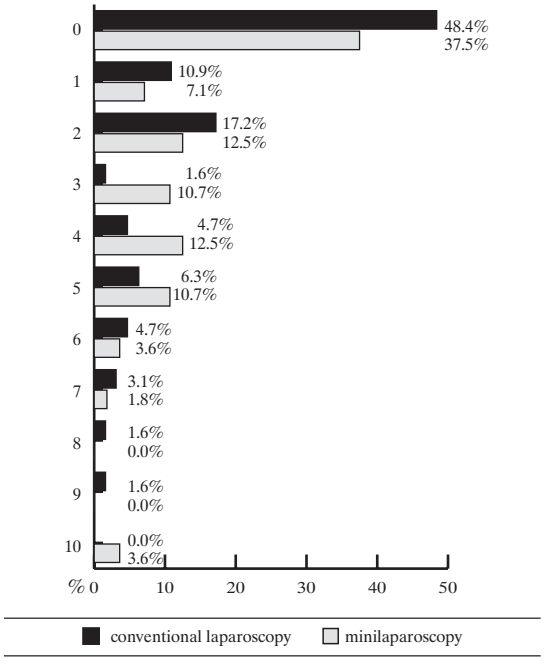


Figure 4. **"What was the grade of discomfort in the two days following laparoscopy (left=no discomfort, right=severe discomfort)?"**



Only 7 patients (5.8%) had undergone ultrasound-guided liver biopsy previously. One patient judged both procedures as comparable and two patients judged laparoscopy less and two patients more inconvenient.

Forty six of 120 patients (38%) could not remember the performance of laparoscopy. The remaining 74 patients (62%) judged the inconvenience of laparoscopy with a mean of 1.6 on a scale from 0 to 10 (visual analog scale, 0=no inconvenience, 10=very unpleasant) (*Fig. 2*). There was no difference between conventional and minilaparoscopy (p=0.15).

One hundred fourteen patients (95%) would – retrospectively – again give their consent to the performance of laparoscopy. Only 2 patients (1.7%) would decline to give their consent again (*Fig. 3*).

One hundred five patients (87.5%) would give their consent to undergo another laparoscopy, if necessary in the future; 10 patients (8.3%) only after the administration of more sedatives. The performance of another laparoscopy was declined by 3 patients (2.5%). Again there was no difference between conventional and minilaparoscopy (p=0.58).

One hundred one patients (84.2%) would prefer the same sedoanalgesia in the case of another laparoscopy. Four patients (3.3%) would prefer less and 15 patients (12.5%) more sedoanalgesics. Again there was no difference between the patients who had undergone conventional and minilaparoscopy (p=0.66).

The discomfort in the two days following laparoscopy was graded with a mean of 2.1 on a scale from 0 to 10 (visual analog scale, 0=no inconvenience, 10=very unpleasant) (*Fig. 4*). There was no difference between both groups (p=0.14).

The answers to question 4 ("How was your overall experience of laparoscopy?") and 8 ("What was the grade of discomfort in the two days following laparoscopy?") were analysed with respect to an influence of: age, gender, body-mass-index and indication for laparoscopy. There was only one significant association: sex and judgement of the discomfort in the two days following laparoscopy. Male patients judged the discomfort with a mean of 1.7 and female patients with a mean of 2.6. This difference was statistically significant (p=0.02).

Discussion

Diagnostic laparoscopy – either as conventional laparoscopy or minilaparoscopy – can be performed under sedoanalgesia. As

the value of a diagnostic tool is dependent on the acceptance by patient we performed a structured questionnaire in a consecutive series of 120 patients undergoing diagnostic laparoscopy. Especially the question whether the reduced invasiveness of minilaparoscopy compared with conventional laparoscopy results in improved patient acceptance was addressed.

Our results demonstrate the overall minor inconvenience of diagnostic laparoscopy under sedoanalgesia. Only 10 percent of patients judged diagnostic laparoscopy more inconvenient than gastroscopy. Fourty eight percent of patients judged the two procedures comparable and 29% experienced gastroscopy even more inconvenient in comparison with diagnostic laparoscopy. Ninety five percent of patients would – retrospectively – give their consent to the performance of laparoscopy again. Eighty four percent of patients were satisfied with sedoanalgesia. Only 12.5% of patients would prefer more sedatives in a future diagnostic laparoscopy and 3.3% of patients less sedatives. However, there was no difference between the patients who had undergone conventional laparoscopy and minilaparoscopy. Only gender was a predictor of the grade of inconvenience after laparoscopy. Male patients experienced significantly less inconvenience.

Discomfort following diagnostic laparoscopy is mainly caused by pneumoperitoneum [19]. Presumably distension by gas insufflation leads to minor injuries of vessels and nerves with the consequence of inflammatory cytokine release [19].

In a placebo controlled study on 110 patients undergoing laparoscopy for fertility reasons the administration of 200 mg celecoxib two hours prior to laparoscopy reduced pain after the procedure [20]. Celecoxib-like rofecoxib – is a cox-II-inhibitor. Whether the cardiovascular side effects of rofecoxib, which has lead to its recall by the pharmaceutical company, are specific for the substance or a class effect, remains unclear [21]. However, a new study using conventional NSAR seems to be necessary.

In a study by Poynard and Lebrec [22] 113 patients and 80 hepatologists were interviewed with respect to the inconvenience of different diagnostic procedures. These procedures included gastroscopy and conventional laparoscopy. Gastroscopy was judged more inconvenient compared with laparoscopy by patients in this study too. The assessment of the hepatologists was quite different from the assessment of patients. Hepatologists deemed laparoscopy a procedure with more discomfort compared with gastroscopy.

A study of 56 patients comparing conventional administration of analgosedation with patient-controlled medication resulted in a similar safety and patient tolerance of colonoscopy [23]. As patient tolerance of laparoscopy was excellent in our study, improvement by patient-controlled drug administration seems unlikely.

Conclusions

Diagnostic laparoscopy under sedoanalgesia is a very well tolerated procedure. It compares favourably with diagnostic gastroscopy. However, there is no difference between conventional laparoscopy and minilaparoscopy.

References

1. Weickert U, Buttmann A, Jakobs R, Schilling D, Eickhoff A, Riemann JF. Diagnosis of liver cirrhosis: A comparison of modified ultrasound and laparoscopy in 100 consecutive patients. J Clin Gastroenterol, 2005; 39: 529-32.
2. Weickert U, Jakobs R, Riemann JF. Diagnostic laparoscopy. Endoscopy, 2005; 37: 33-7.
3. Weickert U, Jakobs R, Siegel E, Eickhoff A, Schilling D, Riemann JF. Komplikationen der internistischen Laparoskopie: Eine Analyse von 675 Laparoskopien in der Übergangszeit von der konventionellen zur Mini-Laparoskopie. Dtsch Med Wochenschr, 2005; 130: 16-20.
4. Feussner H, Omote K, Fink U, Walker SJ, Siewert JR. Pretherapeutic laparoscopic staging in advanced gastric carcinoma. Endoscopy, 1999; 31: 342-7.
5. Hulscher JBF, Nieveen van Dijkum EJM, de Wit LT, van Delden OM, van Lanschot JJB, Obertop H, Gouma DJ. Laparoscopy and laparoscopic ultrasonography in staging carcinoma of the gastric cardia. Eur J Surg, 2000; 166: 862-5.
6. Jimenez RE, Warshaw AL, Rattner DW, Willett CT, McGrath D, Fernandez-del Castillo C. Impact of laparoscopic staging in the treatment of pancreatic cancer. Arch Surg, 2000; 135: 409-15.
7. Reddy KR, Levi J, Livingstone A, Jeffers L, Molina E, Kligerman S, Bernstein D, Kodali VP, Schiff ER. Experience with staging laparoscopy in pancreatic malignancy. Gastrointest Endosc, 1999; 49: 498-503.
8. Gebo KA, Herlong HF, Torbenson MS, Jenckes MW, Chander G, Ghanem KG, El-Kamary SS, Sulkowski M, Bass EB. Role of liver biopsy in management of chronic hepatitis C: A systematic review. Hepatology, 2002; 36: S161-72.
9. Jalan R, Harrison DJ, Dillon JF, Elton RA, Finlayson ND, Hayes PC. Laparoscopy and histology in the diagnosis of chronic liver disease. QJM, 1995; 88: 559-64.
10. Orlando R, Lirussi F, Okolicsanyi L. Laparoscopy and liver biopsy: further evidence that the two procedures improve the diagnosis of liver cirrhosis. A retrospective study of 1,003 consecutive examinations. J Clin Gastroenterol, 1990; 12: 47-52.
11. Poniachik J, Bernstein DE, Reddy KR, Jeffers LJ, Coelho-Little ME, Civantos F, Schiff ER. The role of laparoscopy in the diagnosis of cirrhosis. Gastrointest Endosc, 1996; 43: 568-71.
12. Helmreich-Becker I, Schirmacher P, Denzer U, Hensel A, Meyer zum Büschenfelde KH, Lohse AW. Minilaparoscopy in the diagnosis of cirrhosis: Superiority in patients with Child-Pugh A and macronodular disease. Endoscopy, 2003; 35: 55-60.
13. Weickert U, Siegel E, Schilling D, Eickhoff A, Jakobs R, Bohrer MH, Riemann JF. Die Diagnose einer Leberzirrhose: Ein Vergleich der Wertigkeit von Standardlaparoskopie, Minilaparoskopie und Histologie. Z Gastroenterol, 2005; 43: 17-21.
14. Denzer U, Hoffmann S, Helmreich-Becker I, Kauczor HU, Thelen M, Kanzler S, Galle PR, Lohse AW. Minilaparoscopy in the diagnosis of peritoneal tumor spread: prospective controlled comparison with computed tomography. Surg Endosc, 2004; 18: 1067-70.
15. Denzer U, Helmreich-Becker I, Galle PR, Lohse AW. Liver assessment and biopsy in patients with marked coagulopathy: value of mini-laparoscopy and control of bleeding. Am J Gastroenterol, 2003; 98: 893-900.
16. Helmreich-Becker I, Meyer zum Büschenfelde KH, Lohse AW. Safety and feasibility of a new minimally invasive diagnostic laparoscopy technique. Endoscopy, 1998; 30: 756-62.
17. Jensen MP, Karoly P, Braver S. The measurement of clinical pain intensitiy: a comparison of sic methods. Pain, 1986; 27: 117-26.
18. Myles PS, Troedel S, Boquest M, Reeves M. The pain visual analog scale: is it linear or nonlinear? Anesth Analg, 1999; 89: 1517-20.
19. Alexander JI. Pain after laparoscopy. Br J Aneasth, 1997; 79: 369-78.
20. Phinchantra P, Suwajanakorn S. The preemptive analgesic effect of celecoxib for day-case diagnostic laparoscopy. J Med Assoc Thai, 2004; 87: 283-8.
21. Oberholzer-Gee F, Inamdar SN. Merck's recall of rofecoxib. N Engl J Med, 2004; 351: 2147-9.
22. Poynard T, Lebrec D. The inconvenience of investigations used in hepatology: patient's and hepatologist's opinions. Liver, 1992; 2: 369-75.
23. Stermer E, Gaitini L, Yudashkin M, Essaian G, Tamir A. Patient-controlled analgesia for conscious sedation during colonoscopy: a randomized controlled study. Gastrointest Endosc, 2000; 51: 278-81.

Staphylococcus aureus septicemia in non-neutropenic adult patients hospitalized in internal medicine units

Gallucci F[1], Amato G[2], Esposito P[1], Belli CMC[2], Russo R[1], Uomo G[1]*

[1] Department of Internal Medicine, Cardarelli Hospital, Napoli, Italy
[2] Department of Clinical Pathology, Cardarelli Hospital, Napoli, Italy

Abstract

Purpose: ***Staphylococcus aureus*** **septicemia (*SAS*) is usually described in immunocompromised patients and during serious weakening diseases, associated with a neutropenic condition. Over the last recent years, clinic relevance of *SAS* has become more prominent owing to the progressive rise of methicillin-resistent strains in hospital-acquired infections and to its development in non-neutropenic patients.**

Material and methods: **The aim of our study was to evaluate the clinical features and outcome of non-neutropenic patients with positive blood culture for *Staphylococcus aureus* (*SA*) hospitalized in Internal Medicine Wards of our hospital during 1 year of observation. 24 patients with those characteristics were retrospectively recruited; five of them were then excluded from the analysis because of concomitant oncohematologic disease. The median age of the study group of patients (19 cases) was 56 years (range 18-87); 10 (52.6%) patients were male.**

Results: **Infection was hospital-acquired in 10 patients (52.6%). Predisposing factors were: central venous catheter (CVC) (47.4%), recent surgical intervention (21.0%), drug-addiction (15.8%). Main comorbidities were diabetes mellitus in 10 patients (52.6%), heart disease in 4 (21.0%), chronic renal failure in 3 (15.8%), cerebral vascular disease in 3 (15.8%). Fever >38°C was found in all patients at the moment of *SA* isolation in blood culture. *SA* isolated-strains were methicillin-resistant in 7 patients (36.8%). Complications of bacteremia were: pneumonia in 4, endocarditis in 3, vertebral osteomyelytis in 2, septic splenic embolization in 1 and endophtalmitis in 1 patient. The septicemia-attributable mortality was 36.8% (7 patients).**

Conclusions: ***SAS*** **in non-neutropenic patients observed in Internal Medicine Units are associated with significant morbidity and mortality, closer to that reported for neutropenic illnesses.**

Key words: *Staphylococcus aureus*, sepsis, nosocomial infections.

* CORRESPONDING AUTHOR:
Internal Medicine Department, Cardarelli Hospital
via Cardarelli 9, 80131 Napoli, Italy
Tel: +39 81 7472101; Fax: +39 81 7474042
e-mail: generoso.uomo@ospedalecardarelli.it (Generoso Uomo)

Received 25.07.2005 Accepted 25.07.2005

Introduction

Staphylococcus aureus (*SA*) is one of the most common etiological agents of both endemic and epidemic infection acquired in hospitals and in community. Sustained morbidity and mortality are associated with *SA* infections. In fact, when this microorganism enters the blood, it represents one of the most lethal human pathogens also because it is often characterized by multidrug resistance [1]. Humans are a natural reservoir of *SA* that colonizes the nares, axillae, vagina, pharynx or damaged skin surfaces. Rates of staphylococcal colonization are high among patients with type 1 diabetes, intravenous drug users, patients undergoing hemodialysis, surgical patients and patients with the acquired immunodeficiency syndrome [2].

Infections are initiated when a breach of the skin or mucosal barrier allows staphylococci access to adjoining tissues or the bloodstream. Whether an infection is contained or spreads, it depends on a complex interplay between *SA* virulence determinants and host defense mechanism. Patients with qualitative or quantitative defects in leukocyte function are also at increased risk for staphylococcal disease. The risk of infection is increased by the presence of foreign material (devices and intravenous catheters) by means of several pathogenic factors. Staphylococcal bacteremia may be complicated by endocarditis, metastatic infection, or the sepsis syndrome [3-6].

Over the past last years, the frequency of *SA* bacteremia has increased dramatically. This increasing frequency, also in non-neutropenic patients, coupled with rising rate of antibiotic

resistance, has renewed interest in this serious, common infection [7].

The aim of our study was to evaluate the clinical and microbiological characteristic of *Staphylococcus aureus* septicemia (*SAS*) by means of an investigation in non-neutropenic patients hospitalized in the Internal Medicine wards of a regional referral hospital.

Patients and methods

We performed a retrospective study of all *SAS* in non–neutropenic patients, consecutively hospitalized into the five Internal Medicine wards of Cardarelli Hospital, Naples, Italy, a regional referral hospital with a total of 1100 beds. During the study period (January – December 2003) all patients with positive blood culture (confirmed in two samples) for *SA* were enrolled. We excluded patients less than 18 years old, those who had polymicrobic infection, those with a number of neutrophil cells $<2000/mm^3$ at the hospital admission and/or at blood cultural isolation and those who were affected by oncohematologic disease. Significant data derived from the patients' charts togheter with computer-assisted analysis of all microbiological 2003 data-base of our institution. A specific form elaborated for this study was utilized.

We evaluated the following data for each patient: age and sex, place of infection acquisition (community or hospital), predisposing factors (such as devices or intravenous catheters, pre-existent diseases), fever, white blood cells number at hospital admission and at the time of blood cultural isolation, site of infection, complications, antibiotic therapy and antibiotic resistance of isolated streams, duration of hospital stay, relapses and final outcome.

SAS was ascertained in case of positive blood culture (no less than two) in presence of signs and symptoms of septicemia. Borrowing the criteria of pneumonia classification [8], *SAS* was considered "community-acquired", if the first positive blood culture was found before 48 hours from hospital admission; it was classified "hospital-acquired" when the first positive blood culture was after 48 hours from the hospital admission and no clinical evidence of infection was present at admission.

Predisposing factors were considered all known conditions able to help *SA* to access to the bloodstream and to develop (central venous catheter – CVC, recent surgical intervention, drug-addiction). Metastatic infections and relapses were considered as *SAS* complications.

Recovery was considered the diseapparance of infection clinical signs during antibiotic therapy with negative blood cultures and without complications; relapses were defined the *SAS* recurrences during hospital stay with at least a week of delay after the end of antibiotic treatment. *SAS*-attributable mortality was considered the persistence of infection at the death time without other fortuituos causes of death. *SAS* non-attributable mortality was related to a pre-existent illness or to other causes without clinical evidence of infection at death time and without positive blood culture during the last week before death.

Table 1. **Main clinical characteristics of the study-group of patients**

Patients	19		
Age	56 years (range 18-87)		
Sex	9 F, 10 M	N°	%
Infection acquisition site	nosocomial	10	52.6
	community	6	31.6
	non evaluable	3	15.8
Predisposing factors	Central venous catheter	9	47.4
	Recent surgical intervention	4	21.0
	Toxicomania	3	15.8
	None	4	21.0
Comorbidities	Diabetes mellitus	10	52.6
	Heart disease	4	21.0
	Chronic renal failure	3	15.8
	Cerebral vascular disease	3	15.8
	None	2	10.5
Secondary complications	Pneumonia	4	21.0
	Endocarditis	3	15.7
	Vertebral osteomyelitis	2	10.5
	Septic splenic embolization	1	5.2
	Endophtalmitis	1	5.2
Methicillin resistance		7	36.8
Septicemia attributable mortality		7	36.8

Results

Twenty-four patients with above-mentioned inclusion criteria were enrolled; five of them were excluded from the final analysis either because of oncohematologic disease or because of incompleteness of chart's data. Nine patients were female (47.0%); 10 (53.0%) patients were male; the median age was 56 years (range 18-87) (*Tab. 1*). Predisposing factors were CVC in 9 pts (47.4%), recent surgical intervention in 4 (21.0%), drug-addiction in 3 (15.8%); in 4 cases (21.0%) there was no predisposing factor. The most frequent pre-existent illness were: diabetes in 10 patients (52.6), heart chronic disease in 4 (21.0%), chronic renal failure in 3 (15.8%), cerebral vascular disease in 3 (15.8%). No pre-existent disease was in 2 patients (10.5%).

The median value of neutrophil cells was $11600/mm^3$ (range 5100-27900). Fever (T>38°C) was observed in all patients at positive blood culture time. In 6 of them (31.5%), fever was already existent some days before hospital admission and the patients had already been treated with antibiotics at home.

Infection was hospital-acquired in 52.6% of cases (10 patients), appearing on average 5.7 days after hospital admission (five of them showed infection during treatment with cephalosporins). *SA* strains resulted methicillin-resistant in 36.8% (7 patients).

Relevant complications were pneumonia in 4 patients (21.0%), endocarditis in 3 (15.7%), vertebral osteomyelitis in 2 (10.5%), septic splenic embolization in 1 case (5.2%), endophtalmitis in 1 patient (5.2%). Patients who recovered from *SAS* had not relapses; the median of their hospital stay was 24.6 days (range 10-56). The septicemia attributable mortality was 36.8%

Table 2. **Antibiotic *in vitro* susceptibility in the 19 *SA* strains isolated**

Tetracycline	17 (89%)	Rifampicin	17 (89%)	Methicillin	11 (60%)
Teicoplanin	18 (95%)	Amikacin	16 (84%)	Azitromycin	10 (53%)
Cotrimoxazol	18 (95%)	Ofloxacin	12 (63%)	Clindamycin	9 (47%)
Vancomycin	18 (95%)	Imipenem	11 (58%)	Erythromycin	9 (47%)

(7 patients), occurred on average 20.6 days after hospitalization (range 4-31). *Tab. 2* shows the in vitro susceptibility of the 19 *SA* strains isolated.

Discussion

SAS are rather frequent both in community and in hospital [9]. According to the National Nosocomial Infection Surveillance System of the Centers for Disease Control and Prevention of Atlanta (CDC), 16 percent of hospital-acquired cases of bacteremia in the USA from 1990 to 1995 were due to *SA*, that represents the second most common agent after coagulase-negative staphylococci [1]. In fact, within USA's 5400 acute care hospitals, the three leading causes of nosocomial infections of the bloodstream are coagulase-negative staphylococci (80% of which involve strains resistant to methicillin), *SA* (30% methicillin-resistant) and the enterococci (20% vancomicin-resistant). The rough mortality rate associated with these infections are 21%, 25% and 32%, respectively [10].

Multidrug resistant strains of *SA* have been reported with increasing frequency worldwide, including isolates that are resistant to methicillin, lincosamides, macrolides, aminoglycosides, fluorquinolones or combinations of these antibiotics [2]. The emergence of *SA* strains with intermediate resistance to glycopeptides has aroused concern about the development of strains resistant to all available antibiotics. The CDC estimates that 34% of *SA* isolates from cases of nosocomial bacteremia in USA hospitals in 1995 were resistant to methicillin [11]. Infections with methicillin-resistant *SA* are more likely to originate in patients who are seriously ill, immunocompromised and in intensive care units, than are infections with methicillin-susceptible isolates. Colonized patients are the chief source of *SA* in hospitals (nasal carriage) also in intensive care units where isolation of methicillin-susceptible and methicillin-resistant strains are both frequent.

Because there is an increased use of antimicrobial agents in areas where isolates of methicillin-resistant *SA* are found, and because these isolates are resistant to multiple antimicrobial agents and not exclusively to beta-lactams, selective pressure may promote colonization with methicillin-resistant *SA* to a greater degree than colonization with methicillin-susceptible isolates [12]. Patients may already be colonized with *SA* when they enter the hospital. The rising incidence of "community-acquired" infections with *SA* suggest that the rate of colonization with methicillin-resistant *SA* among outpatients is increasing, particulary among those who live in extended-care facilities or have recently been discharged from hospitals. Patients may be colonized with methicillin-resistant *SA* at sites other than the nose, particulary chronic wounds and dermatitides. It is difficult to eradicate *SA* from these sites, and thay can be sources of reinfection. Report of von Eiff and co-workers [13] documented that in most cases, the infecting *SA* isolates from the blood came from the patients themselves, who carried the bacteria in their anterior nares. In addition, nasal carriage has been associated with an increased risk of patients after surgery, in patients receiving continuous ambulatory peritoneal dialysis and in patients receiving hemodialysis.

Although the contribution of resistance to the outcome of such infections is unclear, nosocomial infections of the bloodstream may represent the eighth leading cause of death in the United States, and the relentless rise of antibiotic resistance has markedly curtailed options for therapy [14].

The prevention of hospital-acquired infections due to *SA* is a major goal of hospital infection-control practitioners. Such prevention requires an understanding of the source of the organisms that may eventually find their way into the blood.

Some additional epidemiological considerations arise from our experience. As concerns predisposing factors, it seems appropriate to underline the role of minor surgical interventions in the days before infection onset, particularly in patients with comorbidities. Two patients with diabetes mellitus died a few days after simple toilet of foot cutaneous ulceration; another patient died after displacement of Kirschner's wires in ambulatory. Diabetes mellitus was the most representative comorbidity in our patients. Very high susceptibility to *SAS* in patients suffering from liver cirrhosis is reported [15]. By contrast, no cirrhotic patients were included in our group of patients; this may be very likely related to the occurrence of neutropenia in these patients (neutropenia was considered an exclusion criterion in our study design). We also noted the absence of predisposing factors in a high percentage of cases.

Several studies demonstrated a contribution of methicillin resistance to morbidity and mortality associated with bacteremia caused by *SA* [16-18]. In our group of patients, this was not verified: methicillin resistance, found in 36.8% of isolated *SA* strains was not associated with deleterious clinical outcome. The results of Harbarth and co-workers [19] agree with our data. These authors stated that methicillin resistance in patients with *SAS* had no significant impact on patients outcome as measured by in-hospital mortality after adjustment was made for major confounders as age, sex, lenght of stay from admission to the onset of bloodstream infection, number of comorbidities, severity of underlying illness. *In vitro*, susceptibility of the *SA* strains isolated in our group of patients is shown in *Tab. 2*. Different classes of antibiotics with different degrees of toxicity were listed, thus representing a quite wide range of therapeutic alternatives. As concerns the methicillin-sensitive *SA* strains, we found sensitivity for the beta-lactamine class in percentage ranging from 60% (ceftriaxone, cefazolin, amoxicillin plus clavulanic acid, cefipime) to 100% (piperacillin plus tazobactam).

Conclusions

SAS is an important and frequent disease that physician must often match in Internal Medicine Units. It affects also non-neutropenic patients with a high morbidity and mortality and frequently originates from community-acquired infections.

References

1. Boyce JM. Epidemiology and prevention of nosocomial infections. In: Crossley KB, Archer GL, editors. The staphylococci in human disease. New York: Churchill Livingstone, 1997; p. 309-29.

2. Franklin D. Staphylococcus aureus infections. N Engl J Med, 1998; 339(8): 520-32.

3. Sanabria TJ, Alpert JS, Goldberg R, Pape LA, Cheesman SH. Increasing frequency of staphylococcal infective endocarditis: experience at a university hospital, 1981 through 1988. Arch. Intern Med, 1990; 150: 1305-9.

4. Sandre RM, Shafran SD. Infective endocarditis: review of 135 cases over 9 years. Clin Infect Dis, 1996; 22: 276-86.

5. Libman H, Arbeit RD. Complications associated with Staphylococcus aureus bacteremia. Arch Intern Med, 1984; 144: 541-5.

6. Bone RC. Gram-positive organisms and sepsis. Arch Intern Med, 1994; 154: 26-34.

7. Petti Cathy A, Fowler Vance G Jr. Staphyococcus aureus bacteremia and endocarditis. Infect Dis Clin North Am, 2002; 16: 413-35.

8. American Thoracic Society. Hospital-acquired pneumonia in adults: diagnosis, assessment of severity, initial antimicrobial therapy, and preventive strategies. A consensus statement. Am J Respir Crit Care Med, 1996; 153: 1711-25.

9. National Nosocomial Infections Surveillance (NNIS) report, data summary from October 1986 – April 1996, issued May 1996. Am J Infect Control, 1996; 24: 380-8.

10. Edmond MB, Wallace SE, McClish DK, Pif MA, Jones RN, Wenzel RP. Nosocomial bloodstream infections in United States hospitals: a three-year analysis. Clin Infect Dis, 1999; 29: 239-44.

11. Muder RR, Brennen C, Wagener MM, Vickers RM, Rihs JD, Hancock GA, Yee YC, Miller JM, Yu VL. Methicillin-resistant staphylococcal colonization and infection in a long-term care facility. Ann Intern Med, 1991; 114: 107-12.

12. Archer GL, Climo MW. Stapylococcus aureus bacteremia – consider the source. N Engl J Med, 2001; 344(1): 55-6.

13. von Eiff C, Becker K, Machka K, Stammer H, Peters G. Nasal carriage as a sorce of Staphylococcus aureus bacteremia. N Engl J Med, 2001; 344: 11-6.

14. Wenzel RP, Edmond MB. The impact of hospital-acquired bloodstream infections. Emerg Infect Dis, 2001; 7(2): 174-7.

15. Campillo B, Dupeyron C, Richardet JP. Epidemiology of hospital-acquired infections in cirrhotic patients: effect of carriage of methicillin-resistant Staphylococcus aureus and influence of previous antibiotic therapy and norfloxacin prophylaxis. Epidemiol Infect, 2001; 127(3): 443-50.

16. Blot SI, Vandewoude KH, Hoste EA, Colardyn FA. Outcome and attributable mortalitality in critically ill patients with bacteremia involving methicillin-susceptible and methicillin-resistant Staphylococcus aureus. Arch Intern Med, 2003; 163: 739-40.

17. Cosgrove SE, Qi Y, Kaye KS, Harbarth S, Karchmer AW, Carmeli Y. The impact of methicillin resistance in Staphylococcus aureus bacteremia on patient outcomes: mortality, length of stay, and hospital charges. Infect Control Hosp Epidemiol, 2005; 26: 166-74.

18. Melzer M, Eykyn SJ, Gransden WR, Chinn S. Is methicillin-resistant Staphylococcus aureus more virulent than methicillin-susceptible S. aureus? A comparative cohort study of British patients with nosocomial infection and bacteremia. Clin Infect Dis, 2003; 37: 1453-60.

19. Harbarth S, Rutschmann O, Sudre P, Pittet D. Impact of methicillin resistance on the outcome of patients with bacteremia caused by Staphylococcus aureus. Arch Int Med, 1998; 158: 182-9.

The effect of green tea on the activity of aldehyde dehydrogenase (ALDH) in the liver of rats during chronic ethanol consumption

Chrostek L*, Tomaszewski W, Szmitkowski M

Department of Biochemical Diagnostics, Medical University of Białystok, Poland

Abstract

Purpose: **Alterations in the redox state during chronic ethanol consumption are associated with the oxidation of ethanol via alcohol and aldehyde dehydrogenase. Among various antioxidants present in food, strong antioxidative effects have been attributed to polyphenols of green tea. The aim of the present study was to investigate the effect of green tea consumption during chronic ethanol intake on the activity of aldehyde dehydrogenase in the liver of rats during maturation and aging.**

Materials and methods: **The activity of ALDH was measured in the livers of rats aged 2 (young), 12 (adult) and 24 months (old). The rats were fed with a control liquid Lieber DeCarli diet, control liquid diet containing green tea (3 g/l), ethanol liquid diet (with increasing ethanol dose from 2.3% to 7%) and ethanol liquid diet containing green tea.**

Results: **Chronic ethanol consumption significantly increased the liver ALDH activity in young and adult rats but decreased this activity in old animals. The drinking of green tea did not alter ALDH activity in ethanol-consuming rats. Drinking green tea alone significantly increased ALDH activity in young and adult rats but did not alter this activity in old rats.**

Conclusions: **These results demonstrate that green tea administered during chronic ethanol consumption does not prevents the changes in the hepatic ALDH activity in the rats at each age.**

* CORRESPONDING AUTHOR:
Department of Biochemical Diagnostics
Medical University of Białystok
ul. Waszyngtona 15A
15-276 Białystok, Poland
Tel: +48 85 74 68 587; Fax: +48 85 74 68 585
e-mail: chrostek@amb.edu.pl (Chrostek Lech)

Received 23.05.2005 Accepted 14.06.2005

Key words: green tea, ethanol, liver ALDH.

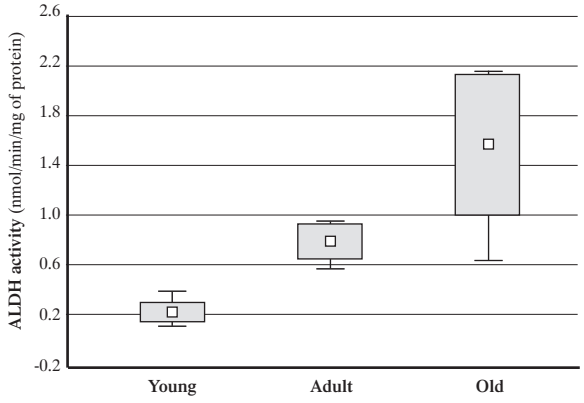
Introduction

Ethanol in mammals is oxidized in the liver to acetaldehyde by alcohol dehydrogenase (ADH), and then to acetate by aldehyde dehydrogenase (ALDH) [1]. In this oxidative metabolic pathway, an excess of reducing equivalents, such as NADH, are generated. Another system capable of carrying out ethanol oxidation is the microsomal ethanol oxidizing system (MEOS) [2]. The altered redox state in the cytosol and mitochondria, and the increase in free-radical production are considered responsible for the different metabolic disturbances following chronic ethanol consumption [1-3].

There are several factors that affect alcohol metabolism. These include food, alcohol abuse, drugs, gender, body weight, body composition and ethnicity. It is generally accepted that age has an effect on alcohol metabolism and that this effect is associated with the activities of alcohol metabolizing enzymes located in the liver and stomach [4-6].

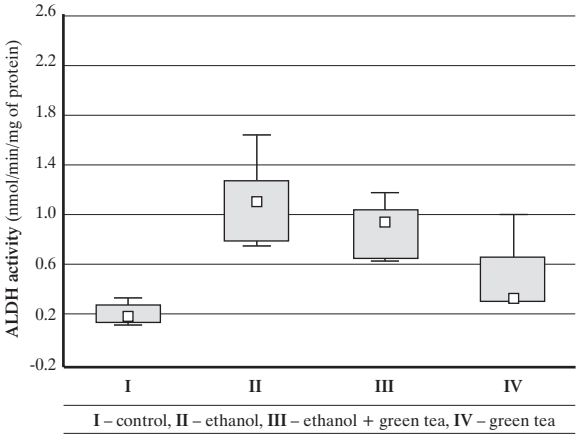
Recently, it has been shown that green tea exhibits antioxidative activity [7,8]. A potential mechanism(s) for such an effect involves radical-scavenging, metal chelating and/or enzyme modulation ability [9,10]. Several compounds of green tea, mainly catechins, have been reported to have a protective action. The molecular mechanisms underlying the effects of catechins in some cases involved inhibition or activation of enzymes. Among other things, it has been observed that green tea inhibits the induction of human cytochrome P450 [11], an enzyme that is induced following chronic ethanol consumption. The objective of the present study was to investigate the effect of green tea drinking together with ethanol on the hepatic activity of ALDH (enzyme involved in redox homeostasis) of rats during maturation and aging. The effect of green tea drinking was also determined separately.

Figure 1. **ALDH activity in the control group according to age of rats**



Squares represent the median values, the boxes represent the second and third interquartile range, and the whiskers represent the overall range

Figure 2. **ALDH activity in the liver of young rats drinking green tea with ethanol**



Squares represent the median values, the boxes represent the second and third interquartile range, and the whiskers represent the overall range

Material and methods

Male Wistar rats aged 2 (200-220 g b.w.), 12 (520-550 g b.w.) and 24 months (750-780 g b.w.) were used in the experiment. The dose of ethanol in this diet was gradually increased from 2.3% (day 1-3), to 4.7% (day 4-6), and to 7% (day 7-28).

The rats were housed in individual cages and pair-fed with either nutritionally control – a liquid Lieber DeCarli diet containing 47% of total energy as carbohydrate, 18% protein, 35% lipid or an identical diet with ethanol substituted isocalorically for carbohydrates (36% of total energy). Liquid diets (control and ethanol) containing 7 g green tea extract/l were also prepared (Camelia sinensis, O. Kuntze, lyophilized extract, TJ Lipton, Englewood Cliffs, NJ). The amount of catechins in this diet, measured by HPLC, was equivalent to the amount of these compounds in 3 g of green tea extract/l water solution, which is frequently the concentration used for human consumption [12]. The content of components in diet with green tea was as follows: epigallocatechin gallate – 337 mg/l, epigallocatechin – 268 mg/l, epicatechin – 90 mg/l, epicatechin gallate – 60 mg/l, and coffeic acid – 35 mg/l. The same age animals were divided into 4 groups (6 animals in each group). The total number of tested rats was 72. All procedures were in accordance with guides for care and use of laboratory animals, and the protocol was approved by the local Animal Care Committee in Białystok.

The control group was fed for 5 weeks a control Lieber DeCarli liquid diet (n=6). The ethanol group was fed for one week the control Lieber DeCarli liquid diet and for the next 4 weeks the ethanol liquid diet (n=6). The green tea group was fed for 5 weeks the control Lieber DeCarli liquid diet containing green tea (n=6). The ethanol and green tea group was fed for one week the control Lieber DeCarli liquid diet containing green tea and for the next 4 weeks the ethanol liquid diet also containing green tea (n=6). Dietary intake was comparable in all groups, with all rats demonstrating consistent weight gain throughout the 5-week feeding period.

The rats were sacrificed with ether anaesthesia, after which the livers were quickly removed and placed in ice-cold 0.15 M NaCl solution, perfused with the same solution to remove blood cells, blotted on filter paper. The organs were weighed and homogenized in 9 ml of 0.25 M sucrose. Homogenates (10%) were centrifuged at 10000 x g for 15 min at 4°C, and the supernatant was kept on ice until assayed.

Aldehyde dehydrogenase (ALDH) activity was measured using the fluorogenic method of Wierzchowski et al. [13] based on oxidation of 6-methoxy-2-naphthaldehyde to the fluorogenic 6-methoxy-2-naphthoate. As described Wierzchowski and co-workers, this naphthaldehyde is very good substrate for liver cytosolic ALDH-1 isoenzyme (class I) and for ALDH-3 isoenzyme (class III), which is not expressed in the liver. The reaction mixture contained 60 µl of 300 µM of 6-methoxy-2-naphthaldehyde, 20 µl of 1 mM of NAD, 2.8 ml of 50 mM Na-pyrophosphate buffer, pH 8.5 and 60 µl of homogenate. The fluorescence was read at an excitation wavelength of 310 nm and an emission wavelength of 360 nm.

Protein concentration was measured according to Lowry using bovine serum albumin as the standard (Sigma kits).

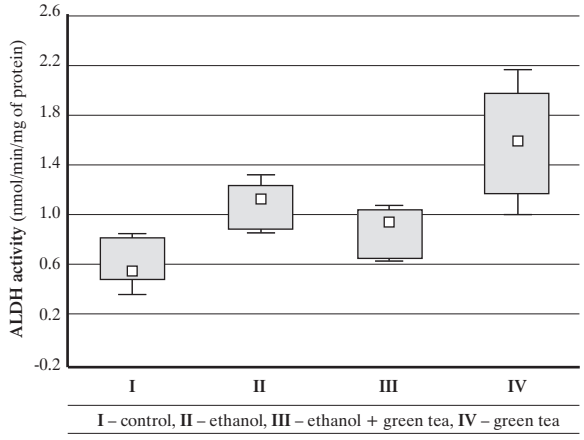
The results are expressed as median and ranges. Statistical analysis was performed using the Mann-Whitney U test. Differences were considered significant at $p<0.05$.

Results

Aldehyde dehydrogenase activity in the liver of rats fed on a control liquid diet increased parallel with the age of the animals, obtaining six-times higher value in old rats than in young (*Fig. 1*). The acitivity in adult rats was 3-times higher than in young.

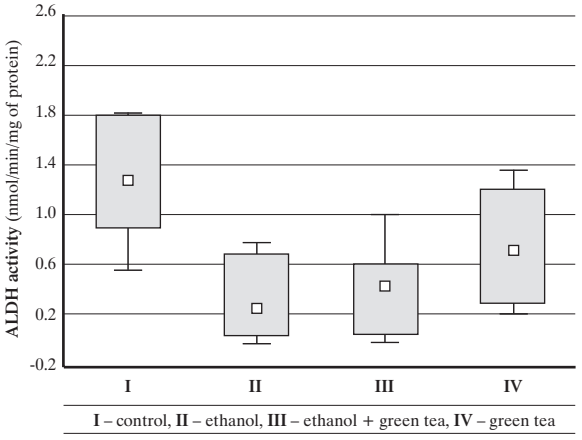
Chronic ethanol consumption caused a significant increase in ALDH activity in the liver of young and adult rats but lead to

Figure 3. **ALDH activity in the liver of adult rats drinking green tea with ethanol**



Squares represent the median values, the boxes represent the second and third interquartile range, and the whiskers represent the overall range

Figure 4. **ALDH activity in the liver of old rats drinking green tea with ethanol**



Squares represent the median values, the boxes represent the second and third interquartile range, and the whiskers represent the overall range

decrease in ALDH activity in old animals when compared to the control group ($p<0.05$) (*Fig. 2*, *3* and *4*). The drinking of ethanol with green tea by young, adult and old rats did not change the activity of ALDH in comparison with the ethanol alone group ($p>0.05$). Administration green tea alone to young and adult rats significantly increased ALDH activity when compared to the control group ($p<0.05$). Drinking green tea alone by old rats did not influence ALDH activity in comparison with the control group ($p>0.05$).

Discussion

The main findings of our study are that chronic ethanol consumption significantly increased the liver ALDH activity in young and adult rats and that drinking ethanol with green tea by rats at each age did not change this activity when compared to the ethanol alone group. These data concerning the effect of ethanol consumption on the activity of enzymes involved in ethanol metabolism are in conflict with some of the literature. Some previous reports have found an increase of ALDH activity in the rodent liver cytosol, mitochondria and microsomes following chronic administration of alcohol [14,15]. Previous works by Guerri et al. also reported a 2-fold increase in the liver ALDH of ethanol fed rats (for 7 week) [16]. As Vaananen et al. noted, even 12 weeks consumption of ethanol did not cause histological changes in the rat liver parenchyma [15]. However, despite the lack of parenchymal lesions they observed an increase in low K_m aldehyde dehydrogenase. In our study, the rats were fed with an ethanol liquid diet for 4 weeks and we did not observe changes in liver histology. In a more recent study, Vidal et al. reported that chronic alcohol abuse reduced ALDH activity (mainly low K_m form) in patients with alcoholic cirrhosis but did not depress ALDH activity in patients without alcoholic liver diseases [17]. It has been established that increases in the rate of ethanol metabolism require the contribution of ethanol metabolizing enzymes. However, on the basis of the results obtained by Lumeng et al., it could be suggested that a reduction in the rate of ethanol elimination in fasted rats may be caused by decreasing ADH activity [18]. The increase in ALDH activity after chronic ethanol drinking in the present study may be caused by the presence of a substrate for this enzyme: acetaldehyde. Recently, Badger et al., for the first time, demonstrated the existence of an ethanol-dependent induction of rat class I hepatic ADH [19]. Enhancing activity of liver class I ADH promotes the generation of acetaldehyde, which may result in an induction of ALDH activity.

Our data show that drinking green tea alone significantly increased ALDH activity in young and adult rats. The flavonoids of green tea have been found to affect (inhibition or activiation) activities of several enzymes, e.g. monooxygenase, lipoxygenases, cyclooxygenase, histydyne decarboxylase, cyclic AMP phosphodiesterase [20]. The increase of ALDH activity observed after green tea consumption may have been caused by the activation of one of these enzymes by the catechins in green tea. The other isoflvonoids isolated from plants that affect the alcohol pharmacokinetics and alcohol-drinking behaviour in rats are daidzin, daidzein and puerarin [21]. Daidzein is a reversible inhibitor of alcohol dehydrogenase [22], and daidzin of mitochondrial aldehyde dehydrogenase (class II ALDH) [23]. In our study we have estimated the activity of ALDH with the substrate for class I ALDH (cytosolic). However, none of the 3 isoflavonoids administrated orally affected liver alcohol dehydrogenase or aldehyde dehydrogenase activities, as it was reported for intraperitoneal administration [21].

When we demonstrated the stimulation of ALDH activity by the consumption of green tea, other authors showed the inhibition of cytochrome P450 2E1 activity under similar conditions [11]. Among the compounds in green tea, the most effective cytochrome P450 2E1 inhibitor was epigallocatechin gallate.

The activation of the MEOS system following chronic ethanol consumption generates free radicals. The green tea antioxidant potential has been attributed to a free radical scavenging effect. Thus, drinking green tea may cause the inhibition of one pathway (MEOS) and the activation of another pathway (ALDH) for hepatic ethanol elimination (our study). The induction of ALDH activity in the liver of rats drinking tea was observed in young and adult rats but not in the old animals. This discrepancy might been caused by differences in ALDH activity at various ages. In partial support of this hypothesis, the activity of ALDH in the old control rats was much higher than the activity in the young and adult animals. Taking into account that the acetaldehyde is the most toxic product generated during ethanol metabolism, these findings suggest that the older subjects have the better defence against the negative consequences of alcohol abuse than the younger subjects.

Conclusions

We conclude that the consumption of green tea following chronic ethanol administration did not prevents the changes in the hepatic activity of ALDH in the rats at each age.

References

1. Lieber CS. Ethanol metabolism, cirrhosis and alcoholism. Clin Chim Acta, 1997; 257: 59-84.
2. Riveros-Rosas H, Julian-Sanchez A, Pina E. Enzymology of ethanol and acetaldehyde metabolism in mammals. Arch Med Res, 1997; 28: 453-71.
3. Lieber CS. Hepatic and metabolic effects of ethanol: pathogenesis and prevention. Ann Med, 1994; 26: 325-30.
4. Seitz HK, Egerer G, Simanowski UA, Woldherr R, Eckey R, Agarwal DP, Goedde HW, von Wartburg J-P. Human gastric alcohol dehydrogenase activity: effect of age, sex, and alcoholism. Gut, 1993; 34: 1433-7.
5. Sjoblom M, Pilstrom L, Morland J. Activity of alcohol and acetaldehyde dehydrogenases in the liver and placenta during development of the rats. Enzyme, 1978; 23: 108-15.
6. Maly IP, Sasse D. Intraacinar profiles of alcohol dehydrogenase and aldehyde dehydrogenase activities in human liver. Gastroenterology, 1991; 101: 1716-23.
7. Mitcher LA, Jung M, Shankel D, Dou JH, Steele L, Pillai SP. Chemoprotection: a review of the potential therapeutic antioxidant properties of green tea (Camellia sinensis) and certain of its constituents. Med Res Rev, 1997; 17: 327-65.
8. Dreosti IE. Bioactive ingredients: antioxidants and polyphenols in tea. Nutr Rev, 1996; 54: S51-S8.
9. Rice-Evans CA, Miller NJ, Paganga G. Structure antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med, 1996; 20: 933-56.
10. Nanjo F, Mori M, Goto K, Hara Y. Radical scavenging activity of green tea catechins and their related compounds. Biosci Biotechnol Biochem, 1999; 63: 1621-3.
11. Obermeier MT, White RE, Yang CS. Effect of bioflavonoids on hepatic P450 activities. Xenobiotica, 1995; 25: 575-84.
12. Maiani G, Serafini M, Salucci M, Azzini E, Ferro-Luzzi A. Application of a new high-performance liquid chromatographic method for measuring selected polyphenols in human plasma. J Chromatogr B, 1997; 692: 311-7.
13. Wierzchowski J, Wroczynski P, Laszuk K, Interewicz E. Fluorometric detection of aldehyde dehydrogenase activity in human blood, saliva and organ biopsies, and kinetic differentiation between class I and II isozymes. Anal Biochem, 1997; 245: 69-78.
14. Kishimoto R, Fujiwara I, Kitayama S, Goda K, Nakate Y. Changes in hepatic enzyme activities related to ethanol metabolism in mice following chronic ethanol administration. J Nutr Sci Vitaminol, 1995; 41: 527-43.
15. Vaananen H, Salaspuro M, Lindros K. The effect of chronic ethanol ingestion on ethanol metabolizing enzymes in isolated periportal and perivenous rat hepatocytes. Hepatology, 1984; 4: 862-6.
16. Guerri C, Wallace R, Grisolia S. The influence of prolonged ethanol intake on the levels and turnover of alcohol and aldehyde dehydrogenases and of brain (Na+K)-ATPase of rats. Eur J Biochem, 1978; 86: 581-7.
17. Vidal F, Toda R, Gutierrez C, Broch M, Fernandez-Muixi F, Lorenzo A, Richart C. Influence of chronic alcohol abuse and liver disease on hepatic aldehyde dehydrogenase activity. Alcohol, 1997; 14: 1-6.
18. Lumeng L, Bosron WF, Li T-K. Quantitative correlation of ethanol elimination rates in vivo with liver alcohol dehydrogenase activities in fed, fasted and food-restricted rats. Biochem Pharmacol, 1979; 28: 1547-51.
19. Badger TM, Hoog JO, Svensson S, McGehee RE, Fang C, Ronis MJJ, Jugelman-Sundberg M. Cyclic expression of class I alcohol dehydrogenase in male rats treated with ethanol. Biochem Biophys Res Commun, 2000; 274: 684-8.
20. Beyeler S, Testa B, Perrissoud D. Flavonoids as inhibitor of rat liver monooxygenase activities. Biochem Pharmacol, 1988; 37: 1971-9.
21. Lin RC, Li T-K. Effects of isoflavones on alcohol pharmacokinetics and alcohol-drinking behavior in rats. Am J Clin Nutr, 1998; 68(Suppl.): 1512S-5S.
22. Keung WM. Biochemical studies of a new class alcohol dehydrogenase inhibitors from Radix puerariae. Alcohol Clin Exp Res, 1993; 17: 1254-60.
23. Keung WM, Klyosov AA, Vallee BL. Daidzin inhibits mitochondrial aldehyde dehydrogenase and suppresses ethanol intake of Syrian golden hamsters. Proc Natl Acad Sci USA, 1997; 94: 1675-9.

Effects of truncal or highly selective vagotomy on the electron microscopic feature of the rabbit pancreas

Głuszek S*, Matykiewicz J

Department of General, Oncological and Casualty Surgery, The Municipal Hospital in Kielce & Institute of Medical Education, Świętokrzyska Academy in Kielce, Poland

Abstract

Purpose: **To perform a comparative study of two types of vagotomy: trunctal vagotomy (TV) and highly selective vagotomy (HSV) effects on pancreatic morphology assessed with light microscope and ultrastructural changes assessed with electron microscope in rabbits 30, 90 and 180 days after the vagotomy.**

Material and methods: **The experiments were carried out on 89 male Popielno white rabbits, aged 4-6 months, 2.5 to 3.0 kg of body weight. On the 30th, 90th and 180th day after the vagotomy, rabbits were sacrificed and the pancreases were taken for the light and electron microscopic evaluation.**

Results: **The regressive changes coexisting with adaptive like renewal of epithelial cells and mild interstitial fibrosis resulting from vagotomy were more pronounced in the early post-operative period and tended to normalize in the later post-operative one. All the changes seen in post-operative period were more prominent after TV than after HSV.**

Conclusions: **Both truncal and highly selective vagotomy affects evidently the morphology and ultrastructure of the pancreas in rabbits however the changes after the first procedure were more advanced than after the latter one. The intensity of the changes is highest early after these operations and tend to normalize in the later post-operative period.**

Key words: highly selective vagotomy, truncal vagotomy, pancreas morphology, pancreas ultrastructure, rabbit.

* CORRESPONDING AUTHOR:
ul. Loefflera 17
25-715 Kielce, Poland
Tel: +48 602 715 889
e-mail: sgluszek@neostrada.pl (Stanisław Głuszek)

Received 24.06.2005 Accepted 27.06.2005

Introduction

The vagal cholinergic pathways play an important role in neurohormonal regulation of pancreatic function. The morphological changes of the pancreas, as a result of vagotomy, for surgical treatment of peptic ulcer have not been adequately investigated.

A decreased size of pancreatic acini and the edema of beta islet cells occurring within the first weeks following total vagotomy with pylorotomy in rats have been described [1]. Lorenz et al. [2] observed a decreased size of pancreatic acini and increased number of alpha cells of the islets 5 weeks after total vagotomy, whereas 3 weeks after operation their number may be significantly reduced in rats.

According to Staszyc and Królikowska-Prasał [3], vagotomy adversely affects the metabolic processes in the pancreas, which manifests with abnormal enzymatic activity. Islet cells were found to regenerate earlier than acinar cells, as it was supported by histochemical examination. Moreover, during the early post-operative period the truncal vagotomy was found to influence the neurogenic secretion. Following truncal vagotomy, the number of zymogen granules was initially decreasing, but subsequently increased in the rats.

Our own experiments performed in dogs, evaluating the morphological and histochemical changes in the pancreas resulting from different types of vagotomy: truncal, and highly selective, showed that the degree of the abnormalities was highest during the early post-operative period. In later periods, the severity of morphological and histochemical changes decreased. The most significant structural abnormalities were observed after truncal vagotomy [4].

In the available literature we could find neither an ultrastructural evaluation of the pancreas following different types of vagotomy, nor the assessment of its influence on the endocrine and exocrine function of this organ. The observations of the neurological changes in the pancreas, as a result of vagotomy, are not consistent. Radke and Stach [5-7] did not find any ultrastructural changes in the pancreatic neurons, which

Table 1. **Experimental and control groups of rabbits**

Type of vagotomy	Duration of the experiment (days)		
	30	90	180
	Number of animals		
Highly selective vagotomy	11	13	11
Total vagotomy	7	12	10
Control I (sham operation)	3	3	3
Control II (no operation)	7	5	4

is inconsistent with the findings of Shashirina [8]. The trophic effect of truncal vagotomy on the rat pancreas, manifesting itself by an increased weight of the organ, was described by Koop et al. [9], Tiscornia et al. [10] and Büchler at al. [11].

The gastrointestinal complications of vagotomy, known from the clinical practice, may result from total or partial parasympathetic denervation. Most of gastrointestinal complications are well known to develop directly after the surgery, and to be of transient nature [3,12-14]. Evaluation of such complications in humans is difficult due to the lack of possibility of morphological, histochemical and ultrastructural assessment in the postoperative period. Therefore, reasonable animal experiments could be helpful to explain the pancreatic complications of the vagotomy. The results of such experiments should be applied with caution in the clinical practice, however, they could bring some clinical implications, allowing the appropriate therapeutic or prophylactic intervention.

Considering the different extent and selectivity of parasympathetic denervation of the stomach and as the consequence of the pancreas after different types of vagotomy the morphological alterations of the pancreas could be less or more advanced

Therefore the aim of the study was to perform a comparative assessment of the influence of different types of vagotomy: truncal vagotomy (TV) and highly selective vagotomy (HSV) on the morphology and ultrastructure of the rabbit pancreas after 30, 90 and 180 days following truncal vagotomy and highly selective vagotomy.

Material and methods

The experiments were carried out on 89 male Popielno white rabbits, aged 4-6 months, with body weight varying from 2.5 to 3.0 kg. The animals were housed in standard laboratory conditions (room temperature, daily light, natural food and tap water ad libitum). The care was provided according to current guidelines for the use of laboratory animals and experiment was performed according to the updated Helsinki Declaration. The study received an approval of local bioethics commission. The animals were allocated to 2 experimental and 2 control groups, depending on the type of vagotomy (*Tab. 1*).

The rabbits were fasted overnight before experiment with free access to tap water. Atropine sulphate was administered subcutaneously in the dose of 0.1 mg per kilogram of body weight. The general anaesthesia was induced by an open-method inhalation with purified ethyl ether. Central superior laparotomy was then performed. In the animals from the control group I (sham operation) laparotomy was performed and a loose catgut ligature was placed on the trunk of the vagus nerve, while no surgery was performed in the control group II.

Total vagotomy was performed by cutting the vagus nerve approximately 0.5 cm below the diaphragm. To perform the highly selective vagotomy after dissection and ligation of blood vessels, the laminae of the lesser omentum were cut approximately 0.5 cm to the lesser curvature of the stomach, with a cut leading from the oesophagus towards the pylorus, reaching the border between the body and the pyloric cavity. Next the small branches leading from the lesser curvature of the stomach to the anterior and posterior groups of the branches of the vagus nerve were cut. This technique saved the branches supplying the celiac plexus, the liver and the pancreas. The abdominal cavity was then closed with two layers of dexon “0” interrupted noose sutures.

The animals were sacrificed on day 30th, day 90th or day 180th after the surgery, and immediate laparotomy was performed with dissection of the pancreas for morphological examination. The weight of the organ was also examined.

Light microscopy

The specimens for morphological examination in the light microscopy were taken from the left and the right portion of each pancreas. The material was fixed for 15 days in a 10% neutral formaldehyde solution; then dehydrated and embedded in the paraffin. The paraffin slices were stained with hematoxylin and eosin (H&E)

Electron microscopy

Following the truncal and highly selective vagotomy in rabbits, specimens for electron microscopy were collected from two random animals in each experimental group (a total of 18 experimental + 4 control specimens).

Small specimens of the rabbit pancreases were stabilized at room temperature, in a 4% glutaraldehyde solution in 0.1 M cacodylate buffer at pH=7.4, for six hours. Scraps were then rinsed for 12 hours in 0.1 M cacodylate buffer at pH=7.4; the buffer solution was changed three times during this process. Subsequently, the preparations were additionally stabilized in a 1% OsO_4 water solution in 0.1 M cacodylate buffer at pH=7.4, for two hours, at the temperature of 4°C. The preparations were then dehydrated in a series of ethanol solutions of increasing concentration (30%, 50%, 70%, 90%, 96%, 99.8%), a mixture of propylene oxide and Spurr resin, a series of volume proportions 1:2, 1:1, 2:1, and twice in pure propylene oxide. Preparations were then covered with Spurr Low Viscosity resin.

Contrast-enhancement of ultra-thin preparations in 8% uranyl acetate solution for 45 minutes and then for ten minutes in plumbum citrate solution, according to Raynolds, was applied. Semi-thin slices, 0.75 μm, were stained in a 1% methylene blue with 1% Azur II solution in 1% water solution of borax. The preparations were evaluated and photographic documentation was made using a TESLA BS-500 electron microscope.

Statistical analysis

The results are descriptive, therefore the statistical analysis is not applicable.

Figure 1. **EM – in acinar cells of the rabbit pancreas, 30 days after TV, we can notice slight widening of smooth and rough endosplasmatic reticulum channels, single myelin figures (arrow) and phagosomes (F). Zymogen granules (Z) have normal ultrastructural feature. Ultimate magnification: x18 000**

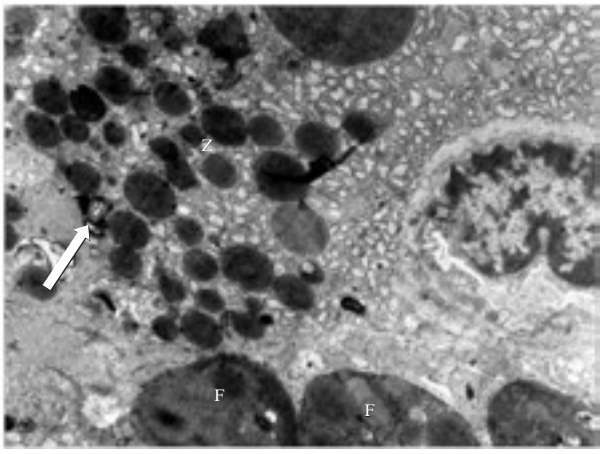
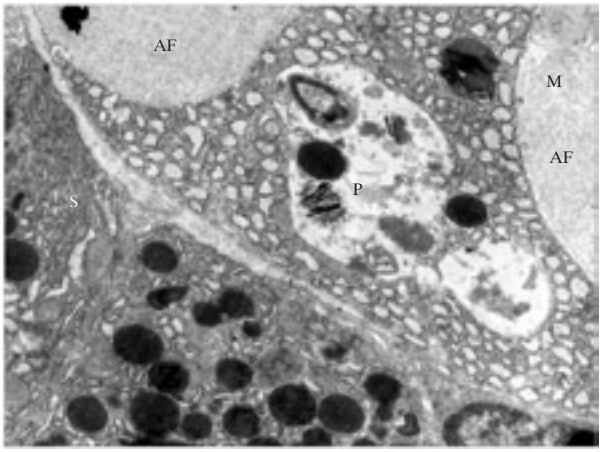


Figure 2. **EM – in one of the cells of the exocrine rabbit pancreas, 90 days after TV, the numerous autophagy vacuoles (AF) containing the material of moderate free-electron density, cell organelles, paracrystalline structures (P) and myelin figures (M) could be seen. In remaining cells there are numerous zymogen granules (Z), and in all cells an abundant rough endosplasmatic reticulum (S) with slight focal broadening of the channels could be noticed. Ultimate magnification: x14 000**



Results

Histological changes

Light microscopy findings comprised foci of dyschylic edema observed in individual rabbits. Another finding was foci of fibrosis within the parenchyma of the pancreas. It needs to be emphasized that – similarly to light microscopic findings – dyschylic edema was found more frequently at earlier post-operative stages. The degree of the phenomenon in the early post-operative period was not very severe. Focal fibrosis of various severity occurred occasionally, similarly to the microscopic findings described above.

Ultrastructure of the rabbit pancreas after the truncal vagotomy (TV)

Thirty days after truncal vagotomy the enlargement of the intercellular spaces, mainly in the exocrine portion of the pancreas, was observed. Within the cytoplasm of the exocrine cells the proliferation and enlargement of tubules of the smooth-surfaced endoplasmic reticulum, mitochondrial edema, numerous myelin figures and autophagic vacuoles were found. Occasionally, myelin figures were found within the enlarged intercellular space, and sometimes within the concavities of the nucleus, filled with cytoplasm. The number and the electron density of zymogen granules varied. Occasionally the degradation resulting in the occurrence of myelin figures in the affected granules could be observed (*Fig. 1*). Besides this ultrastructural features of extensive protein synthesis were observed in numerous cells, including enlargement of the rough-surfaced endoplasmic reticulum tubules, filled with fine fibrous contents and a conspicuous Golgi apparatus. Within the endocrine component of the gland, the ultrastructural findings involved mainly the increased number of granules, observed most frequently in type B cells. Numerous fibroblasts surrounding the blood capillaries were observed, accompanied by fine fibrous matter and collagen fibres.

Ninety days after TV the alterations in the exocrine portion of the pancreas resembled those observed 30 days following the surgery, but were less severe. Large autophagic vacuoles were more frequent, containing cell fragments, crystal-like structures and numerous, well-developed tubules of the rough-surfaced endoplasmic reticulum (*Fig. 2*). In some endocrine cells, mostly type B islet cells, a focal degeneration of the granules was seen. Within the intercellular space, especially in the vicinity of blood vessels, the bundles of collagen fibres, active fibroblasts and macrophages were found. Occasionally, loose cell fragments were detected within the intercellular space, which suggests damage to the cell membranes.

One hundred eighty days after TV a variable, usually big number of zymogen granules in the cells of the exocrine portion of the pancreas and ultrastructural features of extensive protein synthesis were observed. Some cells contained numerous autophagic vacuoles and only few zymogen granules. Sporadically, signs of colliquative necrosis was observed in the exocrine cells (*Fig. 3*). Increased number of granules was observed in type A, B and D endocrine cells, but especially in B cells the granules were markedly condensed and contracted. These changes were accompanied by a marked mitochondrial edema. However, in most cases, the endocrine cells of the pancreatic islets showed a normal ultrastructural pattern. Intercellular spaces, especially the pericapillary area, often contained active fibroblasts and bundles of collagen fibres.

Ultrastructure of the rabbit pancreas following highly selective vagotomy (HSV)

Thirty days after HSV a variable number of zymogen granules of various electron densities was observed in the cells of the exocrine portion of the pancreas. Enlargement of the

Figure 3. **EM – focal colliquative necrosis of pancreatic acinar cells after 180 days from TV. Numerous autophagy vacuoles (AF) are visible. Ultimate magnification: x15 000**

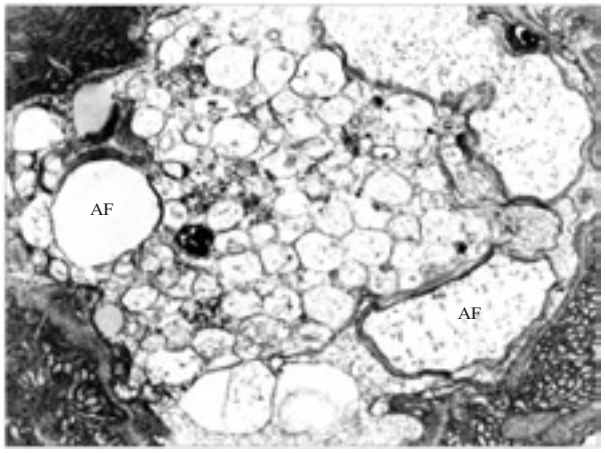


Figure 5. **EM – marked increase of the number of zymogen granules 90 days after HSV. Visible swelling of mitochondria (arrows). Ultimate magnification: x14 000**

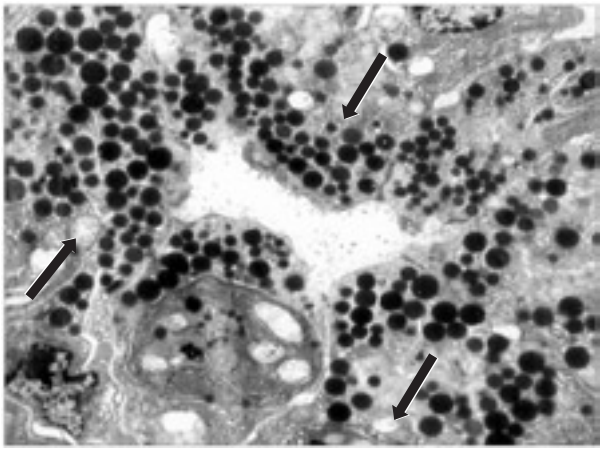
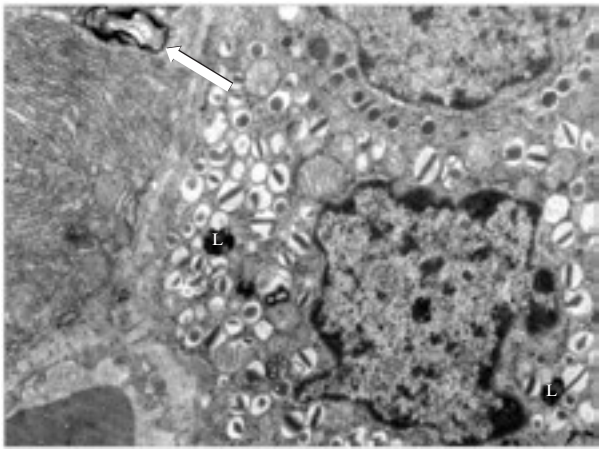


Figure 4. **EM – in beta cell of the rabbit pancreas, 30 days after HSV, shrunk endocrine granules and lysosomes (L) can be noticed. In exocrine pancreas there is a myelin figure (arrow) and abundant rough endosplasmatic reticulum. Ultimate magnification: x16 000**



rough-surfaced endoplasmic reticulum, filled with fine fibrous contents, was a common finding. Moreover, in the cytoplasm of those cells autophagic vacuoles were observed, containing membranous convolutions resembling myelin figures, fragments of organelle and fine granular material. In the endocrine cells, especially type B cells, condensed and contracted granules as well as individual myelin figures were observed (*Fig. 4*). No fibrosis was detected in the evaluated specimens.

Ninety days after HSV the alterations resembled those described in the previous group, but they were significantly less severe. Autophagic vacuoles were occasionally seen in the cytoplasm of the cells of the exocrine portion of the pancreas. Myelin figures could be also observed in the enlarged intercellular space. Well-developed rough-surfaced endoplasmic reticulum, filled with fine fibrous material was seen in many cells of the exocrine portion of the organ. The number and the electron density of the zymogen granules varied. In certain cells, especially those equipped with granular endoplasmic reticulum, zymogen granules were not seen at all, whereas in other cells the number of such granules was moderate or marked (*Fig. 5*). In the endocrine cells, especially type B cells, condensed and contracted granules as well as myelin figures, secondary lysosomes and autophagic vacuoles were found. Chaotically distributed collagen fibres and fragments of fibroblasts were seen in the intercellular space and the interstitium, especially in the vicinity of blood capillaries.

One hundred eighty days after HSV large autophagic vacuoles and myelin figures were only occasionally observed in the cytoplasm of the exocrine portion cells. Individual exocrine cells containing condensed and fragmented nuclear chromatin as well as dark cytoplasm, which might suggest apoptotic processes, were found. Cells with well-developed rough-surfaced endoplasmic reticulum, containing fine fibrous matter in ergastoplastic cisterns, were a frequent finding. Numerous pores of the nuclear membrane were observed. The number of zymogen granules, their shape and density varied. Exceptionally alpha (glycogen) granules were seen in the cytoplasm of the cells of the endocrine part of the pancreas. Some cells, mostly type B islet cells, contained contracted granules and numerous secondary lysosomes.

Discussion

Vagotomy, a conservative surgical procedure used for the treatment of peptic ulcer, remains the subject of experimental research, aimed at the explanation of the enzymatic and hormonal relations and structural changes within the gastrointestinal tract, especially the pancreas, as possible consequences of the surgery. Disturbances concerning innervation of the pancreas and blood supply to the organ result in trophic changes [9,11]. Radke and Stach [5-7] did not find any ultrastructural changes in axons and organelle of the sympathetic and parasympathetic neurons

(vesicles, mitochondria, microtubules) at 14 days and 5 months after truncal vagotomy in dogs. However, Shashirina [8], while evaluating the morphological changes in the neural system of the pancreas of the guinea pig after vagotomy, described chromatolysis and edema in most neurons evaluated 7 days after the surgery and increased number of atrophic cells 14 days after operation. The size of neurons, found to be increased 7 days after surgery, decreased again on day 14th after vagotomy. Similar changes were observed in the nucleus. Sixty days after surgery morphometric indices of neurons were comparable to those observed in the control group. According to this author three post-operative stages can be distinguished for truncal vagotomy:

1. alterations related to disturbed functioning of the cell (7 days after the surgery),
2. destructive changes (approx. 14 days after the surgery),
3. compensatory changes and recovery (60 days after the surgery) [8].

Both Büchler et al. [11] in rats after truncal vagotomy accompanied by pyloroplasty and Koop et al. [9] in rats after truncal vagotomy, observed increased weight of the pancreas. However, they did not find any morphological differences in the exocrine pancreas before and after the operation. The concentrations of DNA and trypsin in homogenized pancreas were increased, but the activity of lipase was found to be decreased in both experiments. Similarly, Tiscornia et al. [10] observed a trophic effect of truncal vagotomy in rats on the exocrine part of the pancreas, accompanied by an increased activity of lipase and unchanged activity of other enzymes. This may prove that the pancreas is controlled by numerous secretory stimuli, which equilibrate abnormal secretion in the early post-operative period. In the early post-operative period (up to 5 months) vagotomy affects also the systems responsible for controlling of the glucose concentration in the blood. The vagus nerve, by stimulating the secretion of insulin, inhibits glycogenolysis.

Koop et al. [9] in their morphometric assessment of the endocrine component of the pancreas of rats after truncal vagotomy, described a relative decrease of the size of this portion in favor of the exocrine portion. The total number of endocrine cells remained unchanged. It suggests a trophic effect of truncal vagotomy on the exocrine, but not on the endocrine portion of the pancreas. In rats, the number of alpha islet cells was found significantly reduced at 3 weeks after truncal vagotomy, while this number was increased at 5 weeks after the surgery. The alpha/beta cell ratio was 1:2 in control animals, 1:5 in experimental animals in 1st week after the surgery, and 1:1 in the 3rd week after the surgery [2]. Truncal vagotomy, apart from its direct effect on the gland cells, affects them by influencing the blood supply, as the pancreatic interstitium – the stroma of the organ – responds to vagotomy with alterations of its enzymatic activity, similarly to the gland tissue.

Available literature indicates on gastrin and the vagus nerve as the two factors bearing most responsibility for the integrity of the mucous membrane of the stomach [15,16]. The function of endocrine cells in the gastrointestinal tract also remains under control of gastrin and the vagus nerve [6,17].

The light and electron microscopy of the peptic cells in patients before and one year after highly selective vagotomy did not show any significant differences within those cells [18].

No ultrastructural alterations were found in the neurons of the submucous ganglions of the intestine of the pig on day 1 after truncal vagotomy. From 3rd day to 5th day after the operation some presynaptic axons of the submucous neurons showed various degree of degeneration. The signs of degeneration involved mostly edema, mitochondrial vacuolization with broken mitochondrial crests, as well as clustering of synaptic vesicles. The 7th day after operation, further degeneration of terminal axons was found. On the 10th day following the surgery the described changes reached their maximal severity, and subsided 30 days after vagotomy. No differences were observed in the number of the axons between day 10th and day 30th after operation. These findings were more frequent as a result of left as compared to right vagotomy.

Huchtebrock et al. [19] observed signs of degeneration of internal pancreatic nerves after truncal vagotomy, resection of the solar and the superior mesenteric ganglions, and a combination of the two procedures. Sixty days after the surgery the hypertrophy of nerve fibers was observed, however, the integrity of the internal pancreatic ganglions remained unaffected. The concentrations of substance P (SP) and neuropeptide Y (NPY) decreased significantly after the denervation, despite the fact that the peptidergic nerves had not been damaged neither by the truncal vagotomy, nor the ganglionectomy, nor the combination of the two procedures. The canine pancreas should be thought to posses peptidergic innervation, independent, except for SP and NPY, of the integrity of the extrapancreatic nerves [19].

Our own studies in dogs did not reveal any significant changes of the pancreas weight as a consequence of truncal and selective vagotomy, compared to control animals [11]. However, in the group of rabbits, 180 days after truncal and highly selective vagotomy, the weight of the organ was significantly increased [20,21]. Our own studies, similarly to other authors [6,11,12], may prove that functional, but not morphological changes of the pancreas are the most significant consequences of vagotomy in the early post-operative period.

The above described electron microscopy findings in rabbits, as well as light microscopy, indicate that:

1. The changes resulting from vagotomy are more pronounced in the early post-operative period.
2. The changes resulting from vagotomy tend to normalize in the later post-operative period.
3. The effects could be mainly of functional nature, including, among others, increased protein synthesis as an electron microscopy finding.
4. Regressive changes coexist with adaptive changes, renewal of epithelial cells and mild interstitial fibrosis; those alterations were seen in all post-operative stages, and were more prominent after TV than after HSV.
5. The evolution of the alterations in the pancreas, evaluated by means of light and electron microscopy, correlates with the neurological complications of vagotomy in the pancreas, described by Shashirina [8].

References

1. Wesołowski H, Snop S. Histochemical studies on the Albino rat pancreas in different periods following vagotomy and simultaneous pyloromyotomy. Folia Histochem Cytochem, 1975; 13: 85-92.
2. Lorenz D, Dorn A, Petermann J, Reding R. Histologische und

histochemische Untersuchungen an den Langerhansschen Insel und am exocrinen Pancreasgewebe von Ratten vor und nach trunkularer Vagotomie. Acta Histochem, 1972; 43: 321-2.

3. Staszyc J, Królikowska-Prasał J. Badania cytochemiczne trzustki po chirurgicznym przecięciu nerwów błędnych. Ann Univ M Curie-Skłodowska sec D Lublin, 1970; 25: 127-40.

4. Głuszek S, Matusiewicz J. Morphology of canine pancreas in electron microscopy following truncal and highly selective vagotomy. Mat Med Pol, 1993; 1: 27-31.

5. Radke R, Stach W. Innervation of the canine pancreas after vagotomy. Acta Anat, 1986; 127: 88-92.

6. Radke R, Stach W. Do B cells periinsular acinar cells of canine pancreas have nerves in common. Acta Anat, 1985; 127: 65-8.

7. Radke R, Stach W. Are the islets of Langerhans neuro-paraneural control axis of the exocrine pancreas. Arch Histol Jpn, 1986; 49: 414-20.

8. Shaschirina MI. Responses of neurons of intramural ganglia of the guinea pig pancreas to distruption of their connectione with CNS. Arkh Anat Gistol Embriol, 1985; 91: 11-5.

9. Koop H, Schwarting H, Trautmann M, Borger HW, Lankisch PG, Arnold Creutzfeldt W. Trophic effect of truncal vagotomy on the rat pancreas. Digestion, 1985; 33: 198-205.

10. Tiscornia DM, Perec CJ, De Lehmann E, Caro L, De Paula Baratti Martinez JL, Dreiling DA. Chronic truncal vagotomy, its effects on weight and the function of the rats pancreas. Mount Sinai J Med, 1981; 48: 295-304.

11. Büchler M, Malfertheiner P, Friess H, Nustede R, Feurle GE, Beger HG. Cholecystokinin influences pancreatic trophism following total gastrectomy in rats. Int J Pancreatol, 1989; 6: 261-71.

12. Hahn VM, Duben W, Menne HJ, Otten G. Die Auswirkungen der trunkularen Vagotomie auf Enzymaktivitaten in Magen, Pankreas und Leber. Zbl Chirurgie, 1984; 109: 199-206.

13. Hahn V, Dorsche H, Schwesinger G, Fischer U, Nowak W, Wolter S. Histotopochemische und elektronemikroskopische untersuchungen am Pankreas von Hunden nach Denervierung des Pankreas. Acta Histochem, 1982; 71: 119-32.

14. Van Hee R, Verhulst D, Van Schill P. Pancreatic function after seromyotomy on the canine stomach. Eur Surg Res, 1988; 20: 59-65.

15. Konturek SJ, Bilski J,Hladij M, Krzyzek E, Cai Ren Z, Schally AV. Role of cholecystokinin, gastrin and gastrin releasing peptide in the regulation of pancreatic secretion in cats. Digestion, 1991; 49: 97-105.

16. Konturek SJ, Dembiński A, Warzecki Z, Jaworek J, Konturek PK, Cai Ren Z, Schally A. Antagonism of receptions for bombesin, gastrin and cholecystokinin in pancreatic and growth. Digestion, 1991; 48: 89-97.

17. Axelson J, Ekelund M, Hakanson R, Sundler F. Gastrin and the vagus interact in the trophic control of the rat oxyntic mucosa. Regul Pept, 1988; 22: 237-43.

18. Aase S, Roland M. Light and electron microscopical studies of parietal cells before and one year after proximal gastric vagotomy in duodenal ulcer patients. Scand J Gastroenterol, 1977; 12: 417-20.

19. Huchtebrock HJ, Niebel W, Singer MV, Forssmann WG. Intrinsic pancreatic nerves after mechanical denervation of the extrinsic pancreatic nerves in dogs. Pancreas, 1991; 6: 1-8.

20. Głuszek S, Kwiecień K, Książka T. Experimental studies on interdependences between gastrin and selected hormones and enzymes in blood serum and on histoenzymatic alterations in pancreatic tissues after vagotomy. Digestive Surgery Stomach, Monduzzi Editore, ed. M Montorsi, P Granelli, Monduzzi Editore Bologne, 1988: 125-9.

21. Głuszek S, Kwiecień K, Książka T. Experimental studies on hormonal changes following various types of vagotomy. Digestive Surgery Stomach ed. M Montorsi, P Granelli, Monduzzi Editore Bologne, 1988: 118-23.

The cumulative effect of nuclear factor-κB (NF-κB) inhibition and endothelins in early cerulein-induced acute pancreatitis in rats

Długosz JW[1*], Andrzejewska A[2], Nowak K[1,3], Wróblewski E[1], Dąbrowski A[1]

[1] Department of Gastroenterology and Internal Medicine, Medical University of Białystok, Białystok, Poland
[2] Department of Medical Pathomorphology, Medical University of Białystok, Białystok, Poland
[3] Department of Invasive Cardiology, Medical University of Białystok, Białystok, Poland

Abstract

Purpose: To assess effects of NF-κB activation inhibitor (pyrrolidine dithiocarbamate – PDTC) alone or with endothelins (ET-1, ET-2, ET-3) in early course of cerulein -induced acute pancreatitis (AP) in rats.

Material and methods: After 4 h of AP in Wistar rats, treated with PDTC 10 or 40 mg/kg or with PDTC 10 mg/kg and ET-1, ET-2 or ET-3, 0.5 or 1.0 nmol/kg twice i.p. in 1 h interval, free active trypsin (FAT), total potential trypsin (TPT) and lipase in 12 000 x g supernatants of pancreatic homogenates, plasma α-amylase and histological changes were assayed. %FAT/TPT was an index of trypsinogen activation.

Results: %FAT/TPT significantly increased to 12.42± 2.14%, lipase to 5.51±0.84 U/mg protein and α-amylase to 28.5±5.61 U/mL in AP vs 1.96±0.31%, 1.29±0.11 U/mg and 5.80±1.38 U/ml in healthy control. Higher dose PDTC attenuated trypsinogen activation to 3.01±0.53% and α-amylase to 15.3±1.38. PDTC and ET-1 attenuated %FAT/TPT to 2.55±0.18% with lower and 2.34±0.44% with higher dose. ET-3 was less effective than ET-1: 6.76±0.46% with lower dose. Lower doses of ET-1 and ET-2 with PDTC, diminished lipase activity to 2.60±0.36 and 2.94±0.33.

Conclusions: Cumulative attenuation of trypsinogen activation after lower dose of PDTC and ET-1 approximated the effect of higher dose of PDTC. Additional effect of ET-3 was weaker than ET-1, and ET-2 was ineffective in this respect. The combination of this NF-κB activation inhibitor and ET-1 could be beneficial in early course of edematous AP by attenuating of trypsinogen activation. However, it should be treated with caution because of some unfavorable effects on histological scores of pancreatic injury.

Key words: cerulein acute pancreatitis, NF-κB, pyrrolidine dithiocarbamate, ET-1, ET-2, ET-3, trypsinogen, rats.

Introduction

Both, premature activation of trypsinogen and an impairment of pancreatic microcirculation are thought to play an important role in the pathogenesis of acute pancreatitis (AP) [1,2]. In recent years a pivotal role of transcriptional nuclear factor-κB (NF-κB) activation in the onset of acute pancreatitis with consecutive expression of inflammatory mediators has been postulated [3,4]. Rapid activation of NF-κB in acute taurocholate pancreatitis in rats, accompanied by an increase of TNF-α gene expression and its inhibition by blocking free radicals formation has been observed [5]. A direct activation of NF-κB within the pancreas increased the infiltration of neutrophils to the pancreas and caused extensive damage to the acinar cells [6]. On the contrary, the inhibition of NF-κB activation with antioxidants: pyrrolidine dithiocarbamate or resveratrol improved the course of taurocholate pancreatitis in rats [7,8].

In mild, cerulein (cholecystokinin analog)-induced AP, the NF-κB activation was correlated with the degradation of its inhibitor IκBα and followed by a rapid appearance of NF-κ B/Rel binding activity [9]. In NF-κB deficient mice, the cerulein-induced pancreatitis was attenuated as evidenced by the reduction of oxidative stress and enzymatic markers [10]. Selective inhibition of NF-κB binding activity or its nuclear import and unspecific inhibition of NF-κB activation, mostly by the reduction of lipid peroxidation, also ameliorated the tissue injury in cerulein-induced pancreatitis [11-14]. There are essential controversies on the relationship between NF-κB

* CORRESPONDING AUTHOR:
Department of Gastroenterology and Internal Medicine
Medical University of Białystok,
ul. M. Skłodowskiej-Curie 24a
15-276 Białystok, Poland
Tel: + 48 85 7468234; Fax: + 48 85 7468506
e-mail: ariosto@amb.edu.pl (Jan W. Długosz)

Received 08.07.2005 Accepted 13.07.2005

and trypsinogen activation in cerulein- or cholecystokinin (CCK)-induced AP. Some authors [15,16] are of opinion, that NF-κB and trypsinogen activation, although temporally closely related, are independent events, whereas others suggest, that intracellular activation of trypsinogen may contribute to NF-κB activation [17].

In severe, necrotizing AP, a pancreatic capillary flow decreased by half during 6h, whereas in mild, cerulein-induced AP it increased almost twice after 3h and remained elevated throughout 6h experiment [18]. The role of endothelins (ETs), an endothelium-derived potent vasoactive peptides, in AP remains not fully elucidated. ETs family consists of three isoforms: ET-1, ET-2 and ET-3. ET-1 is a ligand of two receptors: ET_A – responsible for vasoconstriction and ET_B – linked mostly to vasodilation [19,20]. All forms of endothelins reduced significantly pancreatic blood flow in healthy dogs and rats, however ET-1 was much more effective than ET-2 or ET-3 on a molecular basis [21,22]. Some authors reported a beneficial effect of ET_A receptor blockade and harmful effect of exogenous ET-1 [23,24]. On the contrary, Kogire et al. [25] found a protective effect of ET-1 in cerulein-induced AP on histological changes and a detrimental effect of selective ET_A receptor antagonist in this model of AP. In our own study, both selective ET_A receptor antagonist and nonselective $ET_{A/B}$ antagonist do not exert any positive effects on early course of cerulein-induced AP and even some undesired effects on pancreatic histological changes were noted [26]. On the other side, all forms of endothelins (ET-1, ET-2 and ET-3) decreased inflammatory cell infiltration and attenuated trypsinogen activation in the pancreas in this model of AP [27].

Above data could suggest some positive effects of combined NF-κB inhibition and endothelins in early course of cerulein--induced AP. Therefore, the purpose of present study was to assess the cumulative effect of NF-κB activation inhibitor – pyrrolidine dithiocarbamate (PDTC) and exogenous endothelins (ET-1, ET-2 and ET-3) on trypsinogen activation, enzymatic (pancreatic lipase and plasma α-amylase) and histological changes in early course (4h) of cerulein-induced acute pancreatitis in rats.

Material and methods

Animals

The experiments were carried out on 65 male Wistar rats, weighing 240-300 g, housed individually in wire bottomed cages in a room temperature of 21±1°C using a 12 hours light – dark cycle. The animals were given a standard rat chow diet and fasted overnight before the experiment with free access to water. The care was provided in accordance with the current procedures for the care and use of laboratory animals. The protocol has been approved by the local Bioethical Commission.

Induction of acute pancreatitis

Acute cerulein pancreatitis was induced according to the method of Yamaguchi et al. [28]. The rats were injected i.p. with cerulein (Sigma Chemical Co., St. Louis, MO, U.S.A.) at a dose of 40 μg/kg of body weight (b.w.) twice in 1 hour interval. In control rats, only solvent of cerulein (0.9% NaCl) was given i.p. In the treated rats, the solution of respective endothelins in 0.9% NaCl was given i.p. twice, simultaneously with cerulein. A solution of pyrrolidine dithiocarbamate (PDTC) in 0.9% NaCl was added to the first injection of cerulein.

Experimental design

Rats were subdivided into 10 groups as follows:

Group I. Control group (C), healthy rats, receiving only saline (0.9% NaCl) i.p. at time 0 and 1 hour later (n=7).

Group II. Rats with cerulein-induced untreated acute pancreatitis (AP). The solution of cerulein in equivalent volume of saline was given i.p. at 0 time and 1 hour later (n=9).

Group III. Rats with cerulein-induced AP treated with PDTC at a dose of 10 mg/kg b.w. i.p. once, simultaneously with the first dose of cerulein (n=7).

Group IV. Rats with cerulein-induced AP treated with PDTC at a dose of 40 mg/kg b.w. i.p. once, simultaneously with the first dose of cerulein (n=6).

Group V. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once, as in the group III and ET-1 at a dose of 0.5 nmol/kg b.w i.p. twice, in 1 h interval, simultaneously with cerulein (n=6).

Group VI. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once, as in the group V and ET-1 at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group VII. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-2 at a dose of 0.5 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group VIII. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-2 at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group IX. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-3 at a dose of 0.5 nmol/kg b.w. i.p. twice, as in the group V (n=6).

Group X. Rats with cerulein-induced AP, treated with PDTC at a dose of 10 mg/kg b.w. i.p. once and ET-3 at a dose of 1.0 nmol/kg b.w. i.p. twice, as in the group V (n=6).

The volume of 0.9% NaCl as a solvent was equilibrated in all rats to 3 ml/kg and 2 ml/kg b.w. during the first and the second injection respectively.

Preparation of pancreatic homogenate and the plasma

Four hours after the first cerulein injection (or 0.9% NaCl in C group) a general anesthesia was induced with i.p. ketamine at a dose of 40 mg/kg b.w., supported by pentobarbital at a dose of 20 mg/kg b.w. The blood samples were taken to the heparinized syringe by the cardiac puncture and the rats were sacrificed by decapitation. The pancreases were quickly excised, freed from the peripancreatic tissues and weighed. For light microscopy, representative specimens of the pancreas were fixed and the sections were stained with hematoxylin and eosin.

The remaining portion of the pancreas was processed according to Yamaguchi et al. [28], meaning it was homogenized

in four volumes of ice-cold, 50 mmol/L Tris-HCl buffer (pH 8.0), containing non-organic detergent Triton X-100, 0.5% (v/v) during 1 min by 3 full up and down strokes using a motor driven glass-Teflon homogenizer (Thomas Scientific, New Jersey, U.S.A.) cooled with ice. The resulting homogenate was sonified for 20 seconds in an ice bath using Vibra cell, model VC 50, Sonics and Materials Inc., Danbury, CT, U.S.A. (frequency 20 kHz and amplitude 70). The volumes were then adjusted giving 10% homogenates, placed on ice for 20 min for further extraction of the enzymes, and then centrifuged at 12000 x g for 20 min at 4°C. The supernatants were used for the assays of trypsin activity performed within 6 hours. The remaining portions of the supernatant were frozen at -80°C, for the assay of lipase activity and protein concentration.

The samples of heparinized blood were centrifuged at 4000 rpm with cooling to 4°C, the resulting plasma was collected and frozen at -80°C for the assay of α-amylase activity.

Biochemical assays

1. Trypsin activity: free active trypsin (FAT) and total potential trypsin (TPT) in the supernatants of pancreatic homogenates were estimated according to Yamaguchi et al. [28] with this exception that N_{α}-p-tosyl-L-arginine methyl ester hydrochloride (TAME) 1 mmol/L was used as a substrate and the absorbance of released product was estimated at 247 nm wave length in an automatic spectrophotometer Pye Unicam SP 505 (Cambridge, U.K.) as in our previous study [29].

Total potential trypsin (TPT) in the supernatants was estimated after activation of trypsinogen with enterokinase in 1:1 dilution in 50 mmol/L Tris-HCl buffer (pH 8.0) for 30 min at 37°C. The freshly prepared working solution of enterokinase contained 2 mg of enzyme/mL of the same buffer [28]. The time of this activation proved to be sufficient for maximal activation.

The activity was expressed in μg of trypsin/mg of protein by comparison with the calibration curve of increasing concentrations of bovine trypsin, type I. The %FAT/TPT ratio served as an index of trypsinogen activation [28].

2. Lipase activity in the supernatants of pancreatic homogenates was assayed with tributyrin (1,2,3-tributylglycerol) as a substrate and with the pehametric method using autotitrator (Radiometer, Copenhagen, Denmark) and 0.2 mol/L NaOH, as in our previous study [29].

3. α-amylase activity in the plasma was assayed with colorimetric method with soluble starch as a substrate as in our previous study [29].

All reagents, with the exception of soluble starch were purchased from Sigma Chemicals Co., St Louis, MO, U.S.A.

Histological examination

Ten slides from 5 rats from each group (50 slides per group) stained with hematoxylin and eosin (H&E) were evaluated at a magnification of 200x in light microscopy by an expert pathologist (A.A.), who was not familiar with the experimental code at this time. The edema, inflammatory infiltrate, necrosis and vacuolization were scored from 0 to 3 degrees of severity according to Kyogoku et al. [30]. Generally, the interstitial edema was scored as follows: 0=absent; 1=expansion of interlobular septa; 2=expansion of intralobular septa; 3=separation of acini. The inflammatory infiltrate: 0=absent; 1=less than 20 neutrophils per field; 2=20-50 neutrophils; 3=more than 50 neutrophils per field. Parenchymal necrosis: 0=absent; 1=less than approximately 5%; 2=5-20% ; 3=more than 20% of the involved area. The vacuolization: 0=absent; 1=less than 20% of acinar cells with vacuoles per field; 2=20-50%; 3=more than 50%.

Table 1. **Free active trypsin (FAT), total potential trypsin (TPT) and the index of trypsinogen activation (%FAT/TPT) in the supernatants of pancreatic homogenates in early (4h) cerulein-induced acute pancreatitis (AP) untreated and treated with NF-κB activation inhibitor pyrrolidine dithiocarbamate (PDTC) and different endothelins (ET-1, ET-2. ET-3) vs control group (C) in rats. Means ±S.E.M. are reported**

No	Group	FAT μg/mg protein	TPT μg/mg protein	%FAT/ /TPT
I	Control (C) (n=7)	0.219 ±0.029	11.80 ±1.27	1.96 ±0.31
II	AP untreated (n=9)	1.265••• ±0.103	11.51 ±1.18	12.42••• ±2.14
III	AP+PDTC 10 mg/kg (n=7)	1.507 ±0.486	15.92* ±1.26	10.50 ±3.70
IV	AP+PDTC 40 mg/kg (n=6)	0.434*** ±0.065	15.33 ±1.38	3.01 ±0.53
V	AP+PDTC 10 mg/kg + ET-1 2 x 0.5 nmol/kg (n=6)	0.394*** ±0.106	15.19* ±0.83	2.55*** ±0.18
VI	AP+PDTC 10 mg/kg + ET-1 2 x 1.0 nmol/kg (n=6)	0.385*** ±0.064	16.73** ±0.98	2.34*** ±0.44
VII	AP+PDTC 10 mg/kg + ET-2 2 x 0.5 nmol/kg (n=6)	1.169 ±0.241	11.95 ±1.21	10.78 ±2.56
VIII	AP+PDTC 10 mg/kg + ET-2 2 x 1.0 nmol/kg (n=6)	1.116 ±0.200	11.86 ±0.65	9.24 ±1.22
IX	AP+PDTC 10 mg/kg + ET-3 2 x 0.5 nmol/kg (n=6)	0.918 ±0.144	13.24 ±1.56	6.76* ±0.46
X	AP+PDTC 10 mg/kg + ET-3 2 x 1.0 nmol/kg (n=6)	0.773 ±0.197	11.13 ±0.62	6.88 ±1.68

Statistical significance of differences
between untreated AP group and control group: P<0.001•••, P<0.01••, P<0.05•;
between treated AP groups and untreated AP group: P<0.001***, P<0.01**, P<0.05*.

Statistical analysis

The results of biochemical assays are reported as means ±S.E.M. and after performing an F test for the equality of variances, the means were compared using the *t* test for unpaired data. The differences with P<0.05 were considered statistically significant.

Results

Tab. 1 illustrates the activities of trypsin and the index of trypsinogen activation in the supernatants containing enzymes extracted using organic detergent Triton X-100. FAT in cerulein--induced, untreated AP group has been shown to be 5.8 times higher than in the control group (P<0.001), whereas TPT was

similar in both groups. In the groups with AP treated with lower dose of PDTC, a slight decrease of FAT was not significant, whereas TPT was elevated by 38% ($P<0.05$) in comparison to AP untreated. In the group with AP treated with higher dose of PDTC, FAT achieved only 34% of its value from untreated AP group ($P<0.001$). A slight elevation of TPT was not significant. The combined treatment with lower dose of PDTC and lower or higher dose of ET-1 attenuated FAT elevation to about 30% of its value from untreated AP group ($P<0.001$). Simultaneously, TPT was increased by 32% ($P<0.01$) and by 45% ($P<0.001$) respectively in comparison to untreated AP. The combined treatment with lower dose of PDTC and both, lower and higher doses of ET-2 or ET-3 affected significantly neither FAT nor TPT in comparison to the untreated AP.

The index of trypsinogen activation (%FAT/TPT) in the untreated AP group was markedly (6.3 times) higher than in control group ($P<0.001$). Interestingly enough, lower dose of PDTC did not affect significantly the trypsinogen activation, whereas higher dose of PDTC attenuated it to 24% of its value from untreated AP ($P<0.01$). It is noteworthy, that combined treatment with lower dose of PDTC and both doses of ET-1 resulted in strong attenuation of trypsinogen activation to the values close to that seen after higher dose of PDTC ($P < 0.001$). The combined treatment of AP with lower dose of PDTC and both doses of ET-2 did not affect significantly the trypsinogen activation in comparison to untreated AP group. The attenuating effect of combined PDTC and ET-3 treatment at both doses on the trypsinogen activation to about 55% of its values from untreated AP was significant ($P<0.05$) only for lower dose of ET-3 (*Tab. 1*).

The activity of lipase in the supernatant of pancreatic homogenate from untreated AP was about four times elevated in comparison to control group ($P<0.001$). The treatment with lower dose of PDTC did not affect this activity, whereas after higher dose of PDTC, it was even higher than in untreated AP ($P<0.05$). Only combined treatment with lower dose of PDTC and lower doses of ET-1 or ET-2 attenuated significantly the increase of pancreatic lipase in AP to 47% ($P<0.01$) and to 53% ($P<0.05$) respectively in comparison to untreated AP group. The plasma α-amylase in untreated AP was five times higher as compared to control group ($P<0.001$). In treated AP groups, only higher dose of PDTC decreased plasma α-amylase activity to 54% ($P<0.05$) of its value from untreated AP group (*Tab. 2*).

As can be seen in *Tab. 3*, the increase of edema, inflammatory cell infiltration, necrosis and vacuolization scores after supramaximal cerulein stimulation supports the development of AP. Some shift towards higher scores of edema, necrosis and vacuolization in AP treated with both doses of PDTC can be observed. This trend was even more evident for the necrosis and vacuolization scores after higher dose of PDTC. However, it does not concern the inflammatory infiltration scores, where slight reverse tendency can be seen. The addition of ET-1 at both doses seemed to ameliorate the edema and vacuolization scores in AP treated with lower dose of PDTC, but not below AP untreated, without such an effect on the necrosis scores.

More evident positive shift of inflammatory infiltration scoring after addition of higher dose of ET-1 to PDTC can be seen.

Table 2. **Lipase activity in the supernatants of pancreatic homogenates and plasma α-amylase in caerulein-induced acute pancreatitis (AP) untreated and treated with NF-κB activation inhibitor pyrrolidine dithiocarbamate (PDTC) and different endothelins (ET-1, ET-2, ET-3) vs control group (C) in rats. Means ±S.E.M. are reported**

No	Group	Lipase U/mg protein	α-amylase U/mL
I	Control (C) (n=7)	1.29 ±0.11	5.8 ±1.38
II	AP untreated (n=9)	5.51••• ±0.84	28.5••• ±5.61
III	AP+PDTC 10 mg/kg (n=7)	5.41 ±0.80	24.6 ±3.62
IV	AP+PDTC 40 mg/kg (n=6)	8.69* ±0.77	15.3* ±1.38
V	AP+PDTC 10 mg/kg + ET-1 2 x 0.5 nmol/kg (n=6)	2.60** ±0.36	25.8 ±1.84
VI	AP+PDTC 10 mg/kg + ET-1 2 x 1.0 nmol/kg (n=6)	4.56 ±1.54	27.7 ±6.40
VII	AP+PDTC 10 mg/kg + ET-2 2 x 0.5 nmol/kg (n=6)	2.94 ±0.33	19.7 ±3.04
VIII	AP+PDTC 10 mg/kg + ET-2 2 x 1.0 nmol/kg (n=6)	3.61 ±0.72	26.8 ±2.69
IX	AP+PDTC 10 mg/kg + ET-3 2 x 0.5 nmol/kg (n=6)	4.46 ±0.95	30.1 ±2.86
X	AP+PDTC 10 mg/kg + ET-3 2 x 1.0 nmol/kg (n=6)	4.41 ±1.07	31.9 ±5.79

Statistical significance of differences
between untreated AP group and control group: $P<0.001$•••, $P<0.01$••, $P<0.05$•;
between treated AP groups and untreated AP group: $P<0.01$**, $P<0.05$*

Slight decrease of edema scoring in AP after addition of both doses of ET-3, but not ET-2 to PDTC can be observed, however it remains worse than in untreated AP. The addition of ET-2 and ET-3 does not improve generally the unfavorable effects of lower PDTC dose on the acinar cells necrosis and vacuolization scores in untreated AP. On the contrary, some amelioration of inflammatory infiltration scoring can be seen after addition of ET-2 and ET-3 to lower dose of PDTC in AP, especially after lower dose of ET-3 (*Tab. 3*).

Discussion

Our study shows, that pyrrolidine dithiocarbamate (PDTC) at a dose of 10 mg/kg, known to inhibit almost completely the NF-κB activation, does not attenuate significantly the trypsinogen activation in cerulein-induced AP in rats, but does it markedly at a dose of 40 mg/kg. This higher dose increases the lipase activity in the pancreas but decreases plasma α-amylase activity in AP – the effects not observed after lower dose of PDTC. Beside of enzymatic changes, both doses of PDTC slightly and to a similar extent aggravated the edema, acinar necrosis and vacuolization scores in AP, whereas the inflammatory cell infiltration seemed to be alleviated. The addition of ET-1 at two

Table 3. **The incidence of histological changes of the pancreas, scored at 0-3 scale* in early (4h) cerulein-induced acute pancreatitis (AP) untreated and treated with NF-κB activation inhibitor pyrrolidine dithiocarbamate (PDTC) and different endothelins (ET-1, ET-2, ET-3) vs control group (C) in rats**

Group	No	I Control (C) (n=7)	II AP untreated (n=9)	III AP+PDTC 10 mg/kg (n=7)	IV AP+PDTC 40 mg/kg (n=6)	V AP+PDTC 10 mg/kg + ET-1, 2x0.5 nmol/kg (n=6)	VI AP+PDTC 10 mg/kg + ET-1, 2x1.0 nmol/kg (n=6)	VII AP+PDTC 10 mg/kg + ET-2, 2x0.5 nmol/kg (n=6)	VIII AP+PDTC 10 mg/kg + ET-2, 2x1.0 nmol/kg (n=6)	IX AP+PDTC 10 mg/kg + ET-3, 2x0.5 nmol/kg (n=6)	X AP+PDTC 10 mg/kg + ET-3, 2x1.0 nmol/kg (n=6)
Edema score	0	44	0	0	0	0	1	0	0	0	0
	1	6	12	7	5	5	4	6	0	7	7
	2	0	25	10	20	30	29	15	20	21	20
	3	0	13	33	25	15	16	29	30	22	23
Inflammatory infiltration score	0	47	3	6	10	4	13	11	0	10	6
	1	3	22	27	25	27	24	25	41	33	32
	2	0	19	15	12	15	13	10	6	7	9
	3	0	6	2	3	4	0	4	3	0	3
Necrosis score	0	50	25	11	15	15	15	20	16	18	14
	1	0	21	37	24	32	31	29	31	29	31
	2	0	4	2	11	3	4	1	3	3	5
	3	0	0	0	0	0	0	0	0	0	0
Vacuolization score	0	48	0	0	0	0	2	2	0	0	0
	1	2	21	0	0	9	23	8	12	4	3
	2	0	14	18	5	16	9	16	2	9	11
	3	0	15	32	45	25	16	24	36	37	36

*according to Kyogoku et al. [30] from 50 fields per group are reported

doses, 0.5 or 1.0 nmol/kg b.w. i.p. at 1 h interval to the treatment with lower dose of PDTC attenuated trypsinogen activation to that seen after higher dose of PDTC. Lower dose of ET-1 added to PDTC attenuated significantly the lipase activity in the pancreas without any evident effect on plasma α-amylase activity in AP. Lower dose of ET-1 added to lower dose of PDTC does not affect appreciably histological changes seen after PDTC alone. Higher dose of ET-1 added to lower dose of PDTC does not affect appreciably the necrosis scoring in comparison to PDTC alone, but some amelioration of edema, inflammatory infiltration and vacuolization can be seen.

The addition of ET-2 to PDTC treatment in AP does not exert evident favorable effects on the changes seen after PDTC alone, with the exception of decreasing influence on pancreatic lipase activity and a positive shift of necrosis score after lower dose of ET-2. The addition of ET-3 to PDTC treatment in AP resulted in the attenuation of trypsinogen activation, but to lesser degree than after ET-1 and in slight amelioration of necrosis and inflammatory scoring after lower dose of ET-3. Generally, the inhibition of NF-κB activation by PDTC in rats, despite the extent of trypsinogen activation, leads to slight aggravation of edema, pancreatic acinar cells necrosis and vacuolization but not the inflammatory infiltration in the early (4h) course of cerulein-induced AP in our present study.

Early changes occurring within the first 30 min after the supramaximal cerulein stimulation, leading to AP, include the colocalization of lysosomal hydrolases with digestive zymogens in cytoplasmic vacuoles, intraacinar cell activation of trypsinogen and activation of NF-κB [31,32]. In next hours of cerulein-induced AP, the accumulation of neutrophils [33] and oxidative stress [34] gain an increasing role in acinar cells injury. The activation of NF-κB depends on the phosphorylation and proteolytic degradation of its inhibitors IκBα and IκBβ. Beside early direct effect of cerulein hyperstimulation, another factor contributing to NF-κB activation could be the inflammatory cell infiltration, which becomes pronounced after 2-3 hours of cerulein AP. It is of interest, that known antioxidant, N-acetylcysteine inhibits both NF-κB and trypsinogen activation [3], suggesting an important role of the oxidative stress for both these mechanisms of the pancreatitis progression.

More extensive studies on isolated pancreatic acini have shown that either stimulation or inhibition of acinar cell NF-κB activation did not affect trypsinogen activation. On the other hand, the trypsin activity is not necessary for the NF-κB activation by supramaximal stimulation with cholecystokinin. However, the inhibition of NF-κ-B activation by preincubation with inhibitors of the trypsin activity suggests indirect role of this transcriptional factor in the development of cerulein-induced AP. Known antioxidant, pyrrolidine dithiocarbamate (PDTC) at the concentration of 10 mmol/L strongly inhibits both NF-κB and trypsinogen activation in isolated pancreatic acini [15,17]

Taking into account the key role of NF-κB in the expression of proinflammatory cytokines and their reciprocal effect on the NF-κB activation [6,11,17], it is obvious, that selective inhibition of NF-κB DNA-binding activity [11] or specific inhibition of its translocation into the nucleus by a nuclear import inhibitory peptide [12] could ameliorate the severity of cholecystokinin--induced AP. A controversy exists on the effect of less specific inhibitors of NF-κB activation, like PDTC. We have chosen this agent basing on its positive effects in taurocholate acute

pancreatitis (TCA) in rats at a dose of 10 and 100 mg/kg, abolishing the NF-κB activation in the peritoneal and alveolar macrophages. Lower dose of PDTC injected i.p. 1 hour or just before induction of TCA pancreatitis significantly improved the survival rate. Nevertheless, no significant differences were found in the increase of serum α-amylase or in the histological changes of the pancreas [7].

In cerulein-induced AP in rats, PDTC administered i.p. at a dose of 10 mg/kg, one hour before cerulein injection inhibited NF-κB activation in acinar cells. Unexpectedly, the serum α-amylase and LDH (lactic dehydrogenase) activities were significantly higher at 4h of AP, than after cerulein alone. Moreover, histological changes were aggravated after 24 hours. The authors suggest, that despite the proinflammatory effect of NF-κB/Rel activation, this factor can regulate a self-defending genetic program to prevent a higher degree of the damage to the pancreatic acinar cells. Therefore the final results of the PDTC dependent inhibition of NF-κB activation could be detrimental in rats [9]. On the contrary, in cerulein-induced AP in mice, pretreatment with PDTC at a dose of 30 mg/kg i.p., significantly ameliorated histological changes in the pancreas 6h after the induction of AP [14]. It means, that the effect of PDTC in cerulein-induced AP could be a species dependent.

Our present study have shown that PDTC slightly aggravated the scores of acinar cell injury already in early (4h) cerulein-induced AP in rats both at a dose of 10 and 40 mg/kg b.w., despite the strong attenuation of trypsinogen activation by higher dose of PDTC. Therefore, despite the beneficial effect of PDTC in taurocholate pancreatitis in rats [7] and it doubtless positive effect against NF-κB activation and mob-1 chemokine expression in isolated pancreatic acini [35], the application of this compound in the mild cerulein-induced AP to depress the NF-κB activation requires further evaluation.

In our previous study, we have found an attenuating effect of endothelins on trypsinogen activation in cerulein-induced AP in rats with concomitant improvement of inflammatory infiltration score after higher dose of ET-1 and to lesser extent after higher dose of ET-2 and ET-3. However, it did not prevent other morphological aspects of pancreatic acinar cell injury as slight elevation of necrosis and vacuolization scores after lower dose of ET-2 and ET-3 [27]. In this study, we have used the same doses of endothelins and additionally PDTC at a dose of 10 mg/kg. It appears that only cumulative effect of PDTC and lower dose of ET-1 towards attenuation of increased trypsinogen activation was higher than the effect of ET-1 alone ($P<0.001$). PDTC even depreciated the effect of both doses of ET-2 and higher dose of ET-3 against the trypsinogen activation. The accentuated cumulative effect of PDTC and higher dose of ET-1 to alleviate the inflammatory infiltration and pancreatic acinar cell vacuolization deserves further attention.

There are still controversies on the role of endothelins in AP. Liu et al. [24] observed, that ET-1 administered as a bolus after induction of cerulein AP caused a dose-dependent increase of pancreatic acinar cell damage. On the other hand, Kogire et al. [25] found, that the application of ET-1, infused simultaneously with supramaximal dose of cerulein, resulted in less advanced inflammatory cell infiltration and a decrease in the pancreatic edema index. A secondary stimulation of prostacyclin production by ET-1 has been suggested for this positive effect. In fact, in our previous study, we have found a protective effect of the prostacyclin analog in taurocholate AP in rats [36]. Our present study supports some protective effects of endothelins, which can be not sufficient to abrogate some unfavorable effects of PDTC administration, excluding some positive effects of higher ET-1 dose.

Lipase is known factor in the damage to isolated pancreatic acinar cells to a degree similar to the action of chymotrypsin [37]. The increase of its activity in pancreatic tissue of the rats with cerulein-induced AP treated with higher dose of PDTC over its value in untreated AP, in our study, could participate in the exacerbation of histological changes. On the contrary, its decrease after combined treatment with PDTC and lower dose of ET-1 could be observed, together with marked attenuation of trypsinogen activation. However, these positive trends had no appreciable beneficial influence on histological scores of the pancreatic injury.

In conclusion, the combined treatment of cerulein-induced acute pancreatitis in rats with PDTC and ETs resulted in the attenuation of trypsinogen activation, especially evident in the case of ET-1. The index of trypsinogen activation after ET-1 and lower dose of PDTC was especially diminished and close to the effect of higher dose of PDTC alone. A slight alleviation of the inflammatory cell infiltration in the pancreas was also noted in groups treated with PDTC alone and in combination with ETs. However, some unfavorable shift of the histological scores of edema and pancreatic acinar cell injury could be observed. Therefore, the application of PDTC to inhibit the NF-κB activation in cerulein-induced acute pancreatitis just in rats appears to be questionable, because of other unfavorable effects. Nevertheless, concomitant application of ET-1 could partly counteract these effects.

Acknowledgement

The study was supported by grant of Medical University of Bialystok # 3 515 68.

References

1. Singh VP, Saluja AK, Bhagat L, van Acker GJD, Song AM, Soltoff SP, Contley LC, Steer ML. Phosphatidylinositol 3-kinase-dependent activation of trypsinogen modulates the severity of acute pancreatitis. J Clin Invest, 2001; 108: 1387-95.

2. Plusczyk T, Westermann S, Rathgeb D, Feifel G. Acute pancreatitis in rats: effects of sodium taurocholate, CCK-8, and Sec on pancreatic microcirculation. Am J Physiol, 1997; 272: G310-20.

3. Gukovsky I, Gukovskaya AS, Blinman TA, Zaninovic V, Pandol SJ. Early NF-κB activation is associated with hormone-induced pancreatitis. Am J Physiol, 1998; 275: G1402-14.

4. Vaquero E, Gukovsky I, Zaninovic V, Gukovskaya AS, Pandol SJ. Localized pancreatic NF-κB activation and inflammatory response in taurocholate-induced pancreatitis. Am J Physiol, 2001; 280: G1197-208.

5. Dunn JA, Li C, Ha T, Kao RL, Browder W. Therapeutic modification of nuclear factor κB binding activity and tumor necrosis factor-α gene expression during acute biliary pancreatitis. Am Surg, 1997; 63: 1036-42.

6. Chen X, Ji B, Han B, Ernst SA, Simeone D, Logsdon CD. NF-κB activation in pancreas induces pancreatic and systemic inflammatory response. Gastroenterology, 2002; 122: 448-57.

7. Satoh A, Shimosegawa T, Fujita M, Kimura K, Masamune A,

Koizumi M, Toyota T. Inhibition of nuclear factor κB activation improves the survival of rats with taurocholate pancreatitis. Gut, 1999; 44: 253-9.

8. Meng Y, Ma QY, Kou XP, Xu J. Effect of resveratrol on activation of nuclear factor kappa-B and inflammatory factors in rat model of acute pancreatitis. World J Gastroenterol, 2005; 11: 525-8.

9. Steinle AU, Weidenbach H, Wagner M, Adler G, Schmid RM. NF-κB/Rel activation in cerulein pancreatitis. Gastroenterology, 1999; 116: 420-30.

10. Altavilla D, Famulari C, Passaniti M, Galeano M, Macri A, Seminara P, Minutoli L, Marini H, Calo M, Venuti FS, Esposito M, Squadrito F. Attenuated cerulein-induced pancreatitis in nuclear factor-κB – deficient mice. Lab Invest, 2003; 83: 1723-32.

11. Ethridge RT, Hashimoto K, Chung DH, Ehlers RA, Rajaraman S, Evers BM. Selective inhibition of NF-κB attenuates the severity of cerulein-induced acute pancreatitis. J Am Coll Surg, 2002; 195: 497-505.

12. Letoha T, Somlay C, Takács T, Szaboles A, Jármay K Rakonczay Jr Z, Heggi P, Varga I, Kaszaki J, Krizbay I, Boros I, Duda E, Kusz E, Penke B. A nuclear import inhibitory peptide ameliorates the severity of cholecystokinin-induced acute pancreatitis. World J Gastroenterol, 2005; 11: 990-9.

13. Altavilla D, Famulari C, Passaniti M, Campo GM, Macri A, Seminara P, Marini H, Calo M, Santamaria LB, Bono D, Venuti FS, Mioni C, Leone S, Guarini S, Squadrito F. Lipid peroxidation inhibition reduces NF-κB activation and attenuates cerulein-induced pancreatitis. Free Radic Res, 2003; 37: 425-35.

14. Virlos I, Mazzon E, Serraino I, Di Paola R, Genovese T, Britti D, Thiederman C, Siriwardena A, Cuzzocrea S. Pyrrolidine dithiocarbamate reduces the severity of cerulein-induced murine acute pancreatitis. Shock, 2003; 20: 544-50.

15. Han B, Ji B, Logsdon CD. CCK independently activates intracellular trypsinogen and NF-κB in rat pancreatic acinar cells. Am J Physiol, 2001; 49: C465-72.

16. Hietaranta AJ, Saluja AK, Bhagat L, Singh VP, Song AM, Steer ML. Relationship between NF-κB and trypsinogen activation in rat pancreas after supramaximal caerulein stimulation. Bioch Bioph Res Commun, 2001; 280: 388-95.

17. Tando Y, Algul H, Scheider G, Weber CK, Weidenbach H, Adler G, Schmid RM. Induction of IκB-kinase by cholecystokinin is mediated by trypsinogen activation in rat pancreatic lobules. Digestion, 2002; 66: 237-45.

18. Schmidt J, Ebeling D, Ryshich E, Werner J, Gebhard MM, Klar E. Pancreatic capillary blood flow in an improved model of necrotizing pancreatitis in the rat. J Surg Res, 2002; 106: 335-41.

19. Inoue A, Yanagisava M, Kimura S, Kasuya Y, Miyauchi T, Goto K, Masaki T. The human endothelin family: Three structurally and pharmacologically distinct isopeptides predicted by three separate genes. Proc Natl Acad Sci, 1989; 86: 2863-7.

20. Rossi GP, Seccia TM, Albertin G, Pessina A. Measurement of endothelin: clinical and research use. Ann Clin Biochem, 2000; 37: 608-26.

21. Takaori K, Inoue K, Kogire M, Higashide S-I, Tun T, Aung T, Doi R, Fujii N, Tobe T. Effects of endothelin on microcirculation of the pancreas. Life Sci, 1992; 51: 615-22.

22. Plusczyk T, Bersal B, Menger MD, Feifel G. Differential effects of ET-1, ET-2, ET-3 on pancreatic microcirculation, tissue integrity and inflammation. Dig Dis Sci, 2001; 46: 1343-51.

23. Eibl G, Hotz HG, Faulhaber J, Kirchengast M, Buhr HJ, Foitzik T. Effect of endothelin and endothelin receptor blockade on capillary permeability in experimental pancreatitis. Gut, 2000; 46: 390-4.

24. Liu X-H, Kimura T, Ishikawa H, Yamagouchi H, Furokawa M, Nakano I, Kinjoh M, Nawata H. Effect of endothelin-1 on the development of hemorrhagic pancreatitis in rats. Scand J Gastroenterol, 1995; 30: 276-82.

25. Kogire M, Inoue K, Higashide S-I, Takaori K, Echigo Y, Gu Y-J, Sumi S, Uchida K, Imamura M. Protective effects of endothelin-1 on acute pancreatitis in rats. Dig Dis Sci, 1995; 40: 1207-12.

26. Andrzejewska A, Dlugosz J, Augustynowicz A. Effect of endothelin-1 receptor antagonists on histological and ultrastructural changes in the pancreas and trypsinogen activation in the early course of caerulein-induced acute pancreatitis in rats. World J Gastroenterol, 2005; 11: 1115-21.

27. Dlugosz JW, Nowak K, Andrzejewska A, Wroblewski E, Dabrowski A. The effect of endothelin-1, endothelin-2 and endothelin-3 in early cerulein-induced acute pancreatitis in rats. Rocz Akad Med Bialymst, 2004; 49: 42-9.

28. Yamaguchi H, Kimura T, Mimura K, Nawata H. Activation of proteases in cerulein-induced pancreatitis. Pancreas, 1989; 4: 565-71.

29. Dlugosz JW, Nowak K, Laszewicz W, Andrzejewska A, Wroblewski E. The effect of endothelin receptors antagonists in acute experimental pancreatitis in the rats. Exp Toxic Pathol, 2003; 55: 137-45.

30. Kyogoku T, Manabe T, Tobe T. Role of ischemia in acute pancreatitis. Hemorrhagic shock converts edematous pancreatitis to hemorrhagic pancreatitis in rats. Dig Dis Sci, 1992; 37: 1409-17.

31. Saluja AK, Donovan EA, Yamanaka K, Yamaguchi Y, Hofbauer B, Steer ML. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. Gastroenterology, 1997; 113: 304-10.

32. Hofbauer B, Saluja AK, Lerch MM, Bhagat L, Bhatia M, Lee HS, Frossard JL, Adler G, Steer ML. Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. Am J Physiol, 1998; 275: G352-62.

33. Inagaki H, Nakao A, Kurosawa T, Nonami T, Harada A, Takagi H. Neutrophil behavior in pancreas and liver and the role of nitric oxide in rat acute pancreatitis. Pancreas, 1997; 15: 304-9.

34. Dabrowski A, Konturek SJ, Konturek JW, Gabryelewicz A. Role of oxidative stress in the pathogenesis of caerulein-induced acute pancreatitis. Eur J Pharmacol, 1999; 377: 1-11.

35. Grady T, Liang P, Ernst SA, Logsdon CD. Chemokine gene expression in rat pancreatic acinar cells is an early event associated with acute pancreatitis. Gastroenterology, 1997; 113: 1968-75.

36. Dlugosz JW, Andrzejewska A, Wroblewski E, Poplawski C, Wereszczynska-Siemiatkowska U. Beneficial effect of iloprost on the course of acute taurocholate pancreatitis in rats and its limitation by antecedent acute erhanol intake. Exp Toxic Pathol, 2004; 55: 401-9.

37. Niederau C, Fronhoffs K, Klonowski H, Schulz U. Active pancreatic digestive enzymes show stricking differences in their potential to damage isolated rat pancreatic acinar cells. J Lab Clin Med, 1995; 125: 265-75.

Human red blood cells′ physiological water exchange with the plasma

Kargol M[1]*, Kargol A[2], Przestalski M[3], Siedlecki J[4], Karpińska M[5], Rogowski M[6]

[1] Faculty of Natural Science, Institute of Physics, Świętokrzyska Academy, Kielce, Poland
[2] Department of Physics, Loyola University New Orleans, USA
[3] Department of Laryngology, Medical University, Wrocław, Poland
[4] County Hospital, Kielce, Poland
[5] Department of Biophysics, Medical University of Białystok, Poland
[6] Department of Laryngology, Medical University of Białystok, Poland

Abstract

In the present paper, fundamental issues related to the mechanisms of human red blood cells' physiological water exchange with the plasma (for the stationary conditions) have been discussed. It has been demonstrated, on the basis of mechanistic transport equations for membrane transport that red blood cells are capable of exchanging considerable amounts of water with the plasma. Water absorption is osmosis-driven, and its removal occurs according to the hydromechanics principle, i.e. is driven by the turgor pressure of red blood cells. This newly-acquired knowledge of these issues may appear highly useful for clinical diagnosis of blood diseases and blood circulation failures.

Key words: human red blood cells, cell membrane, water exchange, cytoplasm, plasma, transport equations.

Introduction

Human red blood cells, like any other living cells of the human body, must continue to exchange water, as well as other solutes, with their surroundings. To be precise, the erythrocytes must absorb water as well as other necessary dissolved substances from the plasma (i.e. their surroundings), and simultaneously remove both water and redundant metabolites. This physiological exchange of water and dissolved substances occurs across the erythrocyte cell membrane, with its active participation. It must be stressed here that the mechanisms of this exchange appear to be highly complex [1-7]. This complexity is markedly heightened by the processes related to erythrocyte participation in the removal of carbon dioxide from the entire body, and the supply of oxygen to all the body's living cells. These very problems appear to be very sophisticated, difficult to investigate and little known.

In the present article, which initiates a certain research cycle concerning these issues, we shall necessarily limit our considerations to the issues of red blood cells' exchange of water only with the plasma. We shall be here interested in the so-called stationary water exchange, i.e. the exchange which occurs with the red blood cells maintaining constant volumes (V=const.). This restriction of the research problem results from the fact that the non-stationary exchange (V≠const.) may be explained on the basis of the equations of the Kedem-Katchalsky (KK) thermodynamic formalism [8,9]. However, with the help of these equations, it is not possible to interpret the stationary water exchange [10]. This is caused by the fact that in the KK formalism one does not go into the microscopic structure of porous membranes, whereas real membranes do have specific structures. In fact, the membranes are porous. They have certain pores (channels) which are permeable to water and other solutes. Moreover, porous membranes may be divided into homogeneous and heterogeneous [11-15]. A membrane is homogeneous in terms of transport properties if its pores do not vary in their linear dimensions (cross-section radiuses). A membrane, in turn, whose pores do vary in their linear dimensions, is to be treated as heterogeneous. At this point, it must be explained that cell membranes, erythrocyte cell membranes included, are increasingly perceived as heterogeneous porous structures [16-27]. Under the circumstances, for the purposes of investigation into the stationary physiological water exchange by human red blood cells, the equations of the mechanistic substance transport formalism [11-15] shall be applied. These equations apply unrestrictedly to any porous membranes, both homogenous and heterogeneous ones.

* CORRESPONDING AUTHOR:
Faculty of Natural Science, Institute of Physics
Świętokrzyska Academy
15 Świętokrzyska Str.
25-406 Kielce, Poland
Tel: + 48 041 349 6471; Fax: + 48 041 349 6443
e-mail: kargol@pu.kielce.pl (Marian Kargol)

Received 14.11.2004 Accepted 11.07.2005

These mechanistic transport equations have the following forms:

1) $$J_{vM}^{(1)} = L_p \Delta P - L_p \sigma \Delta \Pi$$

2) $$j_{sM} = \omega_d \Delta \Pi + (1-\sigma)\overline{c}_s L_p \Delta P$$

or $$J_{vsM} = \omega_d \overline{V}_s \Delta \Pi + (1-\sigma)\overline{c}_s \overline{V}_s L_p \Delta P$$

where J_{vM} is the volume flow; J_{vsM} and j_{sM} are solute flows; L_p, σ and ω_d are coefficients (of filtration, reflection and diffusion permeability of the solute); ΔP and $\Delta \Pi$ – pressure differences (mechanical and osmotic); $\overline{c}_s \approx 0.5(C_1 + C_2)$ – mean concentration of the concentrations C_1 and C_2; $\overline{V}$ is the solute molar volume.

The flow J_{vM} is given by the formula:

(3) $$J_{vM} = J_{va} + J_{vb}$$

while (4) $$J_{va} = L_{pa} \Delta P - L_{pa} \Delta \Pi,$$

(5) $$J_{vb} = L_{pb} \Delta P,$$

where $J_{va} = J_{vwa}$ is the volume flow of water which permeates across the semi-permeable pores of the membrane, whose filtration coefficient amounts to L_{pa}. The flow J_{vb}, in turn, is the volume (hydromechanical) flow of the solution pumped across the permeable pores of the membrane (permeable to water and a given solute). The parameter L_{pb} here is the filtration coefficient of these permeable pores. Within the mechanistic transport formalism, the following relations are satisfied:

(6) $$L_p = L_{pa} + L_{pb},$$

(7) $$L_{pa} = \sigma Lp,$$

(8) $$L_{pb} = (1-\sigma)\, L_p$$

and (9) $$\omega_d = (1-\sigma)\overline{c}_s L_p$$

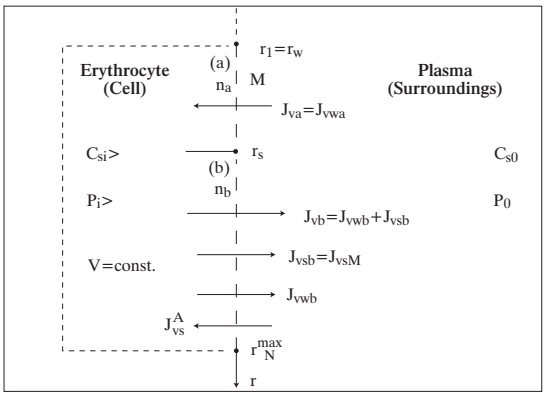
By applying the equations for mechanistic transport formalism, we shall demonstrate in the present paper that human red blood cells can, under stationary conditions, exchange considerable amounts of water with the plasma. This adds a new dimension to the investigations into stationary water exchange by living cells. This aspect of the research appears to be very significant from the medical viewpoint, especially in the diagnosis of blood disease as well as blood circulation failure.

Cell membrane as heterogeneous porous structure

The cell membrane performs several functions which are fundamental to the cell's life. One of these pertains to controlled water permeability. The fundamental character of this function results from the fact that each living cell – in order to live – must (as has been stated in the Introduction) continue to exchange water with its surroundings. This exchange occurs across the cell membrane, and with its active participation. In this context, attention must be drawn to the fact that in the light of the progressing biophysical and biomechanical research, the cell membrane is increasingly perceived as a porous structure. Namely, it has certain channels (pores) which are permeable to water. By these pores, we mean the channels created by transport proteins (aquaporins) [5-7,18-21], the pores created by some antibiotics [5,6,27], as well as the pores which occur in the lipid bilayer (Korohoda [22]). The porous structure of the cell membrane is also provided by ion channels across which (when open) ions may permeate. Suffice it to say at this point that these channels have hydrophilic inner walls and are filled with water [5-7,20,23,25,26]. In view of this, they should be permeable to water (when open), as well as to certain fine-molecule solutes.

From the above-quoted works, it follows that the pores of the cell membrane which are permeable to water vary in their linear dimensions. Consequently, this membrane may be treated as a heterogeneous porous structure. That pertains also to the cell membrane of human erythrocytes.

Figure 1. **Model of an erythrocyte cell and its surroundings (M – replacement cell membrane; C_{si}, C_{s0} – concentrations; P_i, P_0 – mechanical pressures; r_w and r_s – molecule radiuses of water and the solute; r_n^{max} – maximum pore radius; V – cell volume; $J_{va} = J_{vwa}$, J_{vb}; J_{vwb}, $J_{vsb} = J_{vsM}$ – flows)**



Research system. Equation describing water absorption

In order to consider the issue of stationary water exchange by human red blood cells, let us consider a model research system as presented schematically in *Fig. 1*. In this system, the investigated erythrocyte is found in the plasma which constitutes its surroundings. In order to facilitate the solutions, let us assume that the entire cell membrane of the erythrocyte (which has the number N of pores with varying linear dimensions) is represented by the replacement membrane M, which is located to the right of the cell. In this membrane, all the pores (N) have been, for the sake of the model, arranged in such a way that the smallest of them ($r_1 \geq r_w$) are found at the top, while the largest ones r_N^{max} – at the bottom. Let us also assume that the inside of

the erythrocyte is actively penetrated by the volume flow J_{vs}^{A} of a certain given solute (s). Let the radius r_s of this solute's molecules be contained in the value interval $r_1 < r_s < r_N^{max}$.

In the situation at issue, the membrane M may, in accordance with the idea of the mechanistic membrane transport formalism [11-15], be ascribed with the filtration coefficient L_p, the reflection coefficient σ (contained in the interval $0<\sigma<1$) as well as the diffusion permeability coefficient ω_d for the substance (s). It can also be divided into part (a) which contains n_a semi-permeable pores impermeable to the substance (s), and part (b) which contains $n_b = N - n_a$ of pores permeable to the molecules of the said substance. We can also ascribe to these parts the filtration coefficient L_{pa} and L_{pb}, as well as the reflection coefficient $\sigma_a = 1$ and $\sigma_b = 0$ respectively [11-15]. In connection with the existence of the flow J_{vs}^{A}, it is legitimate to assume that the concentration C_{si} of the substance (s) inside the cell is greater than the concentration C_{s0} of this substance in the surroundings ($C_{si} > C_{s0}$). Consequently, on the membrane M, the concentration difference $\Delta C_s = C_{si} - C_{s0}$ will appear, and so will the osmotic pressure difference $\Delta\Pi = RT(C_{si} - C_{s0})$. Driven by the pressure difference $\Delta\Pi$, water shall permeate into the cell, causing an increase in the mechanical pressure P_i inside the cell. Under stationary conditions, P_i shall be constant and greater than the pressure P_0, which occurs in the cell's surroundings ($P_i > P_0$). Suffice it to say that under stationary conditions, on the membrane, a constant osmotic pressure difference shall appear ($\Delta\Pi$=const.), together with the constant mechanical pressure difference ($\Delta P = P_i - P_0$=const.).

The volume flow J_{vwa} of water (w), which permeates across part (a) of the membrane, is given by the formula:

$$J_{va} = J_{vwa} = L_{pa}\Delta P - L_{pa}\Delta\Pi.$$

Considering the formula (7) as well as the formula below, quoted from the work [16], i.e.:

$$(10) \qquad \Delta P = \overline{\sigma}\Delta\Pi, \quad \text{where } \overline{\sigma} = \frac{\sigma + (1-\sigma)c_s V_s}{1-(1-\sigma)c_s V_s}$$

we obtain

$$(11) \qquad J_{vwa} = L_p\sigma(\overline{\sigma}-1)\Delta\Pi = L_p\sigma(\overline{\sigma}-1)RT(C_{si}-C_{s0})$$

This is the sought formula for the flow J_{vwa} of water absorbed from the surroundings by the erythrocyte.

Equation describing water removal

In order to consider the problem of water removal by the investigated model erythrocyte (which functions under stationary conditions, i.e. at constant volume), let us consider the volume flow J_{vb} which permeates across Part (b) of the membrane M (*Fig. 1*). The reflection coefficient of this part of the membrane amounts to $\sigma_b = 0$, and the volume flow which permeates across it is given by the formula:

$$(12) \qquad J_{vb} = L_{pb}\Delta P.$$

Table 1. **Figures and calculation results for cell membranes of human erythrocytes**

No	Solute (s)	$L_p \times 10^{12}$ [$m^3/N{\cdot}s$]	σ	$\overline{V}_s \times 10^3$ [m^3/mol]	Source	$J_{vwa} \times 10^8$ [m/s]	$J_{vwb} \times 10^8$ [m/s]
I	II	III	IV	V	VI	VII	VIII
1	Ethylene glycol	0.92	0.63	0.0566	Katchalsky and Curran [9]	-5.29	5.29
2	Urea	1.27	0.55	0.042	Sha'afi and Gary-Bobo [24]	-7.77	7.77

Other data: C_{si}=150 [mol/m³]; C_{so}=50 [mol/m³]; $\overline{c}_s$=100 [mol/m³]; R=8.3 [N·m/mol·K]; T=300 [K]

Hence, having made allowances for the expression (7), we have:

$$(13) \qquad J_{vb} = (1-\sigma)L_p\Delta P.$$

In the mechanistic formalism for membrane transport, the flow J_{vb} is given by the formula:

$$(14) \qquad J_{vb} = J_{vwb} + J_{vsb},$$

where J_{vwb} is the volume flow of water (w), and J_{vsb} – the volume flow of the solute (s). Therefore, due to introducing the notation $J_{vsb} = J_{vsM}$, the formula (14), having taken into account the expression (13), assumes the following form:

$$(15) \qquad (1-\sigma)L_p\Delta P = J_{vwb} + J_{vsM} = J_{vwb} + J_{vsM}\overline{V}_s$$

since $J_{vsM} = j_{sM}\overline{V}_s$.

Hence, having made allowances for Eqs. (2), (10) and (15), we finally find the sought expression for the flow J_{vwb} of the water removed by the cell. Its form is as follows:

$$(16) \qquad J_{vwb} = (1-\sigma)[(1-\overline{c}_s\overline{V}_s)\overline{\sigma} - \overline{c}_s\overline{V}_s]L_pRT(C_{si}-C_{s0}).$$

Results of quantitative research into water exchange by red blood cells

For the purposes of the present paper, the most reliable experimental figures pertaining to transport properties of the human erythrocytes have been selected from literature. The results of this papers have been presented in *Tab. 1*, and they comprise the numerical values of filtration coefficients L_p and reflection coefficients σ of these cells' membranes for two solutes (ethylene glycol and urea). These figures have been quoted after Katchalsky and Curran [9], as well as Sha'afi and Gary-Bobo [24]. They concern cell membranes of statistical human erythrocytes and may be considered encyclopaedic data. By applying these data, as well as the formulas (11) and (16), the numerical values of the flows J_{vwa} of the absorbed water and the flows J_{vwb} of the removed water for the investigated membranes

have been calculated. These values have been entered into columns VII and VIII of *Tab. 1* respectively.

The obtained values for these flows are relatively large. This testifies to the fact that – in order to perform their life functions – red blood cells must, and can, continue to absorb and remove relatively large amounts of water. Water absorption occurs according to the osmosis principle, and removal is driven by the turgor pressure of the erythrocytes.

Conclusions

If the red blood cells of the human body are to be able to perform their life functions, they must (just like any other living cells of the body) continue to absorb water from their surroundings, and simultaneously remove it into these very surroundings. Within the present paper, we have shown – by applying the mechanistic equations for membrane transport of substances – that human red blood cells are capable of exchanging considerable amounts of water with the plasma under stationary conditions (at its constant volume). Water absorption occurs according to the osmosis principle. Its removal, in turn (realized simultaneously with its absorption), is driven by turgor pressure of the erythrocytes. This interpretation of mechanisms of this exchange is a complete novelty. The following work opens some new research possibilities.

The Authors of the present paper believe that the herein discussed research results may be of interest and significance not only in the medical and cognitive fields, but also in terms of their clinical aspect. The comprehension of the biophysical mechanisms of physiological absorption and removal of water (as well as a variety of solutes) by red blood cells may prove extremely useful for the diagnosis of blood diseases and circulation disorders. A more detailed consideration of these subjects will be presented in the next paper concerning regulation of physiological water exchange between human red blood cells and plasma.

References

1. Januszewicz W, Sznajderman M. Nadciśnienie tętnicze. PZWL Warszawa, 4th ed, 1987; p. 11-272.
2. Begemann H. Hematologia praktyczna. Rozpoznanie różnicowe. Leczenie. Metodyka. Differential diagnosis. Treatment. Methodology, PZWL Warszawa, 2nd ed. 1985; p. 80-93.
3. Horst A. Fizjologia patologiczna. PZWL Warszawa, 9th ed. 1986, 9th ed, p. 475-83.
4. Kiersz J. Fizjologia nerek i dróg moczowych. PZWL Warszawa, 1979; p. 193-274.
5. Miękisz S, Hendrich A, Mozrzymas J. Transport jonów w błonach i potencjały błonowe. Wybrane zagadnienia z biofizyki. Część I. Editors: S. Miękisz i A. Hendrich. Wyd Akad Med, Wrocław, 1996; p. 69-99.
6. Alberts B, Bray D, Johnson A, Lewis J, Raff M, Roberts K, Walter P. Essential cell biology, Garland Publishing Inc., New York and London, 1998; p. 372-406.
7. Stryer L. Biochemistry. Published in the USA by W. H. Freeman and Company, New York, 1995, 4th ed; p. 280-338.
8. Kedem O, Katchalsky A. Thermodynamics of the permeation of biological membranes to nonelectrolytes. Biochim Biophys Acta, 1958; 27: 229-46.
9. Katchalsky A, Curran PF. Nonequilibrium thermodynamics in biophysics, Harvard University Press, Cambridge, Massachusetts, USA, 1965; p. 113-32.
10. Kargol M, Kargol A. Biophysical mechanisms of physiological water exchange with the surroundings by the cells of the Nitella translucens and Chara coralline. Acta Physiologiae Plantarum, 2005; 27: 71-9.
11. Kargol M, Kargol A. Membrane transport generated by the osmotic and hydrostatic pressure. Correlation relation for parameters L_p, σ and ω. J Biol Phys, 2000; 26: 307-20.
12. Kargol A. A mechanistic model of transport precesses in porous membranes generated by osmotic and hydrostatic pressures. J Membrane Sci, 2001; 191: 61-9.
13. Kargol M. Mechanistic approach to membrane mass transport processes. Cellular and Molecular Biol Letters, 2002; 7: 983-93.
14. Kargol M, Kargol A. Mechanistic formalism for membrane transport generated by osmotic and mechanical pressure, Gen Physiol Biophys, 2003; 22: 51-68.
15. Kargol M, Kargol A. Mechanistic equations for membrane substance transport and their identity with Kedem-Katchalsky equations. Bioph Chem, 2003; 103: 117-27.
16. Sha'afi RJ, Gary-Bobo CM. Water and nonelectrolytes permeability in mammalian red cell membranes. Editor: JAV Butler, D Noble, Progress in Biophysics and Molecular Biology, Pergamon Press Oxford and New York, 1973; 26: p. 106-45.
17. Kargol M, Kargol A, Suchanek G, Przestalski M, Dobkowicz M, Dobkowicz J. The influence of some parameters of the water medium on stationary water exchange by plants living therein. Membrane i Procesy Membranowe w Ochronie Środowiska. Monografie Komitetu Inżynierii Środowiska PAN, 2004; 22: 369-77.
18. Agre P, Brown D, Nielsen S. Aquaporin water channels: unanswered questions and unresolved controversies. Current Opinion in Cell Biology, 1995; 7: 472-83.
19. Curry MR, Schachar-Hill B, Hill AE. Single water channels of aquaporin-1 do not obey the Kedem-Katchalsky equations. J Membrane Biol, 2001; 181: 115-23.
20. Crozier PS, Rowler RL. Molecular dynamics simulation of continuous current flow through a model biological membrane channel. Physical Review Letters, 2001; 86: 2467-70.
21. Schütz K, Tyerman SD. Water channel in Chara corallina. J Experim Botany, 1997; 48: 1511-8.
22. Korohoda W. Integrity and dynamics of the cell surface complex. Ninth School on Biophysics of Membrane Transport School Proceedings, 1988; 1; 472-83.
23. Nałęcz KA. Transport of water through biological membranes. Postępy Biochemii, 1996; 42(2): 161-7.
24. Sha'afi RJ, Gary-Bobo CM. Water and nonelectrolytes permeability in mammalian red cell membranes. Progress in Biophysics and Molecular Biology (vol. 26), Eds JAV Butler and D Noble, Pergamon Press Oxford, New York, 1973; p. 105-46.
25. Siebens AW. Cellular volume control. Physiology and pathophysiology. Seldin DW and Glebisch G, Raven Press, New York, 1985; 91-115.
26. Wolf MBA. Simulation study of the anomalous osmotic behaviour of red cells. J Theor Biol. 1980; 83: 687-700.
27. Gruszecki WJ, Gagos M, Kermen P. Polyene antibiotic amphotericin B in monomolecular layers: spectrofotometric and scanning microscopic analysis. FEBS Letters, 2002; 524: 92-6.

The force-frequency relationship in human heart failure: effect of pyruvate and isoproterenol

Martisiene I*, Gendviliene V, Zablockaite D, Gurskaite H

Institute of Cardiology, Kaunas University of Medicine, Kaunas, Lithuania

Abstract

Purpose: **The purpose of present study was to investigate the effect of metabolic substrate pyruvate and β-adrenergic agonist isoproterenol and combination of these agents on the force- and relaxation-frequency relationship in human heart failure.**

Material and methods: **The experiments were performed on isolated human ventricle strips from patients undergoing cardiac corrective open heart surgery, using conventional method of registration of electromechanical activity. The stimulation frequency of myocardial strips was 0.2, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0 Hz.**

Results: **In control, i.e. at perfusion of myocardial strips by Tyrode solution and stimulation frequency 1 Hz, the contraction force (F) was 0.94±0.18 mN, half time of relaxation (t_r) – 178.8±9.3 ms (n=12). Pyruvate (10 mmol/L) increased F to 176.0±13.4%, t_r – 104.6±3.1% (n=8, p<0.05) vs control. By the action of isoproterenol (10^{-5} mol/L) F increased to 122.1±10.2%, t_r decreased to 58.9±3.1% (n=4, p<0.05) vs control. The relationship of F and t_r from stimulation frequency in the absence of pyruvate and isoproterenol was negative. Pyruvate and isoproterenol didn't alter the shape of force-frequency relationships but F was augmented at all stimulation frequencies. The positive inotropic effect of isoproterenol was potentiated by pyruvate.**

Conclusions: **Pyruvate and isoproterenol alone can improve cardiac contractility in wide-range of stimulation frequency. The combination of these inotropic agents results in even more effective increase of contractile performance and therefore may be of therapeutic value in heart failure.**

* CORRESPONDING AUTHOR:
Institute of Cardiology, Kaunas University of Medicine
Sukileliu 17, LT-50009
Kaunas, Lithuania
Fax: 370 37 302872
e-mail: membiof@kmu.lt (Irma Martisiene)

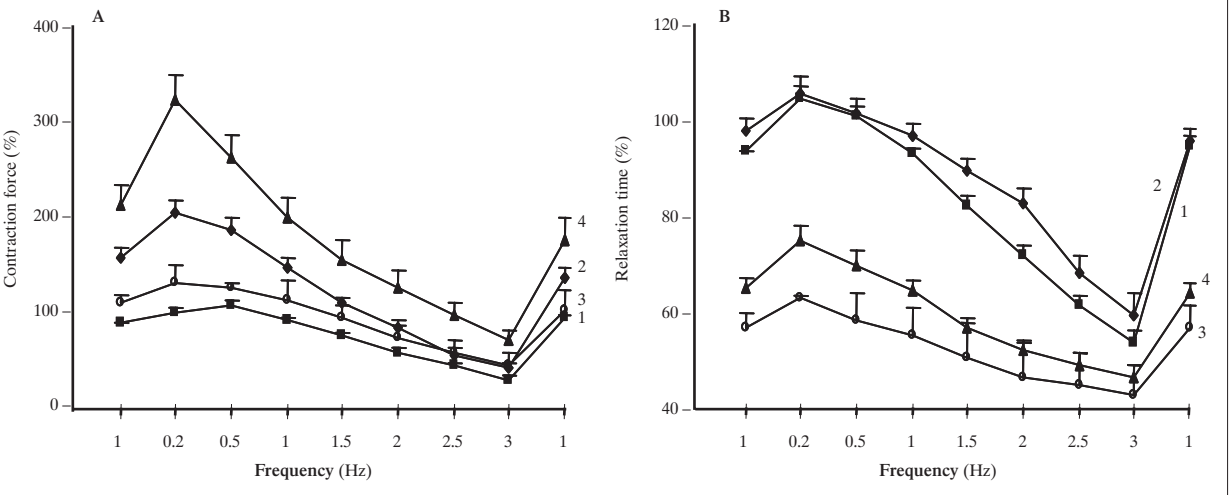
Received 25.05.2005 Accepted 02.06.2005

Key words: failing human myocardium; force-frequency relationship; pyruvate; isoproterenol.

Introduction

The main feature of failing human myocardium is a decline of contraction force caused by disorders of cellular systems regulating intracellular Ca^{2+} concentration, such as sarcolemmal L-type Ca^{2+} channel, Ca^{2+}-ATPase, Na^{+}-Ca^{2+} exchanger, sarcoplasmic reticulum (SR) Ca^{2+}-ATPase (SERCA2) and phospholamban [1]. An informative indicator and convenient methodical tool for evaluating the defects of behavior of these systems and severity of contractile dysfunction, cardiac reserve capacity and effectiveness of therapeutic agents is the force-frequency relationship (FFR). Normal human heart exhibits a positive FFR, i.e. increasing of pacing frequency augments the contraction force of myocardium, while negative FFR is the characteristic of failing myocardium [1,2]. It has been reported that β-adrenergic stimulation of failing human myocardium increases the contractility and partly reverses the negative FFR [2]. However, the increased energy demand (ATP) that is observable during β-adrenergic stimulation limits the efficiency of this inotropic intervention in failing myocardium. It has been recently shown that pyruvate, a natural aliphatic monocarboxylate and central metabolic intermediate in mammalian cells, increases phosphorylation potential, improves contractility and potentiates β-adrenergic inotropism in failing human heart [3,4]. However, the influence of pyruvate and combination of pyruvate with β-adrenergic agonists on the force-frequency relationship in human heart failure is not determined. The purpose of present study was to investigate the effect of pyruvate and isoproterenol and combination of these agents on the force- and relaxation-frequency relationship in failing human heart.

Figure 1. **Influence of stimulation frequency on the contraction force (A) and relaxation time (B) in failing human myocardium: without pyruvate and isoproterenol – curve 1, (n=12); in the presence of pyruvate (10 mmol/L) – curve 2, (n=8), or isoproterenol (10^{-5} mol/L) – curve 3 (n=4), or pyruvate and isoproterenol – curve 4 (n=8) in Tyrode solution. Changes in contraction force and relaxation time are given in % change from the basal value at 1 Hz**



Material and methods

The experiments were performed on strips of human ventricle myocardium from patients undergoing mitral or aortic valve correction surgery in Department of Cardiosurgery of Kaunas University Hospital. Isolated myocardium strips were placed in an experimental chamber and superfused with oxygenated (100% O_2) Tyrode solution (in mmol/L): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.9, glucose 5, Hepes 10, pH 7.4 at 36±0.5°C. The stimulation frequencies was 0.2, 0.5, 1, 1.5, 2, 2.5 and 3 Hz, the duration of pulses – 2-5 ms, amplitude – twice the diastolic threshold. Isometric contraction was recorded using a linear force-displacement transducer (Harvard Apparatus, U.S.A.). The action of pyruvate (10 mmol/L) and isoproterenol (10^{-5} mol/L) on the contraction force (F) and half time of relaxation (t_r) was investigated. Changes of parameters were expressed in percentage in respect to control (Tyrode solution, 1 Hz). All values were presented as means ±SEM. The significance of data was assessed using Student's t-test and the results were considered significant at $p<0.05$.

Results

An average of contraction force of ventricular strips from human failing heart was 0.94±0.18 mN, half time of relaxation – 178.9±9.3 ms (n=12). After addition of pyruvate (10 mmo/L) F increased to 176.0±13.4%, t_r – 104.6±3.1% (n=8, $p<0.05$) vs control. Isoproterenol (10^{-5} mol/L) increased F to 122.1±10.2% and diminished t_r to 58.9±3.1% (n=4, $p<0.05$) vs control. The combination of pyruvate and isoproterenol resulted in an increase of contraction force to 236.9±2.5% (n=8, $p<0.05$), which was higher than the addition of the individual effects of these agents. The half time of relaxation under the action of these agents decreased to 67.7±2.4% (n=4, $p<0.05$) vs control.

Fig. 1 shows the influence of stimulation rate on the contraction force (*A*) and half time of relaxation (*B*) of ventricular strips without (curve 1) and with pyruvate (10 mmol/L) (curve 2) or isoproterenol (10^{-5} mol/L) (curve 3), or combination of these both agents (curve 4) in Tyrode solution. In the absence of pyruvate or isoproterenol the contraction force and relaxation time slightly increased at low stimulation frequency (0.2-0.5 Hz) and continuously declined at higher stimulation rate (curve 1 in *Fig. 1A* and *B*, respectively). Pyruvate as well as isoproterenol didn't alter the shape of FFR, however, contraction force was higher at all stimulation frequencies, as compared to untreated muscles (*Fig. 1A*, curves 2 and 3, respectively). The combination of these inotropic agents resulted in a more significant increase of contraction force at investigated range of stimulation rate as compared to their individual effects (*Fig. 1A*, curve 4). The decrease of relaxation time at high stimulation rate was lesser under the action of pyruvate and higher under the action of isoproterenol as compared to untreated muscles (*Fig. 1B*, curve 2 and 3 respecively). The less significant acceleration of relaxation was observed by combination of pyruvate and isoproterenol as compared to isoproterenol action alone (*Fig. 1B*, curve 4).

Discussion

The present study demonstrates that an increase of stimulation frequency reduced the contraction force and relaxation time in failing human myocardium. The main cause of negative force-frequency relationship may be the alterations in the intracellular Ca^{2+}-handling caused by reduced activity of the SERCA2 in failing myocardium [2]. The metabolic substrate pyruvate and β-adrenoceptor agonist isoproterenol didn't alter the shape of FFR, however, they improved contractile function in wide-range of stimulation frequency in failing human myocardium. The main mechanisms by which pyruvate increases

contractility include the stimulation of SERCA2 activity and an increase of SR Ca^{2+}- uptake due to an increase in phosphorylation potential and free energy available from ATP hydrolysis [4,5]. The positive effect of β-adrenoceptor agonist isoproterenol can be explained by an increase in intracellular cAMP level, phosphorylation of L-type Ca^{2+} channel and phospholamban what removed the inhibition of SERCA2, increased Ca^{2+} load in SR and contractility in failing myocardium [1,2]. Our results show that the combination of these inotropic agents resulted in a more effective increase of contractile performance in wide-range of stimulation frequency. This effect may be interpreted as a potentiation of β-adrenergic inotropism by metabolic substrate pyruvate due to improved cardiac energetic state in failing human myocardium. We conclude that combination of pyruvate and β-adrenergic agents may be of therapeutic value in heart failure.

References

1. Endoh M. Force-frequency relationship in intact mammalian ventricular myocardium: physiological and pathophysiological relevance. Europpean Journal of Pharmacology, 2004; 500: 73-86.

2. Bavendiek U, Brixius K, Münch G, Zobel C, Müller-Ehmsen J, Schwinger RHG. Effect of inotropic interventions on the force-frequency relation in the human heart. Basic Research in Cardiology, 1998; 93: 76-85.

3. Hermann H-P, Zeitz O, Lehnart SE, Keweloh B, Datz N, Hasenfuss G, Janssen PML. Potentiation of beta-adrenergic inotropic response by pyruvate in failing human myocardium. Cardiovascular Research, 2002; 53: 116-23.

4. Hasenfuss G, Maier LS, Hermann H-P, Lüers C, Hünlich M, Zeitz O, Janssen PML, Pieske B. Influence of pyruvate on contractile performance and Ca^{2+} cycling in isolated failing human myocardium. Circulation, 2002; 105: 194-9.

5. Mallet RT, Sun J. Mitochondrial metabolism of pyruvate is required for its enhancement of cardiac function and energetics. Cardiovascular Research, 1999; 42: 149-61.

Changes of β_2-adrenergic stimulation induced by hyperosmosis in human atrium

Zablockaite D*, Gendviliene V, Macianskiene R, Skeberdis VA, Jurevicius J, Kanaporis G, Benetis R

Institute of Cardiology, Kaunas University of Medicine, Kaunas, Lithuania

Abstract

Purpose: The purpose of the present study was to determine whether extracellular osmotic pressure modulates β_2-adrenergic stimulation of the contraction force and L-type Ca^{2+} current in human atrial myocytes.

Material and methods: Experiments were performed on human atrial trabeculae and myocytes isolated from the right atrium. The concentration dependent effect of salbutamol (*SAL*), a β_2-adrenoreceptor agonist, on peak tension (*P*) and L-type calcium current (I_{CaL}) under isoosmolar (345 mOsm) and hyperosmolar (405 or 525 mOsm was achieved by adding of mannitol) conditions was studied.

Results: Salbutamol (10 nmol/L - 10 μmol/L) added to the control solution increased *P* by 180.6±45.8 % over control with a half-stimulation constant EC_{50} = 27±6 nmol/L. Under isoosmolar conditions *SAL* ($0.1 \div 10^3$ nmol/L) increased I_{CaL} by 182.3±19.8% over control with an EC_{50} 2.9±0.9 nmol/L. In hyperosmolar solutions the same concentrations of *SAL* increased *P* and I_{CaL} by 57.2±12.6% and 217.2±70.5% over control with EC_{50}=640±260 nmol/L and 12±5 nmol/L respectively.

Conclusions: These results indicated that hyperosmolarity reduced the effect of β_2-adrenergic stimulation, i.e. the dose-response curve of salbutamol on L-type calcium current was shifted to the higher concentration range and maximal increase in contraction force was diminished in human atrial cells.

* CORRESPONDING AUTHOR:
Institute of Cardiology
Sukileliu 17, LT-50009
Kaunas, Lithuania
Fax: 370 37 302872
e-mail: dangzab@yahoo.com (Danguole Zablockaite)

Received 25.05.2005 Accepted 01.06.2005

Key words: human atrium, salbutamol, contraction force, L-type calcium current, hyperosmosis.

Introduction

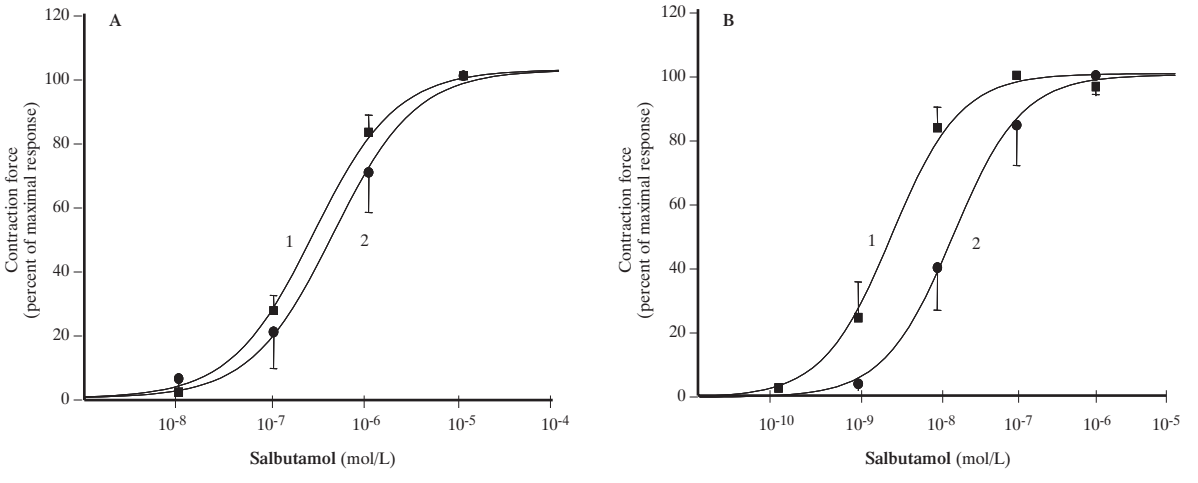
The β-adrenergic receptor system plays a major role in heart failure. β-adrenoreceptors (β-ARs) exist in the heart of various animal species, including man, and relative amount of each receptor subtypes may differ significantly depending on the cardiac tissue, the animal species, the pathophysiological state (many investigators demonstrated substantial loss of β_1-ARs but not β_2-ARs in failing human hearts) and the age. The activation of cardiac β_1- and β_2-ARs mediates inotropic, chronotropic and lusitropic effects in the heart [1]. In some pathological states such as ischemia swelling of myocardial cells is observed but during reperfusion, apoptosis or blockade of sodium pump by ouabain shrinkage of myocardial cells develops [2]. Alterations of cell volume cause the deformation of cell membranes and the underlying cytoskeletal network as well as changes of electrical activity and contractility in the heart [2,3,4]. However, it is few known about dependence of β-adrenergic stimulation of contraction and L-type calcium current in myocardial cells on extracellular osmolarity.

The purpose of the present study was to determine changes of β_2-adrenergic stimulation induced by hyperosmosis on contraction force and L-type calcium current in human atrium.

Material and methods

The experimental pieces of human atrium were obtained from the patient's hearts undergoing coronary bypass surgery in Department of Cardiosurgery of Kaunas University Hospital. The procedure was approved by the Ethical Committee of the University Hospital and conforms to the principles outlined in the Declaration of Helsinki. The standard Tyrode solution was (in mmol/L): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.9, glucose

Figure 1. **Effect of salbutamol on the contraction force (A) and L-type calcium current (B) in human atrial preparations. A. 1 – dose-response curve in the isoosmolar solution (n=8); 2 – dose-response curve in the hyperosmolar solution (n=4). B. 1 – dose-response curve in isoosmolar solution (n=3); 2 – dose-response curve in hyperosmolar solution (n=5). The data are presented as a percentage of the maximal response to the salbutamol effect**



5, Hepes 10; pH 7.4, pO_2 (at 36-37°C) was 77-80 kPa, osmolarity 345 mOsm/L. Hyperosmosis (525 or 405 mOsm/L) was induced by adding 180 or 60 mmol/L of mannitol to the standard Tyrode solution.

The standard electromechanical activity and L-type calcium current registration methods were used [5,6]. The effect of salbutamol on contraction force (P) and L-type calcium current (I_{CaL}) were measured at iso- and hyperosmolar conditions. The dose-response curve was fitted using the Michaelis-Menten equation.

Values are means ±SE Student's t-tests for data group was used for statistical analysis. Differences were considered significant if $p<0.05$.

Results

Under isoosmolar conditions salbutamol (10 nmol/L--10 μmol/L), a selective agonist of β_2-adrenoceptors caused a potent positive inotropic effect on human atrial trabeculae. The maximal increase of contraction force (P_{max}) was 180.6±45.8% (*Fig. 1A, curve 1*). The concentration of salbutamol required for half-maximal stimulation of contraction force (EC_{50}) was 270±60 nmol/L ($p<0.05$) (n=10). Under hyperosmolar conditions (525 mOsm/L) a basal contraction force was decreased to 33.98±5.5% ($p<0.001$) versus isoosmolar conditions. In hyperosmolar solutions the dose-dependent effect of salbutamol on contraction force was: EC_{50}=640±260 nmol/L, P_{max}=57.2±12.6% (n=7) ($p<0.05$). Thus, under these conditions the efficacy of βARs stimulation was 3.14-fold lower increase in contraction force, whereas the dose-response curve was nonsignificantly shifted to the higher concentration range (*Fig. 1A, curve 2*).

A cumulative dose-response curve for the effect of salbutamol on I_{CaL} (0,1-10^3 nmol/L) in isoosmolar conditions is presented in *Fig. 1B* (*curve 1*). Salbutamol increased I_{CaL} with an EC_{50} value of 2.9±0.9 nmol/L and E_{max}=182.3±19.8% (n=3).

In hypertonic solutions dose-response curve of salbutamol on I_{CaL} is presented in *Fig. 1B*, (*curve 2*). EC_{50} and E_{max} were 12±5 nmol/L ($p<0.05$) and 217.2±70.5%, respectively ($p<0.1$) (n=5), i.e. hyperosmolarity increased E_{max} not significantly, but there was significant shift of dose-response curve to the higher concentration range of salbutamol.

Discussion

β-adrenergic stimulation of contraction force (P) and L-type calcium current (I_{CaL}) in myocardial cells is due to the stimulation of adenylate cyclase and a consequent increase in intracellular content of cAMP. cAMP activates protein kinase A resulting in phosphorylation of several proteins involved in the handling of calcium [1]. The same pathway fulfils action of salbutamol as β_2-adrenoceptor agonist.

During hypertonic cell shrinkage water is removed from cells and accumulation of intracellular ions, such as $[Na^+]_i$, $[Ca^{2+}]_i$, $[H^+]_i$ occurres [4,7,8]. In hyperosmolar solution it was shown the inhibition of the delayed rectifier K^+ current, increase of the outward-directed Na^+-Ca^{2+} exchange current, reduction I_{CaL} [4,9] and shift of pH_i to the alkaline, as well as to acidic direction [7,8]. The development of P in cardiac muscle is initiated by the binding of Ca^{2+} to troponin C. This initiation is tightly coherent with $[H^+]_i$, i.e. protons competitively inhibit the extent of Ca^{2+} binding. Negative inotropic effect of hyperosmolarity in our experiments believable was associated with intracellular acidosis [7], and with inhibition of I_{CaL} produced by markedly enhanced of $[Ca^{2+}]_i$ [9]. In shrunken myocytes, as well as in control, salbutamol caused augmentation of I_{CaL}. Less effect of salbutamol on P in hyperosmolar solutions is due to the shift of the dose-response I_{CaL} curve to the higher concentration range of the drug.

In conclusion, our experiments showed that hyperosmosis reduced the effect of β-adrenergic stimulation, i.e. substantially decreased the salbutamol stimulated contraction force and shifted the dose-response curve of salbutamol on L-type calcium current to the higher concentrations range.

References

1. Kaumann A, Bartel S, Molenaar P, Sanders L, Burrell K, Veter D, et al. Activation of β_2-adrenergic receptors hastens relaxation and mediates phosphorylation of phospholamban, troponin I, and C-protein in ventricular myocardium from patients with terminal heart failure. Circulation, 1999; 99: 65-72.

2. Lang F, Busch GL, Ritter M, Völki H, Waldegger S, Gulbins E, et al. Functional significance of cell volume regulatory mechanisms. Physiol Rev, 1998; 78: 247-306.

3. Hermsmeyer K, Rulon R, Sperelakis N. Loss of the plateau of the cardiac action potential in hypertonic solutions. J Gen Physiol, 1972; 59: 779-93.

4. Ogura T, Matsuda H, Shibamoto T, Imanishi S. Osmosensitive properties of rapid and delayed rectifier K^+ currents in guinea-pig heart cells. Clin Exp Pharmacol Physiol, 2003; 30: 616-22.

5. Gendviliene V., Macianskiene R., Kanaporis G., Jurevicius J. Effect of β_3-adrenoceptor agonist BRL 37344 on L-type Ca^{2+} current and contraction force in human myocardium. Acta Medica Lituanica, 2001; 8: 1059-63.

6. Skeberdis VA, Jurevičius J, Fischmeister R. Beta-2 adrenergic activation of L-type Ca^{++} current in cardiac myocytes. J Pharmacol Exp Ther, 1997; 283(2): 452-61.

7. Whalley DW, Hemsworth PD, Rasmussen HH. Regulation of intracellular pH in cardiac muscle during cell shrinkage and swelling in anisosmolar solutions. Am J Physiol, 1994; 266: H658-69.

8. Befroy DE, Powel T, Radda GK, Clarke K. Osmotic shock: modulation of contractile function, pH_i and ischemic damage in perfused guinea pig heart. Am J Physiol, 1999; 276: H1236-44.

9. Smogorzewski M, Galfayan V, Massry SG. High glucose concentration causes a rise in $[Ca^{2+}]_i$ of cardiac myocytes. Kidney Int, 1998; 53: 1237-43.

Blood oxygen-carrying function during the oxidative stress induced by lipopolysaccharide with a modification of the L-arginine-NO pathway

Glebov A, Zinchuk V*

Department of Physiology, Grodno State Medical University, Belarus

Abstract

Purpose: Our aim was to study the blood oxygen-carrying function during the oxidative stress with a modification of the L-arginine-NO pathway.

Material and methods: Oxidative stress was induced by intravenous administration of *Escherichia coli* lipopolysaccharide (LPS) to rabbits. To modify the L-arginine-NO pathway, animals were administered with N^G-nitro-L-arginine methyl ester intravenously 60 min after the LPS. Mixed venous blood was sampled for evaluation of blood oxygen transport before and at 120 and 240 min after the LPS administration; tissue samples (heart, lung, liver, kidney and muscle) were also prepared. The following parameters were measured hemoglobin-oxygen affinity, concentrations of conjugated dienes, Schiff bases, α-tocopherol and activity of catalase.

Results: During the NO synthase inhibition the oxidative stress was characterized by a shift of hemoglobin oxygen dissociation curve rightwards, more prominent activation of lipid peroxidation and decreased tissue levels of antioxidant defense factors.

Conclusions: The inhibition of NO generation induces a shift of prooxidant-antioxidant balance – obviously, not only due its potentially high levels and reactivity with the various target molecules (with a development of oxidative stress), but also because of the lower contribution of other factors including the hemoglobin-oxygen affinity change into the body antioxidant potential.

* CORRESPONDING AUTHOR:
Grodno State Medical University
80 Gorky Str.
230015 Grodno, Belarus.
Fax: +375 152 335341
e-mail: zinchuk@grsmu.by (Vladimir Zinchuk)

Received 22.04.2005 Accepted 18.07.2005

Key words: oxidative stress, lipopolysaccharide, blood, nitric oxide.

Introduction

Under the tissue steady-state conditions, the excessive oxidant generation is counterbalanced by enzymatic and non-enzymatic antioxidants inside and outside the cells; thus the some optimal level of prooxidant-antioxidant balance is created [1]. If this balance is changed due to the excessive free radical production and/or antioxidant system damage, the so-called oxidative stress appears. Under this condition the excessive free radical generation and non-specific tissue impairment occur without a control by antioxidant mechanisms [2]. Experimental evidence suggests that reactive oxygen species may be important mediators of cellular injury during endotoxemia (induced by LPS), either as a result of macromolecular damage or by interfering with extracellular and intracellular regulatory processes [3].

Nitric oxide (NO) is thought to play a key role in the pathogenesis of sepsis. Bacterial endotoxin induces the release of many mediators, including NO, responsible for a late-phase hypotension, vasoplegy, acidosis, hypoxia and multiorgan failure [4,5]. NO is an important participant of this complex system of prooxidant-antioxidant balance. NO is a free radical capable both to ameliorate the oxidative injury (as a chain-terminating radical scavenger) and to be the source of reactive nitrogen species [6]. NO has the complex relationships with the free oxygen radicals – both interaction (NO can react with $O_2^{\bullet-}$ to generate a potent oxidant peroxynitrite) and competition (with a development of oxidative or nitrosative stress in the biological compartment) [7]. In a recent time the interactions between NO and hemoglobin (Hb) were extensively investigated. Three main NO-derivatives of Hb are known: methemoglobin, nitrosylhemoglobin ($HbFe^{2+}NO$), and S-nitrosohemoglobin (SNO-Hb) [8-10]. In a molecule of $HbFe^{2+}NO$ the NO moiety is a ligand to ferrous heme, and SNO-Hb is a result of NO interaction with

cysteine (93) of β-globin chain [11,12]. The presence of different hemoglobin-NO adducts can differently influence on the whole blood hemoglobin-oxygen affinity (HOA). Methemoglobin and SNO-Hb raise the HOA and $HbFe^{2+}NO$ decreases it. Such modulation of the blood oxygen-binding properties may be important for gas exchange and tissue oxygenation [13,14]. Our aim was to study the blood oxygen-carrying function during the oxidative stress with a modification of the L-arginine-NO pathway.

Material and methods

Animals

The adult male Chinchilla rabbits (n=21; body weight 2.5-3.1 kg) were kept for 2 weeks in a constant-climate environment with respect to temperature, humidity and daylight cycle. Animals were fed on a laboratory diet with water and food ad libitum until use and fasted overnight with free access to water before the operation. Operation procedures were performed between 8.00 and 12.00 to avoid the chronobiological variations. All the experimental procedures described in this paper are in accordance with the Guiding Principles for the Care and Use of Animals accepted by the Ethical Committee of Grodno Medical University.

Procedure

Animals were anesthetized with pentobarbital sodium (50 mg/kg). Sham-operated rabbits (1st group) received 1.0 mL saline intravenously (n=5) served as a control for mixed venous blood and tissue sampling. In rabbits of the 2nd group (n=9) the oxidative stress was induced by intravenous administration of 500 μg/kg *Escherichia coli* lipopolysaccharide (LPS) from Sigma Chemical. Animals of the 3rd group (n=7) intravenously received NO synthase inhibitor N^G-nitro-L-arginine methyl ester (L-NAME) from Sigma Chemical, 7.5 mg/kg, 60 min after the LPS. The catheters for mixed venous blood sampling were inserted into the right atrium through the external jugular vein. Blood was collected in heparinized syringes. Such sampling for estimation of the blood oxygen transport was performed before and at 120 and 240 min after the LPS administration. Tissue sampling (heart, lung, liver, kidney and muscle) was performed after 240 min of oxidative stress; tissues were washed with a cold phosphate-buffered saline to remove the blood traces, and then the samples were immediately frozen in liquid nitrogen and stored until analysis.

Lipid peroxidation products

Conjugated diene content was determined by the fluorescence intensity of UV absorption at 232-234 nm, characteristic for the conjugated diene structures [15]. Schiff base level was evaluated by the fluorescence intensity of chloroform extract at excitation and emission wavelengths of 344 and 440 nm, respectively [15] with a spectrofluorimeter "F-4010" (Hitachi).

Antioxidant system

α-tocopherol content was measured by the fluorescence intensity of heptane extract at excitation and emission wavelengths of 292 and 325 nm, respectively [16] with a spectrofluorimeter "F-4010" (Hitachi), using α-tocopherol (Sigma) as a reference. Catalase activity in the biological materials was estimated by the decrease of hydrogen peroxide capable to form a stable stained complex with molybdenum salts, with a spectrophotometer "СФ-46" at 410 nm [16].

Blood oxygen-carrying function

pO_2 were measured with micro gas analyzer ABL-330 (Radiometer) at 37°C with the following correction to actual temperature value. HOA was evaluated by p50 (blood pO_2 at its 50% oxygen saturation) determined by the "mixing" method at 37°C, pH 7.4 and pCO_2 40 mm Hg ($p50_{stand}$) [17]. p50 at actual pH, pCO_2 and temperature ($p50_{act}$) was calculated from $p50_{stand}$ with Severinghaus' formulas [18] using the temperature coefficient of 0.024. Oxygen dissociation curves of Hb (ODCs) were calculated with Hill's equation using n=2.8. The amounts of Hb and methemoglobin were determined spectrophotometrically.

Measurement of plasma NO_3^-/NO_2^-

Plasma samples (50 μl) were deproteinized by incubation with 140 μl of deionized H_2O and 10 μl of 30% $ZnSO_4$ at room temperature for 15 min. Samples were then centrifuged at 2000 g for 10 min. Nitrate was converted to nitrite using cadmium beads, and nitrite was measured spectrophotometrically [19].

Statistical analysis

The data were statistically evaluated by Student's t-test with a significance level of $p<0.05$. The results are presented as mean ± standard error of mean (SE). The analyses and graphs were performed using computer software packages.

Results

LPS administration resulted into the prominent activation of lipid peroxidation processes, with a creation of oxidative stress. Tissue lipid peroxidation activity increased after LPS administration – *Fig. 1* (conjugated dienes in heart, lung, liver, kidney and muscle: by 38.8, 39.2, 48.9, 16.4 and 20.0%, respectively; Schiff bases: by 11.7, 60.1, 309.2, 69.5 and 60.7%, respectively; all $p<0.05$). The largest rises in conjugated diene and Schiff base content under oxidative stress (LPS + L-NAME) comparing with only LPS injection were noted in lungs (16.2 and 14.7%, respectively; both $p<0.05$) and liver (54.3 and 20.6%, respectively; both $p<0.05$). This indicates a higher activity of the free radical lipid oxidation during NO synthase inhibition.

The lowering of the antioxidant defense factors was observed in tissues. Catalase activity and α-tocopherol content in the lung of rabbits with oxidative stress (LPS) were significantly lower than in control – by 24.6 and 18.3%, respectively (all $p<0.05$); in liver the catalase activity decreased only by 6.9% ($p<0.05$). Under NO synthase inhibition such fall of antioxidant defense factors comparing with LPS group was even more significant in all tissues tested (*Fig. 2*).

The content of NO utilization products (NO_3^-/NO_2^-) after LPS administration was larger than at baseline level; and

Figure 1. **Indices of lipid peroxidation in rabbit tissues under the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester (L-NAME): conjugated dienes (A), Schiff bases (B). The values are means ±SE. * – significant difference from the control group ($p<0.05$); # – significant difference from the group of rabbits received LPS ($p<0.05$)**

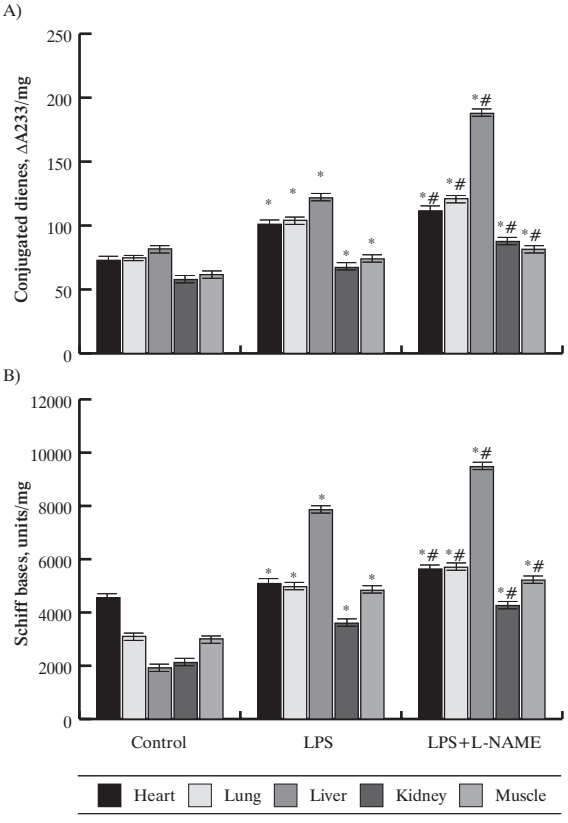
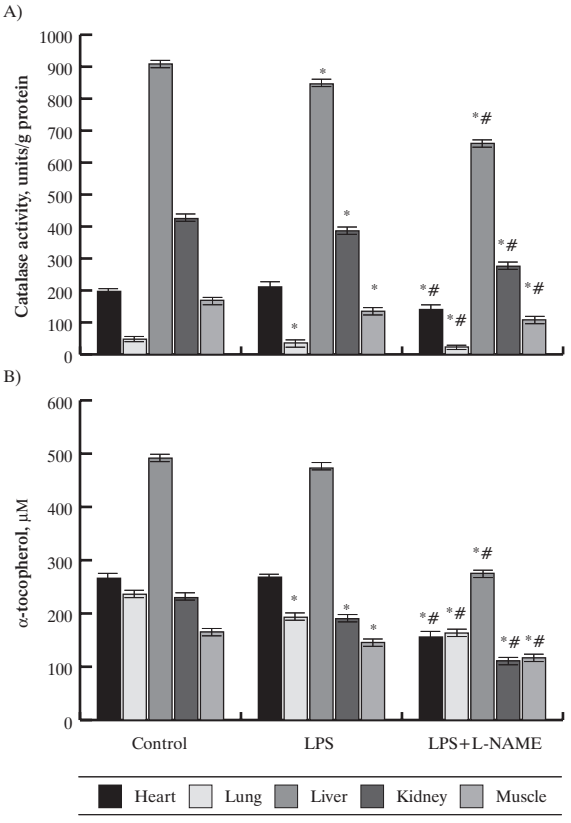


Figure 2. **The changes in catalase activity (A) and α-tocopherol content (B) in rabbit tissues under the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester (L-NAME). The values are means ±SE. * – significant difference from the control group ($p<0.05$); # – significant difference from the group of rabbits received LPS ($p<0.05$)**



administration of L-NAME after LPS increased it at 120 min (less than after LPS only) and at 240 min (*Tab. 1*).

The blood oxygen-binding properties considerably changed after the endotoxin administration (*Tab. 1*). Oxidative stress was accompanied by a development of hypoxia, even more marked during the NO synthase inhibition. The value of $p50_{stand}$ decreased at 120 and 240 min by 9.7 ($p>0.05$) and 10.6% ($p<0.05$), respectively. Meanwhile, $p50_{act}$ rose at 240 min after LPS by 9.3% ($p<0.05$) because of the changes in pH, pCO_2 and body temperature. After the injection of L-NAME the values of $p50_{act}$ increased by 31.3 and 29.5% (both $p<0.001$) after 120 and 240 min of oxidative stress, respectively, reflecting the more prominent shift of actual ODCs rightwards (*Fig. 3*).

Discussion

One can see that oxidative stress combined with NO synthase inhibition was characterized by the actual ODC shift rightwards, more marked lipid peroxidation activation and lowering of antioxidant defense factors in blood and tissues. Hb is an allosterically regulated protein and therefore has many binding sites capable to form the reversible non-covalent bonds with a primary ligands that can result in quaternary conformational changes and their modulation by the secondary effectors [13]. In our investigations the ODC position is dictated by effects of pH, pCO_2 and other factors; but one should take in account also NO and its interactions with Hb. NO is considered as the ligand determining the Hb oxygen-binding properties [14].

Intraerythrocyte interaction between NO and Hb is important for regulation of the both molecules *in vivo*. The red cell properties do not limit such interaction under the physiologic conditions [20]. In arterial blood the reaction between NO and oxyhemoglobin produces nitrate and methemoglobin, and in venous blood $HbFe^{2+}NO$ is generated; under high pO_2 it can be oxidated to met-Hb and NO_3^- [21,22]. The NO-binding site at β-globin chain was also found; such binding results in SNO-Hb [11]. The value of p50 for extracellular SNO-Hb is less than 10 mm Hg [23], and p50 for $HbFe^{2+}NO$ is 39.6±1.5 mm Hg [24]. In our experiments the lowest ODC shift leftwards was observed in animals received L-arginine and exposed to hypothermia [1].

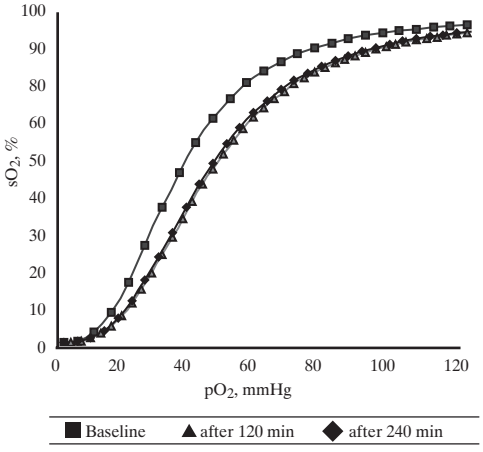
NO can affect tissue oxygenation through its influence on HOA and blood flow regulation. Simultaneously the mechanisms of O_2 transport (including the blood oxygen-binding properties) can modify the activity of the L-arginine-NO pathway. NO can modify HOA through the intraerythrocytic regulatory

Table 1. **The changes NO_3^-/NO_2^- and indices of blood oxygen-carrying function in rabbits during the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester (L-NAME)**

Index	Baseline	After	
		120 min	240 min
oxidative stress (LPS)			
n	9	9	9
NO_3^-/NO_2^-, μM	6.11±0.36	11.41±0.52*	14.27±0.44*#
$p50_{act}$, mm Hg	35.5±0.77	37.1±1.48	38.8±1.13*
$p50_{stand}$, mm Hg	31.0±1.14	28.0±0.53*	27.7±0.91*
Hb, g/dL	9.72±0.20	9.33±0.20	9.30±0.21
pO_2, mm Hg	33.78±2.23	30.94±2.50	28.12±1.34*
methemoglobin, %	0.28±0.09	0.88±0.11*	0.72±0.07*
pH, units	7.322±0.015	7.191±0.035*	7.137±0.037*
pCO_2, mm Hg	47.56±3.12	38.21±2.47*	43.39±1.33
HCO_3^-, mM	24.11±1.08	14.20±1.03*	14.28±0.94*
TCO_2, mM	25.43±1.09	15.37±1.04*	15.51±0.89*
ABE, mM	-1.97±1.37	-12.83±1.43*	-13.71±1.47*
SBC, mM	22.46±1.11	13.81±1.01*	13.14±1.22*
oxidative stress (LPS+L-NAME)			
n	8	8	7
NO_3^-/NO_2^-, μM	6.11±0.26	8.95±0.52*	13.83±0.67*#
$p50_{act}$, mm Hg	33.9±0.95	44.5±2.14*	43.9±1.32*
$p50_{stand}$, mm Hg	31.4±0.74	31.3±0.90	29.8±0.74
Hb, g/dL	10.25±0.25	10.05±0.20	9.99±0.27
pO_2, mm Hg	31.60±2.01	26.15±2.21	20.89±3.04*
methemoglobin, %	0.28±0.08	0.56±0.07*	1.07±0.10*#
pH, units	7.353±0.020	7.103±0.040*	7.029±0.023*
pCO_2, mm Hg	48.65±3.87	47.21±3.36	61.54±2.80*#
HCO_3^-, mM	26.63±1.80	14.27±1.95*	14.75±1.73*
TCO_2, mM	28.11±1.85	15.53±1.93*	16.37±1.59*
ABE, mM	0.98±1.73	-14.98±2.69*	-16.46±1.90*
SBC, mM	24.13±1.65	12.88±2.11*	9.06±1.69*

Note: Data are expressed as mean ±SE.
Significant difference ($p<0.05$) from the baseline level (*) and after the 120 min of received LPS (#)

Figure 3. **Actual oxyhemoglobin dissociation curves for mixed venous blood in rabbits during the oxidative stress induced by LPS combined with administration of N^G-nitro-L-arginine methyl ester**



mechanisms, oxygen-dependent nature of NO production, generation of different NO-Hb derivatives [14]. The concentrations of NO and its derivatives in the microcirculatory part of vascular bed must be much higher than in larger vessels (100-fold and more). NO fraction directly interacting with Hb must also be higher. At the microcirculatory level this may be very important for the change of oxyhemoglobin binding and ultimately for the tissue oxygenation. NO can react with $O_2^{\cdot-}$ to generate a potent oxidant peroxynitrite that can modify the Hb properties [6,7]. Hb may also defend against peroxynitrite, thereby functioning as an intracellular antioxidant. This may also be important for the modification of Hb function and its involvement in formation of O_2 flux to tissues and in maintenance of the body prooxidant-antioxidant balance [1].

Oxidative stress may be considered as the defect of aerobic metabolism – the stochastic process of a free radical production and non-specific tissue damage without the regulation by antioxidant defense mechanisms [2]. Interactions between NO and free oxygen radicals creates the definite balance ($O_2^{\cdot-}$ scavenger system competes with NO for the $ONOO^-$ generation) resulting in the oxidative stress development in a biological object [7]. The HOA change can regulate the oxygen flux to tissues according to requirements, thus preventing its excessive use for the free radical oxidation; therefore, HOA may be considered as one of the factors participating in maintenance of body prooxidant-antioxidant balance. During the oxidative stress the HOA changes mediated by NO-dependent mechanisms (in first turn, endothelial) can affect the oxygen flux to tissues and body prooxidant-antioxidant balance as a whole. Inhibition of NO synthesis induces a shift of this balance – obviously, not only because of potentially high NO levels and reactions with the diverse target molecules, but also due to the lower contribution of other factors including HOA in the antioxidant defense of body. These data support the notion that HOA may alter tissue oxygen supply and may be involved in the pathogenesis of oxidative stress induced by administration of LPS.

Acknowledgement

This work was supported by a grant from the Fund of Fundamental Investigations of the Republic of Belarus.

References

1. Zinchuk VV, Dorokhina LV, Maltsev AN. Prooxidant-antioxidant balance in rats under hypothermia combined with modified hemoglobin-oxygen affinity. J Therm Biol, 2002; 27: 345-52.

2. Hensley K, Robinson KA, Gabbita SP, Salsman S, Floyd RA. Reactive oxygen species, cell signaling, and cell injury. Free Radic Biol Med, 2000; 28: 1456-62.

3. Cadenas S, Cadenas AM. Fighting the stranger – antioxidant protection against endotoxin toxicity. J Toxicology, 2002; 180: 45-63.

4. Wolkow PP. Involvement and dual effects of nitric oxide in septic shock. Inflamm Res, 1998; 47: 152-66.

5. Bateman RM, Sharpe MD, Ellis CG. Bench-to-bedside review: microvascular dysfunction in sepsis-hemodynamics, oxygen transport, and nitric oxide. Crit Care, 2003; 7: 359-73.

6. Klatt P, Lamas S. Regulation of protein function by S-glutathiolation in response to oxidative and nitrosative stress. Eur J Biochem, 2000; 267: 4928-44.

7. Esprey MG, Miranda KM, Feelisch M, Fukuto J, Grisham MB, Vitek MP, Wink DA. Mechanisms of cell death governed by the balance between nitrosative and oxidative stress. Ann NY Acad Sci, 2000; 899: 209-21.

8. Gladwin MT, Ognibene FP, Pannell LK, Nichols JS, Pease-Fye ME, Shelhamer JH, Schechter AN. Relative role of heme nitrosylation and beta-cysteine 93 nitrosation in the transport and metabolism of nitric oxide by hemoglobin in the human circulation. Proc Natl Acad Sci USA, 2000; 97: 9943-8.

9. Joshi MS, Ferguson TB, Han TH, Hyduke DR, Liao JC, Rassaf T, Bryan N, Feelisch M, Lancaster JR. Nitric oxide is consumed, rather than conserved, by reaction with oxyhemoglobin under physiological conditions. Proc Natl Acad Sci USA, 2002; 99: 10341-6.

10. Crawford JH, White CR, Patel RP. Vasoactivity of S-nitrosohemoglobin: role of oxygen, heme, and NO oxidation states. Blood, 2003; 101: 4408-15.

11. Stamler JS, Jia L, Eu JP, McMahon TJ, Demchenko IT, Bonaventura J, Gernert K, Piantadosi CA. Blood flow regulation by S-nitrosohemoglobin in the physiological oxygen gradient. Science, 1997; 276: 2034-7.

12. Gladwin MT, Wang X, Reiter CD, Yang BK, Vivas EX, Bonaventura C, Schechter AN. S-Nitrosohemoglobin is unstable in the reductive erythrocyte environment and lacks O_2/NO-linked allosteric function. J Biol Chem, 2002; 277: 27818-28.

13. Zinchuk V, Borisiuk V. The effect of NO synthase inhibition on blood oxygen-carrying function during hyperthermia in rats. Respir Physiol, 1998; 113: 39-45.

14. Zinchuk VV, Dorokhina LV. Blood oxygen transport in rats under hypothermia combined with modification of the L-arginine-NO pathway. Nitric Oxide, 2002; 6: 29-34.

15. Rice-Evans CA, Diplock AT, Symons MCR. Laboratory techniques in biochemistry and molecular biology: techniques in free radical research. Elsevie, 1991; 125-44.

16. Aruoma OI, Cuppett SL. Antioxidant Methodology: in vivo and in vitro. Concepts AOCSPress, 1997; 152-86.

17. Scheid P, Meyer M. Mixing technique for study of oxygen-hemoglobin equilibrium: A critical evaluation. J Appl Physiol, 1978; 45: 616-22.

18. Severinghaus JW. Blood gas calculator. J Appl Physiol, 1966; 21: 1108-16.

19. Schulz K, Kerber S, Kelm M. Reevaluation of the Griess method for determining NO_3^-/NO_2^- in aqueous and protein-containing samples. Nitric Oxide, 1999; 3: 225-34.

20. May JM, Qu ZC, Xia L, Cobb CE. Nitrite uptake and metabolism and oxidant stress in human erythrocytes. Am J Physiol Cell, 2000; 279: 1946-54.

21. Wennmalm A, Benthin G, Edlund A, Jungersten L, Kieler-Jensen N, Lundin S, Westfelt UN, Petersson AS, Waagstein F. Metabolism and excretion of nitric oxide in humans. Circ Res, 1993; 73: 1121-7.

22. Schechter AN, Gladwin MT. Hemoglobin and the paracrine and endocrine functions of nitric oxide. N Engl J Med, 2003; 348: 1483-5.

23. Bonaventura C, Feruzzi G, Tesh S, Stevens RD. Effects of S-nitrosation on oxygen binding by normal and sickle cell hemoglobin. J Biol Chem, 1999; 274: 24742-8.

Diurnal rhythm of blood pressure, heart rate and adrenergic activity in patients with normotension treated with continuous ambulatory peritoneal dialysis and haemodialysis

Baranowicz-Gąszczyk I*, Jóźwiak L, Bednarek-Skublewska A, Książek A

Department of Nephrology, Skubiszewski Medical School of Lublin, Poland

Abstract

Purpose: People with normotension and with essential hypertension are subjected to the diurnal rhythm of blood pressure (BP) with higher values during the day than during the night. Among dialysed patients nocturnal reduction of BP is blunted. The aim of the study was to evaluate diurnal BP rhythm and adrenergic activity measured as values of catecholamines.

Material and methods: Study was performed among dialysed patients with normotension: 13 haemodialysed patients (HD), 8 patients treated by continuous ambulatory peritoneal dialysis (CAPD) and 10 controls (C). Ambulatory BP monitoring (ABPM) was done by using Micro SJ7400 AMP device. Catecholamines concentrations were measured by HPLC-ED method before and after cold pressure test.

Results: There was no significant difference between manual measurements of BP done by dialysis nurses and mean values of 24-hours ABPM in CAPD group and C group and 48-hours ABPM among HD patients. Diurnal BP was blunted in 80% of HD patients during the day of haemodialysis, 70% during the day without haemodialysis and in CAPD group in 50%. Heart rate (HR) variability was comparable in HD and CAPD groups and significant lower than in C group. Baseline noradrenaline (NA) as well as NA (ng/ml) post cold pressure test levels were significantly higher among HD patients (463±21, 546±31) and CAPD patients (452±76, 527±92) as compared with C (206±53*, 315±61*). (x±SD), *p<0.001

Conclusions: Despite increased adrenergic activity and altered diurnal rhythm of BP and HR exist in dialysed patients we didn't find directly relationship. Another or composed factors could affect diurnal rhythm of BP and HR.

Key words: noradrenaline, dialysis, ambulatory blood pressure monitoring, diurnal rhythm.

Introduction

Blood pressure (BP) among healthy people with normotension and with essential hypertension is commonly subjected to diurnal rhythm with higher values during the day and lower values during the night. Patients with secondary hypertension have the circadian rhythm of BP altered [1,2]. Among dialysed patients nocturnal BP reduction is significantly blunted [3-7]. Abnormal circadian rhythm of BP may be related to a high incidence of cardiovascular disease morbidity and mortality among patients with chronic kidney disease [3,4,8]. The pathogenetic mechanisms of the blunted nocturnal decrease of BP among those patients are still unclear.

Patients with chronic renal failure (CRF) showed increased neural sympathetic activity and elevated levels of catecholamines and it may influence on cardiovascular prognosis, [9-13]. It is not known whether these abnormalities are related to and if they are associated with chronic renal disease [3,14].

The aim of the study was to evaluate the correlation between diurnal rhythm of blood pressure and diurnal heart rate variability, and adrenergic activity measured by levels of catecholamines, among normotensive patients treated with continuous ambulatory peritoneal dialysis and haemodialysis.

* CORRESPONDING AUTHOR:
Department of Nephrology,
Skubiszewski Medical School of Lublin,
ul. Jaczewskiego 8
20-954 Lublin, Poland
e-mail: iwona_gaszczyk@pharmanet.com.pl
(Iwona Baranowicz-Gąszczyk)

Received 10.12.2004 Accepted 09.05.2005

Material and methods

Patients

13 patients undergoing chronic haemodialysis (HD), 8 patients treated with continuous ambulatory peritoneal dialysis (CAPD) and 10 healthy persons (C) were selected to our study. The main selection criterion was normotension without antihypertensive drugs. Patients with a history or physical examination indicative of diabetes mellitus, coronary heart disease, congestive heart failure, cardiac arrhythmias, amyloidosis, infection or other systemic or malignant disease were excluded from the study.

The HD group consisted of 7 men and 6 women, aged between 23-59 years (average 42.2±3.4 years), time of haemodialyses from 7 to 204 months (average 56.1±18.1 months). The etiology of the renal failure included: chronic glomerulonephritis (7 patients), obstructive uropathy (3 patients), interstitial nephritis (2 patients), kidney tuberculosis (1 patient). In the CAPD group there were 3 men and 5 women, aged between 24-59 years (average 38.7±5.4 years), time of dialysotherapy from 4-82 months (average 38.3±17.0 months). The etiology of the renal failure included: glomerulonephritis (4 patients), obstructive uropathy (2 patients), interstitial nephritis (2 patients).

All patients received normal diet with free protein and salt intake and supplementation of calcium carbonate, vitamines, iron and recombinant epoetin in individual doses. The patients were anuric (diuresis <100 ml/day). Routine haemodialysis and peritoneal dialysis procedures were followed for each patient according to their individual orders. All procedures of haemodialysis were performed in the morning hours between 7.00 a.m. and 12.00 a.m. using low-flux, semi synthetic membranes with bicarbonate as the dialysate fluid.

The C group consisted of 5 men and 5 women, aged 16-59 years (average 34.6±4.7 years). They received normal diet and were not treated with medications. Each patient was informed about the nature and purpose of the study and signed written consent.

Ambulatory blood pressure and heart rate measurements (AMBP)

Blood pressure (BP) and heart rate (HR) monitoring was done by Micro-SI 7400 AMP apparatus. In HD patients BP and HR were monitored for 48 hours: during the day of haemodialysis and on the following day without haemodialysis. Among CAPD patients and C group BP and HR were monitored for 24 hours. AMBP measured and recorded systolic (SBP), diastolic (DBP) and HR every 20 minutes during day-time (6.00 a.m. – 23.00 p.m.) and every 60 minutes during night-time (23.00 p.m. – 6.00 a.m.). Diurnal BP rhythm was considered to be lost when night BP was less than 10% of day BP. Diurnal HR profile was blunted, when the difference between day HR and night HR was not significant.

Manual blood pressure and heart rate measurements

The routine BP and HR measurements were performed by dialysis nurses before haemodialysis session (before HD), after haemodialysis session (after HD) and during the day without haemodialysis (day without HD) among HD patients and only once in CAPD and C groups. BP measurements were done using mercury sphingomanometer PRLTH 2.

Cold pressure test (CP)

After an adequate period of rest the hand of the subjects was plunged in icy water (O±C) up to the wrist for 1 minute. BP was measured traditionally before and after CP.

Catecholamines /CA/ (ng/l)

Noradrenaline, adrenaline and dopamine concentrations were determined by HPLC with electrochemical detection method. Blood samples for measurements of CA were obtained, before CP test and after CP test, from venous limb of forearm or arm arteriovenous fistula in HD patients and from forearm vein in CAPD and C groups. Blood collected for CA measurements was immediately transformed into ice-cold tubes with EDTA. Plasma was separated and stored at -70°C until the assay.

In HD patients all the experimental procedure were carried out three times: 1 hour before HD, 1 hour after HD and during the day without HD, in CAPD and healthy persons it was carried out only once.

Statistical analysis

The results are given as mean values ±SD. The statistical evaluation was done using Student's t-test for paired and unpaired data, regression analysis.

Results

We compared results of single manual measurements of BP done by dialysis nurses and average results of 24 hours ABPM in HD, CAPD, C groups. Mean values of ABPM showed a significant correlation with manual values among dialysed patients and healthy people. Among HD patients mean values of SBP in manual measurements were 131±7 mmHg before HD, 124±12 mmHg after HD, 127±10 mmHg during the day without HD and average results of SBP in ABPM were 126±6 mmHg during the day with HD and 122±9 mmHg during the day without HD. In this group mean values of DBP in manual measurements were 85±7 mmHg before HD, 77±10 mmHg after HD, 81±7 mmHg during the day without HD and average results of DBP in ABPM were 79±6 mmHg during the day with HD and 77±6 mmHg during the day without HD. There were no significant differences between manual measurements and ABPM measurements in HD groups. There was significant difference ($p<0.05$) between results of BP manual measurements before and after HD. The mean values of SBP and DBP were higher before HD procedures than after HD procedures. In CAPD group mean values of SBP in manual measurements was 130±20 mmHg and average result of SBP in ABPM was 124±26 mmHg. In this group mean values of DBP in manual measurements were 82±10 mmHg and average result of DBP in ABPM was 79±12 mmHg. There were no significant differences between manual measurements and ABPM measurements in CAPD group. In C group mean values of SBP in manual measurements was 125±11 mmHg and average result of SBP in ABPM was 121±11 mmHg. In this group mean values of DBP in manual measurements was 77±12 mmHg and average result of DBP in ABPM was 72±9 mmHg. There were no significant differences between manual measurements and ABPM measurements in C group (*Tab. 1*).

Table 1. **Single manual measurements and 24-hours ABPM in dialysed patients and control** (x±SD)

BP mmHg		HD group			CAPD group	Control group
		Before HD	After HD	Day without HD		
SBP	Manual measurements	131±7	124±12*	127±10	130±20	125±11
	Automatic measurements	126±6		122±9	124±26	121±11
DBP	Manual measurements	85±7	77±10*	8±7	82±10	77±12
	Automatic measurements	79±6		77±6	79±12	72±9

*p<0.05 compared to values before HD

Table 2. **Diurnal rhythm of BP and HR in dialysed patients and control** (x±SD)

	Diurnal rhythm	HD group		CAPD Group	Control
		Day with HD	Day without HD		
SBP (mmHg)	Day-time	129±12	123±14	127±24	125±11
	Night-time	122±12	118±20	121±30	110±11
	Difference Day/night (%)	6±11	4±12	7±12	12±2^ ^
DBP (mmHg)	Day-time	80±9	78±9	82±12	75±5
	Night-time	76±12	74±6	78±5	62± 9
	Difference Day/night (%)	4±6	6±5	5±9	18±6^ ^
HR (beats/min)	Day-time	80±20	80±18	82±12	79±5
	Night-time	74±24	69±6*	74±12	61±4**
	Difference Day/night	6±18	11±9^	8±8	18±5^ ^

^ p<0.05 compared to day with HD; * p<0.05 compared to day-time; **p<0.001 compared to day-time; ^ ^p<0.01 compared to dialysed patients

We evaluated diurnal rhythm of BP and HR in dialysed patients and control. Diurnal rhythm of BP and HR were blunted in dialysed patients especially in HD patients. In C group all people had normal diurnal rhythm of SBP and DBP and average differences day/night were 14±2% and 13±6% respectively. Among HD group diurnal rhythm of SBP and DBP were blunted in 80% during the day with HD and during the day without HD in 75% and 67% respectively. Average differences of SBP day/night were 6±11% and of DBP 4±10% during the day with HD. Average differences of SBP day/night were 7±12% and of DBP 6±5% during the day without HD. In CAPD group diurnal rhythm of BP (so SBP and DPB) was blunted in 67% Average differences of SBP day/night were 7±12% and of DBP 6±9%.

Heart rate variability was the highest in C group. The significant difference day/night was 18±5 beats/min (p<0.01). In HD group diurnal HR was blunted during the day with HD, difference day/night was 6±18 beats/min. In the same group there was significant difference day/night 11±9 beats/min (p<0.05) during the day without HD. In CAPD group diurnal HR was blunted, difference day/night was 8±8 beats/min (*Tab. 2*).

Noradrenaline (NA) plasma concentration in dialysed patients was higher than in control, both in HD patients before HD, after HD, during the day without HD and CAPD patients (p<0.001). Increase in NA concentration during cold pressor test was smaller in dialysed patients compared with C group. Mean values of NA were similar in HD and CAPD groups. In HD group mean value of NA before CP were 465±38 ng/ml before HD, 463±36 ng/ml after HD, 473±35 ng/ml during the day without HD and 546±54 ng/ml, 557±48 ng/ml, 549±47 ng/ml after CP, respectively (p<0.001). In CAPD group mean value of NA before CP was 452±76 ng/ml and after CP was 527±92 ng/ml (p<0.01). In C group mean value of NA before CP was 206±53 ng/ml and after CP was 315±61 ng/ml (p<0.01) (*Tab. 3*).

The values of plasma adrenaline and plasma dopamine were similar in each group (*Tab. 3*).

There was no correlation between diurnal rhythm of SBP, DBP, HR and the serum levels of NA at the rest and stimulated after the CP test in each study groups.

Discussion

The problem of sympathetic and parasympathetic activity in chronic kidney disease is known in literature for 50 years. In 1968 Hennesey and all reported about autonomic neuropathy in chronic renal failure [15]. Since many authors informed that sympathetic hyperactivity may be associated with mortality and cardiovascular events in patients with CRF. Increased sympa-

Table 3. **Mean values of noradrenaline, dopamine, adrenaline concentration in the blood at the rest and after cold pressure test (CP) of dialysed patients and control group** (x±SD)

Catecholamines		HD group			CAPD group	Control group
		Before HD	After HD	Day without HD		
Noradrenaline (ng/ml)	Before CP test	465±38^	463±36^	473±35^	452±76^	206±53^
	After CP test	546±54*^	557±48*^	549±47*^	527±92*^	315±61*
Dopamine (ng/ml)	Before CP test	84±24	85±22	8822	93±8	90±17
	After CP test	86±24	86±23	90±22	95±5	93±18
Adrenaline (ng/ml)	Before CP test	92±12	89±10	89±9	93±5	84±14
	After CP test	94±6	92±11	90±9	96±5	87±15

* $p<0.001$ compared to „Before CP test"; ^ $p<0.001$ compared to Control group

thetic activity may play a role in raise of the blood pressure and organ damage, heart and vessels, especially [9,12,13,16].

Accepted biochemical marker of sympathetic activity is catecholamines concentration in the blood. Investigators consider that plasma noradrenaline concentration is the best index of adrenergic function [12,13].

In our study we noticed that, the rest levels of NA and levels of NA after stimulation by cold pressure test were higher in dialysed patients with normotension, in CAPD group and HD group as compared with controls. Most authors reported about increased NA level among patients with CRF and dialysed patients treated by HD and CAPD, both with normotension and with hypertension [17-19]. Another authors reported that NA levels remain in normal range [20]. Vlachojanis and all [21] found that highest NA concentration occurred in long-time treated CAPD patients. In our analysis NA plasma levels were unchanged during standard HD procedures similarly to other reports [22,23]. Only some authors informed about decrease NA plasma concentration after HD procedure [16,19]. Long-term nocturnal haemodialyses could reduce plasma NA concentration [24]. In our study dopamine and adrenaline plasma levels remained in normal range among dialysed patients as in another studies [25]. Some authors reported about elevated plasma dopamine concentration in CRF patients compared with control [17,19].

Many studies were performed in order to evaluate the effect of sympathetic activity on BP regulation in dialysed patients. Manual classic measurements of BP were basis in the early studies. Since several years ambulatory blood pressure monitoring is available in medical study and practice. ABPM is a known method which is applied to diagnosis and control of treatment arterial hypertension. ABPM allowed to diagnose "white coach" hypertension. ABPM is used by nephrologists in Dialysis Centre, too [26,27]. It is very important to estimate true values of BP in HD patients. Dialysis nurses exam BP in HD patients several times: before HD, during HD procedure and after HD but there is no control during the day without HD. BP is examined in CAPD patient by dialysis nurse only during control visit in Dialysis Unit once a month. We performed ABPM for 48 hours in HD patients inclusive the day with HD therein HD procedure and the day without HD. In CAPD group and in controls ABPM was performed for 24 hours. We compared mean value of ABPM with single manual measurements done by dialysis nurses. The differences were not significant. According to our results, we suggest that manual measurements performed by dialysis nurses are sufficient to estimate blood pressure in normotensive CAPD and HD patients. Some authors inform that office measurements of BP performed carefully by nurses is sufficient to control BP [28,29].

We estimated diurnal BP rhythm in dialysis patients with normotension. We found altered diurnal rhythm of BP among 80% patients during the day with HD and among 70% patients during the day without HD. In CAPD group 50% of patients were non-dipper. Most of authors examined diurnal BP rhythm in dialysed patients both HD and CAPD and found blunted or even reverted rhythm in hypertensive patients [5,6,30]. Only few authors estimated diurnal rhythm of BP in HD patients during the day with HD and during the day without HD, separately. Some authors noticed significant difference between first and second day [31]. Another didn't find significant difference [32]. Abnormal diurnal rhythm of BP was to be related with organs damage and poor cardiovascular prognosis in patients with secondary hypertension and in patients with CRF [33].

The physiological mechanisms mediating the variability and diurnal rhythm of BP are unclear. Some authors suggest that sympathetic neural function may contribute importantly to the regulation of BP during the day and the night in patients without CRF [34-36].

Some authors reported that altered circadian rhythm of BP is associated with autonomic dysfunction among patients with CRF [3]. Another authors suggest that autonomic dysfunction is not a major contributor to non-dipping in CRF. An altered diurnal rhythm of BP is common after renal transplant but is not related to degree of autonomic dysfunction [14]. Cattone and all present that hypertensive patients with CRF are characterized by higher values of NA, but there are no differences in sympathetic activity between dipper and nondipper subjects [25]. We received the same results among HD and CAPD patients with normotension as Cattone and all.

We estimated diurnal heart rate variability in patients treated by HD and CAPD and compared with controls. We evaluated diurnal rhythm of HR during the day with HD and during the day without HD, separately. There were not significant differences between average values of HR in day-time and night-time in CAPD group and among HD patients during the day with HD.

There was normal diurnal rhythm of HR in HD patients during the day without HD but the difference day-time and night-time was smaller then in control group. We noticed that average values of HR from day-time were no differed in each studies group, but there were no decrease HR during night-time in CAPD group and HD group during the day with HD. Our results were according to the literature [7]. We suppose there is a pseudonormal rhythm of HR during the day without HD, because in the night before next haemodialysis plasma potassium level in HD patients may go high and so decrease HR.

Autonomic dysfunction in most CRF patients, hypertensive or normotensive, treated by HD or CAPD independently, is the fact and abnormal diurnal rhythm of BP and HR in most of them seems to be truth, but there was not strongly correlation.

We didn't find directly relationship between sympathetic hyperactivity and altered diurnal rhythm of BP and HR among HD and CAPD patients with normotension. A small quantity of patients participated in our study. Many dialysed patients have arterial hypertension or hypotension, concomitant diseases and these patients were excluded from the study.

Another studies are need to evaluate what factors play a main role influencing diurnal BP rhythm among dialysed patients.

References

1. Minamisava K, Tochikubo O, Ishii M. Systemic hemodynamics during sleep in young or middle-aged and elderly patients. Hypertension, 1994; 23(2): 167-73.
2. Lurbe A, Redon J, Pascual JM, Tacons J, Alvarez V, Batlle DC. Altered blood pressure during sleep in normotensive subjects with type I diabetes. Hypertension, 1993; 21(2): 227-35.
3. Liu M, Takahashi H, Morita Y, Maruyama S, Mizuno M, Yuzawa Y, Watanabe M, Toriyama T, Kawahara H, Matsuo. Non-dipping is a potent predictor of cardiovascular mortality and is associated with autonomic dysfunction in haemodialysis patients. Nephrol Dial Transplant, 2003; 18(3): 563-9.
4. Amar J, Vernier I, Rossignol E, Bongard V, Arnaud C, Conte JJ, Salvador M, Chamontin B. Nocturnal blood pressure and 24-hour pulse pressure are potent indicators of mortality in hemodialysis patients. Kidney Int, 2000; 57(6): 2485-91.
5. Tonbul Z, Altintepe L, Sozlu C, Yeksan M, Yildiz A, Turk S. Ambulatory blood pressure monitoring in haemodialysis and continuous peritoneal dialysis (CAPD) patients. J Hum Hypertens, 2002; 16(8): 585-9.
6. Shoda J, Nakamoto H, Okada H, Suzuki H. Impact of introduction of continuous peritoneal dialysis on blood presure: analysis of 24-hour ambulatory blood pressure. Adv Perit Dial, 2000; 16: 97-101.
7. Luik AJ, Struijk DG, Gladziwa U, Olden RW, Hoof JP, Leeuw PW, Leunissen KM. Diurnal blood pressure variations in haemodialysis and CAPD patients. Nephrol Dial Transplant, 1994; 9: 1616-21.
8. Covic A, Goldsmith DJ. Ambulatory blood pressure measurement in renal patient. Curr Hypertens Rep, 2002; 4(5): 369-76.
9. Blankestijn PJ. Symphatetic hyperactivity in chronic kidney disease. Nephrol Dial Transplant, 2004, 19: 1354-7.
10. Neumann J, Ligtenberg G, Klein II, Koomans HA, Blankestijn PJ. Sympathetic hyperactivity in renal hypertension: pathogenesis, clinical relevance and treatment. Kidney Int, 2004, 65(5): 1568-76.
11. Koomans HA, Blankestijn PJ, Joles JL. Sympathetic hyperactivity in chronic renal failure: a wake-up call. J Am Soc Nephrol, 2004; 15: 524-37.
12. Zoccali C, Mallamaci F, Tripepi G, Parlongo S, Cutrupi S, Benedetto FA, Cataliotti A, Malatino LS. Norepinephrine and concentric hypertrophy in patients with end-stage renal disease. Hypertension, 2002; 40: 41-50.
13. Zoccali C, Mallamaci F, Parlongo S, Cutrupi S, Benedetto FA, Tripepi G, Bonanno G, Rapisarda F, Fatuzzo P, Seminara G, Cataliotti A, Stancanelli B, Malatino LS. Plasma Norepinephrine predicts survival and incident cardiovascular events in patients with end-stage renal disease. Circulation, 2002; 105: 1354-9.
14. McGregor DO, Olsson C, Lynn KL. Autonomic dysfunction and ambulatory blood pressure in renal transplant recipients. Transplantation. 2001 May 15; 71(9): 1277-81.
15. Hennesey WJ, Siemens AW. Autonomic neuropathy in chronic renal failure. Clin Res, 1968; 16: 385-6.
16. Grekas D, Kalevrosoglou I, Karamouzis M, Geropoulou E, Kabouris H, Tourkantonis A. Effect of sympathetic and plasma renin activity on haemodialysis hypertension. Nephrol, 2001; 55(2): 115-20.
17. Kurata C, Uehara A, Sugi T, Ishikawa A, Fujita K, Yonemura K, Hishida A, Ishikawa K, Tawarahara K, Shouda S, Mikami T. Cardiac autonomic neuropathy in patients with chronic renal failure on haemodialysis. Nephron, 2000; 84(4): 312-9.
18. Brecht HM, Ernst W, Koch KM. Plasma noradrenaline levels in regular haemodialysis patients. Proc Eur Dial Transplant Assoc, 1976; 12: 281-90.
19. Elias AN, Vaziri ND, Maksy M. Plasma norepinephrine, epinephrine, and dopamine levels in end-stage renal disease. Effect of haemodialysis. Arch Intern Med, 1985; 145(6): 1013-5.
20. Campese VM, Romoff MS, Levitan D. Mechanisms of autonomic nervous system dysfunction in uremia. Kidney Int, 1981; 20: 246-53.
21. Vlachojannis JG, Tsaksas S, Alexandri S, Petropoulou C, Goumenos DS. Continuous ambulatory peritoneal dialysis is responsible for an increase in plasma norepinephrine. Perit Dial Int, 2000; 20(3): 322-7.
22. Hegbrant J, Thyssel H, Martensson L, Ekman R, Boberg U. Changes in plasma levels of vasoactive peptides during standard bicarbonate hemodialysis. Nephron, 1993; 63(3): 303-8.
23. Ligtenberg G, Blankenstijn PJ, Boomsma F, Koomans HA. No change in automatic function tests during uncomplicated haemodialysis. Nephrol Dial Transplant, 1996; 11: 651-6.
24. Chan CT, Harvey PJ, Picton P, Pierratos A, Miller JA, Floras JS. Short-term blood pressure, noradrenergic, and vascular effects of nocturnal home hemodialysis. Hypertension, 2003; 42: 925-31.
25. Cottone S, Panepinto N, Vadala A, Zagarrigo C, Galione P, Volpe V, Cerasola G. Sympathetic overactivity and 24-hour blood pressure pattern in hypertensives with chronic renal failure. Ren Fail, 1995; 17(6): 751-8.
26. Sankaranarayanan N, Santos SF, Pexito AJ. Blood pressure measurement in dialysis patients. Adv Chronic Kidney Dis, 2004; 11(2): 134-42.
27. Zoccali C. Arterial pressure components and cardiovascular risk in end-stage renal disease. Nephrol Dial Transplant, 2003; 18: 249-52.
28. Nystrom F, Malmqvist K, Ohman KP, Kahan T. Nurse-recorded and ambulatory blood pressure predicts treatment-induced reduction of left ventricular hypertrophy equally well in hypertension: results from the Swidish irbersartan left ventricular hypertrophy investigation versus atenolol (SILVHIA) study. J Hypertens, 2002; 20(8): 1527-33.
29. Wang MC, Tseng CC, Tsai WC, Huang JJ. Blood pressure and left ventricular hypertrophy in patients on different peritoneal dialysis regimens. Perit Dial Int, 2001; 21(1): 36-42.
30. Covic A, Goldsmith DJA. Ambulatory blood pressure monitoring in nephrology: focus on BP variability. J Nephrol, 1999; 12: 220-9.
31. Narita I, Okada M, Omori S, Nagai M, Sawanaka N, Kondo D, Goto S, Shimada H, Shimotori T, Arakawa M, Gejyo F. The circadian blood pressure rhythm in non-diabetic hemodialysis patients. Hypertens Res, 2001; 24: 111-7.
32. Nishikimi T, Minami J, Tamano K, Takahashi M, Numabe A, Futoo Y, Honda T, Kobayashi T, Uetake S, Mori Y, Saito T, Matsuoka. Left ventrcular mass relates to average systolic blood pressure, but not loss of circadian blood pressure in stable hemodialysis patients: an ambulatory 48-hour blood pressure study. Hypertens Res, 2001; 24: 507-14.
33. Sturrock ND, George E, Pound N, Stevenson J, Peck GM, Sowter H. Non-dipping circadian blood pressure and renal impairment are associated with increased mortality in diabetes mellitus. Diabet Med, 2000; 17(5): 360-4.
34. Narkiewicz K, Winnicki M, Schroeder K, Phillips BG, Kato M, Cwalina E, Somers VK. Relationship between muscle sympathetic nerve activity and diurnal blood pressure profile. Hypertension, 2002; 39: 168-71.
35. Carvalho MJ, Meiracker AH, Boomsma F, Lima M, Freitas J, Veld AJM, Freitas AF. Diurnal blood pressure variation in progressive autonomic failure. Hypertension, 2000; 35: 892-5.
36. Kario K, Mitsuhashi T, Shimada K. Neurohormonal characteristics of older hypertensive patients with abnormal nocturnal blood pressure dipping. Am J Hypertens, 2002; 15(6): 531-7.

Cholecystokinin octapeptide (CCK-8) concentration in plasma is not affected in functional abdominal pain in children

Uścinowicz M*, Kaczmarski M

III Department of Paediatrics, Medical University of Białystok, Poland

Abstract

Purpose: Cholecystokinin regulates gut motility and visceral sensation. The aim of the study was to determine the diagnostic value of plasma cholecystokinin octapeptide (CCK-8) concentration in children with functional abdominal pain (FAP).

Material and methods: Fifty-two children (33 girls and 19 boys) aged 6-17 years with chronic abdominal pain were included in this study. On the basis of clinical data, results of endoscopy and Criteria for Functional Disorders the patients were divided into three groups: group 1 – functional dyspepsia (FD), group 2 – irritable bowel syndrome (IBS), group 3 – non-specific FAP. The control group consisted of children without abdominal pain in anamnesis. CCK-8 concentrations in plasma were measured with radio immunoassay technique, after plasma extraction. In study protocol we analysed CCK-8 levels in fasting state and 15, 30, 60 minutes after a standard test meal.

Results: In the fasting state plasma levels of CCK-8 were similar in each group and in controls. In the IBS patients CCK-8 levels were not increased after meal. In groups 1, 3 and controls postprandial levels were higher when compared to fasting state ($p<0.05$). Area under curve of CCK-8 plasma concentration was the lowest in group 2, but not significant compared to controls and other groups. No correlation was found between main symptoms of FD and IBS and CCK-8 concentration in plasma.

Conclusions: We conclude that gut dysmotility and symptoms of functional abdominal pain in children are not concerned with alteration of plasma CCK-8 levels before and after meal.

Key words: cholecystokinin (CCK-8), functional abdominal pain.

Abbreviations: CCK-8 – cholecystokinin octapeptide, FAP – functional abdominal pain, FD – functional dyspepsia, IBS – irritable bowel syndrome, Δ AUC – Area Under Curve, IBD – inflammatory bowel diseases.

Introduction

The pathophysiology of functional abdominal pain (FAP) in children is complex. It has been revealed that motility disorders and altered visceral sensation are associated with FAP. Motor activity and visceral sensation are thought to be under control by neural mechanisms and regulatory peptides. Cholecystokinin (CCK) is one of the peptides that acts as a hormone when is released from endocrine cells in mucosa of the upper small intestine. CCK as a neurotransmitter is found in nerve fibers in myenteric and submucosal ganglia, and in smooth muscle [1]. Biological role of CCK is related not only to the stimulation of exocrine pancreatic secretion and gall bladder contraction, but also to motility of the alimentary tract. It has been shown previously that CCK inhibited both gastric acid secretion and gastric emptying [2]. This hormone has been known to relax the lower oesophageal sphincter and to stimulate colon motility by gastro-colonic reflex [3]. In experimental study, CCK exhibited potent gastroprotective activity. That protective effect of CCK-8 was accompanied by elevated plasma leptin levels and was involved in the activation of CCK-A receptor localized on vagal sensory fibers [4]. The feeling of satiety was also caused by CCK release and increased CCK activity and its satiating effect were described [5]. CCK might play a key role in gut function control. We hypothesized that disorders of regulatory mechanisms asso-

* CORRESPONDING AUTHOR:
III Department of Paediatrics
Medical University of Białystok
ul. Waszyngtona 17
15-274 Białystok, Poland
Tel: +48 85 7450703; Fax: +48 85 7423841,
e-mail: mirusc@o2.pl (Mirosława Uścinowicz)

Received 28.12.2004 Accepted 26.01.2005

Table 1. **Clinical features of study groups with FAP**

Study group	Diagnosis	Number of patients	Mean age (mean±SD)	Sex (female/male)	Main symptoms (% of group)
1	FD	18	11.7±2.8	12/6	upper abdominal pain (100%) headache (78%) nausea (67%) vomiting (33%)
2	IBS	8	14.1±2.5	7/1	abdominal pain (100%) distension (50%) diarrhea (37.5%) constipation (62.5%) nausea (25%)
3	nFAP	26	12.2±2.6	14/12	abdominal pain (100%) headache (77%) nausea (61.5%)
4	controls	16	11.1±3.5	11/5	-

FD – functional dyspepsia; IBS – irritable bowel syndrome; nFAP – nonspecific functional abdominal pain

ciated with gut hormone dysfunction might play a role in FAP. CCK circulates in plasma in different molecular forms (CCK-8, CCK-22, CCK-33, CCK-39, CCK-58 etc). CCK octapeptide is used as a standard in CCK assay.

The aim of the study was to determine the diagnostic value of plasma CCK-8 concentration in children with FAP. It was analysed whether fasting or postprandial CCK-8 levels in plasma could be changed in functional disturbances of the gut.

Material and methods

Fifty-two children (33 girls and 19 boys) aged 6-17.1 years with chronic abdominal pain lasting over 3 months were included in this study. Preliminary laboratory tests and procedures necessary in differential diagnosis were carried out in all the children. We included patients fulfilled Second Rome Criteria for Functional Disorders [6]. Upper endoscopies with rapid urease test were performed in children with dyspepsia and upper abdominal pain; gastritis was excluded in study group. In children with suspected irritable bowel syndrome (IBS) rectoscopy and laboratory tests were done in order to differentiate inflammatory bowel diseases (IBD). On the basis of clinical data, endoscopy and Criteria for Functional Disorders the study group was divided into three groups presented in *Tab. 1*. The control group consisted of 16 children without abdominal pain in anamnesis or alteration in the laboratory tests. These children were suspected of the respiratory tract allergy. In controls, gastro-oesophageal reflux and inflammation of alimentary tract mucosa were excluded. The study protocol was approved by the Local Bioethics Committee, Medical University of Białystok. Informed consent was obtained from the parents of children who participated in the study.

Cholecystokinin octapeptide (desulfated, CCK-8) concentrations in plasma were measured with a specific radioimmunoassay technique (RIA), using a commercially available rabbit antiserum and kit Peninsula Laboratories, Belmont, CA. Blood samples were taken at 9 a.m. from cubital vein after overnight fasting. Samples were collected in a container with ice, then immediately centrifuged at 4°C (3500 cpmin) and stored at -20°C. The concentration of the hormone was measured after plasma extraction on reverse phase columns. In study protocol we analysed CCK-8 levels in fasting state (0 minute) and 15, 30, 60 minutes after a standard test meal (296 kcal), containing of 11.1 g of proteins, 14.2 g of fat and 40 g of carbohydrates (a roll, butter, ham and tea). The incremental integrated area in the form of Area Under Curve (Δ AUC) of CCK-8 plasma concentration was calculated. Statistical analysis of the data was performed using the Mann-Whitney U-test and Student t-test. Correlations were evaluated using Spearman test and Pearson coefficient. Statistical significance was set at $p<0.05$.

Results

In the fasting state plasma levels of CCK-8 were similar in each group (15.9±12.3 pg/ml in group 1, 14.4±14.9 pg/ml in group 2, 16.5±23.2 pg/ml in group 3, 18.6±19.7 pg/ml in controls). Postprandial peaks were noted at 15 and 60 minutes in groups 1 and 3, at 30 minutes in controls. In the IBS patients (group 2) CCK-8 levels were not significantly increased after meal compared with fasting state. The overall curves of CCK-8 levels are illustrated in *Fig. 1*. We found great ranges among minimum-maximum values of CCK-8 in plasma in study groups. After the standard meal the hormone concentrations were increased in groups 1, 3 and controls. In these study groups postprandial responses of CCK-8 were statistically significant when compared to fasting state at time 0 (15.5±17.2 vs 26.8±51.3 pg/ml, $p<0.05$) (*Fig. 2*).

The incremental integrated area as area under curve (Δ AUC) of CCK-8 plasma concentration was the lowest in the group 2, but the difference was not statistically significant compared to controls and other groups (*Fig. 3*). We analysed whether altered plasma concentrations of CCK-8 might be concerned with symptoms of FAP. In IBS patients the analysis of correlation between main symptoms (dominant diarrhoea

Figure 1. **CCK-8 concentration in plasma at fasting state and after meal in study group**

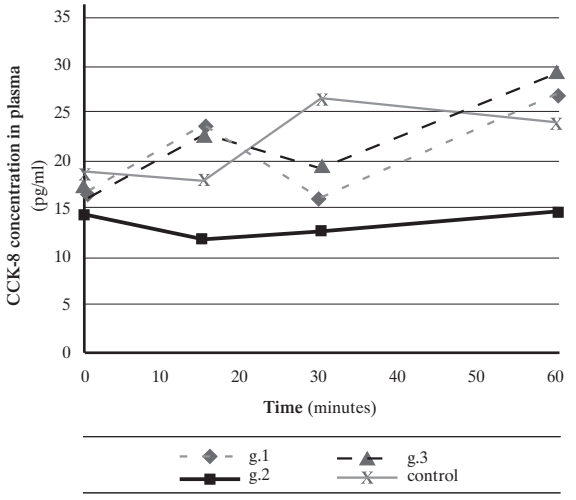


Figure 2. **Correlation between plasma levels of CCK-8 (time: 0 min vs 60 min) Pearson coefficient r=0.47**

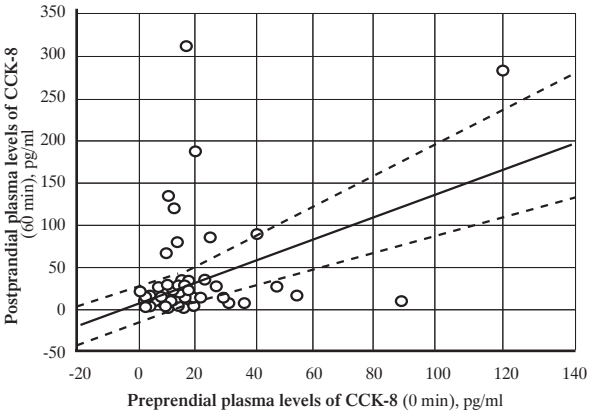
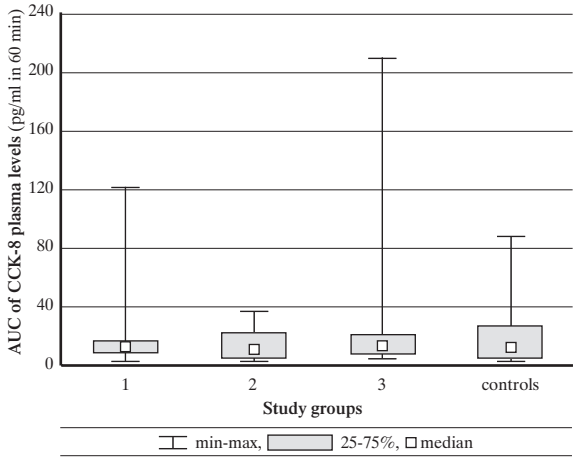


Figure 3. **Range and median value of AUC of CCK-8 in plasma**



or dominant constipation) and Δ AUC of CCK-8 concentrations proved no statistical relationship. Similarly, symptoms of dyspepsia (nausea, vomiting, upper abdominal pain) were not significantly related to CCK-8 release in Spearman test.

Discussion

It was suggested that hormonal aberrations might occur in various types of functional disorders of the gut. In our study, fasting plasma levels of CCK-8 were similar in all groups of children with functional abdominal pain and controls. Alfven and Uvnas-Moberg found significantly higher plasma CCK concentrations in children with recurrent abdominal pain, but no relationships were found between hormone levels and the occurrence of abdominal pain and other symptoms in children investigated twice [7]. The diagnostic criteria of recurrent abdominal pain have been changed and recently named functional abdominal pain (FAP) according to Second Rome Criteria [6]. Pathophysiological mechanisms of FAP were conducted in different subgroups, often in IBS-patients. Sjolund found higher fasting and peak postprandial CCK levels in IBS than in controls [8]. In our study, postprandial release was slightly impaired in IBS-patients compared with others, but not significantly. In the study of Niderau, administration of CCK stimulated motor activity in the colon [1]. However, the use of a blocker of CCK-A receptor, loxiglumide, had no effect on the meal-induced motility in the colon. These diverse results might reflect heterogeneity of IBS-group with predominant diarrhea or constipation. In another functional disorder of colon, encopresis, no significant difference was found in the measurement of CCK-patterns over time between encopretic children and control patients [9]. In the study of Peracchi et al., patients with slow-transit constipation had abnormal postprandial pattern of CCK with delayed postprandial peaks of plasma CCK (99 min in slow-transit constipation and 46 min in controls) [10]. It was previously shown that the phase of intestinal MMC at meal intake could modulate the postprandial endocrine response; plasma CCK increased earlier after intake during late phase II than after phase I [11].

The influence of CCK on motility has been evaluated in various types of gastrointestinal disorders. Elevated CCK plasma levels in patients after cholecystectomy explained the incidence of gastro-oesophageal reflux concerned with lower oesophageal sphincter relaxation [12]. In our dyspeptic patients no significant changes of fasting and postprandial CCK-8 levels were noticed compared with controls and we found no correlation between CCK-8 levels and occurrence of dyspeptic symptoms. Fried and Feinle showed that nutrient fat and distension of the stomach could modulate upper gastrointestinal sensations by postprandial release of CCK and develop dyspeptic symptoms such as nausea, bloating, pain and fullness [13]. CCK acts as mediator of gastric perception. In our study, standard meal containing 14.2 g of fat was used, which caused a postprandial increase in plasma CCK-8 levels in FD, but without influence on severity of dyspeptic symptoms. The action of CCK-8 could be one of the

causes of abdominal pain in the study performed by Chey et al. in patients with IBS [14]. It was explained by dose-dependent muscle contraction via the colon enteric nervous system mediated by CCK-8. From the other point of view CCK-antagonists might have therapeutic potential for the reflux disease, bowel motility disorders and gastroparesis [15]. These arguments explained our study protocol analysing CCK-8 plasma measurements in children with gut motility disorders.

Conclusions

The results of the study indicated no alteration of plasma CCK-8 levels in functional disorders of alimentary tract in children. Predominant symptoms in FAP were not concerned with CCK-8 release impairment. Measurement of CCK-8 plasma levels seems to have poor diagnostic value in FAP.

Acknowledgement

The study was supported by a grant from the Polish Research Committee (No 4PO5E 110 19).

References

1. Niederau C, Faber S, Karaus M. Cholecystokinin's role in regulation of colonic motility in health and in irritable bowel syndrome. Gastroenterology, 1992; 102: 1889-98.

2. Corwin RL, Smith GP. Different effects of CCK A antagonists on gastric-acid response to CCK and pentagastrin. Peptides, 1993; 14: 253-7.

3. Jonkers IJ, Smelt AH, Ledeboer M, Hollum ME, Biemont I, Kuipers F, Stellaard F, Boverhof R, Meinders AE, Lamers CH, Masclee AA. Gall bladder dysmotility: a risk factor for gall stone formation in hypertriglyceridaemia and reversal on triglyceride lowering therapy by bezafibrate and fish oil. Gut, 2003; 52, 1: 109-15.

4. Brzozowski T, Konturek PC, Pajdo R, Kwiecień S, Ptak A, Śliwowski Z, Drozdowicz D, Pawlik M, Konturek SJ, Hahn EG. Brain-gut axis in gastroprotection by leptin and cholecystokinin against ischemia-reperfusion induced gastric lesions. J Physiol Pharmacol, 2001; 52, 4: 583-602.

5. MacIntosh CG, Morley JE, Wishart J, Morris H, Jansen JBM, Horowitz M, Chapman IM. Effect of exogenous cholecystokinin (CCK)-8 on food intake and plasma CCK, leptin, and insulin concentrations in older and young adults: evidence for increased CCK activity as a cause of anorexia of aging. J Clin Endocrinol Metab, 2001; 86, 12: 5830-7.

6. Rasquin-Weber A, Hyman PE, Cucchiara S, Fleisher DR, Hyams JS, Milla PJ. Childhood functional gastrointestinal disorders. Gut, 1999; 45(Suppl II): II60-II8.

7. Alfven G, Uvnas-Moberg K. Elevated cholecystokinin concentration in plasma in children with recurrent abdominal pain. Acta Paediatr, 1993; 82, 967-70.

8. Sjolund K, Ekman R, Lindgren S, Rehfeld JF. Disturbed motilin and cholecystokinin release in irritable bowel syndrome. Scand J Gastroenterol, 1996; 31: 1110-4.

9. Stern HP, Stroh SE, Fiedorek SC, Kelleher K, Mellon MW, Pope SK, Rayford PL. Increased plasma levels of pancreatic polypeptide and decreased plasma levels of motilin in encopretic children. Pediatrics, 1995; 96, 1: 111-7.

10. Peracchi M, Basilisco G, Tagliabue R, Terrani C, Locati A, Bianchi PA, Velio P. Postprandial gut peptide plasma levels in women with idiopathic slow-transit constipation. Scand J Gastroenterol, 1999; 34: 25-8.

11. Medhus AW, Sandstad O, Naslund E, Hellstrom PM, Husebye E. The influence of the migrating motor complex on the postprandial endocrine response. Scand J Gastroenterol, 1999; 34: 1012-8.

12. McDonnel CO, Bailey I, Stumpf T, Walsh TN, Johnson CD. The effect of cholecystectomy on plasma cholecystokinin. Am J Gastroenterol, 2002; 97(9): 2189-92.

13. Fried M, Feinle C. The role of fat and cholecystokinin in functional dyspepsia. Gut, 2002; 51(Suppl 1): 54-7.

14. Chey WY, Jin HO, Lee MH, Sun SW, Lee KY. Colonic motility abnormality in patients with irritable bowel syndrome exhibiting abdominal pain and diarrhea. Am J Gastroeneterol, 2001; 96, 5: 1499-506.

15. Herranz R. Cholecystokinin antagonists: Pharmacological and therapeutic potential. Med Res Rev, 2003; 23, 5: 559-605.

Atopy patch test in the diagnosis of food allergy in children with atopic eczema dermatitis syndrome

Cudowska B*, Kaczmarski M

III Department of Paediatrics, Medical University of Białystok, Poland

Abstract

Puropose: Food allergy has been demonstrated to play an important role in the pathogenesis of atopic eczema dermatitis syndrome (AEDS), affecting often atopic infants and young children. The most commonly offending foods are cow's milk, hen's egg, wheat and soy; implicating immediate (IgE-mediated) and late-phase (T-cells) immunological reactions in the pathogenesis of skin lesions. The diagnostic work-up of suspected immediate food reactions includes skin prick tests (SPT) and the measurement of food-specific antibodies (sIgE). The methodology of atopy patch test (APT) has been reported as a diagnostic tool with high predictive capacity for late-phase clinical reactions in children with atopic dermatitis. Although APT has been introduced into the diagnostic procedure for food allergy, its diagnostic accuracy remains still controversial; especially in older children. The aim of study was to evaluate the diagnostic accuracy of the atopy patch test in the detection of food allergy in correlation with SPT, sIgE and positive oral food challenge to milk, in children suffering from AEDS and to assess the sensitivity and specificity of this method in dependence on the age of investigated children.

Material and methods: 34 children (25 boys, 9 girls) aged 5 months-16 years with suspicion of milk-related AEDS were investigated. These patients were subdivided into 2 age groups: group A – 20 children (<3 years), group B – 14 children (>3 years). The diagnostic procedures as skin-prick tests and atopy patch test were performed. The specific IgE to cow's milk allergens were also measured. The open and blind diagnostic oral food challenge were performed to verify the results of tests. Sensitivity, specificity, positive (PPV) and negative (NPV) predictive value of APT were calculated in both age groups.

Results: A positive challenge response to milk was found in 65.0% of investigated children in group A and in 35.7% in group B. No statistical differences in the prevalence of immediate ($p<0.1905$) and delayed-type ($p<0.409$) reactions has been found between age groups. Positive APT to milk were noticed in 55.0% of patients in group A and in 35.7% of children from group B, that has been in correlation with positive delayed-type reactions in oral food challenge in 72.7% and 80.0% in corresponding age groups. Polysensitization to other food allergens confirmed by SPT and/or sIgE was detected in 35.0% of patients younger than 3 years of age and in 50.0% of older children. The prevalence of positive APT to other foods (soy, rice, maize, cereals) was significantly higher ($p<0.0073$) in the polysensitized children from group A. Sensitivity of SPT/sIgE in children with immediate-type reactions to milk was 100%, specificity 94%. Sensitivity of APT to cow's milk in children with late-phase reactions was 80% in both age groups; specificity 70%/89% with comparable PPV in both groups (73%/80%). Parallel skin testing with combined patch test and evaluation of sIgE enhanced the value of sensitivity to 92% in the group A and specificity to 89% in the group B. For PPV corresponding figures were 85%/80%.

Conclusions: APT was found to be more sensitive and specific method than SPT/sIgE in diagnosing delayed food allergy in children with AEDS. No age correlation between positive results of APT and oral food challenge and higher specificity of APT in older children confirm its accuracy in diagnosing delayed cow's milk allergy in all age groups of children. Combined skin prick and patch testing significantly enhances identification of food allergy in children with AEDS. The outcome of the APT with food does not seem to be influenced by age of children, but because of its variability of sensitivity and specificity, a diagnosis of food allergy should be confirmed by oral food challenge.

* CORRESPONDING AUTHOR:
III Department of Paediatrics
Medical University of Białystok
ul. Waszyngtona 17
15-274 Białystok, Poland
Tel: +48 85 7450704; Fax: +48 85 7423841
e-mail: becud@wp.pl (Beata Cudowska)

Received 18.04.2005 Accepted 25.05.2005

Key words: atopic eczema dermatitis syndrome, food allergy, atopy patch test, skin-prick tests, specific IgE, oral food challenge.

Abbreviations: AEDS – atopic eczema dermatitis syndrome; APT – atopy patch test; CI – confidence interval; DBPCFC – double blind placebo controlled food challenge; IgE – immunoglobulin E; LR – likelihood ratio; NPV – negative predictive value; PPV – positive predictive value; sIgE – specific IgE; SPT – skin-prick tests.

Introduction

Atopic eczema dermatitis syndrome (AEDS) is a form of multifactorial skin disease that generally begins in early infancy and is characterized by extreme pruritus, chronically relapsing course and distinctive morphology and distribution [1]. Frequently there is an association with increased IgE production (I, immediate type of allergic reaction by Gell-Coombs) as well as a local infiltration of T-cells and antigen-presenting cells (IV, cellular type of immunologic reaction) [2]. AEDS is also associated with some of environmental and genetic factors. In older children and adults, exacerbations of eczematous skin lesions have been described after contact with aeroallergens (e.g. house dust mite, pollen or animal dander) [3,4]. Atopic dermatitis is frequently associated with food allergy, but the role of foods in pathogenesis of skin lesions is not well known. A number of early reports suggested that food proteins could provoke eczematous skin rashes, what has been proved by observations performed in the group of cow's milk feeding infants in comparison to breast feeding children [2]. The number of children with AEDS and clinically relevant food allergy is reported to be around 40%. The most commonly offending foods in infants and young children are cow's milk, hen's egg, wheat and soy; in older children the most important role play cereals, citrus fruits, colorants and preservatives [5,6].

In patients with food hypersensitivity and skin lesions, the very small quantity needed for degranulation of IgE-sensitized mast cells probably reaches the tissues via the bloodstream, after entry into body through the mucous membrane of the mouth, stomach or proximal intestine. Antigen which induces local immune-complex reactions or T-cell mediated immunity probably reaches the gut lamina propria through the overlying surface epithelium [2]. After the antigen-specific hypersensitivity response starts, the cytokines and inflammatory mediators may develop a variety of allergic (IgE-mediated and T-cells-mediated) reactions. The eczematous skin lesions of atopic eczema are infiltrated by activated T-cells.

The diagnostic work-up of suspected IgE-mediated food allergy includes skin prick tests (SPT) and the measurement of food-specific IgE antibodies (sIgE) by means of serologic assays. The results of SPT were found to be indicative for early reactions to food challenges. No relationship has been established between reactivity in SPT and delayed-onset clinical reactions. To date, double-blind, placebo-controlled, food challenge (DBPCFC) remains the gold standard for diagnosing clinically relevant food allergy.

Recent clinical and immunologic features indicate that there are atopic patients with no discernible IgE-mediated reactivity, where no detectable IgE antibodies were indistinguishable [7]. During last years, it has been found that about half of children with food allergy have no food-specific IgE as measured by skin tests or sIgE. Some patients with late reactions to foods may when challenged develop immediate reactions [8].

In recent years, the atopy patch test (APT) has been established as a tool in the diagnostic work-up of food allergy in infants and children with AEDS and late phase clinical reactions [9,10]. Although patch testing for foodstuffs has been described by some authors as a method with high sensitivity and specificity to identify delayed hypersensitivity in small children, the others have concluded that the patch test does not be useful in the diagnosis of food allergy; especially in older children [11,12]. Most of data concern the ability of APT in diagnosing allergic sensitization in infants in the first 2 years of life [9,10].

The aim of study was to evaluate the diagnostic accuracy of APT in the detection of food allergy in correlation with SPT, sIgE and positive oral food challenge to milk, in children suffering from AEDS and to assess the sensitivity and specificity of this method in dependence on the age of investigated children. The diagnostic value of APT in diagnosing sensitization to other plant-derived and animal food allergens was also established.

Material and methods

Patients

The prospective, nonrandomized study were carried out on the group of 34 children (25 boys, 9 girls) aged 5 months-16 years, referred to the III Department of Paediatrics in Bialystok in the period from January 2003 to December 2004, for evaluation of atopic eczema dermatitis syndrome (AEDS) suspected of food hypersensitivity. These patients were subdivided into 2 age groups: group A – 20 children (<3 years), group B – 14 children (>3 years). Children with suspicion of milk-related skin symptoms were enrolled to the study according to the inclusion and exclusion criteria.

Inclusion criteria to both groups were: atopic dermatitis as defined by the criteria of Sampson for infants [13], modified from Hanifin and Rajka for children older than 1 years [14], history indicated the time correlation between milk and/or food ingestion and exacerbation of clinical skin symptoms. Exclusion criteria were: active AEDS with skin lesions on the back and forearms, actual and earlier treatment with antihistaminic and antiallergic drugs (in dependence of wash-out period for different types of antihistaminics), treatment with topical corticosteroids applied on the back and forearms (at least 48 hours before testing).

Severity of eczema was scored according to the SCORAD score, with assessment of topography items (affected skin area), intensity criteria (extent of erythema, edema, crusts, excoriations, lichenification and xerosis) and subjective parameters (extent of itch and loss of sleep) [15,16]. Population characteristics are presented in the *Tab. 1*.

Table 1. **Population characteristics**

	Group A (n=20)	**Group B** (n=14)	p
Age (months): Mean: (CI 95%)	5-36 17.1±10.18 (12.33-21.87)	62-192 104.21±43.37 (79.17-129.26)	ns
Boys n (%)	16 (80.0%)	9 (64.3%)	ns
Family history of food allergy n (%)	7 (35.0%)	4 (28.6%)	ns
Family history of atopy n (%)	9 (45.0%)	7 (50.0%)	ns
Breast feeding time Min-max (months): Mean: (CI 95%)	 0-7 3.9±2.17 (2.88-4.92)	 0-7 4.0±2.32 (2.66-5.34)	 ns
Onset of clinical symptoms Min-max (months): Mean: (CI 95%)	 1-12 2.85±2.58 (1.64-4.06)	 1-24 7.07±6.27 (3.45-10.69)	 <0.0069
Gastrointestinal symptoms n (%)	10 (50.0%)	7 (50.0%)	ns
Respiratory tract symptoms n (%)	9 (45.0%)	7 (50.0%)	ns
SCORAD index Min-max: Mean: (CI 95%)	 5-85 36.68±27.31 (23.52-49.85)	 15-57 25.71±17.05 (15.87-35.56)	 ns
Milk-free diet n (%)	15 (75.0%)	5 (35.7%)	ns

Skin prick test

The skin prick tests with cow's milk allergens were performed in all investigated children, by the same person on the volar side of the forearm in accordance with the instructions of the European Academy of Allergy and Clinical Immunology [17]. Milk powder containing 3% of fat was diluted in water (1 g/10 mL) to normal feed concentration. Whisked egg white and yolk was put directly on the skin in the form of a small drop. The SPT with other food allergens (soy, wheat, banana, orange, sesame, arachides, fish, beef, hen) were performed with the same procedure as cow's milk powder, to detect co-sensitization. In all patients, food allergens were tested in native form by means of a modified skin prick technique (prick by prick tests). To perform tests with aeroallergens (mites, tree, grass and weed pollen, dog and cat epithelia, wool, cotton) commercial extracts from Allergopharma Company were used. A 1 mm, single-peak lancet with shoulder was used, and negative (NaCl 0.9%) and positive (9% Codeine) was used as control. Reactions were read after 15 minutes by the author. The mean weal diameter was calculated and a reaction of at least 3 mm was considered as positive without reaction of negative control.

Atopy patch test

Atopy patch tests were applied on uninvolved skin of the child's back, according to the method described by Isolauri and Turjanmaa [18]. A "porridge" was made fresh every day with 0.2 ml isotonic saline and cow's milk powder (300 mg), egg white (40 mg), cereals – wheat, barley, oat, rye (200 mg), gliadin (200 mg), soy (200 mg), maize (200 mg), rice (200 mg). Approximately 20 mg of each porridge was put without filter paper into aluminium test cups on adhesive tape. We used 8 mm diameter aluminium cups for children younger than 3 years and 12 mm aluminium cups for children older than 3 years (Finn Chamber, Epitest Ltd., Finland). Microcrystalline cellulose was used as negative control. Application sites were checked after 20 minutes for immediate reactions. The occlusion time of patch test was 48 hours. The results were read for the first time 15 min after removal of the cups. If irritative redness was found in the test area, the results were read after 30 min. The second evaluation was done 72 hours after attaching the patch tests. Reactions were classified according to standards and considered as: 0 – negative – no reaction, either visible or palpable; + – redness – negative or doubtful reaction; ++ – redness and palpable infiltration with papules – positive reaction; +++ – redness, palpable infiltration with many papules and eczema – strong positive reaction. If the reaction was very intensive as: redness, edema, palpable infiltration and vesicles, the result was marked as ++++. Reactions 0 and + (redness without infiltration) were regarded negative, as redness alone can be the results of local irritation. All tests were prepared and applied by the same nurse and all reactions were classified by the author. The test material used was not standardized as such materials are not available.

Determination of total IgE and specific IgE antibodies

Serum samples (2 ml) were analyzed for concentration of total IgE and specific IgE antibodies to food allergens as: cow's milk, egg white, soy, wheat, maize, rice (Pharmacia Upjohn) with a fluoroimmunoenzymatic essay (UniCAP) according to the manufacture's instruction. The detection limit of the CAP system is 0.35 kU/L IgE; measurable specific IgE was defined as a positive test result if >0.7 kU/L.

Oral food challenge

Standardized oral challenge was performed according to Moneter-Vautrin guidelines [19], with the increasing amounts of the milk at 30 min intervals until intake appropriate for their

age was reached. A challenge was done in all children after 4 week on an elimination milk-free diet. Cow milk challenge was performed as the open challenge being a reliable method in infants and young children (<1 year) and as a blinded test in older children. The foodstuff was blinded in apple pulp or rice if no sensitization to these allergens was detected. Oral food challenges were preceded by labial food challenge. The same doses of milk formula were given in open and blinded challenges. The food challenge was performed in the period of no active AEDS (inclusion criteria was SCORAD index<20) and wash-out period of topical corticosteroids.

Immediate reactions were defined as reactions within 2 hours after the last dose of milk was administered. The challenge was started in the hospital when rising doses of the infant formula were given and continued after 24 hours of observation in the patient's home, where the parents recorded the symptoms of the child being with telephone contact with the author. The challenge was discontinued when a clinical reaction was noticed. If one of the following symptoms such as skin eruptions, pruritus, edema, utricaria, exacerbation of atopic skin lesions, vomiting, diarrhoea, irritability, rhinitis, wheezing, anaphylactic shock, was noticed, the challenge was regarded as positive. The evaluation of symptoms and decision to stop a challenge was made by the investigator. All children were examined once a week to verify reported symptoms and seen 1 month after the beginning of challenge to confirm the diagnosis.

Statistical analysis

Statistical analysis was performed by using SPSS for Windows software (version 8.0; PL). Two-by-two tables were used to calculate sensitivity, specifity, positive (PPV) and negative predictive value (NPV), likelihood ratio (LR) individually for SPT, sIgE for immediate-onset reactions in the group A (in the group B no positive immediate reactions) and for APT in patients with delayed-onset reactions in the group A and B. The analysis was performed for combined SPT, sIgE, APT for immediate and delayed reactions, in both group of children. The Mann-Whitney nonparametric test was used to compare the results of IgE serum concentration in children with/or without cow's milk allergy. The χ^2-test was used for group comparison. Data are presented as mean with ranges or 95% confidence interval (CI). Statistical significance was defined by a level of 0.05.

Results

Cow's milk oral food challenge

A positive challenge response to milk was found in 13/20 (65.0%) of investigated children in group A and in 5/14 (35.7%) in group B (*Tab. 2*). Of the positive reactions, 3/34 (8.8%) involved immediate-type reactions; in the other children, delayed-onset reactions appeared. All positive immediate-type reactions were noticed in the group A of children in the form of urticaria, pruritus and/or exacerbation of atopic dermatitis. Gastrointestinal symptoms (vomiting, diarrhea) occurred in one of patients. No anaphylactic shock was noticed.

In patients with delayed-onset reactions, the symptoms were confined to the skin (exacerbation of atopic dermatitis with pruritus) in 11/15 (73.3%) of children; to the respiratory tract (rhinitis, cough) in 3/15 (20.0%) of patients; to the gastrointestinal tract (vomiting, loss of appetite, irritability) in 4/15 (26.7%) of investigated children. All of mentioned symptoms were observed within 48 hours after the last dose of milk was administered.

Table 2. **Positive results to milk (SPT, sIgE, APT, oral food challenge) in children with AEDS**

	Group A (n=20)	Group B (n=14)	p
SPT n (%)	4 (20.0%)	0 (0.0%)	ns
sIgE n (%)	4 (20.0%)	0 (0.0%)	ns
APT n (%)	11 (55.0%)	5 (35.7%)	ns
Oral food challenge n (%)	13 (65.0%)	5 (35.7%)	
immediate-onset reactions	3/20 (15.0%)	0/14 (0.0%)	ns
delayed-onset reactions	10/20 (50.0%)	5/14 (35.7%)	ns

No statistical differences in the prevalence of immediate ($p<0.1905$) and delayed-type ($p<0.409$) reactions has been found between age groups.

Relationship between clinical response and skin test reactivity (SPT and APT) in children with AEDS and cow's milk allergy

Positive APT to milk were noticed in 11/20 (55.0%) of patients in group A and in 5/14 (35.7%) of children from group B, that has been in correlation with positive delayed-type reactions in oral food challenge in 8/11 (72.7%) and 4/5 (80.0%) of children in corresponding age groups. There was no correlation between positive APT and oral food challenge in 4/34 of children from both age groups. The percentage of false-positive results was 25% for both groups and was comparable (27.3% – group A, 20.0% – group B).

The prevalence of positive SPT and sIgE to milk was higher in the group A, but it was not statistically significant (for SPT and sIgE $p<0.1045$). The results correlated with elevated IgE serum concentration for separate patients. No statistical difference has been found between IgE concentration in both age group ($p<0.6242$). Mostly (75.0%), positive SPT to milk were associated with sIgE to milk.

3/4 (75%) of patients with positive SPT and/or sIgE developed immediate-onset reaction to milk in oral food challenge. The percentage of false-positive results was 25%. Coincidence of IgE-mediated allergy to milk with delayed- type of immunological reactions (IV type) was noticed only in the group A (*Tab. 2*).

No milk hypersensitivity was confirmed by parallel testing (SPT, sIgE, APT, oral food challenge) in 5/20 (25%) of children form group A and in 9/14 (64.3%) of children from group B. These results were in correlation with the percentage of children on the milk-free diet before being enrolled to the study (*Tab. 1*).

8 of children demonstrated positive SPT or APT to other foodstuffs; in 2 of them sensitization to aeroallergens was confirmed.

***Table 3*. Polysensitization to other food and inhalant allergens in children with AEDS**

	Group A (n=20)	Group B (n=14)	p
Food allergy n (%)	16 (80.0%)	9 (64.3%)	ns
Type I (SPT, sIgE)	7 (35.0%)	7 (50.0%)	ns
Type IV (APT)	9 (45.0%)	2 (14.3%)	0.0073
Inhalant allergy (SPT, sIgE) n (%)	3 (15.0%)	6 (42.9%)	0.0789

***Table 4*. Sensitivity, specificity, PPV, NPV, LR for SPT/sIgE in children with immediate-type reactions to milk and for APT in children with delayed-type reactions to milk**

	Group A (n=20)		Group B (n=14)
	SPT/sIgE	APT	APT
Sensitivity (%)	100	80	80
Specificity (%)	94	70	89
PPV (%)	75	73	80
NPV (%)	0	22	11
LR+	17.0	2.67	7.2
LR -	0.0	0.29	0.23

Food and inhalant allergy in SPT and APT

The clinical reaction to cow milk challenge was more frequently noticed in the multisensitized patients (69.2% in the group A and 80.0% in the group B).

Polysensitization to other food allergens confirmed by SPT and/or sIgE (immediate-type reaction) was detected in 7/20 (35.0%) of patients younger than 3 years of age and in 7/14 (50.0%) of older children (group B), but the difference was not statistically significant (*Tab. 3*). SPT were positive to hen's egg white, soy, citrus fruits. The prevalence of positive APT to other foods (soy, rice, maize, cereals) was significantly higher ($p<0.0073$) in the polysensitized children from group A; mostly to soy, cereals, maize, rice. This difference was significant only to cereals ($p<0.0096$) (*Tab. 4*). Although no statistical difference was stated between the prevalence of symptoms from respiratory and gastrointestinal tract in both age groups (*Tab. 1*), inhalant allergy was noticed more often in the group of older children, 42.9% vs 15.0% ($p<0.0789$) (*Tab. 3*).

Sensitivity and specifity of SPT/sIgE, APT in correlation to oral food challenge

Sensitivity of SPT/sIgE in children with immediate-type reactions to milk (group A) was 100%, specificity 94%. No positive immediate-onset reactions in the group B were noticed. Sensitivity of APT in children with late-phase reactions to cow's milk was 80% in both age groups; specificity 70% vs 89% with comparable PPV in both groups (73% vs 80%) (*Tab. 4*). Parallel skin testing with combined patch test and evaluation of sIgE enhanced the value of sensitivity to 92% in the group A and specificity to 89% in the group B. For PPV corresponding figures were 85%/80% (*Tab. 5*).

***Table 5*. The comparison of diagnostic value for combined skin prick and patch testing in correlation to oral food challenge tests (immediate- and delayed-onset reactions)**

	Group A (n=20)	Group B (n=14)
	SPT+sIgE+APT	SPT+sIgE+APT
Sensitivity (%)	92	80
Specificity (%)	71	89
PPV (%)	85	80
NPV (%)	17	11
LR+	3.23	7.2
LR -	0.11	0.23

Disscusion

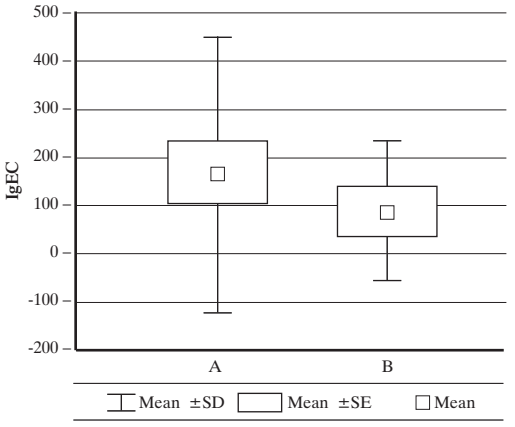
Current data indicate an obvious relation between food allergy and atopic eczema in infants. It has been demonstrated by double-blind placebo controlled food challenges (DBPCFC) that food can exacerbate skin rashes in infants and young children [2,5,6]. Most food allergies involve an IgE-mediated hypersensitivity reaction with positive skin prick test responses, however, no relationship has been established between reactivity in skin prick tests and delayed-onset clinical reactions [18,20]. Patients with atopic eczema who manifest delayed clinical reaction to food challenge have been show to have higher interleukin 2, interferon gamma and TNF-alfa, what confirm the role of lymphocytes T in pathogenesis of AEDS [2,20]. It has been shown that in certain patients with delayed-type reaction eczematous skin lesions can be induced by epicutaneous application of aeroallergens. This procedure was named the atopy patch test (APT).

Identification of offending allergens, food or inhalant, is very important in the management. Accurate and objective demonstration of a casual relationship between the dietary allergen and exacerbation of atopic dermatitis allows for the compliance of the family to the treatment, is the condition of growth in early life and is essential for avoidance of unnecessary elimination diets. Confirmation of the diagnosis and adequate management plays the role in the secondary prevention of multiple food allergies and bronchial asthma.

Other authors suggest that children in whom atopic dermatitis does not improve despite routine treatment with emollients and topical corticosteroids should be tested for allergy to foods, in particular cow's milk [2,5,6,18].

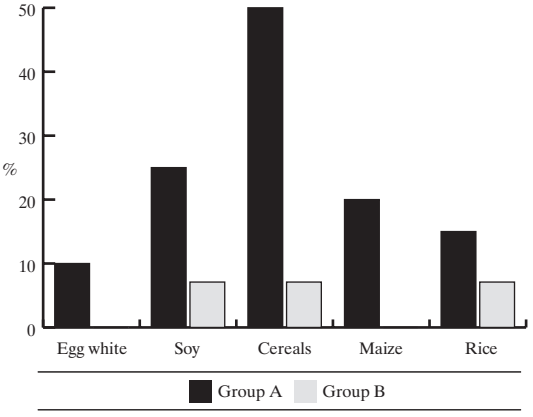
Population of children with AEDS and food hypersensitivity is diversified by age and offending foods. Milk, egg allergy is the most frequent cause of food-induced eczematous symptoms, affecting infants and young children with food hypersensitivity and AEDS. School children are more often polysensitized to soy, arachides, cereals, maize, what has been confirmed in our study. Although approximately 85.0% of patients with AEDS have elevated serum IgE levels, and about 85% of these have evidence of specific IgE antibodies to food and inhalant allergens, not always positive reactions are noticed in DBPCFC [2]. The elevated IgE serum concentration has been confirmed in

Figure 1. **Serum IgE concentration in investigated group of children**



	Group A (n=20)	Group B (n=14)	p
Serum IgE concentration			
Min-max	3-1089	4-575	
Elevated n (%)	10 (50.0%)	7 (50.0%)	ns
Mean (IU/ml)	167.5±282.93	91.57±142.36	
(CI 95%)	(35.08-299.92)	(9.37-173.77)	

Figure 2. **Sensitization to food allergens in APT in children with AEDS**



Foodstuff	Group A (n=20) n (%)	Group B (n=14) n (%)	p
Egg white	2 (10.0%)	0 (0.0%)	ns
Soy	5 (25.0%)	1 (7.1%)	ns
Cereals (barley, wheat, rye, oats)	10 (50.0%)	1 (7.1%)	p<0.0096
Maize	4 (20.0%)	0 (0.0%)	ns
Rice	3 (15.0%)	1 (7.1%)	ns

50.0% of investigated children and no difference between age groups has been noticed (*Fig. 1*). Only three of them presented positive immediate-onset reaction to milk in food challenge. Our results indicated, that as positive SPT/sIgE as immediate-onset reactions in food challenge were observed only in the group of younger children (<3 years). The prevalence of delayed-onset reactions were higher in older children, what was in correlation with positive results of APT. Cow's milk allergy was confirmed in 65.0% of children from group A and in 35.7% from group B, that has been in correlation with positive delayed-type reactions in oral food challenge in 72.7% and 80.0% in corresponding age groups. These results confirm diagnostic accuracy of APT for the diagnosis of food allergy with the elimination-challenge procedure as the most reliable method. Our and previous studies have also shown that immediate-type clinical reactions are more often associated with urticaria and skin prick test positivity and delayed reactions with atopic eczema and patch test positivity [21].

Because more of the current data concern diagnostic accuracy of APT in children less than 2 years [8-10,21], we wanted to assess in our study the relevance of patch testing in the detection of food allergy in older children.

According to recently studies and published data, we expected that the results would show significantly lower sensitivity, specificity and PPV. However, although there was a tendency toward these results, it did not reach statistical significance. Correlation of APT with clinical symptoms and in 80.0% with oral food challenge in the group of older children, indicate the diagnostic value of APT in all age groups. Calculation of PPV and sensitivity of APT showed comparable values in both age groups; specificity was higher in older children. Our observation is in accordance with previous studies by Perackis et al. performed in the group of 498 children in age from 3 to 148 months, indicating that the value of sensitivity, specificity, PPV and NPV concerning cow's milk, hen's egg and wheat does not seem to be influenced by age in infancy and childhood [22].

Our results of sensitivity (100.0%) and specificity (94%) for SPT/sIgE showed a high predictive capacity of these tests for children with immediate-type reactions in food challenge. In the recent study Niggemann and Roehr concluded that the combination of positive APT results and measurement of levels of specific IgE makes DBPCFC superfluous for suspected food allergy [9,23,24]. This and other studies have shown the correlation of SPT/sIgE with immediate-onset reactions and APT with delayed-onset reactions, what confirm the role of these methods in diagnosing of different type of food allergy [10,21,24]. The percentage of false-positive and false-negative results was comparable for SPT, sIgE and APT.

Although the history indicating the time correlation between milk ingestion and exacerbation of clinical skin symptoms, no milk hypersensitivity was confirmed by parallel testing (SPT, sIgE, APT, oral food challenge) in 5/20 (25%) of children form group A and in 9/14 (64.3%) of children from group B. It allows to state that the milk is the main, offending food allergen in infants, but not in older children.

In our study population, some patients were sensitized to soy and cereals and showed symptoms exacerbation after ingesting of foods containing these allergens. Polysensitization was significantly more frequent in children older than 3 years. The results of APT suggest the role of delayed-type reactions in wheat allergy and that sensitization to cereals appears to be more common than generally believed among infants with atopic eczema [25] (*Fig. 2*). Cereal allergy in infants may be the first sign of further pollen allergy and cross-reactivity in older

children. Positive results of APT to cereals should be verified by oral food challenge, but the inclusion criteria to this study was suspicion of cow's milk allergy, so we decided to perform the elimination-challenge procedure to cereals as the next step of diagnosing.

In this investigation, the prevalence of inhalant allergy was statistically higher in children older than 3 years, what confirm the role of aeroallergens in the pathogenesis of AEDS. It is also the risk factor for allergic rhinitis and bronchial asthma. Natural history of AEDS shows that about 80% of children with atopic dermatitis and food allergy will "lose" their clinical reactivity to milk over 1 to 3 years, developing multiple food allergies with allergic rhinitis and/or bronchial asthma [26]. It suggests that the successful management of AEDS and food allergy requires food allergens avoidance and the diagnostic procedures with other foodstuffs.

There are not a lot of study concerning the diagnostic value of combined skin prick and patch testing in correlation to oral food challenge. The findings of Isolauri, Turjanmaa and Majamaa et al. indicate that skin prick and patch testing significantly increases the chances of early detection of food allergy in infants [10,18,23,24]. But there are also the study concluding that the APT was of little value in diagnosing food allergy in small children. In the study of Vanto et al. who investigated 301 children with suspected cow's milk allergy, the PPV was 40% for APT in correlation to immediate-type reactions [12]. The explanation for the divergence and such a low predictive capacity of APT is that the investigations were carried out on the group of unselected children with skin, respiratory and digestive symptoms and that the figures concerned immediate-onset reactions but not delayed-onset reactions.

In our study, in combined skin and patch testing with evaluation of sIgE, the sensitivity was higher in children less than 3 years (92%), but specificity in children older than 3 years (89%). These results and high PPV confirm that APT might be performed in the diagnostic work-up of food allergy in children with atopic dermatitis up to 3 years of age with unimpaired accuracy.

References

1. Kekki OM, Turjanmaa K, Isolauri E. Differences in skin-prick and patch-test reactivity are related to the heterogeneity of atopic eczema in infants. Allergy, 1997; 52: 755-9.
2. Sampson HA. The evaluation and management of food allergy in atopic dermatitis. Clinics in Dermatology, 2003; 21: 183-92.
3. Darsow U, Behrendt H, Ring J. Gramineae pollen as trigger factors of atopic eczema: evaluation of diagnostic measures using the atopy patch test. Br J Deramtol, 1997; 137: 201-7.
4. Ring J, Darsow U, Gfesser M, Vieluf D. The atopy patch test in evaluating the role of aeroallergens in atopic eczema. Int Arch Allergy Immunol, 1997; 113: 379-83.
5. Kaczmarski M. Postaci kliniczne nietolerancji białek mleka krowiego u dzieci. Pol Tyg Lek, 1989; 44: 81-5.
6. Olendzka-Rzepecka E. Uwarunkowania konstytucjonalne i środowiskowe atopowego zapalenia skóry u dzieci w ocenie retro- i prospektywnej. Praca doktorska, 1997.
7. Niggemann B, Reibel S, Roehr ChC, Feler D, Ziegert M, Sommerfeld Ch, Wahn U. Predictors of positive food challenge outcome in non-IgE-mediated reactions to food in children with atopic dermatitis. J Allergy Clin Immunol, 2001; 108(6): 1053-8.
8. Hansen TK, Host A, Bindslev-Jensen C. An evaluation of the diagnostic value of different skin tests with egg in clinically egg-allergic children having atopic dermatitis. Pediatr Allergy Immunol, 2004; 15(5): 428-34.
9. Niggemann B. The role of the atopy patch test (APT) in diagnosis of food alergy in infants and children with atopic dermatitis. Pediatr Allergy Immunol, 2001: 12(Suppl. 14): 37-40.
10. Strömberg L. Diagnostic accuracy of the atopy patch test and the skin-prick test for the diagnosis of food allergy in young children with atopic/eczema dermatitis syndrome. Acta Pediatr, 2002; 91: 1044-9.
11. Osterballe M, Andersen KE, Bindslev-Jensen C. The diagnostic accuracy of the atopy patch test in diagnosing hypersensitivity to cow's milk and hen's egg in unselected children with and without atopic dermatitis. J Am Acad Dermatol, 2004; 51(4): 556-62.
12. Vanto T, Juntunen-Backman K, Kalimo K, Klemola T, Koivikko A, Koskinen J. The patch test, skin prick test, and serum milk-specific IgE as diagnostic tools in cow's milk allergy in infants. Allergy, 1999; 54: 837-42.
13. Sampson HA. Pathogenesis of eczema. Clin Exp Allergy, 1990; 20: 459-67.
14. Hanifin JM. Standardized grading of subjects for clinical research studies in atopic dermatitis work-shop report. Acta Derm Venerol, 1989(Suppl. 144): 28.
15. Stadler JF, Taieb A. Severity scoring of atopic dermatitis: the SCORAD index. Consensus Report of the European Task Force on Atopic Dermatitis. Deramtology, 1993; 186: 23-31.
16. Cudowska B, Kaczmarski M, Wasilewska J, Matuszewska E. Obiektywizacja skuteczności leczenia atopowego zapalenia skóry z wynikami skali SCORAD u dzieci z alergią pokarmową i/lub powietrznopochodną. Postępy Dermatologii i Alergologii, 2002; 3: 136-41.
17. Basomba A, Bousquet J, Dreborg S, Frew A, Haahtela T, Malling HJ. Allergen standarization and skin tests. In: Derborg S, Frew A, editors. Position paper. Prepared by the sub-committee in skin tests within the European Academy of Allergology and Clinical Immunology. Allergy, 1993; 48: 49-82.
18. Isolauri E, Turjanmaa K. Combined skin prick and patch testing enhances identification of food allergy in infants with atopic dermatitis. J Allergy Clin Immunol, 1996; 97: 9-15.
19. Moneret-Vautrin DA. Guide du practicien en immuno-allergologie. Masson, 1994.
20. Kerschenlohr K, Decard S, Przybills B, Wollenberg A. Atopy patch test reactions show a rapid influx of inflammatory dendritic epidermal cells in patients with extrinsic atopic dermatitis and patients with intrisinic atopic dermatitis. J Allergy Clin Immunol, 2003; 4: 869-74.
21. Majamaa H, Moisio P, Holm K, Kautiainen H, Turjanmaa K. Cow's milk allergy: diagnostic accuracy of skin prick and patch tests and specific IgE. Allergy, 1999; 54: 346-51.
22. Perackis K, Celik-Bilgili S, Staden U, Mehl A, Niggemann B. Influence of age on the outcome of the atopy patch test with food in children with atopic dermatitis. J Allergy Clin Immunol, 2003; 112(3): 625-7.
23. Roehr ChC, Reibel S, Ziegert M, Sommerfeld Ch, Wahn U, Niggemann B. Atopy patch tests, together with determination of specific IgE levels, reduce the need for oral food challenges in children with atopic dermatitis. J Allergy Clin Immunol, 2001; 3: 548-53.
24. Niggemann B, Reibel S, Wahn U. The atopy patch test (APT) – a useful tool for he diagnosis of food allergy in children with atopic dermatitis. Allergy, 2000; 55(3): 281-5.
25. Majamaa H, Moisio P, Holm K, Turjanmaa K. Wheat allergy: diagnostic accuracy of skin prick and patch tests and specific IgE. Allergy, 1999; 54: 851-6.
26. Sampson HA, Scanlon SM. Natural history of food hypersensitivity in children with atopic dermatitis. J Pediatr, 1989; 115: 23-7.

Immunoblotting in the diagnosis of cross-reactivity in children allergic to birch

Cudowska B[1]*, Kaczmarski M[1], Restani P[2]

[1] III Department of Paediatrics, Medical University of Białystok, Poland
[2] Department of Pharmacological Sciences, Faculty of Farmacy, State University of Milan, Italy

Abstract

Purpose: The scientific experiments with new immunological methods (immunoblotting, RAST inhibition) and isolation of recombinant allergens suggest structural similarities in the allergenic components responsible for cross-reactions. Immunochemical and molecular biology studies indicate that epitopes of major allergen (Bet v 1, Mal d 1) contain more IgE binding epitopes than minor allergens (Bet v 2, Mal d 2), what explained clinical importance of major birch and apple allergens, but it is individual. The important role in cross-reactivity play also proteins with low molecular weight; a potentially dangerous allergen is lipid transfer protein (LTP) inducing severe systemic reactions in allergic subjects. The recent studies indicate that the IgE cross-reactivity patterns and the clinical relevance is still not clear and that only some of patients with confirmed IgE cross allergy to Bet v 1 and Mal d 1 demonstrated clinical symptoms after ingesting of apple. The aim of study was to establish the pattern of cross-reactivity between major (Bet v 1) and minor (Bet v 2) birch pollen allergens and apple proteins in children allergic to birch using recombinant allergens and immunoblotting method.

Material and methods: The prospective study were carried out on the group of 13 children aged 4-16 years, referred to the IIIrd Department of Paediatrics in Białystok and outpatient clinic with clinical symptoms of food and inhalant allergy. Inclusion criteria to the study were: allergy to birch pollen recombinant allergens and apple, confirmed by presence of specific IgE in the sera of patients. The allergens from peel and pulp of apple and birch were separated and loaded onto the polyacrylamide electrophoretic gel and than transferred to membranes by western blotting. Antigen-IgE complex was detected using goat anti-human IgE antibodies labelled with alkaline phosphatase.

Results: Only few sera presented strong reactions in immunoblotting to birch pollen proteins with a molecular weight of 17-18 kDa, corresponding to the main birch allergen Bet v 1. Most of sera having positive reaction vs Bet v 1 showed cross-reactivity with Mal d 1. All sera recognized specifically the main allergen of apple peal Mal d 3 with molecular weight <10 kDa (Lipid Transfer Protein).

Conclusions: Immunoblotting method allows to verification of cross-reactivity recognized by presence of specific IgE. The nature of proteins responsible for sensitization can influence the spectrum of offending foods and the clinical features of allergic reactions.

Key words: cross-reactivity, birch-apple syndrome, immunoblotting, recombinant allergens, lipid transfer protein.

Abbreviations: IgE – immunoglobulin E; LTP – lipid transfer protein; RAST inhibition – radioallergosorbent test inhibition; SDS-PAGE – sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Introduction

Patients allergic to birch pollen often also react with fruit and vegetables, such as apple and cross-reactivity of allergen-specific antibodies is a well-known phenomenon in food allergy [1,2]. Allergies to plant foods are based on cross-reactive IgE and adverse food reactions are mainly due to a specific family of related proteins. All these proteins seem to possess structural similarities. Proteins that share common epitopes with Bet v 1,

* CORRESPONDING AUTHOR:
III Department of Paediatrics
Medical University of Białystok
ul. Waszyngtona 17
15-274 Białystok, Poland
Tel: +48 85 7450704; Fax: +48 85 7423841
e-mail: becud@wp.pl (Beata Cudowska)

Received 05.05.2005 Accepted 14.06.2005

the major birch pollen allergen, occur in other kinds of tree pollen, apples, stone fruit, celery, carrots and nuts [2]. The major cause of cross-reactivity between birch and apple is biochemical and immunological similarity between the major allergens, Bet v 1 and Mal d 1 [1]. Isolation and sequencing of the major allergen Mal d 1 from apple have shown a high degree of sequential homology with Bet v 1 and it has been demonstrated that Mal d 1 and Bet v 1 cross-react at T-cell level [3]. Bet v 1 belongs to class 10 of pathogenesis-related proteins. Other major allergen from apple (Mal d 2) has been identified as thaumatin-like proteins. In contrast to Bet v 1, two minor allergenic structures which sensitize about 10-20% of all pollen-allergic patients, are named profilins and cross-reactive carbohydrate determinants [2]. Cross-reactivity between birch pollen and apple has been demonstrated using allergen extracts in RAST inhibition, as well as immunoblotting [1]. A common observation of cross-reactivities has shown that a relatively high number of these seem to be without clinical significance [4]. More recently, a lipid transfer protein has been reported to be an important allergen in apple (Mal d 3). Lipid transfer protein (LTP), the major allergen in Rosaceae fruit in areas where the prevalence of birch pollen allergy is low, is a potentially dangerous allergen. Because of its extreme resistance to pepsin digestion, LTP probably reaches the intestinal tract in an almost unmodified form inducing severe systemic reactions in allergic subjects. In fact, lipid transfer proteins from apple showed a homology higher than 90%, which clearly explains immunochemical cross-reactivity in patients not allergic to birch pollen [5].

Food proteins and profilins of the Bet v 1 family are relatively sensitive to heat and can be easily cleaved by proteases. Serological assays and skin prick tests allow no distinction between symptomatic and asymptomatic patients. Positive serological assays should not be taken as indicators for a strict avoidance of foods which are tolerated [2].

Despite the increasing knowledge of cross-reactive structures and the role of recombinant allergens, it is still not clear the IgE cross-reactivity patterns and the clinical relevance [2,4]. According to the published data, sensitization to Bet v 1 or profilin is not always associated with IgE against specific foods. The correlation has been found between Bet v 1 and apple, peach, hazelnut in patients allergic to birch, but the actual question is, if patients allergic to apple demonstrate reactivity to Bet v 1 [2,6].

The aim of our study was to determine the pattern of cross-reactivity between birch pollen and apple in children allergic to birch using recombinant allergens and immunoblotting method. The correlation between immunochemical cross-reactivity to apple and birch was also established.

Material and methods

Patients

The prospective study were carried out on the group of 13 children aged 4-16 years, referred to the IIIrd Department of Paediatrics in Białystok and outpatient clinic with clinical symptoms of food and inhalant allergy. Inclusion criteria to the study were: allergy to birch pollen recombinant allergens (Bet v 1 and Bet v 2) and apple, confirmed by estimation of specific IgE in the sera of patients. Exclusion criteria were: chronic diseases of respiratory and digestive tract with different than allergic background, parasites infections, autoimmunological disorders.

Table 1. **Total and specific IgE in the investigated patients (N=13)**

No	Initials	Age (yrs)	IgE (IU/ml)	Specific IgE to birch allergens (class)			Specific IgE to apple (class)
				Birch	Bet v 1	Bet v 2	
1.	M.W.	14	65	3	3	0	2
2.	K.K.	4	>5000	3	3	3	2
3.	J.M.	12	3293	4	4	3	2
4.	M.R.	8	>5000	2	0	0	3
5.	J.Z.	2	2077	5	5	0	3
6.	P.L.	12	356	2	0	0	2
7.	J.B.	13	965	3	3	0	2
8.	M.W.	13	110	4	4	0	1
9.	J.S.	14	211	3	3	2	2
10.	L.M.	9	1081	3	3	0	4
11.	M.K.	4	141	6	6	0	4
12.	K.K.	13	792	4	4	0	3
13.	E.P.	16	>1000	3	2	0	2

Determination of total IgE and specific IgE antibodies

Serum samples (2 ml) were analyzed for concentration of total IgE and specific IgE antibodies to birch recombinant (Bet v 1 and Bet v 2) and apple allergens (Pharmacia Upjohn) with a fluoroimmunoenzymatic essay (UniCAP) according to the manufacture's instruction. The detection limit of the CAP system is 0.35 kU/L IgE; measurable specific IgE was defined as a positive test result if >0.7 kU/L. The results of total and specific IgE to birch and apple are presented in *Tab. 1*.

Immunoblotting

Immunoblotting has been performed in the cooperation with the Department of Pharmacology in the University of Milan in Italy with the procedures described bellow.

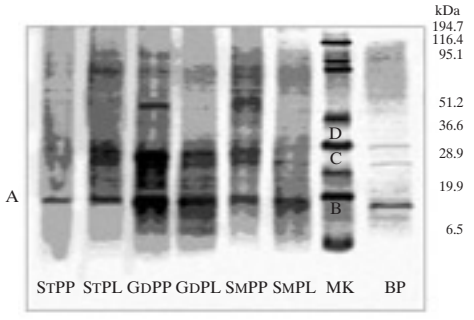
Apple

The apples were purchased from a greengrocer of Milan; they belonged to three varieties: Golden Delicious, Stark Delicious and Smith. Peel and pulp were separated, freeze-dried and suspended in Sample buffer:water (1:1, v:v) at the final concentration of 150 mg/mL. After a night at room temperature, samples were centrifuged at 10 000 rpm and 4°C for 20 minutes. The supernatant was collected and loaded onto the electrophoretic gel. Sample buffer contained 0.25M TRIS-HCl pH 6.8, 7.5% glycerol, 2% SDS, 5% β-mercaptoethanol.

Birch

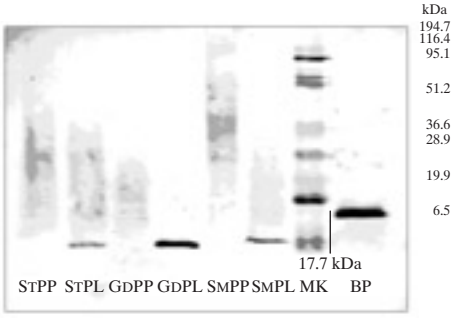
A birch solution for prick test (STALLERGENES SA) was diluted 1:1 (v:v) with Sample buffer and loaded onto a gel.

Figure 1. **SDS-PAGE of apple peel and pulp and birch pollen**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen
Molecular weight:
A: 18.4 kDa; B: 17.7 kDa; C: 31 kDa; D: 35.4 kDa

Figure 2. **Reactivity vs birch pollen proteins 17-18 kDa in one of patient allergic to birch (membrane incubated with serum 11)**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Apple and birch proteins were separated on a gradient polyacrylamide gel with the following characteristics:

1 – Gradient running gel: 12-22% acrylamide; 0.11-0.20% bis-acrylamide; 0.36 M TRIS-HCl buffer pH 8.8; 35% glycerol; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% N,N,N',N'-tetramethylenediamine (TEMED).

2 – Stacking gel: 3.5% acrylamide; 0.09% bis-acrylamide; 0.125 M TRIS-HCl buffer pH 6.8; 0.1% SDS; 0.02% ammonium persulfate; and 0.15% (TEMED).

3 – Running buffer: 25 mM TRIS, 0.19M glycine and 0.1% SDS (w/v), pH 8.8.

After the electrophoretic run (90 V at room temperature, for approximately 6 h) gels were dyed with Coomassie Brilliant Blue G-250. All materials and instruments were purchased from Bio-Rad (Richmond CA, USA).

Molecular Weight Marker Solution (prestained broad range, Bio-Rad) contained myosin (rabbit muscle; 194.7 kDa), β-galactosidase (Escherichia coli; 116.4 kDa), bovine serum albumin (95.1 kDa), ovalbumin (chicken egg; 51.2 kDa), carbonic anhydrase (bovine erythrocytes; 36.6 kDa), soybean trypsin inhibitor (soybean; 28.9 kDa), lysozyme (chicken egg white; 19.9 kDa) and aprotinine (bovine pancreas; 6.5 kDa).

Immunoblotting

After SDS-PAGE, proteins were transferred to polyvinylidene difluoride (PVDF) membrane (PVDF; Immobilon P, Millipore, Bedford, MA, USA) by western blotting in a Trans-blot Electrophoretic Transfer Cell (Bio-Rad). The transfer buffer was 25 mM Tris, 193 mM glycine and 20% methanol. The membranes were blocked with 1% gelatin and washed three times with 0.25% gelatin solution (in 150 mM NaCl, 5 mM EDTA, 50 mM Tris, 0.05% Triton-X) to prevent non-specific adsorption of the immunological reagents. The membrane was then immersed in 10 mL of 0.25% gelatin solution containing 0.5 ml of serum from allergic children. Antigen-IgE complex was detected using goat anti-human IgE antibodies (Sigma Aldrich, Milan, Italy) labelled with alkaline phosphatase; the secondary antibody commercial stock was diluted 1/1000 (v:v) in 0.25% gelatin solution. After incubation for 4 h at room temperature with shaking, membranes were washed twice with 0.25% (2 min each) and once with Tris buffer solution (20 mM Tris and 0.5 M NaCl) for 5 min.

Finally, after incubation in bromochloroindolyl phosphate-nitroblue tetrazolium (BCIP-NBT) solution, an intense black-purple precipitate developed at the site of enzyme binding. The developing solution contained 15% BCIP and 30% NBT in alkaline phosphatase buffer (100 mM Tris, 100 mM NaCl, 5 mM $MgCl_2$, pH 9.5).

Results

The electrophoretic pattern of apple pulp and peel belonging to the three selected varieties (*Malus domestica* var. golden delicious, stark delicious and smith) are shown in SDS-PAGE (*Fig. 1*). In parallel, the profile of pollen birch proteins is shown. The protein profile of apple samples is not well defined; in fact, several proteins are distributed in the range of molecular weights 6.5-95.1 kDa. Same proteins are well defined and among them the most abundant component (both in pulp and peel samples) presents a molecular weight of approximately 17-18 kDa.

The profile of birch pollen presents three major proteins, having molecular weights of approximately 18, 31, 35.5 kDa.

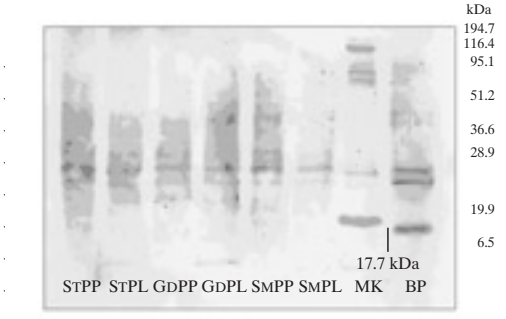
Circulating IgEs from the 13 sera included in this study were tested for reactivity against apple peel and pulp, and birch pollen allergens. The use of prestained Molecular Weight standard solution (broad range) allowed the identification of the proteins recognized by circulating IgEs (positive reaction in immunoblotting).

The main results can be summarized as follows:

1. Reactivity to birch pollen

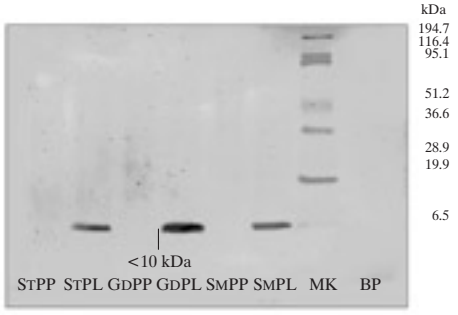
Only sera of 4 patients presented strong reaction to birch pollen proteins (*Fig. 2*). The protein recognized by these sera

***Figure 3*. Reactivity vs the allergens having molecular weight 17-18 kDa and two bands of approximately 30 kDa (membrane incubated with serum 5)**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

Figure 4. Reactivity vs major component of apple peel (Mal d 3 – Lipid Transfer Protein) (membrane incubated with serum 1)



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

presented a molecular weight of approximately 17-18 kDa, corresponding to the main birch allergen Bet v 1. IgEs from the child 5 recognized in the birch pollen sample: a protein having molecular weight of 17-18 kDa (Bet v 1), and two bands of approximately 30 kDa (Bet v 6) (*Fig. 3*). Since allergy to birch pollen was the inclusion criterium for this study we must conclude that birch allergens contain conformational epitopes that loose their reactivity after denaturating processes (treatment with SDS and β-mercaptoethanol). Probably, only the sera from the most reactive subjects are capable to recognize denatured birch allergens.

2. Reactivity to apple peel proteins

All subjects presented reactivity against the major component of peel. Considering its molecular weight (<10 kDa), this protein can be identified as the known allergen Lipid Transfer Protein (Mal d 3) (*Fig. 4*). The reactivity against pulp proteins is characterized by a complex pattern of responses:

Serum 1: no reactivity

Serum 2: no significant reactivity

Serum 3: no significant reactivity

Serum 4: some reactivity to the peel allergen Mal d 1 (MW 17-18 kDa)

Serum 5: reactivity versus two allergens having molecular weights of app. 30 kDa

Serum 6: weak reactivity versus the allergen having molecular weight of 17-18 kDa (Mal d 1). The reaction is stronger versus apple Golden

Serum 7: reactivity versus the allergen having molecular weight of 17-18 kDa (Mal d 1). The reaction is stronger versus apple Golden

Serum 8: no reactivity

Serum 9: reactivity versus the allergen having molecular weight of 17-18 kDa (mainly in apple Golden) corresponding to the major birch pollen allergen (Bet v 1)

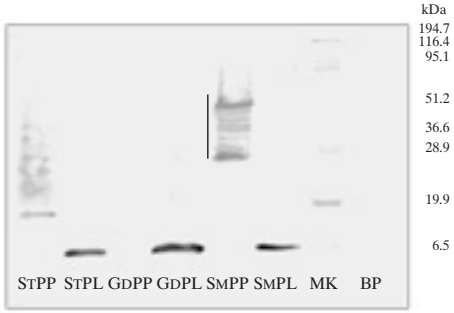
Serum 10: no significant reactivity

Serum 11: low reactivity versus the allergen Mal d 1

Serum 12: strong reaction versus Mad d 1 in the sample containing the apple var Smith (*Fig. 5*)

Serum 13: no significant reactivity.

***Figure 5*. Strong reactivity vs Mal d 1 in the sample containing the apple var. Smith (incubation with serum 12)**



St – *Malus domestica* var. stark delicious; PP – pulp; Gd – *Malus domestica* var. Golden Delicious; PL – peel; Sm – *Malus domestica* var. Smith; MK – molecular weight marker solution; BP – birch pollen

Discussion

Cross-reactivity between birch pollen and apple has been confirmed by analysis of recombinant proteins and using RAST inhibition and immunoblotting. The major cause of birch-apple syndrome is biochemical and immunological similarity between major allergens, Bet v 1 and Mal d 1 [1]. The epitopes of major allergens (Bet v 1, Mal d 1) show the highest IgE-affinity, but the different patterns of IgE-binding were also observed in individuals. Approximately 70% of patients who are allergic to birch pollen may experience symptoms after consumption of apple and fruit from family Rosaceae. Most of patients suffer from local symptoms at the site of the primary allergen contact (oral allergy syndrome) [2]. Some of them are also sensitized to minor birch pollen allergen; profilin Bet v 2, but recent studies suggested that profilin sensitization has little or not clinical relevance [2,7,8]. A possible reason for this fact is monosensitization to profilin or sensitization to other cross-reacting

structures in pollen and vegetables foods or to primary food allergens, such as LTP [2]. The recent studies indicated also that only 75% of patients with confirmed IgE cross-allergy Bet v 1 – Mal d 1, demonstrate clinical symptoms after ingesting of apple. This homology between allergens is clinically irrelevant because of cross-reactive carbohydrate determinants [9]. In our group of children, we observed positive oral food challenge to apple in seven of them; 4 sera showed reactivity to Mal d 1 and all to Mal d 3.

The next problem is that the presence of specific IgE to birch and apple in the sera of patients can not be considered as the predicting factor of clinical symptoms. As was previously described, sensitization to Bet v 1 is specific for birch and apple allergies, whereas sensitization to Bet v 2 is common in polysensitized patients. In the investigations carried out by Rossi et al., more than half of patients with a history of oral allergy syndrome after eating apple reacted to Bet v 1 [10]. In our study only few among 13 children allergic to birch presented in immunoblotting specific IgE capable to recognize birch pollen allergens; probably due to the denaturating processes used in this technique. The protein of birch recognized by the sera of 4 patients has a molecular weight corresponding to the main birch allergen Bet v 1 (17-18 kDa). As we have shown in the *Tab. 1*, all patients were allergic to birch according to the results of specific IgE. These results indicated that there is no correlation between these methods and that the presence of specific antibodies has not the clinical relevance. The great importance has also technical conditions of method. We supposed the influence of denaturating processes on the epitopes structure and the data obtained by other authors, concerning food allergens, confirmed these conclusions. The investigations carried out by Vieths and al. indicated that the proteins in the prick test solution appeared to be strongly degraded and that extraction procedures should be adapted to the specific source material [11].

Our results demonstrated that most of sera having positive reaction vs Bet v 1 showed cross-reactivity with Mal d 1, but some of patients showed reactivity to Mal d 1, even though the reactivity to birch was weak or absent. Detailed analysis of molecular surface areas performed by Holm et al. identifies potential epitopes for cross-reactive antibodies. A minimum of two epitopes would be necessary for cross-linking of receptor bound IgE in histamine release and skin test. The occurrence of limited epitope coincidence between Bet v 1 and Mal d 1 is in agreement with the observation that not all birch pollen allergic patients react with apple and that conformation of epitopes recognized by the IgE of the individual patients determines the degree of cross-reactivity [1].

Very interesting seem to be the results of reactivity to the apple protein with molecular weight <10 kDa, Mal d 3 (Lipid Transfer Protein). Our results showed that all sera, even these without reactivity in immunoblotting to birch, recognize specifically the main allergen of apple peel. LTP is the major allergen in Rosaceae fruit in areas where the prevalence of birch pollen allergy is low and is responsible for severe allergic reactions [5]. This protein show extreme heat stability and the results of investigations performed by Asero et al. suggest that LTP-hypersensitive patients with a history of severe reactions induced by apple should be advised to avoid unpeeled apple even after it undergone thermal processing [5]. Clinical observation of the investigated group of children indicated that only some of children demonstrated symptoms after ingestion of apple, but we didn't notice severe clinical reactions among them. The results of our investigations and the prevalence of sensitization to LTP is the indication for considering the spectrum of offending foods and dietetic restrictions, because of the risk of potentially life-threatening reactions relevant to heat stable and pepsin resistant proteins [12].

The recently studies indicated that the expression of Mal d 1 varies between different apple strains. Golden Delicious and Granny Smith apples have a high expression of Mal d 1 compared to Jamba and Gloster. It is with correlation of clinical symptoms occurred more often after ingestion of green apples than red ones. Hansen and al. described the seasonal variation in food allergy to apple. Although specific IgE against Golden delicious increased during season, neither skin test nor immunoblotting could confirm an increase in reactivity. The results of immunoblotting performed with the sera of our patients, showed stronger reactions vs apple Golden.

The analysis of the immunoblotting results in correlation to clinical observations of investigated children generally agreed that the diagnosis of food allergy must rely on the outcome of oral food challenge [13]. Because clinical aspect of cross reactivity was not the subject of this paper, we didn't compare the diagnostic accuracy of immunoblotting with clinical symptoms. But according to our experiences, we can suggest that immunoblotting is useful to verification of cross-reactivity recognized by the presence of specific IgE.

Acknowledgement

The study was supported by grant of Ministry of Scientific Research and Information Technology No 0742/PO5/2001/21.

References

1. Holm J, Baerentzen G, Gajhede M, Ipsen H, Larsen JN, Lowenstein M, Wissenbach M, Spangfort MD. Molecular basis of allergic cross-reactivity between group 1 major allergens from birch and apple. J Chromatogr B, 2001; 756: 307-13.

2. Vieths S. Allergenic cross-reactivity, food allergy and pollen. Env Toxic Pharmac, 1997; 4: 61-70.

3. Hansen KS, Vieths S, Vestergaard H, Skov PS, Bindslev-Jensen C, Poulsen LK. Seasonal variation in food allergy to apple. J Chromatogr B, 2001; 756: 19-32.

4. Wensing M, Akkerdaas JH, van Leeuwen WA, Stapel SO, Bruijnzeel-Koomen CA, Aalberse RC, Bast BJ, Knulst AC, van Ree R. IgE to Bet v 1 and profilin: Cross-reactivity patterns and clinical relevance. J Allergy Clin Immunol, 2002; 9: 435-42.

5. Asero R, Mistrello G, Roncarolo D, Amato S, Zanoni D, Barocci F, Caldironi G. Analysis of the heat stability of lipid transfer protein from apple. J Allergy Clin Immunol, 2003; 112: 1009-10.

6. Rodriguez J, Crespo JF, Lopez-Rubio A, Cruz-Bertolo J, Ferrando-Vivas P, Vives R, Daroca P. Clinical cross-reactivity among foods of the Rosaceae family. J Allergy Clin Immunol, 2000; 106: 183-9.

7. Cudowska B, Kaczmarski M. Diagnostic value of birch recombinant allergens (rBet v 1, profilin rBet v 2) in children with pollen-related food allergy. Ann Ac Med Bial, 2004; 49: 111-5.

8. Pauli G, Oster JP, Deviller P, Heiss S, Bessot JC, Susani M, Ferreire F, Kraft D, Valenta R. Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. J Allergy Clin Immunol, 1996; 97: 1100-9.

9. Son DY, Scheurer S, Hoffmann A, Haustein D, Vieths S. Pollen-related food allergy: cloning and immunological analysis of isoforms and mutants of Mal d 1, the major apple allergen, and Bet v 1, the major birch pollen allergen. Eur j Nutr, 1999; 38(4): 201-15.

10. Rossi RE, Monasterolo G, Operti D, Corsi M. Evaluation of recombinant allergens Bet v 1 and Bet v 2 (profilin) by Pharmacia CAP system in patients with pollen-related allergy to birch and apple. Allergy, 1996; 51(12): 940-5.

11. Vieths S, Scheurer S, Reindl J, Lüttkopf D, Wangorsch A, Kästner M, Haase T, Haustein D. Optimized allergen extracts and recombinant allergens in diagnostic applications. Allergy, 2001; 56(Suppl 67): 78-82.

12. Asero R, Mistrello G, Roncarolo D, Amato S, Zanoni D, Barocci F, Caldironi G. Detection of clinical markers of sensitization to profilin in patients allergic to plant-derived foods. J Allergy Clin Immunol, 2003; 8: 427-32.

13. Cudowska B, Wasilewska J, Kaczmarski M. Alergia krzyżowanowe aspekty. Alergia Astma Immunologia, 2002; 7(Suppl 4): 47-51.

Hypersensitivity to hydrolyzed cow′s milk protein formula in infants and young children with atopic eczema/dermatitis syndrome with cow′s milk protein allergy

Kaczmarski M, Wasilewska J*, Lasota M

III Department of Pediatrics, Medical University of Białystok, Białystok, Poland

Abstract

Purpose: Atopic eczema/dermatitis syndrome (AEDS) is often the first manifestation of atopic disease in children. Food hypersensitivity should be considered in approximately 40% of these patients. AEDS children with cow's milk allergy are commonly prescribed a hydrolyzed formulas or amino acid-based formulas for an alternative protein source. The aim of this study was to investigate hypersensitivity to extensive hydrolyzed casein and whey proteins in AEDS children with cow's milk protein allergy (CMA).

Material and methods: The study included 67 hospitalized children with AEDS (m/f – 43/24), aged 1-28 months (mean 11.34±8.52) and CMA confirmed by oral food challenge. All patients were treated with extensively hydrolyzed formulas: 48/67 children with casein hydrolysates and 19/67 children with whey hydrolysates.

Results: In most of studied children we recognized severe AEDS (SCORAD Index: mean 55.41±17.4; 95% CI 51.17-59.66) with elevated total IgE (mean 432.98±1030.46; 95% CI 181.63-684.33). In 22/67 children (32.8%) we established diagnosis of hypersensitivities to hydrolyzed formula (HHF): in 17/22 to casein hydrolysates, in 4/22 to whey hydrolysates and in 1/22 to amino-acid based formula. Children with HHF did not differ in the severity of AEDS evaluated by SCORAD (57.18±16.59 vs 54.56±17.90), the serum level of total IgE (603.9±1253 vs 349.4±906.1) and the time of breast-feeding (4.4±4.0 months vs 6.8±7.28). They differ in the number of plasma eosinophils and positive correlation between number of eosinophils and serum level of total IgE ($p<0.05$, $r=0.46$ vs $r=0.07$).

Conclusions: Children with moderate or severe atopic eczema/dermatitis syndrome can demonstrate hypersensitivity to hydrolyzed formula recommended for therapeutic indications.

Key words: atopic eczema/ dermatitis syndrome, extensively hydrolyzed formula, cow's milk protein, amino acid-based formula.

Abbreviations: AAF – amino acid-based formula; AEDS – atopic eczema/dermatitis syndrome; CI – confidence interval; CM – cow's milk; CMA – cow's milk allergy; DBPCFC – double-blind, placebo-controlled, food challenge; eHC – extensive hydrolysates of casein; eHW – extensive hydrolysates of whey.

Introduction

Atopic eczema/dermatitis syndrome (AEDS) is a common ailment in children, affecting 10% to 12% of infants [1]. It is often regarded as the first manifestation of atopic disease in children. Patients with AEDS have in general an elevated total and food-specific level of IgE antibodies and food hypersensitivity should be considered in approximately 40% of these children [1,2]. Sensitivity to foods is seen in the first few years of life, while older children and adults with AEDS are more predisposed to developing respiratory allergy. The most commonly offending foods are cow's milk (CM), hen's egg, wheat and soy [3]. Cow's milk allergy affects 2% to 3% of infants in unselected cohorts [4]. Approximately one-third of the children with AEDS have been diagnosed with cow's milk allergy or cow's milk intolerance, as determined by means of an elimination diet and challenges, and approximately 40% to 50% of children <1 year of age with cow's milk allergy or cow's milk intolerance have AEDS [5]. Hill et al. found AEDS in 57% of the children with IgE-mediated cow's milk allergy [6].

* CORRESPONDING AUTHOR:
III Department of Pediatrics, Medical University of Białystok
ul. Waszyngtona 17
15-274 Białystok, Poland
Fax: +48 85 742 38 41
e-mail: jolanta@amb.edu.pl (Jolanta Wasilewska)

Received 31.05.2005 Accepted 08.07.2005

Table 1. **Characteristics of AEDS children with or without hypersensitivity to hydrolyzed formulas**

	Children with hypersensitivity to hydrolysates formulas (n=22)	Children without hypersensitivity to hydrolysates formulas (n=45)	p value
Sex (f/m)	8/14	16/29	
Age, mo (mean ±SD) (median) (95% CI)	11.95±8.3 (9.5) (8.3-15.6)	11.04±8.8 (7.0) (8.4-13.7)	<0.69=ns
SCORAD (mean ±SD) (median) (95% CI)	57.18±16.6 (58.5) (49.8-64.5)	54.56±17.9 (52.0) (49.2-59.9)	<0.57=ns
IgE IU/ml (mean ±SD) (median) (95% CI)	603.9±1253.5 (75.5) (48.14-1159.7)	349.4±906.1 (34) (77.2-621.7)	<0.35=ns
Number of peripheral eosinophils (mean ±SD) (median) (95% CI)	10.3±8.2 (8.0) (6.7-13.9)	5.7±4.0 (4.0) (4.5-6.9)	<0.02
Time of breast-feeding in months (mean ±SD) (median) (95% CI))	4.4±4.0 (4.0) (2.9-5.9)	6.8±7.28 (4.5) (4.6-9.0)	<0.29=ns

An avoidance diet plays an important role in the treatment of AEDS particularly in small children. Formulas containing hydrolyzed cow's milk proteins are used to treat these infants. Partially or extensively hydrolyzed formulas or amino acid-based formulas can be used depending on the severity of the clinical course of AEDS. The incidence of adverse reactions or allergy to these formulas in infants has been a new clinical problem in recent years.

The aim of this study was to investigate hypersensitivity to extensive hydrolysates of casein and to extensive hydrolysates of whey protein in children up to 3 years of age with AEDS.

Material and methods

The study included 67 hospitalized children with AEDS (43 boys, 24 girls), aged 1-28 months (mean 11.34±8.52; 95% CI 9.24-13.44). The inclusion criteria were: 1. diagnosis of AEDS, 2. age up to 3 years, 3. case history of CMA. The exclusion criteria: 1. concomitance of other organ diseases, 2. lack of parents consent to the study.

We used the diagnostic criteria of atopic dermatitis by Hanifin and Rajka [7] and SCORAD Index adapted by European Task Force on Atopic Dermatitis [8]. Scoring of AEDS included: assessment of topography items (affected skin area), intensity criteria (extent of erythema, edema, crusts, excoriations, lichenification and xerosis), and subjective parameters (extent of itch and loss of sleep). The maximum score was 92 points.

Cow's milk allergy (CMA) was recognized: 1) on the basis of case and family history, 2) by prick skin tests with native food allergens, 3) laboratory data: total IgE, food-specific IgE (cow's milk proteins: casein and whey protein: α-lactoalbumin and β-lactoglobulin) and 4) oral open CM challenge. The total IgE concentration and serum-specific IgE antibodies to CM allergens were measured with a fluoroimmunoenzymatic assay (UniCAP Pharmacia & Upjohn Diagnostics, Uppsala, Sweden) as detailed by the manufacturer's details and the cut-off point for positivity was set at 0.7 kU/l. Open challenge was chosen, since it has been shown to be reliable method in young children. Children taking antihistamine were advised to avoid it for 72 hours before provocation. The challenge was started in the hospital and then continued in the patient's home where the parents recorded the symptoms. The challenge period was 2 weeks. During the first day successive and increasing doses (0.1, 1.0, 3.0, 10.0, 30.0 and 50 or 100 ml – according to age of life) low-lactose CM (Bebilon Nutricia) were administered. Challenge was performed with the access to full emergency equipment with antianaphylactic drugs. After CMA diagnosis, all patients were treated with cow's milk protein hydrolyzed formula: 48/67 (71.6%) children with extensive hydrolysates of casein (eHC) (Nutramigen; Mead Johnson) and 19/67 (28.4%) children with extensive hydrolysates of whey (eHW) (Bebilon pepti 1 or 2; Nutricia). Introduction of hydrolyzed formula and monitoring of its clinical tolerance was started during hospitalization and continued in the out-patient department. Intolerance symptoms were indication to changing formula (eHC or eHW or AAF).

Statistical analyses

SCORAD, IgE, plasma eosinophils and the time of breast feeding data are expressed as mean (95% CI) and median values. The Mann-Whitney nonparametric test was used to compare the results of patients with those of controls. Results were considered statistically significant at $p<0.05$.

Ethics

Informed consent was obtained from the parents. The study was approved by the Ethics Committee of Medical University of Białystok.

Results

Tab. 1 shows the characteristics of the patients examined. In most of studied patients we recognized severe AEDS: SCORAD Index: range 18-92, mean 55.41±17.4 (95% CI 51.17--59.66) (*Fig. 1*) with elevated total IgE: range 2-5000IU, mean 432.98±1030.46 (95% CI 181.63-684.33). In 22 of 67 AEDS children (32.8%) we established diagnosis of hypersensitivities to hydrolyzed formulas (HHF): in 17/22 to extensive hydrolysates of casein (17 of 48 treated with eHC), in 4/22 to extensive hydrolysates of whey protein (4 of 19 treated with eHW) (*Fig. 2*). One of 22 children had reaction to amino acid-based formula. Children with hypersensitivity to hydrolyzed formulas did not differ in the intensity of AEDS evaluated by SCORAD (57.18±16.59 vs. 54.56±17.90) (*Tab. 1*). In HHF group the serum level of total IgE was higher than in children without HHF, but the difference was not significant (*Fig. 3*). In these both studied groups, we did not find the significant correlation between the serum IgE and SCORAD (*Fig. 4*, *Fig. 5*). Exclusively in children with HHF we noted a significant higher number of eosinophilic granulocytes in peripheral blood ($p<0.02$) (*Tab. 1*) and high positive correlation between number of eosinophils and serum level of total IgE ($p<0.05$, $r=0.46$ vs. $r=0.07$) (*Fig. 6*, *7*).

Figure 1. **SCORAD index in studied AEDS children**

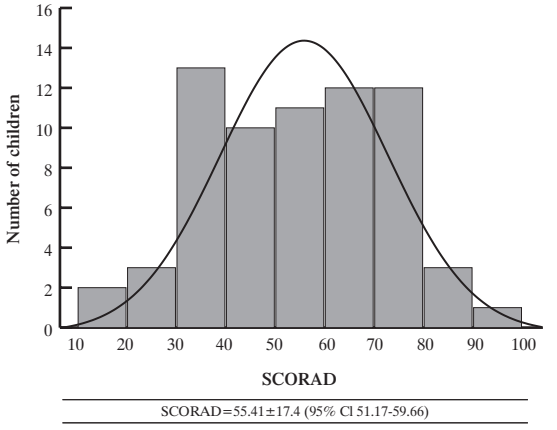


Figure 2. **Hypersensitivity to hydrolysates in AEDS children**

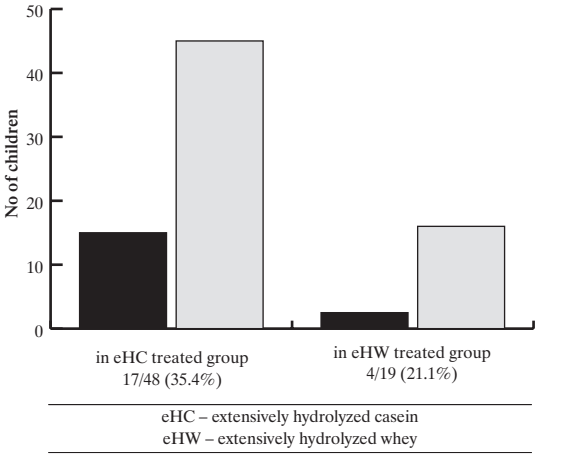


Figure 3. **Total serum IgE in AEDS children with or without hypersensitivity to hydrolyzed formula**

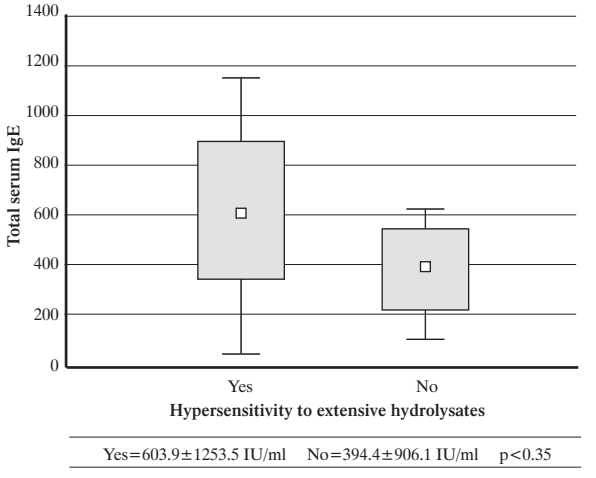


Figure 4. **IgE vs SCORAD in children with hypersensitivity to hydrolyzates** (r=0.27)

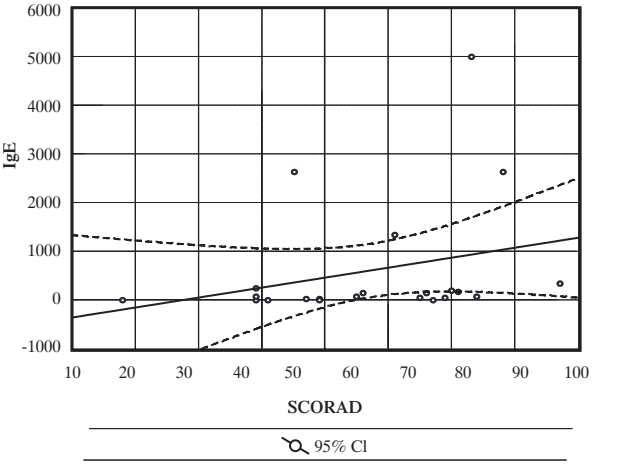
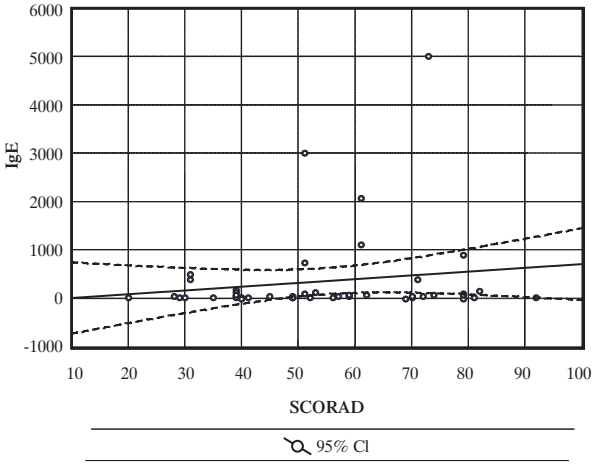


Figure 5. **IgE vs SCORAD in children without hypersensitivity to hydrolyzates** (r=0.15)



Clinical symptoms of hypersensitivity (dermatological, gastrointestinal and from nervous system) were observed in 17 of 48 (35.4%) AEDS patients treated with eHC and in 4 of 19 (21.1%) treated with eHW, 4 children did not tolerate as well eHC as eHW and were successfully treated with AAF (*Tab. 2*).

In studied group of 67 children mean time of breast feeding was 5.84±6.01 months (95% CI 4.13-7.55). The shortest time of breast feeding we noted in 4 infants with hypersensitivity to both hydrolysates (3.6±2.7) but the difference between patients with and without HHF was insignificant (*Tab. 1*, *Fig. 8*).

Discussion

Food allergy plays a role in at least 20% of the cases of AEDS in children younger than 4 years and in about 30% of AEDS children CMA is recognized [5]. All of AEDS children included to our study presented clinical symptoms of CMA confirmed by open oral cow's milk challenge. The complete elimination of cow's milk protein from the child diet for a variable period time is vital to the management of cow's milk allergy and extensively hydrolyzed formulas are the first-line therapy for routine use [9,10]. In this study most of patients (71.6%) were treated with eHC in the beginning. Protein hydrolysates are used to the

Figure 6. **Plasma eosinophils vs serum IgE in AEDS children with HHF** (r=0.46)

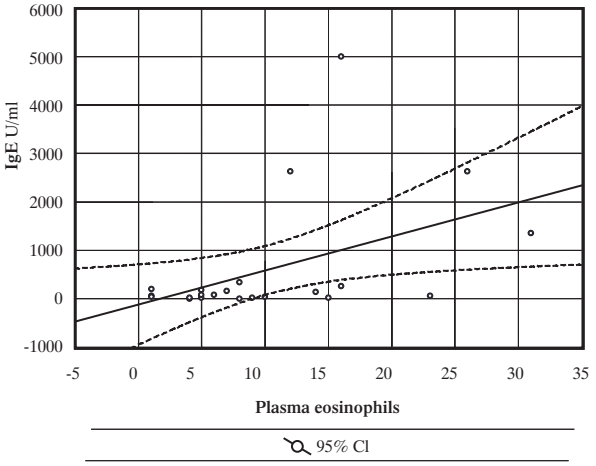


Figure 7. **Plasma eosinophils vs serum IgE in AEDS children without HHF** (r=0.07)

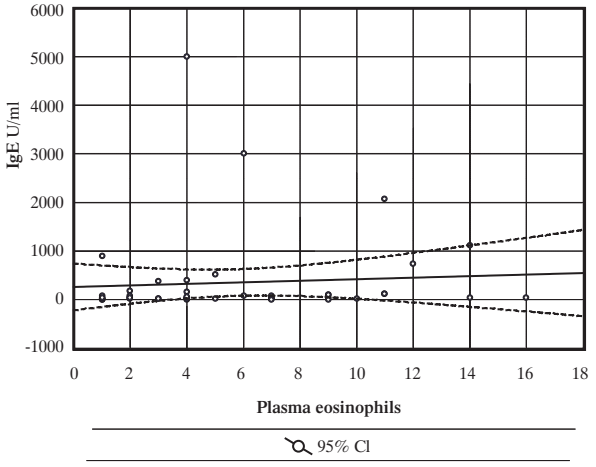


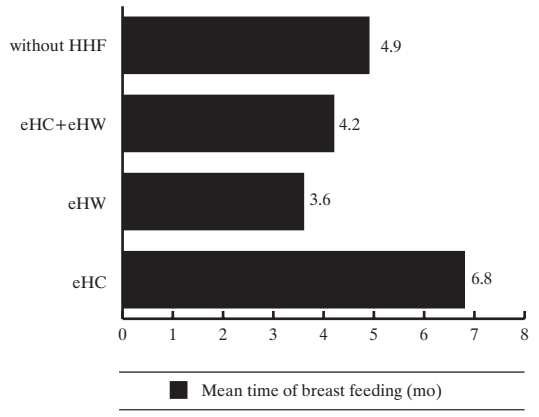
Table 2. **Characteristics of AEDS children with cow's milk allergy and hypersensitivity to hydrolyzed formula**

Patient	Age (mo)	Sex (F/M)	No tolerated extensively hydrolyzed protein	Clinical symptoms of hypersensitivity					Total IgE (UI/ml)	IgE caseine (class)	IgE whey (class)	
				Dermato-logical	Gastrointestinal			Nervous				
				Exacer-abtion of AEDS	GER	Colic	Diarrhea	Irritability			α-La	β-Lg
1	16	M	Casein	+	-	-	+	+	26.0	3	0	1
*2	3	M	Casein	-	+	+	+	-	4.0	3	3	1
3	6	M	Casein	+	-	+	-	+	195.0	3	0	2
4	6	F	Casein	+	-	-	-	-	66.0	2	0	2
5	9	F	Casein	+	-	-	-	-	74.0	4	2	1
6	6	M	Casein	+	+	-	-	+	257.0	2	1	0
7	24	M	Casein	+	-	-	-	-	5000.0	2	1	0
8	14	F	Casein	+	-	-	-	+	2633.0	4	0	1
9	28	F	Casein	+	+	-	+	-	12.0	3	0	3
10	7	M	Casein	-	+	-	-	+	2.0	3	2	0
11	11	F	Casein	+	+	-	-	-	342.0	ND	ND	ND
*12	18	M	Casein	+	-	-	-	-	177.0	4	3	2
13	6	M	Casein	+	+	+	+	-	1354.0	2	2	1
14	20	F	Casein	-	-	-	-	+	33.0	ND	ND	ND
*15	6	M	Casein	+	+	-	+	+	141.0	4	2	3
16	10	F	Casein	+	-	-	+	-	149.0	2	2	0
17	28	M	Casein	+	-	-	+	-	2634.0	2	1	2
18	11	M	AAF	+	+	-	-	-	12.0	3	4	2
*19	6	M	Whey	+	-	+	-	+	39.0	2	3	0
20	24	F	Whey	+	+	-	-	-	77.0	1	3	3
21	2	M	Whey	+	+	+	+	+	9.0	ND	ND	ND
22	2	M	Whey	-	+	+	-	+	50.0	ND	ND	ND

AAF – amino acid-based formula; ND – not done; α-La – alfa-lactoalbumin; β-Lg – beta lactoglobulin; * – children with hypersensitivity to both hydrolysates

treatment of cow's milk protein allergy and are well tolerated by 90% allergic children [9,11-13]. In recent times there have been increasing reports of intolerance to hydrolysates in infants and young children [14-16]. Allergy to extensive hydrolyzed formula may be part of more severe syndrome, multiple food allergy (MFA) in highly allergic children. According to Sampson et al., 8-10% of CMA children have hypersensitivity to casein [17]. In our study hypersensitivity to casein or whey proteins or both was find in 32.8% of AEDS children. In retrospective study by Latcham et. al, 43 of 121 (36%) children with multiple food allergy had intolerance to standard cow's milk hydrolysate formulas and did well with an amino acid formula (Neocate) [18]. In the

Figure 8. **Mean time of breast feeding in AEDS children with or without hypersensitivity to hydrolysates** (HHF) (n=67)



presence of allergy to other foods, tolerance of eHF and of CM occurs later and a restricted diet based on AABF is required for a longer duration [14].

The skin is one of the target organs involved in food hypersensitivity reactions. The most recent studies have shown that the IgE response after allergen-induced mast cell activation is characterized by skin infiltration of monocytes and lymphocytes [19]. Although eosinophils are not predominent in histologic sections of AEDS lesions, as seen in allergen-induced asthma, immunohistochemical staining of AEDS skin has revealed prominent deposition of eosinophil major basic protein and eosinophil-derived neurotoxin in active eczematous lesions [20]. Major basic protein is a cytolytic protein secreted almost exclusively by eosinophils capable of damaging skin epithelial cells and promoting mast cell degranulation and its deposition is not find in uninvolved skin sites. We have studied plasma eosinophils and higher number of these cells indicates an active allergic component in AEDS children with HHF.

The potential antigenicity and allergenicity of hydrolysates is caused by residual immunologically active protein and its immunoreactive epitopes. Residual allergenic activity has been described in partially and in extensively hydrolyzed formulas and indicates that technological separation of casein from whey proteins is unsatisfactory and it may cause anaphylactic reactions in sensitized children [21].

Accepting that none of hydrolysate-based products is completely safe, the American Academy of Pediatrics (AAP), the European Society of Paediatric Gastroenterology and Nutrition (ESPGAN) and the European Society of Paediatric Allergy and Clinical Immunology (ESPACI) recommend that introduction of these formulas in CMA children should be carried out under a doctor's supervision and tested in double DBPCFC [22]. As indicates our study children with AEDS accompanied by CMA are highly predisposed to hypersensitivity reactions to hydrolysate-based formulas, but randomized study is necessary to verify these results.

Acknowledgement

This study was supported by Medical University of Białystok project 3-43-647.

References

1. Sampson HA, McCaskil CC. Food hypersensitivity and atopic dermatitis: evaluation of 113 patients. J Pediatr, 1985; 107: 669-75.
2. Eigenmann P, Sicherer S, Borowski T, Cohen B, Sampson HA. Prevalence of IgE-mediated food allergy among children with atopic dermatitis. Pediatrics, 1998; 101: 8-13.
3. Burks AW, James JM, Fiegel A. Atopic dermatitis and food hypersensitivity reactions. J Pediatr, 1998; 132:132-6.
4. Moneret-Vautrin DA. Cow's milk allergy. Allerg Immunol, 1999; 31(6): 201-10.
5. Oranje A, Wolkerstorfer A, de Waard-van der Spek FB. Natural course of cow's milk allergy in childchood atopic eczema/dermatitis syndrome. Ann Allergy Asthma Immunol, 2002; 89(Suppl.): 52-5.
6. Hill DJ, Bannister DG, Hosking CS, Kemp AS. Cow's milk allergy within the spectrum of atopic disorders. Clin Exp Allergy, 1994; 24: 1137-43.
7. Hanifin JM, Rajka G. Diagnostic criteria of atopic dermatitis. Acta Dermatovener, 1980; 92(Suppl.): 44-7.
8. European Task Force on Atopic Dermatitis Severity scoring of atopic dermatitis: The SCORAD index. Dermatology, 1993; 186: 23-31.
9. Terracciano L, Isoardi P, Arrigoni S, Zoja A, Martelli A. Use of hydrolysates in the treatment of cow's milk allergy. Ann Allergy Asthma Immunol, 2002; 89(Suppl. 1): 86-90.
10. Walker-Smith J. Hypoallergenic formulas: are they really hypoallergenic? Ann Allergy Asthma Immunol, 2003; 90(Suppl. 3): 112-4.
11. Businco L, Dreborg S, Einarsson R, Giampetro PG, Keller KM. Hydrolysed cow's milk formulae. Allergenicity and use in treatment and prevention. An ESPACI position paper. Pediatr Allergy Immunol, 1993; 4: 101-11.
12. De Boisseau D, Matarazzo P, Dupont C. L'allergie aux hydrolysates de proteins. Rev Fr Allergol, 2000; 40: 98-104.
13. Fiocchi A, Travaini M, D'Auria E, Banderali G, Bernardo L, Riva E. Tolerance to a rice hydrolysate formula in children allergic to cow's milk and soy. Clin Exp Allergy, 2003; 33(11): 1576-80.
14. De Boisseau D, Matarazzo P, Dupont C. Allergy to extensively hydrolysed cow milk proteins in infants: identification and treatment with amino acid-based formula. J Pediatr, 1997; 131: 744-7.
15. Hill DJ, Cameron DJ, Francis DE. Challenge confirmation of late-onset reactions to extensively hydrolysed formulas in infants with multiple food intolerances. Allergy Clin Immunol, 1995; 96: 386-94.
16. Vanderhoof JA, Murray ND, Kaufmann SS. Intolerance to protein hydrolysate infant formulas: an under recognized cause of gastrointestinal symptoms in infancy. J Pediatr, 1997; 131: 741-4.
17. Hoffman KM, Sampson HA. Serum specific IgE antibodies to peptides detected in a casein hydrolysate formula. Pediatr Allergy Immunol, 1997; 8: 185-9.
18. Latcham F, Merino F, Lang A, Garvey J, Thomson MA. A consistent pattern of minor immunodeficiency and subtle enteropathy in children with multiple food allergy. J Pediatr, 2003; 143: 39-47.
19. Burks W. Skin manifestations of food allergy. Pediatrics, 2003; 111: 1617-24.
20. Gleich G, Frigas E, Loegering DA. Cytotoxic properties of eosinophilic major protein. J Immunol, 1979; 123: 2925-7.
21. Docena G, Rozenfeld P, Fernandez R, Fossati CA. Evaluation of the residual antigenicity and allergenicity of cow's milk substitutes by in vitro tests. Allergy, 2002; 57(2): 83-91.
22. Giampietro PG, Kjellman NI, Oldaeus G, Wouters-Wesseling W, Businco L. Hypoallergenicity of an extensively hydrolyzed whey formula. Pediatr Allergy Immunol, 2001; 12(2): 83-6.

Acute phase proteins serum concentrations in children are related to urinary iodine excretion

Lialikau SA[1], Haurylik LL[1], Sobieska M[2]*, Klachko NM[1], Samborski W[2]

[1] Chair of Pediatrics N1, State Medical University, Grodno, Belarus
[2] Department of Physiotherapy, Rheumatology and Rehabilitation, University of Medical Sciences, Poznań, Poland

Abstract

Purpose: The paper presents links between iodine provision and selected acute phase proteins' (APP) serum concentrations as well as their glycosylations profiles (investigated with the use of affinity immunoelectrophoresis with Concanavalin A as ligand) in children.

Material and method: 116 children (58 girls and 58 boys) were enrolled. Iodine level was measured in the morning (7:30-8:30) urine portion, using Cr-As method. According to iodine level children were divided into two groups. The first one consisted of 56 children with decreased iodine level (lower than 100 micrograms/L), second – 60 children with iodine level higher than 100 micrograms/L. In serum the concentration of ferritin, beta2-microglobulin (beta2-MG), thyroxin (T_4), triiodothyronin (T_3), thyrotrophic hormone (TSH) were measured by radioimmunoassay (BELORIS, Belarus). Concentrations of APP: C-reactive protein (CRP), alpha1-acid glycoprotein (AGP), alpha1-antichymotrypsin (ACT), alpha1-antitrypsin (AT), haptoglobin (Hp), alpha2-macroglobulin (A2-M), ceruloplasmin (Cp) and transferrin (Tf) were measured in sera samples by rocket immunoelectrophoresis acc. to Laurell with antibodies and standard from DakoCytomation, Denmark. Microheterogeneity of AGP, ACT and Tf was estimated using affinity immunoelectrophoresis with ConA as a ligand, acc. to Bøg-Hansen.

Results: It was established, that CRP level was lower than upper limit of normal range. Levels of other investigated proteins were reliably dependent on the level of iodine. Especially for AGP lower level was observed for children of the group with low iodine level. In children with low iodine level along with the decrease of serum AGP concentration altered glycosylations profile was observed, namely decrease in the content of variant non-reactive to ConA (W0) and increase in content of weakly reactive (W1) and reactive (W2) variants content, which resulted in increase of the reactivity coefficient (AGP-RC). Similar tendency in alterations of distinctly glycosylated variants in relation to iodine level could be shown for ACT. Serum concentration of any investigated protein was not dependent on the concentration of the hormones of pituitary-thyroid system.

Conclusions: It seems that the influence of the iodine level is direct, not via thyroid hormones. It could be suggested that in euthyroid children with low iodine excretion with urine a hidden iodine deficiency is already registered by the regulatory mechanisms and a kind of acute phase reaction is started, may be in order to increase iodine uptake and storage.

Key words: iodine provision, thyroid hormones, acute phase proteins.

Introduction

A problem of environmental too low iodine level exists in 140 countries all over the world. According to the reports of WHO about 1.5 milliard people live under iodine deficiency conditions [1]. It is known that iodine deficiency may result in several pathological changes, not only in traditionally reported goiter, and ongoing not necessarily along with thyroid enlargement. Iodine plays the most important biological role as a part of thyroidal hormones that take part in regulation of all metabolic processes, physical and mental development, reproductive functions and immunity [2]. But thyroid is not the only organ that makes use of iodine. Also the cells of the immune system use this microelement. As example the bactericidal activity

* CORRESPONDING AUTHOR:
Department of Physiotherapy, Rheumatology and Rehabilitation,
University of Medical Sciences
ul. 28. Czerwca 1956 roku 135/147
61-545 Poznań, Poland
Tel/Fax: +48 61 8310244
e-mail: magdula2811@poczta.onet.pl (Magdalena Sobieska)

Received 11.04.2005 Accepted 12.07.2005

of iodides and peroxiiodides produced by phagocytes may be mentioned [3]. However, no reports were found on relationship between iodine provision and acute phase proteins (APP) level or their microheterogeneity.

APP belong to the most ancient part of the unspecific immunity and contribute markedly to the keeping of homeostasis. As much as 30 various proteins are for the moment regarded as APP. Being multifunctional regulators and effectors APP stay in multiple relations to practically all types of cells and molecules. Among APP following functional groups may be described: transport proteins (transferrin, ceruloplasmin and haptoglobin), clotting factors (fibrinogen), antiproteases (α_1-antitrypsin, α_1-antichymotrypsin, α_2-macroglobulin), complement components (C3, C4) and several proteins of hardly known function, like C-reactive protein (CRP), serum amyloid A (SAA), α_1-acid glycoprotein (AGP) and others. Majority of those proteins are produced in hepatocytes, some of them to lesser extent also in lymphocytes, macrophages and endothelial cells, and their synthesis is mediated by interleukin-1 (IL-1), IL-6 and glucocorticosteroids in response to trauma, tissue injury, physical or psychic stress, but also autoimmune, allergic and neoplastic disorders.

Almost all APP (except albumin, CRP and SAA) are glycoproteins. It means that in specific glycosylation sites they bear side oligosaccharide chains, forming bi-, tri- or tetraantennary glycans. The molecules of the same glycoprotein may differ in the structure of side oligosaccharides and this feature is referred to as major microheterogeneity. Molecules with similar sugar chains form so-called variants of the glycoprotein. In physiological conditions the proportion of variants remains stable. There are several reports on microheterogeneity alterations in various pathological conditions, all reporting posttranslational changes in the side sugar structure due to activity of cytokines, such as IL-6, TGFβ, TNFα [4-6].

For the most widely investigated glycoprotein – AGP, the following alterations were described: in acute inflammatory conditions (trauma, acute bacterial infection and exhausting physical stress) there is an increase in biantennary sugar structures along with increase in protein concentration. Chronic inflammation results in increase or decrease of total AGP concentration along with marked increase in tri- and tetraantennary glycans [7,8]. Similar changes were also reported for some other APP.

The aim of the study was to investigate possible links between iodine provision and selected acute phase proteins' serum concentrations as well as their glycosylations profiles (investigated with the use of affinity immunoelectrophoresis with Concanavalin A as ligand) in children.

Material and methods

The study was performed with children whose places of residence are situated on areas affected by the Chernobyl disaster and who were spending their summer vacations in a spa. The whole treatment and investigation performed during this stay were a subject of Ethic Committee Consent and parents of all children expressed in written their approval for the routine laboratory investigation as screening tests for the general health status (also vitamin provision, hormones levels etc.). For the purpose of this study 116 children (58 girls and 58 boys), of them 57 aged from 7 to 12 years and 59 – 12-15 years were enrolled. In all investigated children neither any symptoms of iodine deficiency diseases, acute inflammation nor signs of deterioration of any chronic diseases were found, thus all children were found clinically healthy at the moment of enrollment. Clinical investigation showed also that all children were euthyroid and any substitution of iodine was used prior to investigation.

Iodine level was measured in the morning (7:30–8:30) urine portion, using Cr-As method [9]. According to iodine level children were divided into two groups. The first one consisted of 56 children with decreased iodine level (lower than 100 micrograms/L), second – 60 children with iodine level higher than 100 micrograms/L. In both groups similar proportion of boys and girls was noticed (girls to boys 26:30 in the 1st and 32: 28 in the 2nd group).

Parallel to urine sample and at the same time a sample of peripheral blood was drawn for the routine laboratory investigations. The rests of sera samples were used. In serum the concentration of thyroxin (T4), triiodothyronin (T3), thyrotrophic hormone (TSH) were measured by radioimmunoassay (BELORIS, Belarus). Concentrations of APP: CRP, AGP, alpha1-antichymotrypsin (ACT), alpha1-antitrypsin (AT), haptoglobin (Hp), alpha2-macroglobulin (A2-M), ceruloplasmin (Cp) and transferrin (Tf) were measured in sera samples by rocket immunoelectrophoresis acc. to Laurell [10] with antibodies and standard from DAKOPATTS, Denmark. Microheterogeneity of AGP, ACT and Tf was estimated using affinity immunoelectrophoresis with ConA as a ligand, acc. to Bøg-Hansen [11] with modification of Mackiewicz [12]. For AGP and ACT the so-called reactivity coefficients were calculated acc. to formula: for AGP-RC variants reactive with ConA (W1+W2+W3)/variant non-reactive (W0). For ACT-RC – variants reactive to ConA (A2+A3+A4+A5)/weakly reactive variant A1. Statistical analysis of data was performed using Statistica 6.0 Software. For the differences between the groups the Student t-test was performed. Prior to any other tests the distribution of variables was measured by K-S and Lillefors' tests. To evaluate the influence of a given factor to the alterations of other parameters one way Anova with Fisher's test was performed. Pearson's r was used to expressed significant correlations between the parameters. For all test the level of significance was established for $p<0.05$.

Results and discussion

The investigation of thyroid hormones showed normal values for all children. No differences were found if children were divided according to age, sex or iodine urine excretion. For the group with low iodine level slightly higher level of TSH was observed than in the group with normal iodine level, but the difference was not significant (3.7 ± 0.7 versus 3.1 ± 0.3 microunits/mL, respectively).

It was established, that CRP level was lower than upper limit and no differences were found between the groups. This could

Table 1. **Serum concentration of APP and percentage of their variants (mean ± SD) in relation to iodine excretion in urine, in children with low iodine level and with normal iodine level. Statistical significance of the difference between the groups is given in the last column and "NS" means p>0.1**

Parameters	1st group – low iodine	2nd group – normal iodine	p
	Mean ± SD	Mean ± SD	
AGP (mg/L)	603.79 ± 211.25	829.71 ± 397.26	0.0004
W0 (%)	41.05 ± 7.84	44.10 ± 5.29	0.02
W1 (%)	45.97 ± 6.06	43.59 ± 5.23	0.03
W2 (%)	11.28 ± 3.22	9.68 ± 3.37	0.01
W3 (%)	1.72 ± 2.04	1.93 ± 1.81	NS
AGP-RC	1.46 ±0.41	1.28 ± 0.26	0.009
ACT (mg/l)	279.32 ± 108.50	362.67 ± 142.98	0.001
A1 (%)	19.34 ± 4.09	21.53 ± 4.48	0.03
A2 (%)	29.02 ± 2.94	30.44 ± 5.59	NS
A3 (%)	32.27 ± 4.79	21.59 ± 7.01	0.000001
A4 (%)	16.38 ± 5.36	23.54 ± 11.44	0.001
A5 (%)	2.75 ± 2.41	5.13 ± 2.63	0.00008
ACT-RC	4.41 ± 1.34	3.93 ± 1.10	0.08
TF (mg/l)	2325.68 ± 478.54	2627.87 ± 935.70	0.06
T1 (%)	5.65 ± 3.80	6.29 ± 3.26	NS
T2 (%)	16.88 ± 5.27	17.99 ± 7.39	NS
T3 (%)	65.66 ± 8.53	63.91 ± 9.53	NS
T4 (%)	12.78 ± 4.54	14.07 ± 7.68	NS
A2-M (g/l)	4.84 ± 1.36	5.20 ± 1.62	NS
Cp (mg/l)	412.64 ± 112.39	525.41 ± 162.99	0.0001
Hp (mg/l)	0.58 ± 0.43	0.79 ± 0.48	0.05
AT (g/l)	1.72 ± 0.38	2.09 ± 0.59	0.0007

Table 2. **ANOVA analysis of the influence of selected factors (age, sex and iodine in urine) on investigated APP. Shown is only those parameters for which the influence of iodine level was proved. "NS" means p>0.1**

Parameters	Sex		Age		Iodine in urine	
	F	p	F	p	F	p
AGP	2.77	NS	3.40	0.07	14.99	0.0002
W1 (%)	1.33	NS	3.40	0.07	4.17	0.04
AGP-RC	0.96	NS	0.56	NS	4.78	0.03
ACT	2.12	NS	3.82	0.06	12.80	0.0005
A3 (%)	0.44	NS	0.01	NS	44.35	0.000001
A4 (%)	0.13	NS	0.01	NS	8.66	0.004
A5 (%)	0.92	NS	0.06	NS	16.43	0.0001
Tf	0.51	NS	0.13	NS	3.85	0.06
Cp	2.62	NS	8.28	0.005	19.07	0.00003
Hp	3.98	0.05	0.58	NS	6.62	0.01
AT	0.84	NS	0.04	NS	12.56	0.0006

be regarded as exclusion of any inflammatory processes in all investigated children.

Levels of other investigated proteins were reliably dependent on the level of iodine. Especially for AGP lower level was observed for children of the first group (low iodine level), (*Tab. 1*).

In children with low iodine level along with the decrease of serum AGP concentration altered glycosylation profile was observed, namely decrease in the content of W0 variant (non-reactive with ConA) and increase in W1 and W2 content, which resulted in increase of AGP-RC. Similar tendency in alterations of distinctly glycosylated variants in relation to iodine level could be shown for ACT. In children with iodine deficiency total serum ACT concentration was decreased, along with decrease in percentage of A1 variant and significant increase in A3 variant. Percentages of A4 and A5 variants decreased in comparison to group 2. All these alterations together resulted in increase of ACT-RC value. Thus, similarly as for AGP, decreased total ACT concentration was accompanied by higher reactivity with ConA, i.e. relative domination of biantennary glycans. For both proteins the decrease of ConA non-reactive variants (W0 of AGP and A1 of ACT) was the common feature. This tendency of decreasing ConA non-reactive variants seemed to be the most probable candidate for a link between iodine and APP.

Mean serum transferrin concentration was slightly (not significantly) lower for children with iodine deficiency than in group 2. The percentages of its variants did not differ significantly, T1, T2 and T4 being slightly lower and T3 slightly higher than for group 2. Also serum A2-M concentration did not differ between the groups. On the contrary, serum concentrations of Cp, Hp and AT seemed tightly related to iodine level, with normal concentrations in group 2 and decreased below the normal values in group 1.

It is known that during development in children several processes take place that influence basic biochemical parameters. Especially during sex maturation these changes are caused not only by age, but mainly by sex. To determine the influence of all factors mentioned (age, sex and iodine deficiency) on the investigated parameters, the multi-factorial analysis of dispersion (ANOVA) was conducted. The influence of each factor was calculated according to Fisher's test (F). The results of this analysis showed that the influence of iodine deficiency on AGP, ACT, Tf, Cp, and Hp and AT was independent from age and sex (*Tab. 2*), but when the influence of sex and age was excluded, the link between iodine deficiency and the concentration of variants W0, W2 of AGP and A1 of ACT lost its statistical significance.

The level of iodine in the organism is tightly bound to the production of thyrotrophic hormone and the function of thyroid gland. This could explain the results obtained in the correlation analysis, showing that majority of the investigated proteins' serum concentrations correlated significantly (and with the same sign) with both urinary excretion of iodine and the concentrations of pituitary-thyroid hormones (*Tab. 3*).

As conclusion one question appeared: which of the investigated factors – iodine level or hormones influenced the concentration and glycosylation of the investigated APP. And again the dispersion analysis was performed for each pair of factors: TSH and iodine, T_3 and iodine, T_4 and iodine. It was shown that the level of iodine with high significance and directly influenced the serum concentration of AGP, the percentage of its ConA strongly reactive variants and the AGP-RC value, as well as the serum concentration of ACT and the percentage of its variants A3, A4 and A5 (also strongly reactive with ConA), and concentration of Cp, Hp and AT (*Tab. 4*). Serum concentration of any

Table 3. **The correlation (Pearson's r) between TSH, T_3, and T_4 in the peripheral blood, iodine in urine and serum APP concentrations. Shown are only those parameters for which the correlation was proved to be statistically significant. "NS" means p>0.1**

Parameters	Iodine in urine		T_3		T_4		TSH	
	r	p	r	p	r	p	r	p
AGP	0.32	0.001	0.31	0.001	0.28	0.002	-0.06	NS
W1 (%)	-0.12	NS	-0.18	0.05	-0.04	NS	0.09	NS
W2 (%)	-0.19	0.05	-0.03	NS	0.06	NS	-0.16	0.08
ACT	0.36	0.001	0.30	0.001	0.32	0.001	0.04	NS
A1 (%)	0.12	NS	0.23	0.02	0.22	0.03	-0.08	NS
A3 (%)	-0.47	0.0001	-0.59	0.0001	-0.42	0.0001	-0.11	NS
A4 (%)	0.20	0.05	0.11	NS	0.20	0.05	0.05	NS
A5 (%)	0.43	0.0001	0.48	0.0001	0.33	0.001	0.17	0.09
ACT-RC	-0.11	NS	-0.25	0.01	-0.20	0.05	0.07	NS
Tf	0.30	0.003	0.16	0.08	0.05	NS	0.15	NS
T2 (%)	0.12	NS	0.14	NS	0.04	NS	-0.19	0.05
T4 (%)	0.14	NS	0.18	0.06	-0.03	NS	0.24	0.01
A2-M	0.24	0.01	0.06	NS	-0.13	NS	-0.06	NS
Cp	0.35	0.001	0.37	0.001	0.14	NS	-0.06	NS
AT	0.34	0.001	0.33	0.001	0.001	NS	0.20	0.05

Table 4. **Investigation of the link between thyroid hormones and APP. The F value and significance (p) of the influence of TSH, T_3, T_4 and iodine on the investigated parameters. Shown are only those parameters for which the influence was proved. "NS" means p>0.1**

Parameter	TSH		Iodine		T_3		Iodine		T_4		Iodine	
	F	p	F	p	F	p	F	p	F	p	F	p
AGP	0.39	NS	9.07	0.001	1.32	NS	9.63	0.001	1.31	NS	5.42	0.005
W1 (%)	0.76	NS	3.84	0.02	0.74	NS	2.41	0.09	1.51	NS	1.80	NS
W2 (%)	0.37	NS	2.58	0.08	0.13	NS	2.59	0.08	2.52	0.08	4.41	0.01
W3 (%)	1.95	NS	3.33	0.04	0.47	NS	4.65	0.01	0.02	NS	2.92	0.05
AGP-RC	1.38	NS	3.29	0.04	0.31	NS	3.01	0.05	1.26	NS	6.22	0.003
ACT	0.42	NS	10.5	0.001	0.61	NS	11.1	0.001	1.44	NS	6.87	0.001
A3 (%)	0.67	NS	28.9	0.001	5.12	0.01	17.3	0.001	3.32	0.04	13.5	0.001
A4 (%)	2.30	NS	5.48	0.005	0.21	NS	4.76	0.01	0.01	NS	2.36	NS
A5 (%)	1.49	NS	10.5	0.001	2.60	0.08	4.60	0.01	0.08	NS	5.01	0.01
T2 (%)	3.29	0.04	0.26	NS	1.69	NS	0.28	NS	1.65	NS	0.56	NS
Cp	0.31	NS	10.4	0.001	1.00	NS	9.27	0.001	0.06	NS	15.3	0.001
Hp	1.83	NS	5.42	0.005	0.04	NS	4.71	0.01	0.42	NS	1.36	NS
AT	2.40	NS	5.82	0.004	0.21	NS	6.82	0.001	0.21	NS	6.74	0.001

investigated protein was not dependent on the concentration of the hormones of pituitary-thyroid system. However, the percentage of variant A3 of ACT was dependent on triiodothyronin and the percentage of T2 variant of Tf was dependent on TSH.

Thus, low iodine in urine was associated with decreased AGP, ACT, along with altered glycosylation of those glycoproteins, with decreased Cp, Hp and AT. The investigated proteins were not reliably bound to the hormones of pituitary-thyroid system but to the level of iodine in the organism, which could be shown by the high correlation values. Also the glycosylation profiles of the investigated proteins correlated with iodine provision, not with the hormones. This was mainly true for the AGP and ACT variants strongly reactive with ConA, thus possessing mainly biantennary glycans. All the described links were not dependent on age or sex of the enrolled children and were stable for the whole investigated group. The iodine provision seemed not to influence the serum concentration of Tf and the percentage of its variants, the serum concentration of A2-M nor the AGP or ACT variants non-reactive with ConA (with mainly triantennary glycans).

The explanation of these associations observed between iodine and investigated APP is difficult. It could be suggested that in euthyroid children with low iodine excretion with urine a hidden iodine deficiency is already registered by the regulatory mechanisms and a disturbance of liver protein synthesis appears, first for the most important acute phase reactants. It may be postulated that these alterations appear in order to increase iodine uptake and storage or they play a regulatory role. It cannot be stated that such a small iodine deficit is already causing protein deficiency as general health status of the investigated children was satisfying. Besides the observed alterations of the glycosylations profiles were rather similar to what was previously described as acute inflammatory changes rather than a sign of deficit (according to our previous observations liver cirrhosis caused dramatic decrease of ConA reactivity along with decreased APP concentrations; unpublished data). According

to our own experience any inflammatory process would cause elevation in at least AGP level (if not other glycoproteins, even in absence of elevated CRP), along with elevated AGP-RC in acute inflammation or decreased in chronic inflammatory processes. Finally, decreased levels were also found in pregnancy and in allergy, mainly in allergy to food, but in this cases reactivity to ConA was also diminished. Thus, this is the first report on decreased AGP level with increased AGP-RC. Though we are not able to show the exact mechanism of iodine influence on the synthesis and glycosylation of acute phase proteins, the proven links seem interesting and we would like to describe them as preliminary study.

Acknowledgement

The paper was based on the common research between the University of Medical Sciences, Poznań, Poland and the State Medical University, Grodno, Belarus. No extra funds were obtained for the reported investigation. All research was done in accordance with national and international guidelines for the protection of human subjects welfare. Blood sample drawn was intended for routine laboratory investigation and the rests were used for the investigation of acute phase proteins.

References

1. Health for all in the 21st century / HEALTH 21. Copenhagen, WHO Regional Office for Europe, 1999. European Health for All Series 6, 28.
2. Lektorov VN. Diseases caused by iodine deficiency: problems and decisions. Public Health, 2002; 3: 2-4.
3. Zaichik AS, Churilov LP. Bases of Pathochemistry. S. Petersburg, 2000; 688.
4. Bierhuizen MFA, De Wit M, Govers, C. Glycosylation of three molecular forms of α_1-acid glycoprotein having different interactions with Concanavalin A. Variations in the occurrence of di-, tri- and tetraantennary glycans and the degree of sialysation. Eur J Biochem, 1988; 175: 387-94.
5. Mackiewicz A, Kushner I. Role of IL-6 in acute phase proteins glycosylation. Ann N Y Acad Sci, 1989; 557: 515-6.
6. Novorytko J, Guzdek A. Potranslacyjna modyfikacja glikoprotein. Post Bioch, 1987; 33: 65-7.
7. Bręborowicz J, Mackiewicz A. Affinity electrophoresis for diagnosis of cancer and inflammatory conditions. Electrophoresis, 1989; 10: 569-70.
8. Kushner I, Mackiewicz A. Acute phase proteins as disease markers. Dis Markers, 1987; 5: 1-11.
9. Dunn JT, Crutchfield HE, Gutekunst R, Dunn AN. Methods for measuring iodine in urine. International Council for control of Iodine deficiency Disordes. Netherlands, 1993; 1: 18-29.
10. Laurell CB. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Scand J Clin Invest, 1972; 124; 21-8.
11. Bøg-Hansen TC. Crossed immuno-affinoelectrophoresis: an analytical method to predict the result of affinity chromatography. Anal Biochem, 1973; 56: 480-8.
12. Mackiewicz A, Mackiewicz S. Determination of lectin-sugar dissociation constans by agarose affinity electrophoresis. Anal Biochem, 1986; 156, 481-8.

Extraction of nerve cells in images with herpetic infections

Nedzved A[1]*, Ablameyko S[1], Oczeretko E[2]

[1] United Institute of Informatics Problems of the National Academy of Sciences of Belarus, Minsk, Belarus
[2] Institute of Informatics, University in Białystok, Poland

Abstract

In this paper, algorithms for extracting and diagnosis of nerve cells with herpetic infections are proposed. Degree of herpetic lesions is divided into four classes. Morphological characteristic of herpetic lesions for cell is accomplished by analyze cells structure. Because virus of herpes change shape of nucleus. Therefore automated analysis of herpetic lesion carry out by morphology segmentation and cells structure identification. The algorithms have been successfully used in practical systems and showed good results.

Key words: herpes, nervous system, diagnostic, image processing.

Introduction

The herpes simplex virus has been studied actively comparatively not long ago, although it is a well-known disease since antiquity. It is connected with the fact, that prevalent majority of the world population which is older 15 years being infected is enduring quite safely to the most widespread and harmless local forms of herpetic infections such as herpes nasalis and labialis [1-3]. Only in the past decade the role of VHS was established in the formation of the secondary immunodeficiency at the patients. The deterioration of ecological situation, the increase of oncological diseases, the expansion of human immunodeficiency virus (HIV) and extension of infections caused by AIDS-virus are result in the growth of the human herpetic lesions. Physicians of all over the world note the significant increase of complicated forms of herpetic infection.

Number of publications about various aspects of herpetic infections, concerned only the local VHS presentations (f.e. stomatitis, oftalmoherpes, herpes of genitals, skin diseases, etc.), has sharply increased during last ten years. However, the most terrible form of this pathology is disseminated herpes infection (DHI). If one of these forms is a disseminated infection of newborns which has described in the literature many years ago, the questions about an opportunity and mechanisms of DHI development at children under 1 year and adults are discussed. It is explained by the absence of sufficient number of clearly diagnosis cases of disease and sufficient qualified medical supervision and investigation [2,3].

Diagnosis of this disease is very complicated task since the main methods of immunofluorescence and polymerase chain reaction used in practice bring to positive results in the acute period of disease or exacerbation of chronic current of DHI only. At the same time, it frequently give false results, because of about 90% population of our planet has this infectious agent. Therefore the basic diagnostic method is morphological, i.e. the image analysis of the cells. It makes possible to reveal the presence of inflammatory process and etiological factor as original herpes inclusions.

Beside that the problem of diagnostics of herpes viruses is very important in the biopsy material from the brain. It is very important in a choice of the treatment methods and definition of diseases prognosis. The establishment of the diagnosis of the herpetic infection allows to escape of fatal diagnostics mistakes. Analysis of biopsy brain material is based on extracting and diagnosis of nerve cells with herpetic infections.

Modern tools like digital microscopy and developed mathematical methods allow an automatization of cell image analysis. There are known cell image analyzers [4] although most of them are developed and used mainly for image segmentation but not for diagnosis of disease.

The analysis of cell images can be considered as image segmentation problem where cells or their kernels should be

* CORRESPONDING AUTHOR:
United Institute of Informatics Problems of the National Academy of Sciences of Belarus
6 Surganov Str, 220012, Minsk, Belarus
e-mail: NedzvedA@newman.bas-net.by (Alexander Nedzved)

Received 25.05.2005 Accepted 31.05.2005

Figure 1. **Image of good neuron**

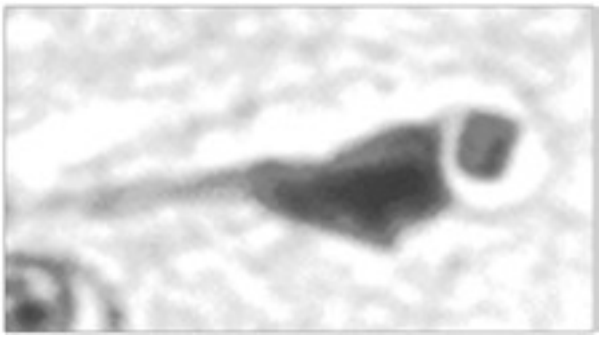
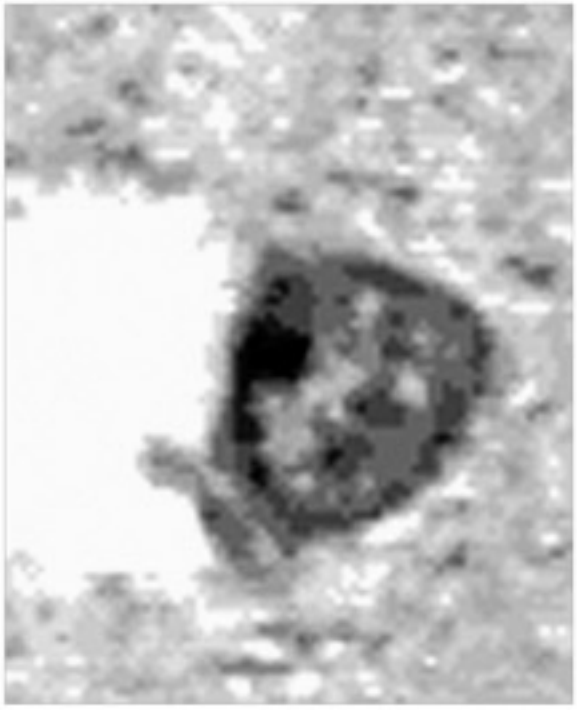


Figure 3. **Nerve cells with the second type of affection**

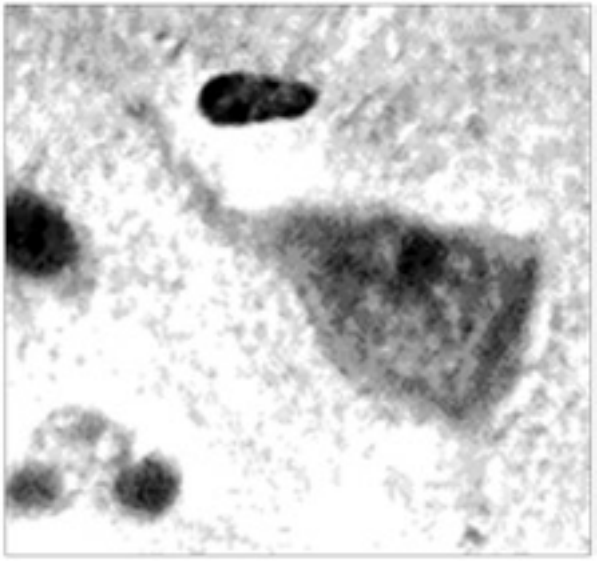


extracted. Being extracted, object characteristics should be computed, analysed and used by doctors.

There were already made many attempts to solve this problem. Review of cell image segmentation methods can be found in papers [5-7]. However, due to the very complex nature of brain images, it is not possible to select or develop automatic segmentation and recognition methods that could be applied for any type of such images. This is why most of the papers consider separate features of cell images and methods of their segmentation. The results also depend on a cell image quality. If the difference between cell or kernel and background is small, most of the methods do not work properly.

In this paper, algorithms for extracting and diagnosis of nerve cells with herpetic infections are proposed. The algorithms have been successfully used in practical systems and showed good results.

Figure 2. **Nerve cells with the first of affection**



Nerve cells images with herpetic infections

The neurons in human brain affected tissues are classified as healthy ones (*Fig. 1*), and cells of the first type (*Fig. 2*) and second type of affection (*Fig. 3*). One of the main features of a cell is its diaphragm, which outlines its boundary. The task is to extract nerve cells with herpetic infections.

Since an image background is inhomogeneous, and the image includes separately positioned objects of the same type, and, besides, the grayscale value for the pixels of the background varies uniformly and does not have abrupt jumps, good results are obtained by the morphological segmentation. The designed algorithm is based on the grayscale thinning of the morphological gradient, accompanied by tailing at each iteration which allows one to obtain closed contours bounding the regions corresponding to the objects. Taking into account the fact that the areas of the pyramidal neurons vary from 8 to 2000 μ^2, the objects which do not fall into this interval are eliminated. Thereafter, a hierarchical image of the cells for the cleared nuclei is constructed.

Morphological segmentation of cell images

Fig. 4 shows a gray scale image of neuron of head brain with indistinct boundaries and noisy background. Our task is to find a cell boundary, which would best correspond to its diaphragm.

According to our approach, at a first stage, the morphological gradient is computed that is the difference of the outputs of erosion and dilation in one iteration (*Fig. 4*). Obviously, an optimal border should pass on the sharpest overfall of brightness in a diaphragm, which corresponds to highest grayscale values on the image obtained with the help of morphological gradient. The obtained borders usually have more than one pixel thickness. Therefore, thinning of border lines is applied.

Figure 4. **Result of morphological gradient extraction of nerve cells**

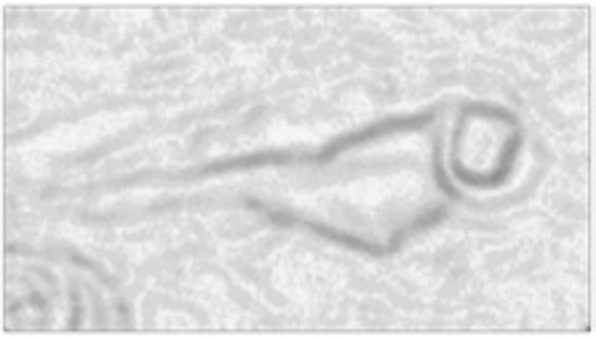
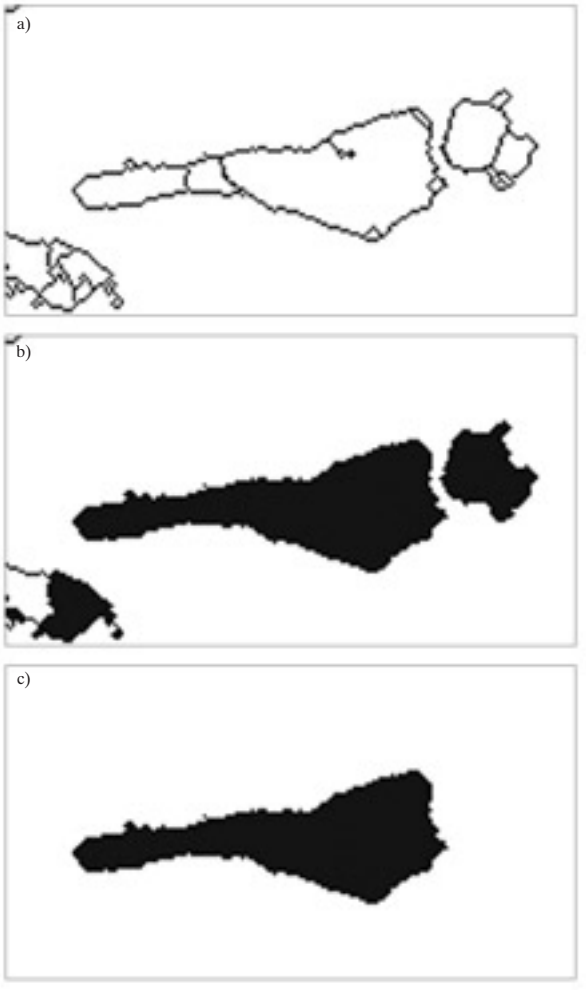


Figure 5. **Extraction of neuron: a) contour extraction after thinning operation, b) object filling, c) a binary image of a neuron obtained in the result of area measurements**



However, quite many false branches are obtained after thinning that should be deleted. To avoid this problem, we use two possibilities. The first one is to reduce noise at the preprocessing stage. Morphological operations can be used for this aim. For example, closing operation allows smoothing object contours that give better processing result. The second possibility is that we check and delete false branches after thinning. The result contains only closed contours, where the exterior contour corresponds to the border of a cell (*Fig. 5a*). To delete interior contours in binary image, contour filling is performed. As a result of all these operations, the binary cell image is obtained (*Fig. 5b*).

To delete objects not being cells, the computation of their area parameters is performed. The objects that do not satisfy apriori given geometric or optical parameters are deleted from the image (*Fig. 5c*).

Cell structure identification

Morphological characteristic of herpetic lesions for cell is accomplished by analyze cells structure. Because virus of herpes change shape of nucleus.

The algorithm of cells structure identification consists of two branches (*Fig. 6*). The first is intended for cells with a unpainted nucleus, another for the painted nucleus. In them according to hierarchical features binary images of cells, nucleus, nucleolus and inclusions are created. For reception of topological characteristics the cell is convenient for presenting as hierarchy of binary images. Where radically the column lays the binary image of a cell, at the following level there is an image of a nucleus, in branches the column lay nucleolus and various cellular inclusions.

Reception of each image is accompanied by logic operations of association and crossing of images, filling of holes in objects and the control on the area and diameter with the purpose of removal of noise. During identification cytoplasm of a nucleus, the certain bats mark nucleolus and cellular inclusions. In computer facilities for the characteristic of pixel the byte that is realized eight by byte more often is used. About eight levels of hierarchy, therefore, are optimum to allocate. Thus, all identified images are kept in the multiphase image. It makes presentation of result and ease of the automatic analysis of various cellular components and their analysis. Advantage of this method consists that under one multiphase image is possible to draw conclusions on all of a level of classification. Namely to receive the full information about structure of nerve cells.

Extracting of nerve cell types

All nerve cells are divided into four classes. First class consists of the healthy cells (*Fig. 7*). They have a triangular body. Therefore, a value of the factor of the shape is used, which vary in the range from 0.001 to 0.7, in order to classify these cells. The nuclei of these cells are well dyed and are darker than the cytoplasm, therefore they are not distinguished on the hierarchical image. Hence, the second parameter which specify these cells is Euler number (E8). In our case, it is equal to 0 for healthy neurons.

Second class consists of the cells with inclusions of the first kind (*Fig. 8*). Their shapes vary from triangular to the circular. Therefore, the factor of the shape for these cells vary from 0.001 to 1 and cannot serve as a basic classification parameter. The nucleus affected by the virus is not dyed and is lighter. Therefore, it is not distinguished by a separate color on the hierarchi-

Figure 6. **Block diagram of algorithm of construction of the hierarchical image of cells structrure from binary, where I – the binary image, G – the color image, C – the binary image of a cell, N – binary nucleus, V – the binary image nucleolus or inclusions, B – a morphological structural element. SCRAP (X) – function of removal of objects on the sizes of the area and diameter on image X. FILL (X) – function of filling of apertures in objects on image X. DIST (G) – adaptive threshold segmentation of image G**

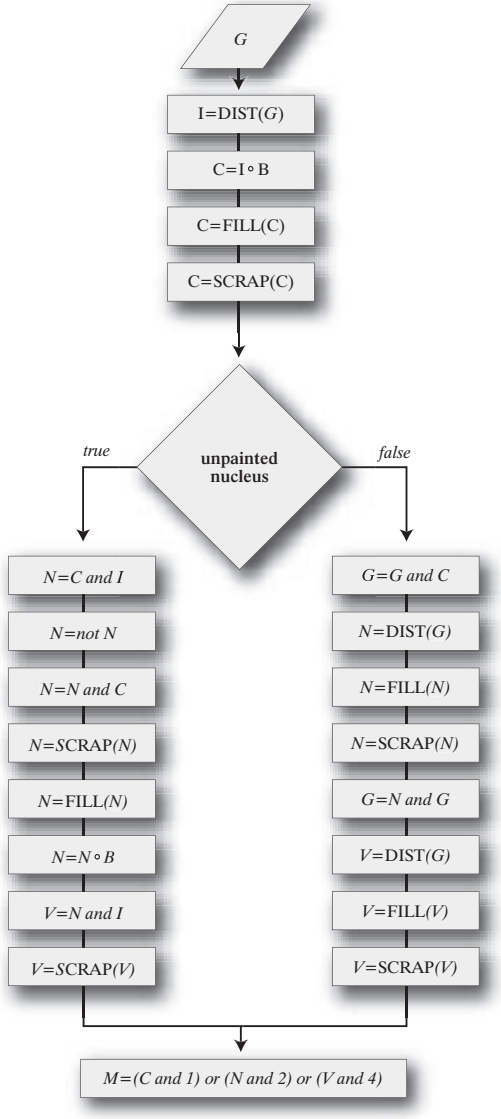


Figure 7. **Binary images of good neuron**

Figure 8. **Binary hierarchical image of nerve cells with the first type (second class) of affection**

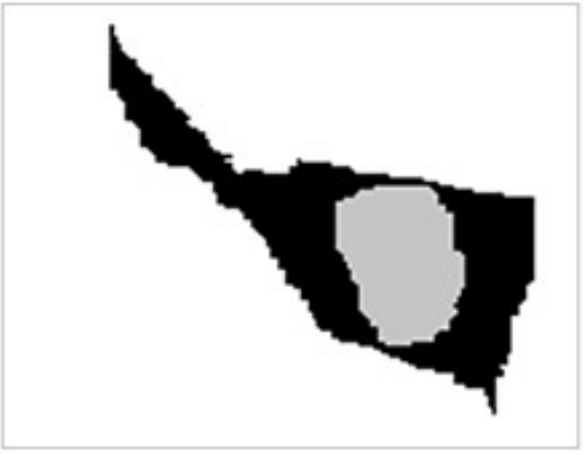


Figure 9. **Binary hierarchical image of nerve cells with the second type (third class) of affection**

cal image. In this case, the Euler number is equal to 1. Basic peculiarity of this class is characterized by the nucleus-cell ratio which is less than 0.8.

The third class consists of the cells with inclusions of the second type (*Fig. 9*). The shape of these cells is nearly circular, and there is practically no cytoplasm in the cell. Therefore, the following parameters are used for this class: the shape factor varies from 0.8 to 1, the Euler number is equal to 1, the nucleus-cell ratio varies from 0.8 to 1.

The fourth class includes all other objects which are eliminated immediately after their identification.

The result is an image containing healthy neurons and cells of the first and second kind. They have geometric and optical parameters which can be useful not only for the scientific research, but also in the problems of the practical diagnosis of a disease.

Conclusions

Algorithms for extracting and diagnosis of nerve cells with herpetic infections have been proposed in the paper.

These algorithms allow to extract nerve cells and make its classification by type of herpetic lesions. The algorithms have been successfully used in practical systems and showed good results.

Their usage allows to improve quality of diagnostic of herpetic lesions and exclude mistakes of human factor and decrease expended time for process of verification diagnosis.

Acknowledgement

This work is partially supported by INTAS 04-77-7067.

References

1. Vannier MW, Haller JW. Biomedical image segmentation. Proc of IEEE International Conference on Image Processing, 1998; 2: 20-4.

2. CIRES – Cell Image Retrieval and Evaluation System. Magazine for users of image analysis systems, Sept. 1992, pp. 18-9.

3. Walker RF, Jackway P, Lovell B,.Longstaff ID. Classification of cervical cell nuclei using morphological segmentation and textural feature extraction. Second Australian and New Zealand Conference on Intelligent information systems, Brisbane, 1994, pp. 183-9.

4. Vazquez L, Sapiro G, Randall G. Segmenting neurons in electronic microscopy via geometric tracing. Proc of IEEE International Conference on Image Processing, 1998; 3: 814-8.

5. Nedzvedz MK, Nedzvedz GK, Nedzvedz GE, Petrovich TM, Kulichkovskaja IV. Morphological peculiarities of demyelination in chronic herpetic meningoencephalitis. Russian scientific-practical conference of neurologists. Neuroimmunology, St.Petersburg, 2002; 193-5.

6. Nedzvedz MK, Guzov SA. Morphological characteristic of CNS lesions stipulated by VHS. Achievements of medical science and technology. Minsk, 1999; 1: 76-7.

7. Nedzvedz MK, Fridman MV, Nedzvedz AM. Modern views about morphogenesis of herpetic infection. Infection and immunity. Materials of Republican scientific-practical conference devoted to 75 years of BSRIEM. Minsk. 1999: 309-13.

8. Nedzved A, Ablameyko S, Pitas I. Morphological segmentation of histology cell images. Proc Int Conf on Pattern Recognit, Barcelona, Spain; 1: 500-3.

Alterations of lymphocyte subpopulations in choroidal melanoma patients undergoing surgery

Manyś-Kubacka K[1], Kostrzewa A[2], Sobieska M[3]*, Samborski W[3]

[1] Department of Ophthalmology, University of Medical Sciences, Poznań, Poland
[2] Department of Biology and Environmental Studies, University of Medical Sciences, Poznań, Poland
[3] Department of Physiotherapy, Rheumatology and Rehabilitation, University of Medical Sciences, Poznań, Poland

Abstract

Purpose: The alterations of lymphocyte subpopulations assessment after surgery in choroidal melanoma patients compared to cataract patients.

Material and methods: 12 patients with malignant melanoma of the choroid, 10 patients subjected to surgery due to cataract. Methods – flow cytometric measurement of absolute lymphocyte count, the number of all T cells (CD3+), T helper lymphocytes (CD3+CD4+), T cytotoxic lymphocytes (CD3+CD8+), B lymphocytes (CD19+), NK cells (CD3-CD16+) and T cells (CD3+) cells with γδ TCR, on the day of surgery and two days after it.

Results: Comparable numbers of cells were observed in both groups prior to surgery, but the behavior of some populations differed: CD3+, CD3+CD4+ cells increased in melanoma patients whereas they decreased in reference group, the number of T lymphocytes with γδ TCR was significantly higher in melanoma patients before surgery and it did not differ after it.

Conclusions: Though there were no significant differences in lymphocyte subpopulations between melanoma patients and the reference group, it seems that the presence of tumour influences the reactivity of the immune system to the trauma (surgery).

Key words: choroidal melanoma; flow cytometry; lymphocyte subpopulations; T lymphocytes with γδ TCR.

* CORRESPONDING AUTHOR:
Department of Physiotherapy, Rheumatology and Rehabilitation,
University of Medical Sciences
ul. 28. Czerwca 1956 roku 135/147
61-545 Poznań, Poland
Tel/Fax: +48 61 8310244
e-mail: magdula2811@poczta.onet.pl (Magdalena Sobieska)

Received 11.04.2005 Accepted 14.07.2005

Introduction

The study was designed to assess the reaction of the organism of melanoma patients to the surgery, in comparison to reaction of relatively healthy controls, undergoing surgery due to cataract. The study was also intended to investigate the numbers of lymphocytes in those patients and to compare them with values described by other investigators.

The results presented in the literature show static values of lymphocyte subpopulations in peripheral blood of uveal melanoma patients. Earlier reports suggested an increase in T helper, T supressor and B lymphocytes [1], but when the data were analyzed in comparison to healthy, age and sex matched controls no differences were found [2]. It is known, however, that both the total number and the proportions of lymphocytes may long stay within normal values independently of the ongoing pathological process. Besides as the lymphocytes in peripheral blood constitute as little as 1% of all present in the organism, the measurements of their absolute numbers or percentages hardly inform about the reaction to local processes. Therefore we decided to investigate the influence of surgery on the above mentioned parameters.

A homogenous group of patients with similar localization of the tumour (exclusively choroidal tumours) was chosen to diminish the known factors influencing the parameters under study. It was previously described that for example involvement of the ciliary body may contribute to the inflammatory response, normally not noticed when only lymphocytes were investigeted [2]. The patients enrolled into the study group presented the same grade as assessed in TNM scale [3]; due to the advanced clinical status they were classified to enucleation. The control group was adjusted in age.

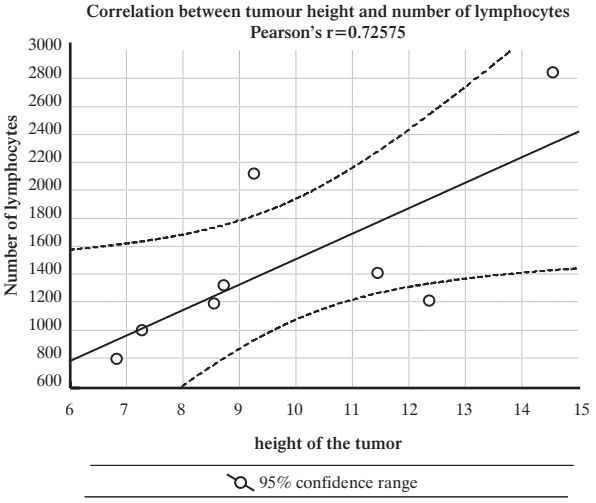
Material and methods

Twelve patients, five women and seven men, aged from 29 to 80 years, mean age 55.8. Tumour localization was assessed to

Table 1. **The medians with quartils of all investigated parameters for the choroidal melanoma patients (n=14) and cataract patients (n=10). Results are expressed as absolute number of cells**

Cells Before surgery After surgery	Melanoma patients n=12		Cataract patients n=10	
	median	quartils	median	quartils
Total lymphocyte count	1465 1726	1212-2121 1153-2026	1574 1480	1096-2022 1254-1861
CD3+ T cells	1021 1386	852-1194 840-1539	1150 1122	707-1383 832-1397
CD3+CD4+	631 889	453-903 439-1134	728 617	522-830 494-924
CD3+CD8+	311 384	252-447 280-541	357 406	278-568 268-618
CD19+ B cells	161 138	105-205 120-262	209 207	103-239 155-321
CD3-CD16+ NK cells	208 235	101-323 154-264	207 156	159-228 125-213
CD4/CD8 ratio	1.8 1.6	1.4-2.4 1.4-2.0	1.7 1.6	1.2-2.0 1.0-2.4
T cells with γδ TCR – %	7.6 2.9	3.1-8.9 2.1-3.3	2.8 2.8	0.9-4.7 1.8-5.2

Figure 1. **The correlation between the height of tumor and the total number of lymphocytes in the choroidal melanoma patients**



be exclusively choroid. In routine clinical examination the size of tumour (height and basis diameter) was assessed. Tumour infiltration towards sclera was found in two patients. All choroidal melanoma patients were subjected to surgery (enucleation). Histopathological investigation allowed to classify all patients as T3 grade acc. to TNM classification. In all patients a sample of peripheral blood was taken on the day of surgery and two days after.

As reference, a group of ten persons, five women and five men, aged from 44 to 80 years, mean age 65, undergoing surgery due to cataract was subjected to similar investigation. In all patients enrolled into the control groups any malignant conditions, inflammatory disorders or immunosuppresive treatment were excluded.

In all blood samples lymphocyte subpopulations were investigated using flow cytometry with a panel of monoclonal antibodies. Following subpopulations were analyzed and expressed in absolute count: the number of all lymphocytes, T lymphocytes (CD3+), T helper lymphocytes (CD3+CD4+), T cytotoxic lymphocytes (CD3+CD8+), B lymphocytes (CD19+), and NK cells (CD3-CD16+), as well as CD4/CD8 ratio. Additionally, the percentage of T lymphocytes (CD3+) with γδ TCR was assessed. All these investigations were performed on flow cytometer (Cytoron) from ORTHO Diagnostic Systems and the analysis was performed using ImmunoCount 2 Software.

The data obtained were analyzed using STATISTICA Software, and the results were expressed as medians because of non-normal distribution. Non-parametric tests were used to assess the significance of differences. The alterations within the groups were tested with Wilcoxon test, whereas the comparison between the groups was estimated using Wald and Wolfowitz test.

Results

Number of lymphocytes was comparable in both groups. A small increase was observed after surgery in the melanoma group and a small decrease in the reference group. The number of CD3+ lymphocytes was lower in melanoma patients and it increased after surgery, whereas no change was observed in reference group. The number of CD4+ cells was similar in both groups before surgery but an increase after surgery was observed in melanoma patients and a decrease in the reference group. In contrast, the number of CD8+ cells increased slightly in both groups. The number of B lymphocytes (CD19+) was lower in melanoma patients and showed a slight decrease in both groups. After surgery an increase in the number of NK cells was shown in melanoma patients and a decrease in the reference group, but the number of cells was similar and the differences were not significant. The percentage of γδ TCR T lymphocytes was within normal values (up to 10% in healthy individuals), but significantly higher in melanoma patients than in control group before surgery (p=0.0352). After surgery it decreased in both groups and the difference was no longer significant. All results (expressed as medians and 25-75% quartils) are given in the *Tab. 1*.

In the choroidal melanoma patients the correlation between all cell numbers and the size of tumour basis as well as the height of tumour were investigated. The statistically significant correlations are shown as *Fig. 1*, *2* and *3*, respectively.

Discussion

The aim of the study was to follow the changes in major lymphocyte subpopulations in patients undergoing enucleation

Figure 2. **The correlation between the height of tumour and the number of CD3-CD16+ NK cells in the choroidal melanoma patients**

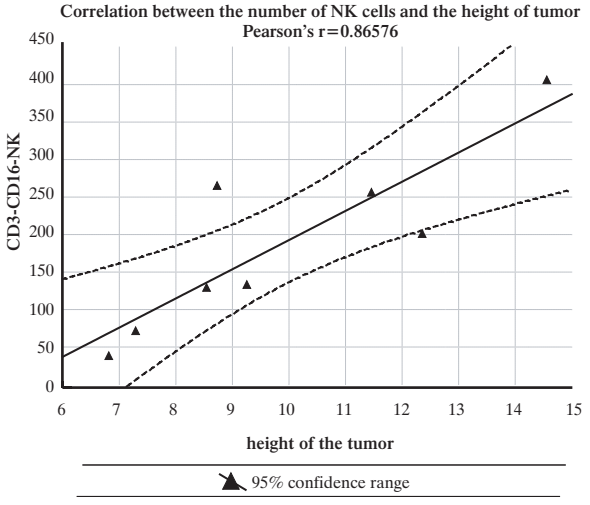
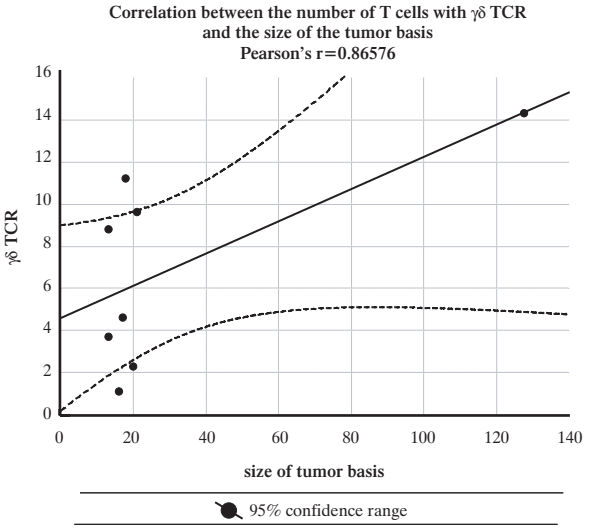


Figure 3. **The correlation between the size of tumour basis and the percentage of T cells with γδ TCR in the choroidal melanoma patients**



due to the presence of tumour. As the growth of melanoma itself may influence the immune status of the patient, similar investigations were performed in a group of patients also undergoing surgery but with no malignant background. This was intended to compare both the values before surgery to see whether the presence of tumour may influence lymphocyte subpopulations, and the alterations occurring in both groups as a result of surgery. Even if the size of tumour (both basis and height) was reliably bound to the number of lymphocytes, number of NK cells and the percentage of γδ TCR T lymphocytes, the melanoma group did not differ from cataract patients, presumably due to large range of results. Nevertheless, the alterations of several investigated parameters were distinct in both investigated groups. It may be concluded that these differences may be due to the influence of tumour on the immune system.

There were reports on the production of cytokines [4-7] in melanoma cells which could be responsible for the differences observed. Our preliminary data showed also changes in acute phase proteins concentrations and glycosylations profiles, both processes mediated by cytokines, probably mainly by interleukin-6 [8]. All these data taken together suggest that the presence of tumour alters the regulatory mechanisms in the cellular immunity.

In earlier reports some data concerning the influence of the tumour localisation on the inflammatory response were presented. It is possible that differences in numbers obtained for particular patients may reflect this influence, especially in case of NK cells. However there was no clear tendency in patients under study which would allow any hipothesis.

The number of T cells with γδ TCR decreased after enucleation in melanoma patients. Such a decrease of cells with γδ TCR was not noticed for the reference group. This could suggest the involvement of this population in the reaction with tumour. Changes in γδ TCR expression may be relevant as the cause or consequence of several diseases. The accumulation of cytotoxic TCR γδ+ cells at the sites of inflammation may suggest their involvement in the local injury process, as it was reported e.g in Behçet disease [10].The presence of of γδ TCR T cells was shown within uveal melanoma in immunohistochemical staining.There were few reports on infliltration of uveal tumours [9]. No characteristic pattern of α/β chains of the TCR was detected but the mortality was associated with advanced stage, patient age and extent of necrosis, whereas survival was increased with evidence of Vγ1 and Vδ1 TCR positive T cells [11]. The data indicate that while tumour infiltrating lymphocytes have a capacity to locate selectively within the tumour they nonetheless comprise a population expressing a diversity of TCR V β genes, showing no clonal expansion. All this is in agreement with the data presented in this paper.

References

1. Flynn K, Felberg NT, Koegel A, Hager R, Shields JA, Augsburger JJ, Donoso LA. Lymphocyte subpopoulations before therapy in patients with uveal malignant melanoma. Am J Ophthalmol, 1986; 101: 160-3.

2. Haynie GD, Shen TT, Gragoudas ES, Young LH. Flow cytometry analysis of peripheral blood lymphocytes in patients with choroidal melanoma. Am J Ophthalmol, 1997; 124: 357-61.

3. Harmer MH, Oesterhius JA. TNM Classification of ophthalmic tumors. International Union Against Cancer, Geneva 1985. Spiessl B. Beahrs OH, Hermanek P, Hutter RVP, Scheibe O, Sobin LH, Wagner G. TNM Atlas Illustrated Guide to the TNM/p TNM Classification of Malignant Tumours. Springer-Verlag Berlin, Heidelberg, New York, London, Paris, Tokyo, Hong Kong, Barcelona, Budapest, 1990.

4. Graves DT, Barnhill R, Galanopoulos T, Antoniades HN. Expression of monocyte chemotactic protein-1 in human melanoma in vivo. Am J Pathol, 1992; 140(1): 9-13.

5. Kock A, Schwartz T, Urbański A, Peng Z, Vetterlein M, Mickschke M, Ansel JC, Fung HF, Luger TA. Expression and release of interleukin-1 by different human melanoma cell lines. J Natl Cancer Inst, 1989; 81(1): 6-12.

6. Mackiewicz K, Karczewska A, Kortylewski M, Pecold K,

Kocięcki J, Bręborowicz D, Mackiewicz A. Expression of IL-6 type cytokines and their receptors in uveal melanoma (UM) microenvironment. The Immunologist, Hogrefe and Huber Publishers, 1998(Suppl. 1), p. 308.

7. Reed JA, Mc Nutt NS, Prito VG, Albino AP. Expression of transforming growth factor beta 2 in malignant melanoma correlates with the depth of tumor invasion. Am. J Pathol, 1994; 145(1): 97-103.

8. Manyś-Kubacka K, Kostrzewa A, Sobieska M, Pecold K, Wiktorowicz K. Selected immune parameters in patients with choroidal malignant melanoma. In: EFIS 2000. 14th European Immunology Meeting. Poznań, Poland, September 23-27, 2000. Ed.: Andrzej Mackiewicz, Maciej Kurpisz, Jan Żeromski. Bologna, 2000; pp. 815-20.

9. Durie FH, George WD, Campbell AM, Damato BE. Analysis of clonality of tumour infiltrating lymphocytes in breast cancer and uveal melanoma. Immunol Lett, 1992; 33(3): 263-9.

10. Hamzaoui K, Hamzaoui A, Hentati F, Kahan A, Ayed K, Chabbou A, Ben Hamida M, Hamza M. Phenotype and functional profile of T cells expressing gamma delta receptor from patients with active Behcet's disease. J Rheumatol, 1994; 21(12): 2301-6.

11. Bialasiewicz AA, Ma JX, Richard G: Alpha/beta and gamma/delta TCR (+) lymphocyte infiltration in necrotising choroidal melanomas. Br J Ophthalmol, 1999; 83(9): 1069-83.

Phagocytic and bactericidal activity and morphological parameters of blood platelets in patients with *Trichinella spiralis* infection

Matowicka-Karna J*, Kemona H,
Dymicka-Piekarska V, Butkiewicz A

Department of Clinical Laboratory Diagnostics, Medical University of Białystok, Poland

Abstract

Purpose: The production of IgE increases in parasitic invasions, triggering local or systemic inflammatory response with the involvement of blood platelets. The aim of the study was to assess the number and morphological parameters of blood platelets as well as their phagocytic and bactericidal activity in patients with *Trichinella spiralis* infection. It is interesting to investigate the blood platelet response following *Trichinella spiralis* in order to elucidate possible effects on non-specific immunity.

Material and methods: Twenty-six patients with *Trichinella spiralis* (before and after antiparasitic therapy) and forty healthy subjects were examined. The platelet count and morphological parameters were determined using a hematologic analyzer Technicon H-1 System. The platelet phagocytic activity was determined by measuring the percentage of phagocytizing cells and the phagocytic index. The bactericidal activity was assessed measuring the percentage of the bacteria killed by platelets and plasma. The strain *Staphylococcus aureus* ATCC 6538P was used for this purpose.

Results: In patients infected with *T. spiralis* morphological parameters do not change, except for the percentage of large platelets. In the course of trichinellosis the phagocytic index of platelets is statistically significantly decreased and platelet bactericidal activity is impaired, while the bactericidal activity of the plasma is statistically significantly increased, compared to healthy subjects.

Conclusions: The present study has revealed that due to *T. spiralis* infection, the percentage of large, young blood platelets is decreased. The parasitic infection causes impairment of non-specific immunity through decreased bactericidal activity of blood platelets.

Key words: blood platelets, phagocytic activity, bactericidal activity, trichinellosis.

* CORRESPONDING AUTHOR:
Department of Clinical Laboratory Diagnostics
Medical University of Białystok
ul. Waszyngtona 15a
15-274 Białystok, Poland
Tel./Fax: +48 85 7468584
e-mail: matowic@amb.edu.pl (Matowicka-Karna Joanna)

Received 24.03.2005 Accepted 15.07.2005

Introduction

Increased IgE production is the characteristic feature of parasitic invasions. This defect is due to disorders in the regulation of antibody production by T helper cells. Through the release of mediators from mast cells IgE promotes local inflammatory reaction and participates in antibody-dependent cellular cytotoxicity (ADCC) [1]. Parasitic invasions are the source of foreign antigens and exotoxins that trigger local or systemic inflammatory reactions. Blood platelets initiate and maintain inflammatory processes through the secretion of PDGF, platelet activating factor (PAF), platelet factor 4, β-thromboglobulin and IL-1 [2-4]. In the course of some parasitic infections, platelet activation is followed by the release of α-granular contents and granular membranes are fused with the platelet membrane [4]. Cytotoxic properties of blood platelets are induced by such cytokines as IFN-γ, TNF and IL-6. The mechanism of adhesion between platelets and parasites has not been fully elucidated although it is known to depend on platelet surface receptors. With GPIIb-IIIa glycoprotein deficiency, platelets show markedly lower cytotoxic activity against parasites [5].

The aim of the present study was to evaluate platelet count and morphological parameters, and to assess the phagocytic and bactericidal activity of blood platelets in patients with *T. spiralis* infection. It is interesting to investigate the blood platelet response following *T. spiralis* infection in order to elucidate possible effects on non-specific immunity.

Material and methods

The study group included 26 patients (12 women and 14 men, aged 19-65 years) hospitalized in the Department of Infectious Diseases, Medical University of Białystok. The patients (without overweight and hypertension) infected with *Trichinella spiralis* were examined twice (T1 – before treatment, T2 – after antiparasitic therapy). Trichinosis was diagnosed based on the clinical data and immunoserological tests (antibody titre was determined).

Control group (C) consisted of 40 healthy subjects (22 women and 18 men, aged 18-40 years, without overweight and hypertension). The concentration of C-reactive protein was 0-5 mg/l (in each healthy subjects).

As anticoagulants, dipotassium versenate (K_2EDTA – 1.5 mg/ml blood) was used to determine platelet count and morphological parameters, while heparin (50 IU/ml blood) to estimate the phagocytic and bactericidal activity. ACD (a mixture of citric acid and glucose) was added to prevent thromboxan synthesis by platelets, aggregation and secretion, and to reduce plasma pH to 6.5. To determine platelet count (PLT) and morphological parameters (MPV, PDW, LPLT) hematologic analyzer Technicon H-1 System was employed. The phagocytic activity was measured by determining the percentage of phagocytizing platelets and the phagocytic index. The bactericidal activity was assessed by determining the percentage of the bacteria killed by platelets and by plasma [6,7]. *Staphylococcus aureus* ATCC 6538P strain was used to assess the phagocytic and bactericidal activity of blood platelets. Following the Guidelines for Good Clinical Practice all the patients gave consent to the examination.

Student's t test for matched pairs was used for statistical analysis. Values are means ±SD. The means between examined groups were compared using the unpaired Student's t test. All tests of significance were two-tailed, with $P<0.05$, considered to indicate significance.

Results

No statistically significant differences were found in the platelet count between patients with *T. spiralis* infection, both before treatment (T1) and after antiparasitic therapy (T2), and healthy subjects. A statistically significant difference was noted in the mean platelet volume (MPV) between group T2 and C (*Tab. 1*). The lowest MPV was observed in patients after antiparasitic therapy (T2), perhaps due to platelet activation [8], which seems to be confirmed by the slightly increase index of anizocytosis (PDW) noted in this group. However, no statistically significant differences in PDW were found between study groups (T1 and T2) and control group (C). PDW and MPV are means of estimating the thrombocytopoietic activation. The slightly increase of PDW was observed in trichinellosis after antiparasitic therapy (T2), which may point to a slight increase in blood platelet production by megakaryocytes. PDW characterises the intensity of blood platelet production by "megathrombocytes". A statistically significant reduction was noted in the percentage of large young platelets (LPLT), both in group T1 and T2, compared to healthy subjects. This may be the effect *T. spiralis* exerts on the host – blood platelets become activated and undergo exhaustion, and in this way the number of large, metabolically more active platelets gets reduced [9]. Therefore, the lowest percentage of LPLT was observed in the group of patients after antiparasitic therapy (T2).

Table 1. **Statistical analysis of morfological parameters of blood platelets and their number in patients infected with *T. spiralis* before (T1), and after treatment (T2), and in the control group (C)**

Parameters	N-26 T1 X±SD	N-26 T2 X±SD	N-40 C X±SD	P
PLT	218.8±59.7	243.6±66.7	229.2±52.5	T1:T2 $0.4<p<0.5$ T1:C $0.6<p<0.7$ T2:C $0.7<p<0.8$
MPV	8.28±0.9	7.87±0.9	8.66±1.0	T1:T2 $0.3<p<0.4$ T1:C $0.2<p<0.3$ T2:C $p<0.05$
PDW	51.9±5.1	53.4±4.7	48.2±3.7	T1:T2 $0.6<p<0.7$ T1:C $0.7<p<0.8$ T2:C $0.7<p<0.8$
LPLT	2.9±2.7	2.3±2.1	4.8±1.9	T1:T2 $0.1<p<0.2$ T1:C $p<0.05$ T2:C $p<0.05$

The highest percentage of phagocytizing platelets (3.13) was noted in *T. spiralis* patients before antiparasitic therapy (*Tab. 2*). It seems that the presence of the parasite has a stimulatory effect on platelets, increasing their phagocytic activity but decreasing their bactericidal activity. The phagocytic indices in groups T1 and T2 are almost identical, but significantly decreased in comparison to the values noted in healthy subjects (*Tab. 2*). Plasma bactericidal activity in patients with *T. spiralis* infection was significantly higher compared to the values revealed in healthy subjects (*Tab. 2*). Not only was this activity elevated in group T1 (vs control group), but it also slightly increased after antiparasitic therapy (T2). On the contrary, the bactericidal activity of blood platelets decreased statistically significantly both before and after antiparasitic therapy, compared to control group (*Tab. 2*). The percentage of the bacteria killed by platelets in *T. spiralis* patients was three times as low compared to healthy subjects. Application of the antiparasitic treatment only slightly increased the bactericidal activity of blood platelets (*Tab. 2*). Perhaps, the impaired bactericidal activity of blood platelets is compensated by the increased bactericidal activity of the plasma.

***Table 2*. Statistical analysis of phagocytic and bactericidal activity of blood platelets in patients infected with *T. spiralis* before (T1), after antiparasitic treatment (T2), and in the control group (C)**

Parameters	N-26 T1 X±SD	N-26 T2 X±SD	N-40 C X±SD	P
Percentage of phagocytic platelets (%)	3.13 ± 1.5	2.27 ± 0.9	2.26 ± 0.6	T1:T2 0.05<p<0.1 T1:C p<0.05 T2:C 0.8<p<0.9
Phagocytic index	1.44 ± 0.3	1.42 ± 0.4	1.83 ± 0.4	T1:T2 0.7<p<0.8 T1:C p<0.05 T2:C p<0.05
Bacteria killed by plasma (%)	28.47 ± 26.6	35.5 ± 30.7	19.8 ± 10.8	T1:T2 0.1<p<0.2 T1:C p<0.05 T2:C p<0.05
Bacteria killed by blood platelets (%)	6.40 ± 6.3	8.03 ± 7.2	20.5 ± 12.9	T1:T2 0.3<p<0.4 T1:C p<0.05 T2:C p<0.05

Discussion

In patients infected with parasites, e.g. *T. spiralis*, Th1 lymphocytes through the released cytokines (IL-2, IFN-γ, IL-12) induce differentiation and proliferation of cytotoxic cells and activate macrophages enhancing their ability to kill parasites [10]. It is suggested that certain manifestations in the pathology of trichinosis are due to cytokine-induced production of nitrogen oxide. Nitrogen oxide kills parasites and its concentration correlates with evident inflammatory reactions observed in the intestines and muscles in *T. spiralis* infection [11,12].

The antibody-dependent antiparasitic immunity involves some mechanisms which cause blockade of the receptors present on the parasite surface. On the one hand, these mechanisms lead to parasite damage through the complement system, but on the other hand increase the production of IgE antibodies [13]. However, the main antiparasitic mechanism is based on antibody-dependent cellular cytotoxicity which is also demonstrated by blood platelets [4,14]. Blood platelets as the effector cells are involved in parasitic diseases. They release various inflammatory mediators, are capable of phagocytosis and get in interactions with the cells of the immune system. In the course of parasitosis, platelets have a cytotoxic effect, but then increased concentrations of IgE, lymphokines (TNF, IFN-γ) and IL-6 are required [15-22].

The clinical course of trichinosis may vary and largely depends on the host response [23,24]. The present study has revealed that *T. spiralis* infection causes a slight decrease in platelet count and volume (MPV), and a statistically significant reduction in the percentage of large platelets, the so-called "megathrombocytes". These patients did not develop coagulation disorders that could result in platelet count decrease and therefore in trichinosis platelets seem to be destroyed mainly on "the periphery". The literature data suggest that platelet count reduction is characteristic of infectious diseases, viral infections and many parasitic infections [3,25-27].

As platelet activation leads to the release of α-granular contents and to thrombocytopoiesis stimulation, it is reflected in morphological parameters [8]. The decrease in platelet volume and in the percentage of large platelets seems to confirm the presence of the platelet-activating factor. The decrease MPV, observed in trichinellosis after antiparasitic therapy (T2), may be connected with their activation and release of platelet factor 4, β-thromboglobulin, platelet derived growth factor, fibrinogen, fibronectin, thrombospondin [2,4,8].

In patients with *T. spiralis* infection, platelet bactericidal properties were evidently impaired, constituting only 30% of the value obtained in healthy subjects. This impairment may be associated with the blocking of bactericidal mechanisms, with changes in arachidonic acid metabolism and with the action of T cells on blood platelets through PCIF lymphokines. Platelets participate in bactericidal mechanisms via peroxidase present in lysosomes and membranes of dense canaliculi and thanks to cationic proteins. Degradation of phagocytized bacteria involves proteolitic enzymes located in the lysosomal fraction: cathepsins and acid phosphatase [3,26,27]. The changes in the bactericidal activity of blood platelets may be related to bactericidal mechanism failure, which is indicated by the increased percentage of phagocytizing platelets and the decreased phagocytic indices. Most likely the impaired bactericidal activity of blood platelets and reduction in the percentage of LPLT are compensated by the increased bactericidal activity of the plasma.

The assessment of morphological parameters and phagocytic and bactericidal activity of blood platelets can be used to evaluate their functional condition and thus to determine platelet involvement in the mechanisms of non-specific immunity in the course of trichinosis.

Conclusions

1. The present study revealed a decrease in the percentage of large, young blood platelets in *T. spiralis* infection.

2. In patients with *T. spiralis* infection non-specific immunity is impaired due to reduced bactericidal activity of blood platelets.

References

1. Venturiello SM, Malmassari SL, Constantino SN, Nunez GG. Cytotoxicity-blocking antibodies in human chronic trichinellosis. Parasitol Res, 2000; 86: 762-7.

2. Blockmans D, Deckmyn H, Vermylen J. Platelet activation. Blood Reviews, 1995; 9: 143-56.

3. Klinger MHF. Platelets and inflammation. Anat Embryol, 1997; 196: 1-11.

4. Polack B, Peyron F, Auriault C. Platelet cytotoxicity against parasites. Nouv Rev Fr Haematol, 1991; 33: 317-22.

5. Ameisen JC, Joseph M, Caen JP, Kusnierz JP, Capron M, Boizard B, Wautier JL, Levy-Toledano S, Vorng H, Capron A. A role for glycoprotein IIb-IIIa complex in the binding of IgE to human platelets and platelet IgE-dependent cytotoxic functions. Br J Haematol, 1986; 64: 21-32.

6. Mantur M, Wołosowicz N, Prokopowicz J, Kemona H. System for testing the phagocytic capacity of human blood platelets. Folia Haematol Frankf, 1986; 113: 691-5.

7. Mantur M, Wołosowicz N, Kemona H, Prokopowicz J. Aktywność bakteriobójcza płytek krwi, oznaczenia i wartości prawidłowe. Acta Med Pol, 1987; 28: 45-9.

8. Softeland E, Fromstad T, Thorsen T, Holmsen H. Porcine platelets in vitro and in vivo studies: Relevance to human thrombosis research. Eur J Haematol, 1992; 49: 161-73.

9. Matowicka-Karna J, Panasiuk A, Kemona H. Estimation of platelet function state in the course of Trichinella spiralis infection. Rocz Akad Med Bialymst, 1998; 43: 110-6.

10. Helmby H, Grencis RK. IFN-gamma-independent effects of IL-12 during intestinal nematode infection. J Immunol, 2003; 171: 3691-6.

11. Boczoń K, Wandurska-Nowak E, Rarus M, Szulc M. Wolnorodnikowe mechanizmy azotowe obrony żywiciela jako pole działania wybranych leków przeciwrobaczych we włośnicy doświadczalnej. Wiad Parazytol, 2001; 47: 6-10.

12. Wandurska-Nowak E, Wiśniewska J. Release of nitric oxide during experimental trichinellosis in mice. Parasitol Res, 2002; 88: 708-11.

13. Negrovo-Correa D. Importance of immunoglobulin E (IgE) in the protective mechanism against gastrointestinal nematode infection: look at the intestinal mucosae. Rev Inst Med Trop Sao Paulo, 2001; 43: 291-9.

14. Souza-Atta MLB, Araujo M, D'Oliveira Junior A, Ribeiro-de Jesus A, Almeida RP, Atta AM, Carvalho EM. Detection of specific IgE antibodies in parasite diseases. Braz J Med Biol Res, 1999; 32: 1101-5.

15. Bout D, Joseph M, Pontet M, Vorng H, Desl D, Capron A. Rat resistance to schistosomiasis: platelet-mediated cytotoxicity induced by C-reactive protein. Science, 1986; 231: 153-6.

16. Brattig NW, Lepping B, Timmann C, Buttner DW, Marfo Y, Hamelmann C, Horstmann RD. Onchocerca volvulus-exposed persons fail to produce interferon-gamma in response to O. volvulus antigen but mount proliferative responses with interleukin-5 and IL-13 production that decrease with increasing microfilarial density. J Infect Dis, 2002; 185: 1148-54.

17. Damonneville M, Wietzerbin J, Pancre V, Joseph M, Delanoye A, Capron A, Auriault C. Recombinant tumor necrosis factors mediate platelet cytotoxicity to Schistosoma mansoni larvae. J Immunol, 1988; 140: 3962-5.

18. Joseph M, Auriault C, Capron A, Vorng H, Viens P. A new function for platelets: IgE-dependent killing of schistosomes. Nature, 1983; 303: 810-2.

19. Joseph M, Capron A, Ameisen JC, Capron M, Vorng H, Pancre V, Kusnierz JP, Auriault C. The receptor for IgE on blood platelets. Eur J Immunol, 1986; 16: 306-12.

20. Pancre V, Auriault C, Joseph M, Cesbron JY, Kusnierz JP, Capron A. A suppressive lymphokine of platelet cytotoxic functions. J Immunol, 1986; 137: 585-91.

21. Pancre V, Joseph M, Mazingue C, Wietzerbin J, Capron A, Auriault C. Induction of platelet cytotoxic functions by lymphokines: role of interferon gamma. J Immunol, 1987; 138: 4490-5.

22. Pancre V, Monte D, Delanoye A, Capron A, Auriault C. Interleukin-6 is the main mediator of the interaction between monocytes and platelets in the killing of Schistosoma mansoni. Eur Cytokine Network, 1990; 1: 15-9.

23. Dupouy-Camet J, Kociecka W, Bruschi F, Bolas-Fernandez F, Pozio E. Opinion on the diagnosis and treatment of human trichinellosis. Expert Opin Pharmacother, 2002; 3: 1117-30.

24. Kocięcka W. Trichinellosis: human disease, diagnosis and treatment. Vet Parasitol, 2000; 93: 365-83.

25. Gerardin P, Rogier C, Ka AS, Jouvencel P, Brousse V, Imbert P. Prognostic value of thrombocytopenia in African children with falciparum malaria. Amer J Tropical Med Hyg, 2002; 66: 686-91.

26. Yeaman MR. The role of platelets in antimicrobial host defense. Clin Infectious Diseases, 1997; 25: 951-70.

27. Yeaman MR, Bayer AS. Antimicrobial peptides from platelets. Drug Resist Updat, 1999; 2: 116-26.

Development and psychokinetic therapy of children suffering from West Syndrome – an overview

Gajewska E[1]*, Mojs E[2], Sobieska M[1], Samborski W[1]

[1] Department of Physiotherapy, Rheumatology and Rehabilitation, Karol Marcinkowski University of Medical Sciences, Poznań, Poland
[2] Organization and Management Unit, Karol Marcinkowski University of Medical Sciences, Poznań, Poland

Abstract

Paper presents reasons leading to the West Syndrome, disturbances occurring in the child's development and possible rehabilitation programmes. Psychological rehabilitation of a child with West Syndrome is multilateral, but its main aim is to improve the quality of life, social adaptation and optimizing of the cognitive functioning. Taking into consideration the psychokinetic retardation occurring in the West Syndrome because of prenatal, perinatal or postnatal disturbances apart from pharmacotherapy an intensive psychokinetic stimulation based on plasticity of the brain should be stressed out. Only the complex, intensive and long lasting rehabilitation of a child may secure to it the best possible development.

Key words: West Syndrome, psychological and physiotherapeutic diagnosis, rehabilitation methods.

Introduction

The presented paper is a result of cooperation between psychologist and physiotherapist, therefore it presents more therapeutic than diagnostic approach and has a form of a review.

The West Syndrome (WS) is one of the catastrophic epileptic syndromes in infancy characterised by triad of infantile spasms, psychomotor deterioration and hypsarhythmic EEG pattern [1,2]. WS is commonly associated with poor long-term prognosis outcome, especially in symptomatic cases, with development of other seizure types, impaired motor, cognitive and psychosocial functioning. The aim of our study was to present the therapeutic methods most commonly used in diagnosing and rehabilitation of a child with WS. Early physiotherapy is necessary in all children with WS, but according to the clinical status and possibilities of both therapist and parents various methods may be used.

* CORRESPONDING AUTHOR:
Department of Physiotherapy, Rheumatology and Rehabilitation
Karol Marcinkowski University of Medical Sciences
ul. 28. Czerwca 1956 roku 135/147
61-545 Poznań, Poland
e-mail: karasinska1@wp.pl (Ewa Gajewska)

Received 11.04.2005 Accepted 08.07.2005

Definition of the West Syndrome

West Syndrome is one of the most severe early childhood epilepsy, in which three major components appear: spasms, the EEG pattern of hypsarhythmia and mental deterioration or retardation. Spasms consist of short bend and straighten up or of mixed bend – straighten up movement of trunk, neck or extremities, they may be accompanied by a short shout. Spasms have tendency to appear in clusters of 20 to 40, sometimes up to 100; they are usually symmetric in typical WS. Interictal and ictal EEG [3] consist of generalized high-voltage slow waves and spikes. The treatment of WS is difficult, because the most conventional antiepileptic drugs are ineffective [1]. According to the classification of the International League Against Epilepsy of 1989, WS is classified as general epilepsies with cryptogenic or symptomatic aetiology [4].

According to Smith and Wallace [1] such seizures begin in 90% during the first year of life, usually between 3rd and 9th month, and the morbidity accounts for 1 for 1 900 to 1 for 3 900 children. WS may also present a hidden form in which prognosis both concerning results of the future treatment and first of all proper psychomotor development is much better than in symptomatic forms. Concerning the outcome in WS, the classification in symptomatic and non-symptomatic WS were proposed [5]. Cerebral malformations and neurocutaneous syndrome mainly tuberous sclerosis are the most common causes of WS [6,7].

Early age of occurrence of bend seizures is usually combined with structural disturbances (e.g. cortical displasia), chromosomal or metabolic anomalies (e.g. leukodystrophy) or perinatal aetiology, e.g. anoxia, [2,8,9]. WS may be a consequence of

perinatal hypoxic encephalopathy in about 15% of symptomatic cases [10]. Rarely metabolic encephalopaties are causes of WS, high proportion of these patients have Menkes disease [11] or NARP mutation [12]. Prenatal infections, especially cytomegaloviral infection can cause WS [13]. Matsuo et al. were able to show that in children who were born with low weight (which often means preterm labour) the frequency of West Syndrome is higher than in children with appropriate birth weight [14].

Prognosis in West Syndrome is rather poor, mainly because of diverse aetiology and possible early appearance of seizures of different morphology [15,16]. Prognosis concerning seizures is not optimistic as in about 60% of children epilepsy never cease and in some the Lennox-Gastaut Syndrome with tonic attacks may develop. Psychomotor retardation and other cognitive deficits occur in 80-85% of children in whom West Syndrome was noticed early, before 2nd year of life [1,8]. In 25% of those children additionally a cerebral palsy is diagnosed [8,9].

Despite low mortality, about 10% of all affected children [8,15] the course and effects of the disease make the realization of the developmental tasks and adaptation to the social environment really difficult. Attacks occur in early childhood when the central nervous system is very susceptible to unfavourable external factors [17].

WS symptoms may appear after several months of apparently proper development of the child and then the deficits will be insignificant, noticeable only in specialized diagnostic procedures, psychological or neurodevelopmental.

In therapy of the West Syndrome pharmacotherapy is used most often. Dosing should be adjusted to the patient to affect least possibly its mood and life course. Due to low efficacy of medication also other forms of treatment are used: surgery, ketogenic diet, vagal nerve stimulation [15,18,19].

Evaluation of patients with WS before complex, intensive and long-lasting rehabilitation

Analysis of the reasons of the bend seizures is important mainly for recognition of mechanisms of their occurrence and may have prognostic value, allowing for settings aims of the neuropsychological therapy and kinetic development with higher probability. Mental handicap and other cognitive deficits are caused by the same reason that has lead to epilepsy, but are also a consequence of the disease itself – progressive damage of neurons. Significant diversification of the aetiology of the West Syndrome and of its clinical image – number of seizures, their form, psychokinetic deficits – forms a basis to distinguish a group of patients with better prognosis, so-called gentle bend syndrome [8]. In this group seizures are not so intensive, they occur in later years of life, psychokinetic development is normal or only slightly impaired.

EEG also shows just minor disturbances or is even described as normal. In this patients' group prognosis is rather good, usually the ceasing of symptoms is combined with normalization of the bioelectric activity of the brain and normal psychokinetic development, and thanks to intensive stimulation and neuropsychological therapy even acceleration of selected functions, e.g. speech may occur.

Aim of the treatment process in WS

The aim of the therapy in this particular and in other groups of patients with epilepsy is not just minimising of seizures, but also optimizing of emotional and social functioning of the child.

The treatment process is long-lasting and proceeds in constantly changing psychophysical conditions, also in altering cognitive conditions and interests. Thus psychological proceedings in the West Syndrome should contain systematic and several times repeated diagnostic procedures of the cognitive, emotional and social fitness.

This diagnosis of the cognitive, emotional and social fitness is particularly important in cases of low efficacy of pharmacotherapy, when new anti-epileptic drugs are introduced, mainly because of their side effects, occurring both during monotherapy and in combined treatment due to drug-drug interactions [20,21].

Psychological diagnosis

Psychological diagnosis, particularly neuropsychological gives opportunity of individualized therapeutic programmes and setting aims [22]. Aims of the diagnostic of the cognitive functions are based on monitoring of the course of the disease. Also localizing diagnostics of the brain damages and hemisphere domination is very important. In the psychological investigation special attention should be paid to intelligence level, as according to it further education and its aims are set.

Short-term rehabilitation planning

Short term rehabilitation planning is also one of the aims of the diagnostic [23-25]. Psychological rehabilitation of a child with West Syndrome is multilateral, but its main aim is to improve the quality of life, social adaptation and optimizing of the cognitive functioning. It may be deducted from the clinical practice that parents in the beginning often set too far going aims, often too ambitious and demand to determine advantages and possible probability of their realization. It is not possible because of the diverse clinical course of epilepsy in the West Syndrome, multitude of mechanisms and factors remaining in close dependency and affecting the development.

Psychological diagnosis allows mainly short-term and direct aims. These are set individually and adjusted both to developmental possibilities (developmental plasticity), social possibilities and to the structural limits. Thus the neuropsychological therapy is a form of dynamic interaction of the above mentioned factors.

Learning processes

Learning processes are the main mechanism the neuropsychological therapy in its behavioural aspect is based on. Also some developmental processes, in which cognitive training is a recapitulation of developmental phases of the cognitive

functions, are taken into consideration. A pedagogical approach with mainly behavioural methods, a neuropsychological approach, basing on reorganisation of the functional system, neurolinguistical trying to amplify potential ability to language communication psychosocial aimed on social adaptation and a complex approach, taking into consideration all the above elements [26]. Such an integrated therapeutic process has a long-term character and demands a close cooperation of neurologists, rehabilitants and psychologists.

Physiotherapy

A main task of kinetic rehabilitation is a removal or possibly maximal diminishment of the movement disturbances, as well as gaining control of the kinetic functions in the further development of a child within its largest biologic and social possibilities. Special attention should be paid to locomotive activities, like crawling, or finally walking, manipulative activities and everyday functions. That is why early beginning of rehabilitation of a child with West Syndrome is so important, along with gaining contact with the child itself and its family. A decision about the beginning of physiotherapeutic rehabilitation and about its shape depends on detailed neurological assessment of the child and its behaviour. The whole therapeutic programme must be prepared and modified in cooperation with neurologist and psychologist, because only after complex activity the best results may be expected. Detailed and reliable developmental diagnostics is needed to start physiotherapy, as the individual training programme should be based on the actual assessment.

Munich Functional Developmental Diagnostics

For the evaluation of the development as a marker of complex neurological functions of a child, some methods of assessment of the spontaneous behaviour are used, among others a Munich Functional Developmental Diagnostics, based of the description of the age of: crawling, sitting, walking, grasping, perception, speech development and social contacts development. This Diagnostics is a result of long-term cooperation between paediatricians and psychologists from Children's Centre in Munich. It encompasses experiences based on examination of healthy babies and children. Also data from results of examination of thousands babies with developmental handicap were collected. Basic statement of this method is that every deviation from accepted time ranges or behavioural forms is regarded as a symptom of psychokinetic development disturbance [27].

Philadelphia Profile of the Development

Another diagnostic method is Philadelphia Profile of the Development, prepared by The Institute for the Achievement of Human Potential (IAHP) as a tool serving to set a diagnosis, to work out a programme and to assess the effects of the therapy. It is a table allowing assessing the development of a child concerning perception (seeing, hearing and sense of touch) and executive functions (kinetic, speech and manual fitness) in seven stages of determined age ranges. These ranges correspond to functional levels in the hierarchic structure of the central nervous system [28]. According to this diagnostic a detailed rehabilitation programme is constructed, in which therapy forms a complex stimulation. It relates not only to kinetic, but also to fitness of eyesight, hearing, sense of touch speech and manual. Programme workout for a child with West Syndrome must be done individually. According to Favata et al. the prognosis is generally poor, but thanks to complex diagnostics even slightest deviations may be recognized and that allows the therapists to start rehabilitation in the most crucial phases of the child's development [29].

Importance of individual treatment programme

According to own observation these children present kinetic retardation, often show hypotonia and low spontaneous activity, what causes later problems with head control, intentional grasp, and further with turning, independent sitting down and sitting. They also have difficulties with (crossed crawling and creeping), which is the most important element in the development of the child as it affects not only the development of both hemispheres, but also grasping, sense of touch and eyesight. The therapeutic choice is so rich nowadays that physicians and therapists often make a mistake of showering parents with various training methods, suggesting large number of means that should theoretically help in regaining fitness. It is definitely wrong way. Thus an individual treatment programme should be constructed for each child, with precise description of short-term aims. Such programme constructing often takes a lot of time. Together with parents the therapist has to analyze which therapy should be used as basic, and which one as additional.

The term programme

It should be noticed that better results are often achieved by smaller amount of exercises but conducted systematically and consequently. The term programme means frequency, intensity and time of a stimulus used to create an opportunity for a child to learn and repeat a skill. As every child is different, every programme is different, too, adjusted to particular, individual needs. The highest priority should be given to the skills a child must possess. Then the time needed for every recommended technique should be set.

Doman's method

Doman's method suggests setting a sequence of exercises conducted during each day. Such setting should help parents to conduct exercises systematically and to plan the whole day for their child. Rehabilitation serves to adjust such exercises that by intensity, repetition and action of a stimulus in particular

time should contribute to the child's development. As Glenn Doman states this influence (encompassing intensity, time and frequency) that should compensate at least in part the neurological deviations should be much stronger than the influence of the normal environment on a healthy child. That is why programmes set according to Doman's method may take up to dozen or so hours per day.

Vojta's method

Another rehabilitation programme may be built up according to Vojta's method [30]. It is a rehabilitation less time consuming that may be applied right after the birth if only any deviations are noticed. Vaclaw Vojta, neurologist of Czech origin introduced a method of early rehabilitation in the years 1959-69 already during the stay on Paediatric Intensive Medicine Ward. This method is widely used in rehabilitation of children with various neurological syndromes, in cases of peripheral nerve system damages, in genetic syndromes, asymmetric position or tension. The rules of kinetic complexes described by Vojta apply not only to kinetics, but also on the whole body, its sensual and psychic sphere. Rehabilitation of a child according to Vojta's method starts with an analysis of position and determining of the proper zones of movement initiation that are then stimulated with stimulus of appropriate strength. A child experiences formerly unknown activities, cooperation of whole groups of muscles. It may feel its body much better and builds up a schema of its own body. Thanks to Vojta's methods a reflective turn and crawling may be triggered of. This method is based on the statement that initializing of one kinetic activity and elimination of all pathologic features allows removal of deviations in the kinetic development [8,31]. Building up a programme based on this method one should remember the most basic rules of rehabilitation: definition of the starting position, applying of the adequate stimuli on the precisely determined zones of movement liberation, definition and description of the kinetic response, recurrent character and individual dosage.

Bobath method

Bobath method is another rehabilitation programme, introduced by Karel and Bertha Bobath. This method is very useful in rehabilitation of children with WS and very popular also in children with other developmental abnormalities. The method is based on plasticity of brain and aim to stimulate the development of the child.

The main rationale of this method is based on the statement that the central nervous system regulates the movements, not the muscles, thus one may achieve best results teaching the movements that were initiated by the child itself. Special attention should also be paid to the development of the sensory system that influences the mobility, taking into consideration all kinds of stimuli like proprioceptive or visual. The development of healthy children is regarded as model pattern to assess abnormalities observed in children with disturbances.

Only a therapist with proper education should build up a programme using Vojta's or Doman-Delacato's method, dosing time of exercises and individually set the kind of stimulation.

Conclusions

Proper postural function of the central nervous system is a condition of normal psychomotoric development. This function encompasses automatic, invariable maintenance of the chosen body position. Such a control of body position is inborn and genetically encoded in every human being like also a normal psychic development [30]. Taking into consideration the psychokinetic retardation occurring in the West Syndrome because of prenatal, perinatal or postnatal disturbances (bacterial or viral infections) apart from pharmacotherapy an intensive psychokinetic stimulation based on plasticity of the brain should be stressed out. Only the complex, intensive and long-lasting rehabilitation of a child may secure to it the best possible development. Basing on the experience of many centres the basic form of work of a psychologist and a therapist is to educate parents to make them well prepared therapists of their own child. They – in home conditions – bear the whole task of complex rehabilitation, formerly built up and learnt, both in the sphere of movement and in psychic development. Only thanks to close cooperation of all specialists and parents children suffering from West Syndrome have a chance for better psychokinetic development, and thus – for independency.

References

1. Smith PEM, Wallace SJ. Padaczka – kliniczny przewodnik. 2003.
2. Guberman A, Bruni J. Essentials of clinical epilepsy. Butterworth, Henemann, 1999.
3. Dulac O. What is West Syndrome? Brain Dev, 2001; 23: 447-52.
4. Commission of Classification and Terminology of the International League Against Epilepsy. Proposal for revised clinical classification of epilepsies and epileptic syndrome. Epilepsia, 1989; 30: 389-99.
5. Osborne JP, Lux A. Towards an international consensus on definitions and standardised outcome measures for therapeutic trials (and epidemiological studies) in West Syndrom. Brain Dev, 2001; 23: 677-82.
6. Ohtahara S, Ohtsuka Y, Yamatogi Y, Oka E, Ypshinaga H, Sato M. Prenatal etiologies of West Syndrome. Epilepsia, 1993; 34: 716-22.
7. Rikkonen R. A long-term follow-up study of 214 children with the syndrome of infantile spasms. Neuropediatrics, 1982; 13: 14-23.
8. Michałowicz R, Jóźwiak S. Zespoły padaczkowe i inne stany padaczkowe w zależności od wieku. In: Michałowicz R, ed. Padaczka i inne stany padaczkowe u dzieci. PZWL, Warszawa, 1992.
9. Watanabe K. West Syndrome: etiological and prognostic aspects. Brain Development, 1998; 20: 1-8.
10. Aicardi J, Chevrie JJ. Les spasms infantiles. Arch Fr Pediatr, 1978; 35: 1015-23.
11. Sztriha L, Janaky M, Kiss J, Buga K. Ele and 99 mTc-HMPAO-SPECT studies in Menkes disease. Brain Dev, 1994; 16: 224-8.
12. Makela-Bengs P, Suomalainen A, Majander A, Rapola J, Kalimo H, Nuutila A, Phihko H. Correlation between the clinical symptoms and the proportion of mitochondrial DNA carrying the 8993 point mutation in the NARP syndrome. Pediatr Res, 1995; 37: 634-9.
13. Rikkonen R. Infantile spasm: infectious disorders. Neuropediatrics, 1993; 24: 274-80.
14. Matsuo A, Matsuzaka T, Tsuru A, Moriuchi H, Nakashita Y, Tanaka C, Tomimasu K. Epidemiological and clinical studies of West syndrome in Nagasaki Prefecture, Japan. Brain Development, 2001; 23(7): 575-9.
15. Appleton R. West syndrome: long term prognosis and social aspects. Brain Development, 2001; 7: 688-91.

16. Glauser TA. Following catastrophic epilepsy patients from childhood to adulthood. Epilepsia, 2004; 45: 23-6.

17. Kułakowska Z. Wczesne uszkodzenie dojrzewającego mózgu. Od neurofizjologii do rehabilitacji. Folium, Lublin, 2003.

18. Wheless JW. Nonpharamacologic treatment of the catastrophic epilepsies in childhood. Epilepsia, 2004; 45: 17-22.

19. Campistol J, Garcia-Cazarola A. West's syndrome. Analysis, aetiological factors and therapeutic options. Rev Neurol, 2003; 37(4): 345-52.

20. Mojs E. Ocena funkcji poznawczych u dzieci i młodzieży z padaczką leczonych lamotryginą lub wigabatryną w systemie mono lub politerapii. Epileptologia, 2001; 2: 143-67.

21. Mojs E. Wybrane zagadnienia rozwoju poznawczego a padaczka i jej leczenie. Epileptologia, 2001; 9: 347-58.

22. Schopler E, Reichler R, Dansing M. Techniki nauczania dla rodziców i profesjonalistów. SPOA, Gdańsk, 1995.

23. Mojs E, Kozak I, Brajer S, Zgorzalewicz M. Rola badania psychologicznego w neurologii dziecięcej. Med Dypl, 2000; special edition.

24. Kozak I, Mojs E, Brajer S, Zgorzalewicz M. Diagnostyka neuropsychologiczna zaburzeń poznawczych u dzieci i młodzieży. Med Dypl, 2001; 10: 13-22.

25. Mojs E, Białkowska E. Zaburzenia funkcji psychicznych w padaczce a możliwości edukacji i rehabilitacji psychologicznej w świetle obowiązującej ustawy oświatowej. Pielęgniarstwo polskie, 2002; 1: 95-100.

26. Ognik P. Terapia neuropsychologiczna w klinice uszkodzeń ośrodkowego układu nerwowego – historia poglądów. In: Waligóra B. ed. Elementy psychologii klinicznej. UAM, Poznań, 1995.

27. Hellbruge T, Lajosi F. Monachijska Funkcjonalna Diagnostyka Rozwojowa. Antykwa, Kraków, 1994.

28. Doman G. Jak postępować z dzieckiem z uszkodzeniem mózgu" Poznań. Protez, 1996.

29. Favata I, Leuzzi V, Curatalo P. Mental outcome. In: West Syndrome: prognostic value of some factors. J Mental Defic Res, 1987; 31: 9-15.

30. Vojta V, Die Posturale Ontogenese als Basis der Entwicklungediagnostik. Kinderarzt, 1989; 5: 669-74.

31. Feldkamp M. Krankengymnastik nach Bobath oder Vojta. Kinderarzt, 1983; 14: 857-60.

The duration of breastfeeding and attention deficit hyperactivity disorder

Kądziela-Olech H*, Piotrowska-Jastrzębska J

Department of Pediatrics and Developmental Disorders of Children and Adolescents Children Hospital of Medical University of Białystok, Poland

Abstract

Purpose: To examine whether duration of the breastfeeding is associated with the symptoms of attention deficit hyperactivity disorder in children.

Material and methods: A total of 100 children aged 4-11 years were divided into two groups: 60 children with ADHD symptoms (based on ICD-10) and 40 subjects of normal control grup. The structured interview and the retrospective questionnaire (including the items: number of pregnancy, pregnancy course, gestational age, status of newborn, birth weight, duration of breastfeeding: <3 months; 3-6 months; 6-12 months; >12 months) were used during the study of the both examined groups to indicate the risk factors of development.

Results: No significant differences in the percentages of duration of pregnancy, pregnancy complications, delivery complications, condition of the newborn, and birth weight were found between the two groups. The mean of the duration of breastfeeding for group with ADHD was 0.45 year: 5 months and 9 days (median 0.25 year: 3 months). The mean of the duration of breastfeeding of control group was 0.55 year: 6 months and 18 days (median 0.46 year: 5 months) and was significantly greater than that of group with ADHD ($p<0.04$). The 36 (60%) children with ADHD were breast fed less than 3 months. For comparison 13 (32.5%) controls were breast fed less than 3 months. Significant differences were found among the two children groups ($p<0.05$).

Conclusions: The short duration of breastfeeding as environmental factor may be considered a risk factor of ADHD symptoms. However, the further studies are nedeed.

* CORRESPONDING AUTHOR:
Department of Pediatrics and Developmental Disorders
of Children and Adolescents
Children Hospital of Medical University of Białystok
ul. Waszyngtona 17, 15-274 Białystok, Poland
Fax: +48 85 7450644
e-mail: halkolech@mediclub.pl (Halina Kądziela-Olech)

Received 01.06.2005 Accepted 18.07.2005

Key words: ADHD, hyperactivity, children, breastfeeding, risk factor.

Introduction

Attention deficit hyperactivity disorder (ADHD) is the most commonly diagnosed neurobehavioral disorder in childhood. Children with ADHD also had problems with sustained attention, impulse control and their motor hyperactivity. Different symptoms may appear in different settings, depending on the demands the situation may pose for the child's self-control. Children with ADHD show difficulties with social contacts. Its onset is in early childhood: by definition before the age of 6, nearly always before the age 5 and frequently before the age of 2 years [1-3]. Clinical studies indicate that the inattentive and restless behavior is a developmental risk. Of children referred to clinics for ADHD, 30-60 percent will continue to have symptoms of the disorder into their adolescence and adulthood. Patients diagnosed with ADHD are at higher risk for learning, behavioral and emotional problems [3,4]. Scientific data suggested that ADHD is due to neuroanatomical or neurochemical abnormalities that result in the inconsistent meta-regulations of brain chemicals [1,3]. The exact aetiological pathways of ADHD are unknown. This is disorder with an aetiology in a combination of genetic and environmental factors. Molecular genetic studies have found associations with variations in genes for the dopamine receptors: DRD4, DRD5, and the dopamine transporter: DAT1[5-9]. The central dopamine receptors participate in the control of locomotor, cognitive and emotional funcions in the brain. Studies using structural and functional brain imaging and transcranial magnetic stimulation have shown various abnormalities in prefrontal, temporal, parietal cortical regions and striatum [1,3]. Nonetheless, researches have not discovered a unique brain pattern with ADHD.

There is also association with a variety of environmental factors, including prenatal and perinatal obstetric complications, low birth weight, prenatal exposure to alcohol or nicotine, viral

infections, and brain diseases and injuries [10-12]. Idiosyncratic reactions to food and exposure to toxic levels of lead are also considered to have aetiological importance[1]. There may be multiple developmental pathways from aetiological factors to behavioral symptoms.

ADHD impairs specific aspects of cognition, including the ability to sustain attention in early childhood [2,13]. On the other hand several studies have shown a positive correlation between breastfeeding and cognitive development. Breastfeeding is the optimal mode of feeding for the infant. Human milk composition knowledge has been basis for recommended dietary allowances for infants, contains just the right amount of long-chain polyunsaturated fatty acids (LC-PUFA), such as docosahexaenoic acid (DHA, C22:6n3) and arachidonic acid (AA, C20:4n6), lactose, different oligosaccharides, water, and amino acids for human brain development [14-17]. Docosahexaenoic acid (DHA) is a major component of neuronal membranes. In rats, low brain levels of DHA during development produce alterations in the mesocortical and mesolimbic dopamine systems [18]. It is also well known that sensory stimulation is like a nutrient which is essential for the normal growth, development and functioning of the brain and that sensory deprivation during the formative periods of brain development induces developmental brain abnormalities of both structure and function including the neurochemical activity. Clinical literature provide support for the hypothesis that breast feeding benefits mental development. On the hand, biochemical components of human milk affect particular elements of the neural circuitry that contribute to information processing. On the other hand, in addition to the emotional ties that arise between mother and infant from suckling, it is plausible that breastfeeding helps the development of interpersonal communication between infant and caretaker [14,17,19-22].

The aim of the study was to investigate whether is a link between duration of breastfeeding and ADHD symptoms in children.

Material and methods

The specific method of laboratory, psychological or biological research enough to make an accurate diagnosis of the ADHD is unknown. Observation of the subject's behavior and the disease is still the basic procedure for determining the presence of this mental disorder. The DSM-IV or ICD-10 criteria are the fundamental elements of diagnosis of ADHD [23,24].

The ICD-10 and DSM-IV diagnostic criteria for ADHD require symptoms or impairment in two or more settings. Thus, information on children's behaviors at school or kindergarten is usually required.

Sifting examination was conducted in 6 randomly chosen kindergartens and elementary schools of Białystok. The object of the research was a group of 1180 children aged 4-11 years (591 boys and 589 girls). The initial selection of children was conducted using the ICD-10 criteria (*Tab. 1*). The study was preceded by meetings with parents and teachers that detailed directions to the investigation to inquire into the qustionnaire were imparted. The consent was obtained from parents or legal guardians. It contains statements regarding the observed behavior of a child in 3 categories: motor restlessness, impulsiveness, and concentration problems. Children were divided into two groups.

Table 1. **The Behavioral Scale of ICD-10 criteria**

	Hyperactivity	yes	no
1.	Often fidges with hands or feet		
2.	Often leaves seat in classroom or in other situation in which remaining seated is required		
3.	Often runs about or climbs		
4.	Often has difficulty engaging in leisure activities quietly		
5.	Is often "on the go" or often acts as if "driven by a motor"		
	Innatention		
1.	Is often forgetful in daily activities		
2.	Often fails to give close attention to details or makes careless mistake in schoolwork or other activities		
3.	Often has difficulty sustaining attention to tasks or play activities		
4.	Often does not seem to listen when spoken to directly		
5.	Often has difficulty organizing tasks and activities		
6.	Often loses things necessary for tasks or activities		
7.	Often does not follow through on instructions and fails to finish schoolwork (not due to oppositional behavior or failure to undersand instructions)		
8.	Is often easily distracted by external stimuli		
9.	Often avoids or is reluctant to engage in tasks that require sustained mental effort		
	Impulsivity		
1.	Often talks excessively		
2.	Often blurts out answers before questions have been completed		
3.	Often has difficulty one's waiting turn		
4.	Often interrupts or intrudes on others		

Subjects

Group I with ADHD (N=60) selected a group of 60 children (51=85% boys and 9=15% girls), aged mean age 7 years and 3 months, who had been referred to them by parents and teachers of difficulties learning in an ordinary classroom setting. All children were of 10-18 points according to ICD-10 classification (at least 3 symptoms of hyperactivity, at least 1 symptom of impulsivity and at least 6 symptoms of concentration problems). Stated disorders disturbed the functioning of children, out of proportion to their development. All the children of this group were subjected to psychiatric examination. Any neurological disease, mental handicap, head injury, anxiety, or general developmental disorder (according to ICD-10) were exclusion criteria.

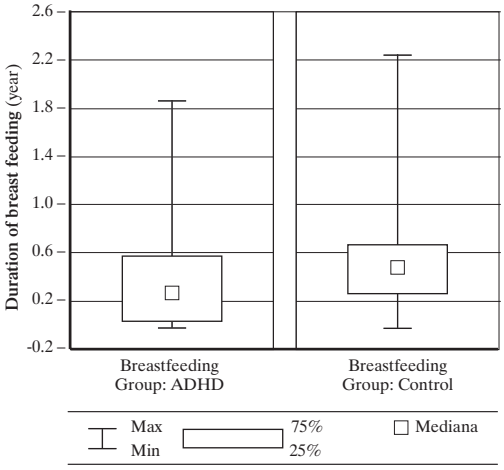
Group II, normal control children (N=40): 34=85% boys and 6=15% girls, mean age 7 years and 8 months were selected as normal controls, who in the parent's and teacher's opinion, had no significant learning difficulty, no know hearing loss and no significant behavioral nor emotional problems.

Table 2. **The perinatal state of study groups**

Examined group	with ADHD		control group		
	N= 60		N=40		p
	N	%	N	%	
Number of pregnancies					
I	24	40.00	15	37.50	ns
II	21	35.00	14	35.00	ns
III	10	16.67	5	12.50	ns
≥IV	5	8.33	6	15.00	ns
Pregnancy complications	12	20.00	3	7.50	ns
Gestational age (wk)					
<37	7	11.67	3	7.50	ns
37-40	52	86.67	37	92.50	ns
>40	1	1.67	-	-	
Delivery complications	7	11.67	2	5.00	ns
Status of newborn					
(Apgar test point)					
10-7	53	88.33	38	95.00	ns
6-4	6	10.00	2	5.00	ns
3-1	1	1.67	-	-	
Birth weight (g)					
<2500	6	10.00	1	2.50	ns
2500-3500	34	56.67	25	62.50	ns
>3500	20	33.33	14	35.00	ns

p – ns – not significant difference between the children with ADHD and the control group

Figure 1. **The assesment of the duration of breastfeeding in examined groups**



Group with ADHD: median – 0.2500; mean – 0.4462 SD – 0.50355; Control group: median – 0.4583; mean – 0.5498; SD – 0.50067 p<0.04 Significant difference between the children with ADHD and the control group

Procedure

The structured interview and the retrospective questionnaire (including the items: number of pregnancy, pregnancy course, gestational age, status of newborn, birth weight, kind of nutrition, duration of breastfeeding: <3 months; 3-6 months; 6-12 months; >12 months) were used during the study of the both examined groups to indicate the risk factors of development.

Data of the quantity variables as birth weight and duration of breastfeeding were expressed as mean, median and standard deviation (SD). The U Mann-Whitney test was used to examine the differences between children with ADHD and control group. Results were expressed using the percentages for discrete variables (number of pregnancy, pregnancy complications, getational age, status of newborn, delivery complications). Groups were compared the Chi-square test or Fisher precision test for discrete variables. P value was considere statistically significant at the level <0.05. The data was analysed using Statistica 5.0 Stat-Soft program.

Results

The retrospective study of perinatal and postnatal period of a child from both examined groups was evaluated. Six (10%) children with ADHD had low birth weight: <2500g. Pregnancy complications were refered to 12 (20%) children with ADHD, and to 3 (7.5%) controls, but no significant differences were observed in the number and duration of pregnancy, pregnancy complications, delivery complications, condition of the newborn (Apgar test), and birth weight between the two groups (*Tab. 2*).

The mean of the duration of breastfeeding for group with ADHD was 0.45 year: 5 months and 9 days (median 0.25 year: 3 months). The mean of the duration of breastfeeding of control group was 0.55 year: 6 months and 18 days (median 0.46 year: 5 months) and was significantly greater than that of group with ADHD (*Fig. 1*). The 36 (60%) children with ADHD were breast fed less than 3 months. For comparison 13 (32.5%) controls were breast fed less than 3 months. There was significant difference between two groups (*Tab. 3*).

Discussion

The results of the children with ADHD in the present study differed from those of the control children in duration of breastfeeding and number of pregnancy complications and in birth weight, but duration of breastfeeding was evident in significant diference. The children of control group have been breast fed longer than children with ADHD. WHO and Work Group on Breastfeeding of American Academy of Pediatrics conclude exclusive breastfeeding is ideal nutrition and sufficient to support growth and development for approximately the first 6 months after birth. It is recommended that breastfeeding continue for at least 12 months [17,21,22].

Children who were breast fed for less than three months had a higer risk, compared to children who were breast fed at least six months, of a lower mental developmental index [19,21]. Human milk contain many biological factors that may be beneficial for mental development, including biologically active peptides and the long chain polyunsaturated fatty acids [25-29]. Dietary fats may affect the brain composition and func-

Table 3. **Duration of breastfeeding (BF) of examined group with ADHD and control group**

Duration of BF	<3		3 – 6		6 – 12		>12		Total	
(months)	n	%	n	%	n	%	n	%	n	%
With ADHD	36	60.00	9	15.00	7	11.67	8	13.33	60	100
Control group	13	32.50	15	37.50	8	20.00	4	10.00	40	100
p value		0.05*		ns		ns		ns		

* p – significant difference between the children with ADHD and the control group; ns – not significant difference

tion in early life. Breast fed infants receive docosahexaenoic acid (DHA) and arachidonic acid (ARA) in their diet, which are highly concentrated in the central nervous system and they are important components of it's [26,29]. Upon weaning, infants lose this dietary source of long-chain polyunsaturates because many commercial formulas do not contain these important fatty acids [29]. The amount of these fatty acids in the central nervous system increases dramatically during the last intrauterine trimester and the first of life. DHA and ARA are transferre across the placenta, are present in human milk, and are accumulated in the brain and retina during fetal and infant development [25,29]. The high of DHA and ARA concentrations in brain gray matter suggest that these fatty acids have important roles in neural functions. Lipids are essential for brain development and function thoughout the life course. Over the first 6 months of life, DHA accumulates at about 10 mg/d in the whole body of breast fed infants with 48% of that amount appearing in the brain [29]. The latter effects may be explained by changes in the membrane bilayer that alter membrane – associated receptors and signal transduction systems, ion channel activity, or direct effects on gene expression [25]. May by possbility that deficiency of docosahexaenoic acid and arachidonic acid in diet of children have been breast fed short may be played any role in ADHD pathogenesis. Maher et al. [9] indicate that the dopamine system play a major role in the development of attention deficit hyperactivity disorder. Farooqu and Horrocks suggest that deficiencies of DHA and plasmalogenes in ADHD may be responsible for abnormal signal transduction associated with learning disability and cognitive deficit. These abnormalities in the signal – transduction process can be partially corrected by supplementation with a diet enriched with DHA [31]. Several studies have identified abnormalities in membrane fatty acids in some subjects with ADHD, and some success has been reported using dietary treatment with supplementation DHA of children with ADHD [30,32].

The brain develops intensively in the first two years. Infant interacts with environment every time and its brain makes a new connection. At the first months of age there is intensive activity in the cortical and subcortical regions that control sensory-motor functions. The sensory stimulation is like the biochemical components of human milk which is essential for the normal growth, development and functioning of the brain [33]. Breastfeeding has lots of skin to skin contact and interaction between child and with his mother. Breastfeeding itself with its the emotional senses of body touch, and the closeness between mother and baby is usually a more interesting, more interactive experience than bottle-feeding this is nature's way of insuring that babies get the stimulation they need for optimal brain development. The closeness of breastfeeding is an important bridge between baby's intrauterine life and his new experience of being out in the world. Studies have shown that babies who receive lots of closeness with their breastfeeding mothers and lots of stimulating eye contact and conversation are getting important brain stimulation that gadgets and and toys cannot produce [22,33,34]. The contact between mothers and their infants who are formula-fed is short. These sensory deprivation process that involve the emotional senses of body touch, movement and smell have been well described in failed affectional bonding in the mother-infant/child relationship [35].

Conclusions

The results of this study suggest that the short duration of breastfeeding beside others may be considered a risk factor of ADHD development. However, the further studies are needed to understanding of this problem better.

References

1. Taylor E, Döpfner M, Sergeant J, Asherson Ph, Banaschewski T, Buitelaar J, Coghill D, Danckaerts M, Rothenberger A, Sonuga-Barke E, Steinhausen HCh, Zuddas A. European clinical guidelines for hyperkinetic disorder – first upgrade. Eur Child Adolesc Psychiatry, 2004; 13(Suppl. 1): I7-I30.

2. Barkley RA, Shelton TL, Crosswait C, Moorehouse M, Fletcher K, Barrett S, Jenkins L, Metevia L. Preschool children with distruptive behavior: three-year, outcome as a function of adaptive disability. Dev Psychopathol, 2002; 14(1): 45-67.

3. Sergeant J. Eunethydis – searching for valid aetiological candidates of Attention-Deficit Hyperactivity Disorder or Hyperkinetic Disorder. Eur Child Adolesc Psychiatry, 2004; 13(Suppl 1): I/43-I/9.

4. Searight RH, Nahlik JE, Campbell DC. Attention-Deficit/Hyperactivity Disorder: Assessment, diagnosis and management. J Fam Pract, 1995; 40: 270-9.

5. Thapar A, Holmes J, Poulton K, Harrington R. Genetic basis of attention deficit and hyperactivity. British Journal of Psychiatry, 1999; 174: 105-11.

6. Comings DE, Gade-Andavolu R, Gonzalez N, Wu S, Muhleman D, Blake H, Dietz G, Saucier G, Macmurray JP. Comparison of the role of dopamine, serotonin and noradrenaline genes in ADHD, ODD and conduct disorder: multivariate regression analysis of 20 genes. Clin Genet, 2000; 57(3): 178-96.

7. Fisher SE, Francks C, McCracken JT, McGough JJ, Marlow AJ, MacPhie IL, Newbury DE, Crawford LR, Palmer CG, Woodward JA, Del'Homme M, Cantwell DP, Nelson SF, Monaco AP, Smalley SL. A genomewide scan for loci involved in attention-deficit/hyperactivity disorder. Am J Hum Genet, 2002; 70(5): 1183-96.

8. Winsberg BG, Comings DE. Association of the dopamine transporter gene (DAT1) with poor methylphenidate response. J Am Acad Child Adolesc Psychiatr, 1999; 38(12): 1474-7.

9. Maher BS, Marazita ML, Ferrell RE, Vanyukov MM. Dopamine

system genes and attention deficit hyperactivity disorder: a metaanalysis. Psychiatr Genet, 2002; 12(4): 207-15.

10. Milberger S, Biederman J, Faraone SV. Pregnancy delivery and infancy complications and attention deficit hyperactivity disorder: issues of gene-environment interaction. Biol Psychiatry, 1997, 41(1): 65-75.

11. Botting N, Powls A, Cooke RW. Attention deficit hyperactivity disorder and other psychiatric outcomes in very low birth weight children at 12 years. J Child Psychol Psychiatry, 1997; 38(8): 931-41.

12. Kuperman S, Schlosser SS, Lidral J. Reich W. Relationship of child psychopathology to parental alcoholism and antisocial personality disorder. J Am Acad Adolesc Psychiatry, 1999; 38(6): 686-92.

13. Landgren M, Kjellman B, Gillberg Ch. Attention deficit disorder with developmental coordination disorders. Arch Dis Child, 1998; 79: 207-12.

14. Golding J, Rogers IS, Emment PM. Association between breast feeding, child development and behaviour. Early Hum Dev, 1997; 29(Suppl. 49): S175-84.

15. Angelsen NK, Vik T, Jacobsen G, Bakketeig LS. Breast feeding and cognitive development at age 1 and 5 years. Arch Dis Child, 2001; 85: 183-8.

16. Quinn P, O'Callaghan M, Williams GM, Najman JM, Andersen MJ, Bor W. The effect of breastfeeding on child development at 5 years: a cohort study. J Paediatr Child Health, 2001; 37(5): 465-9.

17. Mortensen EL, Michaelsen KF, Sanders SA, Reinisch JM. The association between duration of breastfeeding and adult intelligence. JAMA, 2002; 287(18): 2365-71.

18. Levant B, Radel JD, Carlson SE. Decreased brain docosahexaenoic acid during development alters dopamine – related behaviors in adult rats that are differentialy affected by dietary remediation. Behav Brain Res, 2004; 152(1): 49-57.

19. Agostoni C, Marangoni F, Lammardo AM, Giovannini M, Riva E ,Galli C. Breastfeeding duration, milk fat compsition and developmental indices at 1 year of life among breasted infants. Prostaglandins Leukot Essent Fatty Acids, 2001; 64(2): 105-9 .

20. Bakker EC, van Houwelingen AC, Hornstra G. Early nutrition, essential fatty acid status and visual acuity of term infants at 7 months of age. Eur J Clin Nutr, 1999; 53(11): 872-9.

21. Agostoni C, Marangoni F, Giovannini M, Galli C, Riva E. Prolong breast-feeding (six months or more) and milk fat content at six months are associated with higer developmental scores at one year of age within a breast-fed population. Adv Exp Med Biol, 2001; 501: 137-41.

22. American Academy of Pediatrics. Breastfeeding and the use of human milk. Pediatrics, 1997; 100 (6): 1035-9.

23. Statistical manual of mental disorders, 4th ed. American Psychiatric Association. Washington, 1994.

24. The ICD-10 classification of mental and behavioural disorders: clinical description and diagnostic guidelines. World Health Organization Geneva, 1992.

25. Innis SM. Perinatal biochemistry and physiology of long-chain polyunsaturated fatty acids. J Pediatr, 2003; 143(Suppl. 4): S1-8.

26. Jorgensen MH, Hernell O, Hughes E, Michaelsen KF. Is there a relation between docosahexaenoic acid concentration in mothers' milk and visual development in term infants? J Pediatr Gastroenterol Nutr, 2001; 32(3): 293-6.

27. Willatts P. Long chain polyunsaturated fatty acids improve cognitive development. J Fam Health Care, 2002; 12(Suppl. 6): 5.

28. Hoffman DR, Birch EE, Castaneda YS, Fawcett SL, Wheaton DH, Birch DG, Uauy R. Visual function in breast-fed term infants weaned to formula with or without long-chain polyunsaturates at 4 to 6 months: a randomized clinical trial. J Pediatr, 2003; 142(6): 669-77.

29. Cunnane SC, Francescutti V, Brenna JT, Crawford MA. Breast-fed infants achieve a higher rate of brain and whole body docosahexaenoate accumulation than formula-fed infants not consuming dietary docosahexaenoate. Lipids, 2000; 35(1): 105-11.

30. Ross BM, Mc Kenzie I, Glen I, Bennet CP. Increased levels of ethane, a non-invasive marker of n-3 fatty acid oxidation, in breath of children with attention deficit hyperactivity disorder. Nutr Neurosci, 2003; 6(5): 277-81.

31. Farooqu AA, Horrocs LA. Plasmalogenes, phospholipase A2, and docosahexaenoic acid turnover in brain tissue. J Mol Neurosci, 2001; 16(2-3): 263-72.

32. Richardson AJ, Puri BK. A randomized double-blind, placebo-controlled study of the effects of supplementation with highly unsaturated fatty acids on ADHD-related symptoms in children with specific learning difficulties. Prog Neuropsychopharmacol Biol Psychiatry, 2002; 26(2): 233-9.

33. Lanting DI, Fidler V, Huisman M, Touwen BC, Boersma ER. Neurological differences between 9-year old children fed breast-milk or formula-milk as babies. Lancet, 1994; 344(8933): 1319-22.

34. Prescott JW. The origins of human love and violence. Pre- and Perinatal Journal of Psychology, 1996; 10(3): 143-88

35. Cook PS. Early Child Care: Infants & Nations At Risk. News Weekly Books, Melbourne, 1996.

Chlamydia trachomatis infection in chronically hemodialyzed patients

Bednarek-Skublewska A[1]*, Majdan M[2], Dryglewska M[2], Książek A[1]

[1] Department of Nephrology, University Medical School, Lublin, Poland
2 Department of Rheumatology and Connective Tissue Diseases, University Medical School, Lublin, Poland

Abstract

Purpose: In the general population there is association between *Chlamydia trachomatis (Ch. trachomatis)* infection and reactive arthritis (RA). RA is a systemic illness characterized by inflammatory synovitis. Arthritis tends to be oligoarticular and involves mainly the lower limbs. The aim of this study is to assess the age and sex specific prevalence of *Ch. trachomatis* infection in dialysis population and to find possible relationship between manifestation of infection and renal osteodystrophy.

Material and methods: The study was conducted in 53 patients: 22 women (W) and 31 men (M), with a mean age of 58.1 ± 15 years, treated with HD for 28.5 ± 28.2 months. The *Ch. trachomatis* infection was assessed by the detection IgG antibodies for *Ch. trachomatis*. Also some other biochemical parameters of osteodystrophy, inflammation and malnutrition were measured.

Results: The presence of a high titre of anti-*Ch. trachomatis* antibodies was found in 22 patients – 41% [G IgG (+)]. Mean level of anti-*Ch. trachomatis* antibodies was significantly higher in G IgG (+) than in seronegative patients [G IgG (-)]: 19.0 ± 8.6 vs 4.0 ± 2.1 U/ml, p<0.001. There was no difference in mean age of seropositive and seronegative patients for *Ch. trachomatis* (62.4 ± 13.1 vs 56.2 ± 15.9 years). We did not observe in both groups of patients any differences in mean level of C-reactive protein (CRP): 12106.2 ± 10791.0 vs 14015.3 ± 11194.3 ng/ml. The mean ferritin level was significantly higher in G IgG (+): 624.3 ± 375.7 vs 418.3 ± 341.4 ng/ml, p<0.05. Significant negative correlations were found in G IgG (+) between IgG antibodies and transferrin saturation (r=-0.645719, p<0.001) and between CRP and calcium (r=-0.4526, p<0.05). IgG antibodies were detected frequently in W (60%) than in M (29%). Mean level of IgG was significantly higher in seropositive W than in seropositive M (23.3 ± 7.8 vs 12.1 ± 4.2 U/ml, p<0.0001). The seropositive W were older (67.9 ± 11.8 vs. 53.8 ± 11.0 years, p<0.02) and seropositive W were shorter treated with HD (18.1 ± 16.6 vs 43.7 ± 30.6 months, p<0.02). The mean serum calcium conc. and phosphorus were significantly lower in seropositive W (2.1 ± 0.1 vs 2.3 ± 0.2 mmol/l, p<0.05 and respectively 1.3 ± 0.3 vs 1.8 ± 0.2 mmol/l, p<0.005). Likewise the mean transferrin saturation (TS) was significantly lower in that group (25.7 ± 7.3 vs 38.0 ± 11.3%, p<0.01). There were no differences between seropositive men and women in mean serum concentrations of CRP, iPTH, albumin and hemoglobin. We found in seropositive W significant negative correlation between IgG antibodies and age (r=-0.633, p<0.02).

Conclusions: The patients treated with HD were quite frequently shown significantly elevated level of IgG antibodies for *Ch. trachomatis*. It could have be connected with past infection. The antibodies were more commonly detected in women, particularly in younger patients. No relationship between osteodystrophy and *Ch. trachomatis* infection was found.

Key words: hemodialysis, *Chlamydia trachomatis* infection, renal osteodystrophy.

Introduction

Chlamydia trachomatis (Ch. trachomatis) infection is sexually transmitted disease with chronic and often asymptomatic course [1,2]. The infection causes major complications, especially in the eye and urinogenital tract [3,4]. In the general population there

* CORRESPONDING AUTHOR:
Department of Nephrology, University Medical School
ul. Jaczewskiego 8
Lublin, Poland
Fax: + 48 81 7425 334
e-mail: anna.bednarek@gambro.pl (Anna Bednarek-Skublewska)

Received 13.09.2004 Accepted 03.01.2005

is a relationship between *Ch. trachomatis* infection and reactive arthritis (RA) [5]. RA is a systemic illness characterized by inflammatory synovitis. Arthritis tends to be oligoarticular and involve mainly the lower limbs [5,6]. It is known, that patients treated hemodialysis (HD) are more susceptible to all kinds of infections [7]. The bone disorders in dialysis population are first of all caused by renal osteodystrophy, spectrum of skeletal disorders that range from high turnover to low turnover lesions [8]. One must take into consideration that musculosceletal system complaints in patients treated HD may be caused by *Ch. trachomatis* infection as well. In the accessible literature we didn't find data about prevalence the *Ch. trachomatis* infection in patients treated HD and association between *Ch. trachomatis* infection and process of renal osteodystrophy. The aim of this study was to assess the age and sex specific prevalence of *Ch. trachomatis* infection in dialysis population. Likewise we sought possible relationship between *Ch. trachomatis* infection and renal osteodystrophy.

Table 1. **Biochemical parameters in serum of seronegative and seropositive haemodialysis patients for *Chlamydia trachomatis***

Parameter	G-IgG(+) n=22	G-IgG(-) n=31	Statistical significance
IgG (U/ml)	19.0±8.6	4.0±2.1*	p<0.001
hemoglobin (mmol/l)	6.6±0.8	6.8±0.9	N S
albumin (g/l)	34.0±3.0	34.0±4.0	N S
CRP (ng/ml)	12106.2±10791.0	14015.3±11194.3	N S
TS (%)	25.7±7.3*	32.1±18.7	p<0.05
ferritin (μg/l)	624.3±375.7	418.3±341.4*	p<0.05
Ca (mmol/l)	2.2±0.2	2.2±0.2	N S
P (mmol/l)	1.5±0.4	1.6±0.6	N S
iPTH (μg/l)	463.4±424.3	378.6±231.2	N S
nPCR (g/kg/day)	1.1±0.2	1.0±0.3	N S

G IgG (+) seropositive patients for *Ch. trachomatis*;
G IgG (-) seronegative patients for *Ch. trachomatis*;
Ca – calcium; nPCR – normalized protein catabolic rate;
P – phosphorus; CRP – C-reactive protein;
TS – transferrin saturation; *– statistical significance

Material and methods

The study was conducted in 53 patients: 22 women (W) and 31 men (M), with a mean age of 58.1±15 years, treated with HD for 28.5±28.2 months. The cause of end stage renal failure was: glomerulonephritis in 16 cases, diabetic nephropathy in 12 cases, interstitial nephritis in 9 cases, hypertensive nephropathy in 5 cases, connective tissue diseases in 4 cases, polycystic renal diseases in 4 cases and 3 underterminated cases. All patients were dialyzed with bicarbonate based dialysate, average time's session was 4±0.3 minutes, low-flux polisulfone and hemophan membranes were reused. The *Ch. trachomatis* infection was assessed by the detection IgG antibodies against *Ch. tachomatis*. Some other biochemical parameters of osteodystrophy, inflammation and malnutrition were also measured: intact parathormon (iPTH), calcium (Ca), phosphorus (P), hemoglobin (Hb), albumin, normalized protein catabolic rate (nPCR), ferritin, C-reactive protein (CRP) and transferrin saturation (TS). Likewise Kt/V and mean arterial pressure (MAP) were determined. The specific IgG antibodies to *Ch. trachomatis* were detected by ELISA method with Genzyme Virotech kit. The specific IgG antibodies above 9 U/ml were considered to be positive for *Ch. trachomatis*. CRP were measured by high-sensitive ELISA method with Eucardio kit. iPTH was measured by immunochemiluminescence method with Nichols Inst kits. The normal value of iPTH in patients treated with HD was assumed to be 3-4 fold higher than normal for the healthy controls (10-65 pg/ml). nPCR and Kt/V were calculated by the Daugirdas formula according to DOQI GUIDELINES [9]. Other parameters were measured by standard automated techniques.

Statistical analysis

The results were expressed as the arithmetic mean ± standard deviation (SD). The data were analyzed statistically. To compare groups U-test of Mann-Whitney was used because of diagonal distribution of the parameters tested. Nonparametric r-Spearman test was used to define correlations. The test χ-square was used to examine correlation between non measured features. Values of $p<0.05$ were considered to be statistically significant.

Results

The mean concentrations (conc) of measured parameters in patients were shown in *Tab. 1*. We observed that 22 patient (41%) were seropositive for *Ch. trachomatis* infection [G-IgG (+)]. The mean level of IgG antibodies for *Ch. trachomatis* was significantly higher in G-IgG (+): 19.0±8.6 vs 4.0±2.1 U/ml, $p<0.001$. There was no difference in mean age in seropositive patients and seronegative patients [G-IgG(-)]: 62.4±13.1 vs. 56.2±15.9 years. Likewise we did not find any differences in mean time of HD treatment and Kt/V in both groups of patients (27.8±25.6 vs 29.8±27.1 months and 1.3±0.2 vs 1.2±0.2 respectively). The serum CRP above correct level (4000 ng/ml) was in 41 patients (77%). There was no any significant differences in mean serum level of CRP in seropositive and seronegative patients (12106.2±10791.0 vs 14015.3±11194.3). The mean serum ferritin level was significantly higher in G-IgG (+): 624.3±375.7 vs 418.3±341.4 ng/ml, $p<0.05$. We did not find any significant differences in both groups of patients in mean arterial pressure (MAP): 91.2±15.8 vs 97.2±10.4 mmHg and mean serum levels of other measured parameters. We found in G IgG (+) significant negative correlations between level of IgG antibodies and TS ($r=-0.6457$, $p<0,001$) and between CRP and Ca ($r=-0.4526$, $p<0.05$).

Antibodies for *Ch. trachomatis* were detected generally in group of W. The mean serum levels of measured parameters in seropositive W and seropositive M for *Ch. trachomatis* were shown in *Tab. 2*. We found that 60% of W and 29% of M were seropositive for *Ch. trachomatis*. Mean level of IgG antibodies was significantly higher in seropositive W: 23.3±7.8 vs 12.4±4.2 U/ml, $p<0.0001$. The seropositive W were older then

Table 2*. Biochemical parameters in serum seropositive women and seropositive men for *Chlamydia trachomatis

Parameter	G IgG (+) women n=13	G IgG (+) men n=9	Statistical significance
IgG antibody for *Ch.trachomatis* (U/ml)	23.3±7.8	12.1±4.2*	p<0.0001
hemoglobin (mmol/l)	6.5±0.6	6.9±0.9	N S
albumin (g/l)	30.3±.3.0	35.0±0.3	N S
CRP(ng/ml)	12490.3±12024.4	11482.0±9171.0	N S
TS (%)	25.7±7.3*	38.0±11.3	p<0.01
ferritin (μg/l)	604.6±312.7	660.8±499.0	N S
calcium (mmol/l)	2.1±0.1	2.3±0.2*	p<0.05
phosphorus (mmo/l)	1.3±0.3 *	1.8±0.2	p<0.005
iPTH (μg/l)	426.2±412.1	367.0±349.3	N S
nPCR (g/kg/day)	1.0±0.2	1.1±0.2	N S

G IgG (+) – seropositive patients for *Ch. trachomatis*;
G IgG (-) – seronegative patients for *Ch. trachomatis*;
nPCR – normalized protein catabolic rate;
CRP – C-reactive protein; iPTH – intact parathormone;
TS – transferrin saturation; * – statistical significance

seropositive M (67.9±11.8 vs 53.8±11.0 years, p<0.02) and they were shorter treated with HD (18.1±16.6 vs 43.7±30.6 months, p<0.02). The mean serum levels of Ca and P were significantly lower in seropositive W (2.1±0.1 vs 2.3±0.2 mmol/l, p<0.05 and respectively 1.3±0.3 vs 1.8±0.2 mmol/l, p<0.05). Likewise the mean TS was significantly lower in seropositive W (25.7±7.3 vs 38.0±11.3%, p<0.01). There were no differences in mean MAP in seropositive men and seropositive women (92.1±15.1 vs 89.6±17.8 mmHg). We did not observe in both groups of patients any significant differences in mean serum levels of other measured parameters (*Tab. 2*). We found in seropositive W significant negative correlation between IgG antibodies for *Ch. trachomatis* and age (r=-0.633, p<0.02).

Discussion

Ch. trachomatis is a powerful immunogen, which stimulates the host's immunological processes [10]. The primary infection leads to a local inflammatory reaction due to penetration and reproduction of the bacteria in the epithelial cells. The IgA and IgG antibodies neutralize the primary infection. The serological tests are useful to identify the specific IgG antibodies in diagnosis of *Ch. trachomatis* infection [11,12]. The antibodies can not be used as a sign of current infection, they often persist for years after the infection has resolved. But in most cases the host's reaction to the primary infection is transient and does not causes tissue damage [10]. Locally produced antibodies limit the spread of *Chlamydia* infection, but do not eliminate the bacteria completely. The chronic infection leads to progressive damage of epithelial cells and can lead to serious and costly sequels.

There is a high prevalence of inflammation in the hemodialysis population [7]. The source of inflammation in dialysis patients is multifactorial. The immunodeficiency state recognized in hemodialysis patients can promote the development of *Ch. trachomatis* infection. We observed that in our hemodialysis patients 41% were infected with *Ch. trachomatis*. Prevalence of *Ch. trachomatis* infection among young adults in Poland is high, the Ig G antibodies against this pathogen were detected in 26% young adults (13). In general population there is high prevalence of *Ch. trachomatis* in younger women [1,2,13,14]. Likewise we observed that *Ch. trachomatis* antibodies were detected in women, but the seropositive women were older and shorter treated with HD then the seropositive men. We observed that the immunological reaction presence of this pathogen was significantly more expressed in younger subjects. We have found in seropositive women lower serum concentrations of calcium and phosphorus and transferrin saturation than in seropositive men. There was no link between time of HD treatment and titre of anti-*Ch. trachomatis* antibodies.

Ch. trachomatis infection is frequently asymptomatic, the sequels are common and could be a serious health problem: urinogenital tract complains, acute or chronic renal interstitial inflammation, infertility, increased risk of ectopic pregnancy and ocular damage [15,16]. The *Chlamydia* infection may be a problem in patients after renal transplantation, so one must examine the presence of *Ch. trachomatis* infection in patients prepared to transplantation.

The local inflammatory reaction is most intense on days 2-4 after *Ch. trachomatis* infection onset [10]. The infected epithelial cells secrete numerous proinflammatory chemokines and cytokines including TNF-alfa, interleukin IL-1, IL-6, which in turn activate the liver to secrete CRP and other acute-phase protein like ferritin. It is now known that CRP may act as a clearance factor for endotoxin and opsonized bacterial products. Serum CRP levels in dialysis population are 5 to 10 times higher than in healthy controls [17]. But elevated CRP values are frequently found in the absence of apparent infection or inflammation. Bacterial contamination during the extracorporal circulation and bioincompatiblity explain only a very small part of high prevalence of inflammation as defined by raised CRP in those patients.

We observed that CRP concentration in our studied patients was markedly increased and exceeded the upper limit of the normal range. But we didn't observe any differences in mean level of CRP in all seropositive and seronegative patients for *Ch. trachomatis*. We didn't find association between CRP and antibodies for *Ch. trachomatis*.

The inflammation is strongly linked to atherosclerosis in dialysis population [18]. The cardiovascular risk in the dialysis population is extremely high. Cardiovascular diseases and infections remain the main mortality causes in hemodialysis patients. There are relationships between some infection factors and presence of coronary heart disease [18,19]. Increased levels of CRP and evidence of chronic *Chlamydia pneumoniae (Ch. pneumoniae)* infection have been identified as risk factors for cardiovascular diseases in the general population and in dialysis population [20,21]. The patients *Ch. pneumoniae* antibodies are associated with the severity of atherosclerosis, but this relationship was not observed for *Ch. trachomatis*. The levels of IgG, IgM, IgA antibodies for *Ch. pneumoniae*, *Ch. trachomatis* and *Ch. psittaci* were measured in blood serum patients with myocardial infarction compared with control. Patients with coronary heart disease had higher frequency of seropositivity

to *Ch. pneumoniae* and similar levels of seropositivity to *Ch. trachomatis* and *Ch. psittaci* [22]. We didn't observe any differences in MAP in seronegative and seropositive patients for *Ch. trachomatis*. The malnutrition and inadequate dialysis dose contribute to reduced immune responsiveness in HD patients [7]. We didn't find any association between Kt/V and any well recognized nutritional parameters (albumin, nPCR) or levels of IgG antibodies. We didn't observe in our groups of patients any influence of *Ch. trachomatis* on nutritional status. We have found in seropositive patients a negative link between anti-*Ch. trachomatis* antibodies and ferritin. The *Ch. trachomatis* infection causes major complications, especially in the eye and urinogenital tract [3,4]. We didn't observe any ocular damage in seropositive patients.

In the general population there is a relationship between *Ch. trachomatis* infection and musculoskeletal system disorders, especially with reactive arthritis. In reactive and postinfectious arthritis the joints are generally sterile, although the presence of bacterial antigens has been reported. In vessel walls of synovial membranes IgG and IgA deposits are found [23]. Taking this fact into consideration we tested hypothesis that seropositivity for *Ch. trachomatis* is associated with the osteodystrophy process. We haven't find any typical clinical symptoms of rheumatological disorders in our population studied. In that group of patients we recognized process of osteodystrophy with high bone turnover. Seropositivity to *Ch. trachomatis* did not significantly increase the risk associated with hyperparathyroidism. In seropositive women we found lower mean serum concentrations of calcium and phosphorus and transferrin saturation than in seropositive men.

Conclusions

The patients treated with HD have high frequency of significantly elevated level of IgG antibodies for *Ch. trachomatis*. It could have be connected with infection in the past. The antibodies were more commonly detected in women, particularly younger one. No relationship between osteodystrophy and *Ch. trachomatis* infection was found.

References

1. Stokes T. Screening for Chlamydia in general practice: a literature review and summary of the evidence. J Public Med, 1997; 19: 222-32.

2. Taylor-Robinson D. Chlamydia trachomatis and sexually transmitted disease. BMJ, 1994; 308: 150-1.

3. Weinstock H, Dean D, Bolan G. Chlamydia trachomatis infections. Infect Dis Clin North Am, 1994; 8: 797-819.

4. Peipert JF. Clinical practice. Genital chlamydial infection. N Engl J Med, 2003; 18: 2424-30.

5. Kuipers JG, Koohler L, Zeidler H. Reactive or infectious arthritis. Ann Rheum Dis, 1999; 58: 661-5.

6. Barth W, Segal K. Reactive arthritis. Am Fam Physicisn, 1999; 60: 499-503.

7. Cohen G, Haag-Weber M, Horl WH. Immune dysfunction in uremia. Kidney Int, 1997; 62(Suppl): 79-82.

8. Ferreira MA. Diagnosis of renal osteodystrophy: when and how use biochemical markers and non-invasive methods; when bone biopsy is needed. Nephrol Dial Transplant, 2000; 15(Suppl 5): 8-14.

9. NKF-KDOQI. NKF-K/DOQI Clinical Practice Guidelines for Hemodialysis Adequance: update 2000. Am J Kidney Dis, 2000; 37 (Suppl 1): 7-64.

10. Zdrodowska-Stefanow B, Ostaszewska-Puchalska I, Pucioło K. The immunology of Chlamydia trachomatis. Arch Immunol Ther Ex, 2003; 51: 289-94.

11. Bas S, Vischer TL. Chlamydia trachomatis antibody detection and diagnosis of reactive arthritis. Br J Rheumatol, 1998; 37: 1054-61.

12. Barnes RC. Laboratory diagnosis of human Chlamydia infections. Clin Microb Rev, 1989; 4: 119-36.

13. Swierkot J, Choroszy-Krol I, Marczyńska-Gruszecka K, Teryks-Wołyniec D, Czepulkowska J, Szechinska J. The role of the diagnostic tests in the identification of Chlamydia trachomatis infection in reactive arthritis. Pol Arch Med Wew, 2003; 110: 711-8.

14. Miller WC, Ford CA, Morris M, Handcock MS, Schmitz JL, Hobbs MM, Cohen MS. Prevalence of Chlamydia and Gonococcal infection among young adults in the United States. JAMA, 2004; 12: 2229-36.

15. Gonzales GF, Munoz G, Sanchez R, Henkel R, Gallegos-Avila G, Diaz-Gutierrez O, Vigil P, Vasquez F, Kortabani G, Bustos-Obregon E. Update on the impact of Chlamydia trachomatis infection on male fertility. Andrologia, 2004; 36: 1-23.

16. Idahl AS, Boman J, Kumlin U, Olofosson JI. Demonstration of Chlamydia trachomatis IgG antibodies in the male partner of the infertile couple is correlated with a reduced likehood of achieving pregnancy. Hum Reprod, 2004; 19: 1121-6.

17. Iseki K, Tozawa M, Yoshi, Fukiyama K. Serum C-reactive (CRP) and risk of death in chronic dialysis population. Nephrol Dial Transplant, 1999; 14: 1956-60.

18. Zimmermann J, Herrlinger S, Pruy A, Metzger T, Wanner C. Inflammation enhances cardiovascular risk and mortality in haemodialysis patients. Kidney Int, 1999; 55: 648-58.

19. Bellomo G, Lippi G, Sarion P, Reboldi G, Verdura C, Timio F, Timio M. Inflammation, infection and cardiovascular events in chronic hemodialysis patients: a prospective study. J Nephrology, 2003; 16: 245-51.

20. Zoccali C, Mallamaci F, Tripepi G. Atherosclerosis in dialysis patients: does Chlamydia pneumoniae infection contribute to cardiovascular damage? Nephrol Dial Transplant, 2002; 17(Suppl 8): 25-8.

21. Haubitz M, Brunkhorst R. C-reactive protein and chronic Chlamydia pneumoniae infection long-term predictors for cardiovascular disease and survival in patients on peritoneal dialysis. Nephrol Dial Transplant, 2001; 16: 809-15.

22. Basinkevich AB, Shakhnovich RM, Martynova VR, Kolkova NI, Rakovskaia I. Role of Chlamydia, Mycoplasma and Cytomegalovirus infection in development of coronary artery disease. Kardiologiia, 2003; 43: 4-9.

23. Pavlica L, Tatic V, Draskovic N, Nikolic D. Diagnostic significance of synovial biopsy in patients with Reiter's syndrome. Med Preg, 2003; 56: 403-8.

Increased serum levels of troponin I and lesions in coronary angiography in hemodialysed patients

Popowska-Drojecka J, Muszytowski M*, Sobiło-Jarek L, Majewska A

Department of Nephrology and Internal Medicine, Rydygier Hospital, Toruń, Poland

Abstract

Purpose: Calcium-phosphate disorders are a frequent finding in HD patients, and, in some cases, may cause an abnormal coronary calcification. Some of the HD patients have increased serum cTnI level without evidence of acute coronary syndrome. The aim of this study was to determine if there is a correlance between increased cTnI levels and presence of stenotic changes in coronary arteries in asymptomatic HD patients.

Material and methods: In 13 of 119 HD patients (M:F 10:3) a coronary angiography was performed. The mean age of the patients was 53 years (33-76) and the mean HD duration was 55 months (3-156). cTnI was analyzed by AxSYM system and, subsequently, by VIDAS system.

Results: A constant or intermittent elevation of cTnI was detected in 5 of 13 patients. In 10 of 13 pts a critical stenosis of at least 1 coronary artery was found. A critical stenosis was found in 4 of 5 cTnI (+) patients and in 6 of 8 cTnI (-) patients. An excess calcification of coronaries was observed in 7 patients, including 1 cTnI positive patient with no evidence of coronary stenosis.

Conclusions: 1. The elevation of cTnI in asymptomatic HD patients is observed when there is: (I) excess calcification accompanied by a critical stenosis of at least 1 coronary artery, (II) a critical stenosis of 2 or more coronaries with no evidence of calcification. 2. We suggest that excess cardiovascular calcification in HD patients may be one of the major factors responsible for the troponin release.

* CORRESPONDING AUTHOR:
Department of Nephrology and Internal Medicine
Rydygier Hospital
ul. Św. Józefa 53
87-100 Toruń, Poland
Tel: + 48 56 6101310; Fax: + 48 56 6544060
e-mail: muszyt@i.uni.torun.pl (Marek Muszytowski)

Received 04.01.2005 Accepted 25.05.2005

Key words: cardiac troponin I, artery calcification, hemodialysis.

Introduction

Cardiovascular diseases are a main cause of mortality among end-stage renal disease (ESRD) patients. There are methods and markers to be found that could possibly predict the risk of life threatening cardiovascular events, especially in HD patients. In the latest data, troponin I (cTnI) was found to be one of the most specific markers of cardiac injury [1,2]. Troponin I is a marker with high specifity to cardiomyocyte injury and plays a major role in acute myocardial infarction (AMI) diagnosis [2]. Some of the HD patients have increased serum cTnI levels without evidence of acute coronary syndrome (ACS) [3,4]. The reason for this phenomenon is unknown. Factors that may possibly predict the elevation of serum cTnI are left ventricular hypertrophy (LVH) [5,6], coronary artery disease (CAD) [5,7], HD duration and calcium-phosphate disorders [3,8]. In HD patients with a long history of therapy, asymptomatic cardiomyocyte injury caused by heart calcification may occur [9]. In some cases, an abnormal calcification of coronary arteries was observed in this group of patients. The aim of the study was to determine if there is a correlance between increased cTnI levels and stenotic changes in coronary arteries in asymptomatic HD patients.

Material and methods

We examined a total of 119 patients (M:F 69:50, age range 40.5 (20-78) years) haemodialyzed in the dialysis unit in Toruń. In patients with no history of angina during the previous one month, serum concentration of cTnI was determined before dialysis. During the 28 month follow-up period, five determinations of cTnI were performed at 6 month intervals. In 13 of 119 patients (M:F 10:3) a coronary angiography (CA) was

Table 1. **Comparison of coronary artery stenosis (CAS) and coronary artery calcification (CAC) in analyzed group of HD patients**

	cTnI (+) positive pts n = 5	cTnI (-) negative pts n = 8
Sex (m:f 10/3)	4 / 1	2 / 6
Age, median (years)	60.0	49.8
HD duration, median (months)	70.8	45.1
Coronary artery lesions:		
CAS only	1	3
CAS and CAC	3	3
CAC only	1	-
no lesions	-	2

performed. The indications for CA were ischaemic heart disease (IHD) history or a positive exercise ECG test in asymptomatic potential renal recipients. The mean age of the patients was 53 years (33-67) and the mean HD duration was 55 months (3-156). The following kidney diseases as a cause of renal failure were noted: diabetes – 2, glomerulonephritis – 7, tubulointerstitial nephropathy – 2 and others in 2 patients. The results of the CA were compared with serum cTnI concentrations. The Troponin I level was analyzed by MEIA technology with AxSYM system (Abbott Diagnostic, IL, USA) and, subsequently, by ELFA technology with VIDAS system (Bio-Merieux). Concentrations of cTnI >0.3 μg/L for the AxSYM method and >0.1 μg/L for the VIDAS method were concluded to be positive.

Results

A constant or intermittent elevation of serum cTnI concentration was detected in 5 out of 13 patients (38%). In 10 of 13 patients (77%) a critical stenosis of at least 1 coronary artery was found. After comparing the results, a critical stenosis was found in 4 out of 5 cTnI (+) positive patients and in 6 out of 8 cTnI (-) negative patients. An excess calcification of coronary arteries was observed in 7 patients, including 1 cTnI (+) positive patient with no evidence of coronary stenosis. In 2 cTnI (-) negative patients no coronary lesions were found (*Tab. 1*).

Discussion

Studies carried out in medical centers around the world indicate a prognostic value of the cTnI increase in HD patients. According to some authors, the increased cTnI level predicts ACS and one year cardiac death [6-8,10]. Our findings indicated that 77% of the HD patients with an ischaemic heart disease history or with a positive exercise ECG test had a significant lesions in coronary arteries. An increase of serum cTnI levels was found in 38% of the patients. This suggests that not in all of the patients with lesions narrowing coronary arteries, a constant or intermittent increase of cTnI occurs. There are also other factors that may damage the cardiomyocytes, independently of the coronary artery lesions. It seems that cardiomyocyte injury in HD patients occurs when a long-lasting effect of a number of kidney disease related factors as the course and HD complications take place. It should be noted that a serum increase of cTnI appeared in our patients almost in all cases when CA revealed coronary artery calcification. Arterial calcification is a frequent finding in ESRD patients. Calcification develops at two sites of the arterial wall; arterial intima calcification (AIC) represents an advanced stage of atherosclerosis and is associated with the development of plaques and occlusive lesions [11]; arterial media calcification (AMC) is commonly associated with aging, presence and duration of diabetes and is common in ESRD [11,12]. In ESRD patients, AMC may occur without AIC, and this phenomenon is a consequence of calcium-phosphate disorders. Also, arterial calcification may cause arterial stiffness; however, the mechanism of disturbances varies from obstructive coronary artery disease. It has been shown that arterial wall stiffness, as assessed by an aortic pulse wave velocity is correlated independently with vascular calcification [9]. The elevation in serum phosphate, calcium-phosphate product and increased calcium load are found to be a risk factors for vascular calcification [13,14]. The progress of arterial calcification occurs especially when mineral metabolism is not well controlled and the intake of calcium-based phosphate binders as well as sevelamer is inadequate [15].

Conclusions

The elevation of cTnI level in asymptomatic HD patients is observed when there is: (I) an excess calcification accompanied by a critical stenosis of at least 1 coronary artery, (II) a critical stenosis of 2 or more coronaries with no evidence of calcification. We suggest that excess cardiovascular calcification in HD patients may be one of the major factors responsible for the troponin release.

Acknowledgement

The paper was presented at the 8th Congress of the Polish Society of Nephrology in Białystok, 2-5 June 2004.

References

1. Adams JE, Bodor GS, Davila-Roman VG, Delmez JA, Apple FS, Ladenson JH, Jaffe AS. Cardiac troponin I. A marker with high specificity for cardiac injury. Circulation, 1993; 88: 101-6.

2. Myocardial infarction redefined – A consensus document of The Joint European Society of Cardiology / American College of Cardiology Committee for the Redefinition of Myocardial Infarction. Eur Heart J, 2000; 21: 1502-13.

3. Muszytowski M, Popowska-Drojecka J, Wojnicz M, Moczyński P, Normantowicz K, Brygiert-Jarząbek H. Factors associated with increased serum levels of troponin I in hemodialyzed patients without evidence of acute coronary syndromes. Nephrol Dial Transplant, 2003; 18(Suppl. 4): 716-20.

4. Watnick S, Perazella MA. Cardiac troponins: utility in renal insufficiency and end-stage renal disease. Sem Dial, 2002; 15: 66-70.

5. Iliou MC, Fumeron C, Benoit MO, Tuppin P, Le Courvoisier C, Calonge VM, Moatti N, Buisson C, Jacquot C. Factors associated with increased serum levels of cardiac troponins T and I in chronic haemodialysis patients: Chronic Haemodialysis And New Cardiac Mark-

ers Evaluation (CHANCE) study. Nephrol Dial Transplant, 2001; 16: 1452-8.

6. Mallamaci F, Zoccali C, Parlongo S, Tripepi G, Benedetto FA, Cutrupi S, Bonnano G, Fatuzzo P, Rapisarda F, Seminara G, Stancanelli B, Bellanuova I, Cataliotti A, Malatino LS. Troponin is related to left ventricular mass and predicts all-cause and cardiovascular mortality in hemodialysis patients. Am J Kidney Dis, 2002; 40: 68-79.

7. Martin GS, Becker BN, Schulman G. Cardiac troponin-I accurately predicts myocardial injury in renal failure. Nephrol Dial Transplant, 1998; 13: 1709-12.

8. Apple FS, Murakami MM, Pearce LA, Herzog CA. Predictive value of cardiac troponin I and T for subsequent death in end-stage renal disease. Circulation, 2002; 106: 2871-5.

9. Blacher J, Guerin AP, Pannier B, Marchais SJ, London GM. Arterial calcifications, arterial stiffness and cardiovascular risk in end-stage renal disease. Hypertension, 2001; 38: 938-42.

10. Freda BJ, Tang WHW, Van Lente F, Peacock WF, Francis GS. Cardiac troponins in renal insufficiency. J Am Coll Cardiol, 2002; 40: 2065-71.

11. Shenahan CM, Carry NRB, Salisbury JR. Medial localization of mineralization-regulatind proteins in association with Monckeberg's sclerosis: evidence for smooth muscle cell-mediated vascular calcification. Circulation, 1999; 100: 2168-71.

12. Letho S, Nishanen L, Suhonen M. Medial artery calcification. A neglected harbinger of cardiovascular complications in non-insulin dependent DM. Atheroscler Thromb Vasc Biol, 1996; 16: 978-83.

13. Goodman WG, Goldin J, Kuizon BD. Coronary-artery calcification in young adults with end-stage renal disease who are undergoing dialysis. N Engl J Med, 2000; 342: 1478-83.

14. Raggi P, Boulay A, Chasan-Taber S. Cardiac calcification in adult hemodialysis patients. A link between end-stage renal disease and cardiovascular disease? J Am Coll Cardiol, 2002; 39: 695-9.

15. Chertow GM, Burke SK, Raggi P. Sevelamer attenuates the progression of coronary and aortic calcification in hemodialysis patients. Kidney Int, 2002; 62: 245-52.

Results of improvement in adequacy of intermittent hemodialysis in uremic patients

Grzegorzewska AE[1,2]*, Banachowicz W[2], Leander M[1]

[1] Department of Nephrology, Transplantology and Internal Diseases, Karol Marcinkowski University of Medical Sciences, Poznań, Poland
[2] International Dialysis Center, Rawicz, Poland

Abstract

Purpose: Increasing number of patients, who need intermittent hemodialysis (IHD), is a great challenge for every society. The aim of study is to look if small increase in IHD adequacy is able to improve standard medical parameters.

Material and methods: In 40 patients, Kt/V was monitored on-line during the middle IHD session in the week, 4 times in each of 6 consecutive months. In the first month of observation Kt/V was lower (1.09±0.02) than in the further months, in which Kt/V was increasing to 1.17±0.01. Blood count was estimated every month. At the beginning of study period, after 3 months and at the end of studies, dry body mass, body mass index (BMI), the blood pH and serum concentration of calcium, phosphate, intact parathormone (iPTH), total protein, albumin, cholesterol, iron, ferritin, urea and creatinine were determined.

Results: The increase in Kt/V was accompanied by rising values of hemoglobin (Hb), hematocrit (Hct), mean corpuscular volume, iron, blood pH before and after IHD session as well as by decreasing values of PTH. Statistically unchanged parameters included dry body mass, BMI, serum concentration of total protein, phosphate, cholesterol and ferritin as well as white blood cells and platelet count. There were correlations between Kt/V and serum concentrations of phosphate, PTH, ferritin, Hb and Hct, indicating that higher IHD doses were provided to patients in more advanced uremic state.

Conclusions: Even small increase in IHD adequacy leads to beneficial changes in management of uremic patients.

* CORRESPONDING AUTHOR:
Department of Nephrology, Transplantology and Internal Diseases
ul. Przybyszewskiego 49.
60-355 Poznań, Poland
Tel: +48 61 8691700; Fax: +48 61 8691688
e-mail: alicja_grzegorzewska@yahoo.com (Alicja E. Grzegorzewska)

Received 30.03.2005 Accepted 12.07.2005

Key words: Kt/V, blood morphology, pH, PTH, iron.

Introduction

Increasing number of uremic patients, who need dialysis treatment, is a great challenge for every society but especially for poor and developing countries. In Poland, number of dialyzed patients increased from 497 in 1981 to 11440 in 2003, that is over 20-times [1]. Estimated number of patients on renal replacement therapy is 27500 in 2010 [2]. Thus, a question how to counterbalance medical expectancies and costs is of special importance.

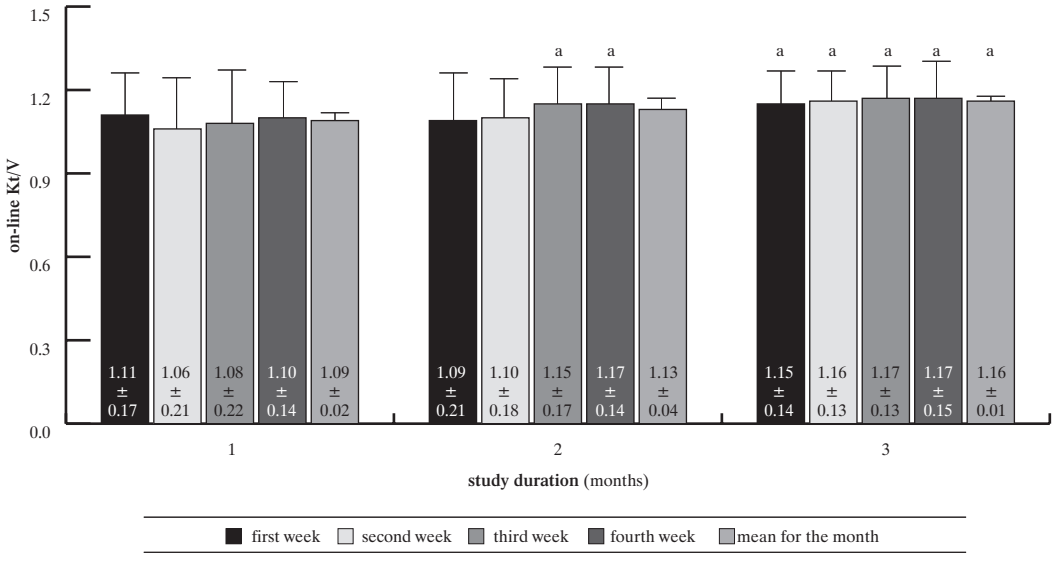
The aim of our study is to look if small (not very expensive) increase in intermittent hemodialysis (IHD) adequacy, expressed by Kt/V, is able to improve standard medical parameters.

Material and methods

Studies were carried out during six months in 40 uremic patients (23 women, 17 men) in the age of 58.9±14.7 years. All patients were stable in the month proceeding the study beginning. Causes of end-stage renal disease included: diabetic nephropathy (12 patients), chronic glomerulonephritis (7 patients), chronic tubulointerstitial nephritis without renal stones (4 patients) or with renal stones (3 patients), polycystic kidney disease (2 patients), amyloidosis, renal cirrhosis, lupus nephritis, myeloma multiplex, chronic lymphatic leukemia (one case each). In 7 patients etiology of end-stage renal disease remained unknown. Arterial hypertension occurred in 25 patients, cardiovascular disease – in 20 patients, diabetes mellitus type I – in 2 patients, diabetes mellitus type II – in 10 patients.

Patients were treated with dialyses for 21 (1-185) months. One patient before starting IHD program was treated with continuous ambulatory peritoneal dialysis for 14 months. Those, who were dialyzed longer than one month but shorter than 6 months (n=6) were also included into the study, because they

Figure 1. **Values of on-line Kt/V during the first three month of study**



* $p<0.05$ as compared to the mean for the first month of study

had started their IHD program in planned manner with mature arterio-venous fistulas and did not show any problems related to dialysis initiation.

Patients (n=36) were taken erythropoietin beta intravenously in the individual doses ranging from 1 000 to 8 000 units per week (3 375±2 371 units per week including four patients not receiving erythropoietin). Iron was supplemented intravenously in 27 patients in the doses of 100 mg per week (67.5±47.4 mg per week including 13 patients not receiving iron). Alfa-calcidol was applied orally to 12 patients in the individual doses of 0.25 to 100 μg per day. Calcium carbonate was administered in 38 patients in the doses of 1.0 to 9.0 g per day. Doses of intravenous drugs were stable. Prescriptions of oral drugs were also unchanged, but probably influenced by patients' compliance.

In all patients, Fresenius dialysis machines type 4008 S and polysulfone-based membranes were used. Dialyzers were not reused. Composition of dialysis solution was not changed during the study.

On-line monitoring of Kt/V was repeatedly performed during the middle IHD session in the each week in six consecutive months. Measurements of Kt/V based on the conductivity method. Total body water, which is assumed the equal the urea distribution volume, was calculated using the formula of Watson et al. [3] for women and men, respectively.

In the first month of study, IHD schedule was not changed as compared to that used in the few previous months in respect of blood and dialysate flows, duration of IHD session and selection of a dialyzer. Values of on-line Kt/V were stable in weekly evaluations. In the next weeks, efforts were made to obtain significantly higher Kt/V by an increase in blood flow and/or dialysate flow. The increase in blood flow was preferable but not always sufficiently possible due to poor blood access or patients' intolerance. In such cases, an increase in dialysate flow (from 500 ml/min to 800 ml/min) accompanied trials to enhance blood flow.

In the second month of study, on-line Kt/V in the third and fourth week was significantly higher than mean on-line Kt/V for the first month of study, but mean values for both months were still not different.

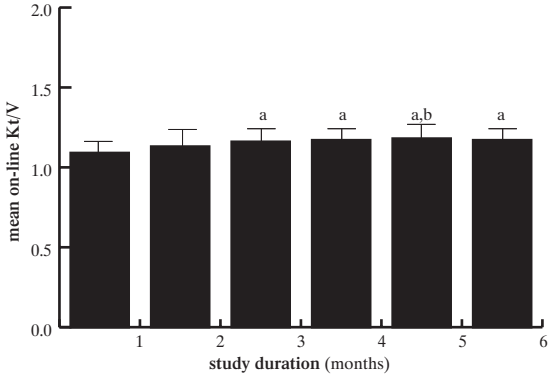
In the third month of study, values of weekly Kt/V estimations and mean monthly Kt/V were stable and significantly higher than mean value for the first month (*Fig. 1*). Thus, our aim to obtain slightly, but significantly higher Kt/V was achieved. During the next three months of study the more effective IHD schedule was maintained, but further efforts to improve adequacy of IHD were not undertaken.

Hemodialyses were performed three times a week. The average duration of IHD session was not significantly different in consecutive months, ranging from 4.19±0.32 hours to 4.24±0.32 hours. The majority of the patients (n=34) were dialyzed through arteriovenous fistulae (85%). Permanent vascular catheters were used in 6 patients (15%). Effective blood flow increased from 216±26 ml/min to 234±15 ml/min. The dialysate flow increased from 545±108 to 665±151 ml/min.

Results of white blood cell (WBC) count, hemoglobin – Hb, hematocrit – Hct, mean corpuscular volume – MCV and platelet (PLT) count were analyzed every month. At the beginning of study, after 3 months and at the end of studies, dry body mass, body mass index (BMI), the blood pH and serum concentration of ionized calcium, phosphate, intact parathormone (iPTH), total protein, albumin, cholesterol, iron, ferritin, urea and creatinine were determined. Standard laboratory methods were applied for blood analysis. BMI was calculated by the formula of post-dialysis weight in kilograms per height in square meters.

Results are expressed as mean and one standard deviation or median and range of values. Distribution of values was

Figure 2. **Values of mean on-line Kt/V during six months of study**



[a]p<0.0009 as compared to result obtained in the first month of study
[b]p<0.041 as compared to result obtained in the second month of study

checked using Kołmogorow–Smirnov test. Statistical analysis included ANOVA test for repeated evaluations and ANOVA Friedman test with post hoc Scheffe test. Results of examined parameters were correlated to Kt/V values, using Spearman or Pearson correlation coefficients as appropriate. Two values of on-line Kt/V were used for correlations: simultaneously obtained Kt/V (simultaneous Kt/V) and mean Kt/V shown in the month proceeding blood analysis. A p value below 0.05 was considered as statistically significant.

Results

Values of Kt/V, obtained during six months of studies, are presented on *Fig. 2*. Mean on-line Kt/V was increasing from 1.09±0.16 in the first month to 1.16±0.13 in the third month (*Tab. 1*) and this higher value was maintained in the next three months of study. As assumed, there were no further significant changes in the mean on-line Kt/V after the second month of study.

The increase in Kt/V over six months period was accompanied by rising values of Hb, Hct, MCV, serum iron concentration, blood pH before and after IHD session as well as by decreasing values of serum iPTH. Statistically unchanged parameters included dry body mass, BMI, serum concentration of total protein, albumin, ionized calcium, phosphate, cholesterol, urea, creatinine and ferritin as well as WBC and PLT (*Tab. 2*).

There were correlations between Kt/V and serum concentration of examined parameters. For one parameter only the highest significant correlation coefficient shown with respective Kt/V during the entire study is presented. Positive correlations were found for phosphate (r=0.324, p=0.041 for simultaneous on-line Kt/V; r=0.370, p=0.019 for mean on-line Kt/V), iPTH (r=0.312, p=0.049 for simultaneous Kt/V; r=0.314, p=0.048 for mean Kt/V) and ferritin (r=0.417, p=0.007 for simultaneous Kt/V), whereas negative correlations – for Hb (r=-0.369, p=0.019 for simultaneous Kt/V; r=-0.376, p=0.017 for mean Kt/V) and Hct (r=-0.365, p=0.021 for simultaneous Kt/V; r=-0.374, p=0.017 for mean Kt/V).

Table 1. **Changes in effective blood flow and dialysate flow during three months of studies**

	First month			Third month		
Patient	Effective blood flow ml/min	Dialysate flow ml/min	Mean monthly Kt/V	Effective blood flow ml/min	Dialysate flow ml/min	Mean monthly Kt/V
1	237	500	1.09	239	800	1.10
2	190	500	1.06	234	500	1.07
3	190	500	0.98	233	500	1.03
4	233	500	1.06	250	500	1.08
5	207	800	1.19	233	800	1.30
6	234	500	1.15	236	800	1.23
7	237	500	1.42	240	800	1.45
8	234	500	1.01	237	800	1.11
9	188	500	1.11	206	500	1.17
10	235	500	1.19	237	800	1.24
11	244	500	1.27	245	800	1.37
12	228	500	0.94	232	800	1.06
13	188	500	0.88	208	500	1.00
14	237	500	1.16	242	800	1.17
15	235	500	1.09	247	800	1.11
16	188	500	1.22	210	500	1.30
17	235	500	1.24	236	800	1.36
18	235	500	1.03	242	800	1.06
19	242	500	0.94	243	800	1.07
20	186	800	1.14	225	800	1.32
21	236	500	1.22	248	800	1.32
22	225	500	1.40	231	500	1.38
23	200	500	1.11	214	500	1.12
24	227	500	1.06	231	800	1.09
25	187	500	0.59	222	500	0.87
26	138	500	0.91	187	500	1.02
27	189	500	0.84	231	500	1.02
28	185	500	1.01	232	500	1.11
29	234	500	1.22	249	800	1.36
30	224	500	0.84	232	500	1.10
31	234	800	0.96	256	800	1.05
32	190	500	1.22	233	500	1.19
33	239	500	1.05	257	500	1.07
34	244	500	1.24	260	500	1.28
35	172	500	1.08	208	500	1.13
36	191	800	1.05	234	800	1.19
37	228	800	1.11	234	800	1.14
38	236	500	1.09	261	500	1.12
39	227	500	1.11	234	800	1.14
40	239	800	1.32	234	800	1.33
mean	216	545	1.09	234	665	1.16
SD	26	108	0.16	15	151	0.13
median	228	500	1.09	234	800	1.125
range	138-244	500-800	0,59-1,42	187-261	500-800	0,87-1,45

Simultaneous on-line Kt/V showed correlation with the difference between post- and pre-dialysis serum creatinine level (r=0.354, p=0.025).

A positive correlation was shown between duration of treatment with IHD and on-line Kt/V (r=0.572, p=0.000 for simultaneous Kt/V; r=0.606, p=0.000 for mean Kt/V) and ultra-

Table 2. **Changes in examined parameters in the course of treatment with intermittent hemodialysis (IHD) with increasing values of on-line Kt/V**

Parameter	The beginning of study	The end of study	p value
hemoglobin (g/l)	99.1±16.6	105.1±12.5	0.002
hematocrit (%)	31.6±5.2	33.8±3.6	0.004
mean corpuscular volume (fl)	95.9±7.7	100.7±5.7	0.000
iron (μg/dl)*	58.2±29.6	73.2±27.8	0.002
pH before IHD session	7.26±0.04	7.41±0.04	0.000
pH after IHD session	7.34±0.05	7.48±0.05	0.000
intact parathormone (pg/ml)*	918 (38-3500)	420 (15-4341)	0.036
total protein (g/l)*	69.1±5.7	70.9±4.8	NS
albumin (g/l)	40.0±3.3	40.9±3.2	NS
ionized calcium (mmol/l)*	1.13±0.13	1.13±0.10	NS
phosphates (mmol/l)*	1.85±0.48	1.74±0.65	NS
cholesterol (mmol/l)*	5.26±1.27	4.95±1.60	NS
urea (mmol/l)*	19.1±4.0	17.9±3.7	NS
creatinine (μmol/l)*	752±179	742±178	NS
ferritin (ng/dl)*	740±558	632±346	NS
white blood cells (K/ml)	8.60±3.89	6.52±1.50	NS
platelet count (K/ml)	251±91	195±60	NS
dry body mass (kg)	70.4±15.6	70.9±16.1	NS
body mass index (kg/m^2)	28.2±6.7	28.2±6.8	NS

* serum concentration
NS – non significant

filtration volume per dialysis session (r=0.370, p=0.018). Duration of dialysis session was positively related to patients' height (r=0.428, p=0.006) and dry body mass (r=0.547, p=0.0001).

Inverse correlations were found for adequacy parameters and patient's characteristics: height (r=-0.510, p=0.000 for simultaneous Kt/V; r=-0.436, p=0.005 for mean Kt/V) and dry body mass (r=-0.362, p=0.021 for simultaneous Kt/V; r=-0.399, p=0.011 for mean Kt/V).

Discussion

The technique of Kt/V estimation, based on the use of a conductivity method, is at present possible in the newer dialysis machines. It enables frequent precise Kt/V monitoring, adjusted to patient's needs. In our study we could observe beneficial blood changes, occurring with the Kt/V increase.

Already, in 1983, Harter [4] have found that reducing dialysis dose as reflected by increasing the blood concentration of urea nitrogen averaged with respect to time (TAC_{urea}) significantly reduced Hct and Hb and increased the transfusion requirements. In 1996 Ifudu et al. [5] showed positive effect of higher URR (72%) on Hct compared to their standard URR (61%). Like in our study, patients were treated with a fixed dose of erythropoietin. Ifudu et al. [5] analyzed two different groups of patients with similar Hct at the start of observation, whereas in our study the dialysis dose was increasing in the same patients. Results from the United States Renal Data System, published in 1997, confirmed a correlation between dialysis dose and Hct level in IHD patients treated with erythropoietin [6]. In 2001, it was shown that adequate IHD diminished requirement for recombinant erythropoietin, even in cases in which celulose dialysis membranes were used [7]. In mentioned study, Hct did not correlate with Kt/V, whereas erythropoietin dose and Kt/V were inversely correlated [7]. In 2003, Salahudeen et al. [8] presented data showing Hct in patients with second generation spKt/V ranging from <1.23 to >1.68. There were no significant differences in Hct between groups, however, patients with highest Kt/V had significantly lower serum concentration of creatinine and pre-albumin, what may indicate undernutrition, although serum albumin level was also similar in all spKt/V groups [8]. In our study, an increase in Kt/V was not accompanied with decreased nutritional parameters like dry body mass, BMI, or serum concentration of total protein, albumin, urea, creatinine and cholesterol. Serum ionized calcium concentration was also insensitive to changes in Kt/V. In studies of Harter [4] plasma calcium, cholesterol and triglyceride levels did not show significant changes with higher TAC_{urea}, but less adequate IHD correlated with increased risk from cardiovascular morbidity.

When patients with known causes of resistence to erythropoietin treatment (iron depletion, aluminium overload, severe hyperparathyroidism, acute or chronic infections) are excluded from the study, the beneficial effect of higher IHD adequacy on Hct may be related to removal of dialysable low-molecular-weight inhibitors of erythropoiesis, like spermine and/or polyamine [9,10]. In our study, this effect is also possible, but additionally the influences of serum iPTH decrease, serum iron concentration increase and less pronounced metabolic acidosis have to be considered as contributing factors. Beneficial effects of more effective IHD on plasma PTH and correction of metabolic acidosis were also seen by Harter [4], when results obtained with high TAC_{urea} (100 mg/dl) were compared to respective values shown with TAC_{urea} of 50 mg/dl.

Significant increase in MCV may be related to higher serum iron level as well as to probably greater removal of folic acid with higher IHD adequacy, but with fixed vitamin supplementation.

Correlations between Kt/V and below mentioned parameters indicate that higher IHD adequacy was applied to uremic patients in poorer clinical status. These patients showed higher serum concentrations of phosphate, iPTH, and ferritin as a marker of inflammation, and lower Hb concentration and Hct. Greater IHD adequacy in underdialyzed patients could evidently contribute in clinical improvement.

In our study, higher doses of dialysis and greater ultrafiltration volumes per session were provided to patients treated longer with IHD than those being on IHD on shorter period of time. Such clinical intervention may be related to decrease in residual renal function and to increase in inflammatory catabolic state over time.

In IHD patients, dry body mass and BMI were progressively lower with values of the second generation spKt/V increasing from <1.23 to >1.68 [8]. In other study, overweight patients received less dialysis as measured by spKt/V, and conversely, those with lower BMI received higher spKt/V [11]. In our study, Kt/V was inversely correlated with dry body mass and height, what is in agreement with cited data. Such negative correlation occurred despite longer dialysis sessions provided to patients

with greater dry body mass and height. As expected, Kt/V was greater in patients who received longer dialysis sessions.

Increases in dialysate flow and/or in dialysis duration lead to higher costs of IHD session, but optimizing erythropoietin responsiveness and iron utilisation an adequate dialysis treatment can contribute to a reduction of the costs of maintenance dialytic therapy.

In summary, our results indicate that even small increase in IHD adequacy may be accompanied by beneficial changes in management of uremic patients (better response on erythropoietin, diminished laboratory features of secondary hyperparathyroidism, better iron utilisation). Correlation between Kt/V and examined parameters indicate that higher IHD doses were provided to patients in more advanced uremic state. It may partially explain advantages observed with incremental IHD adequacy.

Acknowledgement

The paper was orally presented at the 11th International Symposium on Hemodialysis, Tampa (Florida, USA), 28.02 – 2.03.2005.

References

1. Puka J, Rutkowski B, Lichodziejewska-Niemierko M, Grenda R, Czekalski S, Lao M, Rowiński W, Bautembach S. Raport o stanie leczenia nerkozastępczego w Polsce – 2003 (Report on the renal replacement therapy in Poland – 2003). Ed. MAKmedia, Gdańsk, 2004.

2. Puka J, Rutkowski B, Lichodziejewska-Niemierko M, Lao M, Rowiński W, Grenda R, Czekalski S, Bautembach S. Raport o stanie leczenia nerkozastępczego w Polsce – 2002 (Report on the renal replacement therapy in Poland – 2002). Ed. Akademia Medyczna w Gdańsku, Gdańsk, 2003.

3. Watson PE, Watson ID, Batt RD. Total body water volumes for adult males estimated from simple anthropometric measurements. Am J Clin Nutr, 1980; 33: 27-39.

4. Harter HR. Review of significant findings from the National Cooperative Dialysis Study and recommendations. Kidney Int, 1983; 23(Suppl. 13): S-107-12.

5. Ifudu O, Feldman J, Friedman EA. The intensity of hemodialysis and the response to erythropoietin in patients with end-stage renal disease. N Eng J Med, 1996; 334(7): 420-5.

6. Young EW, Woods JW, Segieda GF, Held PJ, Port FK, Bloembergen WE. Predictors of target hematocrit among erythropoietin-treated HD patients. Am Soc Nephrol, 1997; 8: 259A.

7. Movilli E, Cancarini GC, Zani R, Camerini C, Sandrini M, Maiorca R. Adequacy of dialysis reduces the doses of recombinant erythropoietin independently from the use of biocompatibile membranes in haemodialysis patients. Nephrol Dial Transplant, 2001; 16(1): 111-4.

8. Salahudeen AK, Dykes P, May W. Risk factors for higher mortality at the highest levels of spKt/V in haemodialysis patients. Nephrol Dial Transplant, 2003; 18(7): 1339-44.

9. Radtke HW, Rege AB, La Marche MB, Bartos D, Campbell RA, Fisher JW. Identification of spermine as an inhibitor of erythropoiesis in patients with chronic renal failure. J Clin Invest, 1981; 67(6): 1623-9.

10. Vanholder R, De Smet R, Hsu C, Vogeleere P, Ringoir S. Uremic toxicity: the middle molecule hypothesis revisited. Semin Nephrol, 1994; 14(3): 205-18.

11. Salahudeen AK, Fleischmann EH, Bower JD. Impact of lower delivered Kt/V on the survival of overweight patients on hemodialysis. Kidney Int, 1999; 56(6): 2254-9.

Patients′ quality assessment of ambulatory obstetric and gynaecological services

Bojar I*, Wdowiak L, Ostrowski T

Management and Economics of Health Care Department, Lublin Medical University, Poland

Abstract

Purpose: The quality could be assessed from two perspectives: internal and external. From the internal perspective the quality means being consistent with particular conditions and standards. The external perspective is based on relative assessment of the product made by a client who is also aware of other competitors' offer. Despite the professional assessment which is focused on providing health services according to medical and managerial correctness, patient's assessment is also relevant. Measuring patient's satisfaction is additional method of health services quality assessment.

The aim of the study was to estimate patients' opinion on quality of ambulatory obstetric and gynaecological services.

Material and methods: The study was conducted in 11 obstetric and gynaecological out-patient clinics of Lublin in September and October 2003. The study tool was an author's questionnaire. Patients were asked to assess such areas as registration before visit, their relationship with nurses and gynaecologists and other aspects of services provision like intimacy assurance and respecting Patient's Rights. The collected data was statistically analysed.

Results: 635 patients took part in the study. The biggest groups in the studied population were women at the age of 20-30 years, married, living in cities and secondary educated. It was found that around half of the population is satisfied and 2.2% of them are unsatisfied with the fact that they have chosen particular out-patient clinic. More than 70% of women had positive opinion of following aspects influancing general opinion about service quality: politness of reception desk staff, opening hours, the length of time before a patient is seen by the specialist, intimacy assurance and respect for Patient's Rights in the practice. 80% of patients were satisfied with the relationship with nurses and 3.3% were unsatisfied with it. Eight patients out of ten were satisfied with gynaecological care, less than 2% were unsatisfied. The studied women had also high opinion of the course of visit. More than 90% of studied patients trust their gynaecologists.

Conclusions: More satisfied with the services provided by the obstetric and gynaecological out-patient clinics were women living in cities than in villages and those visiting nonpublic than public practices.

Key words: quality assessment, obstetric and gynaecological services, patient's satisfaction.

Introduction

The quality could be assessed from two perspectives: internal and external. From the internal perspective the quality means being consistent with particular conditions and standards. The external perspective is based on relative assessment of the product made by a client who is also aware of other competitors' offer. Despite the professional assessment which is focused on providing health services according to medical and managerial correctness, patient's assessment is also relevant. Measuring patient's satisfaction is additional method of health services quality assessment.

Present interest in quality of health care is connected with standardization of offered services, patient's right to choose, increased patients' expectations and increasing requirements of auditing organisations regarding health care services.

The aim of the study was to estimate patients' opinion on quality of ambulatory obstetric and gynaecological services.

* CORRESPONDING AUTHOR:
Management and Economics of Health Care Department,
Lublin Medical University
ul. Chodźki 1, 20-093 Lublin
Tel/Fax: +48 81 7405752
e-mail: ibojar@poczta.onet.pl (Iwona Bojar)

Received 25.05.2005 Accepted 03.06.2005

Figure 1. **Patients' satisfaction with the choice of out-patient obstetric and gynaecological clinic**

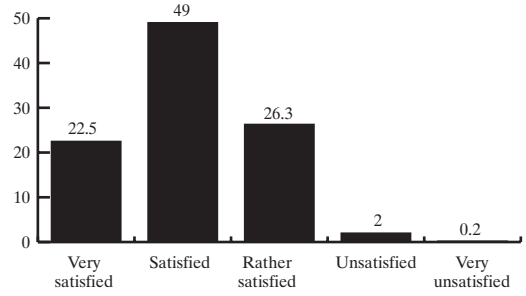


Figure 2. **The relations with nurses and midwives in patients' opinion**

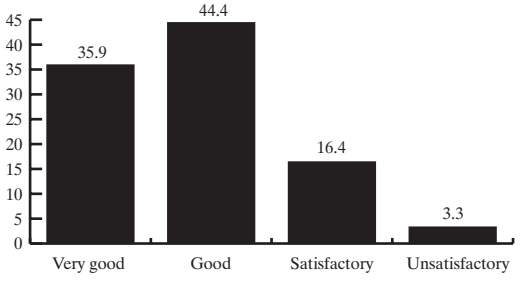
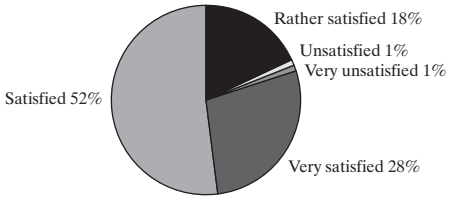


Table 1.

	Opening hours	Possibility of phone registration	Awaiting time for a visit	Politness of reception desk staff	Intimacy assurance	Respect for Patient's Rights
Yes	92%	87.7%	80.9%	87.7%	95.8%	87.6%
No	8%	12.3%	19.1%	12.3%	4.2%	12.4%

Figure 3. **Patients' satisfaction with gynaecological care of out-patient clinics**



Material and methods

The study was conducted in randomized obstetric and gynaecological out-patient clinics of Lublin in September and October 2003. Public (5) and non-public (6) clinics that had contract for 2003 with Lublin Department of National Health Fund were included in the study. The study tool was an author's questionnaire. Patients were asked to assess such areas as registration before visit, their relationship with nurses and gynaecologists and other aspects of services provision like intimacy assurance and respecting Patient's Rights. The collected data was statistically analysed using computer programme Statistica 6.0. Chi-square test was used to find significant correlation between two values. P value <0.05 was set up as being statistically significant.

Results

635 patients took part in the study. The biggest groups in the studied population were women at the age of 20-30 years, married, living in cities and secondary educated. Before detailed analysis of satisfaction with various aspects of organisation functioning patients could estimate their general satisfaction with the choice of particular obstetric and gynaecologic clinic they made. Around half of the population (49%) is satisfied, some of them even very satisfied and 2.2% are unsatisfied with the fact that they have chosen particular out-patient clinic (*Fig. 1*).

More than 70% of women had positive opinion of following aspects influencing general opinion about service quality: politness of reception desk staff, opening hours, the length of time before a patient is seen by the specialist, intimacy assurance and respect for Patient's Rights in the practice (*Tab. 1*).

In patients' opinion the most inconvenient aspect is too long waiting time before a patient is seen by a specialist.

In most patients' opinion the relationship with nurses of out-patient clinics (e.g. in treatment room) is good (44.4%) or very good (35.9%) with. For 16,4% studied women the relationship is satisfactory and 3.3% patients are unsatisfied with it (*Fig. 2*).

All divorced and widows had no remarks on nurses and midwives' work. Single patients were more critical than the others regarding nurses ($p<0.05$).

University and elementary educated women are the most satisfied with nurses' work. Less satisfied were secondary and technical educated women ($p<0.05$).

More patients living in voivodship city and any other city than those from villages considered that relationship with nurses of clinics were only satisfactory ($p<0.05$).

Most patients of non-public clinics evaluated the relationship with nurses and midwives as very good (41.83%) whereas patients of public clinics evaluated it as good (47.85%).

Eight patients out of ten were satisfied with gynaecological care (28% – very satisfied), every fifth woman is rather satisfied less than 2% are unsatisfied (*Fig. 3*).

The studied women had also high opinion of the course of visit. Almost all patients considered that a doctor carefully listened to them during a visit (93.5%). Nearly 92% of women are always informed about planned treatment and 94% patients said that a doctor gives information on how to take prescribed medications. Patients had a little worse opinion on examination during the visit. Every third said that a doctor not always examined her by during the visit. More than 90% of studied patients trust their gynaecologists.

The most satisfied with the services provided by the obstetric and gynaecological out-patient clinics were women living in voivodship city, less satisfied were those living in any other cities and women coming from villages were the least satisfied. Also those who have good financial conditions were more satisfied with the quality of gynaecological care.

The type of out-patient clinics had an influence on patients' satisfaction with obstetric and gynaecological services. Patients visiting non-public are generally more satisfied with services than patients of public clinics. It can be seen both in higher satisfaction with the choice of particular clinic and general satisfaction with gynaecological care.

Discussion

Most patients of obstetric and gynaecological clinics (88%) said that the staff of reception desk was nice and polite. These opinions are better than those observed in the study carried out by CBOS in which half of the population has no critical remarks on health care administrative workers attitude towards patients. 40% of patients said that they were treated politely and nicely. However, 9% of women are unsatisfied with the way they were treated by administrative staff of health care organisations [1].

The relations with nurses and midwives of clinics were evaluated as good and very good by most patients. The results are similar to those obtained in the study of CBOS in which 60% of population said that they were always treated properly by doctors and nurses, 36% considered that the way they were treated was usually proper. Few patients have critical opinion about medical staff attitude towards them (3%). [1] It should emphasized that according to U.S. studies nearly 80% of patients' satisfaction depends on nurses [2].

Patients expect professionalism and kindness from a doctor which is emphasized by different authors [3,4]. The ability of accurate communication with a patient has greater meaning than duration of visit and their recurrence. Patients satisfaction – measure of quality of care is closely connected with doctors' communicative skills [5].

The course of visit was evaluated quite high by patients. The results are similar to those obtained in CBOS study in which 90% of studied group said that they were always or usually clearly informed by physicians, nurses or other health care workers [6].

According to Hupert [7] 94% of patients considered that a doctor of out-patient clinic is friendly and disinterested and examines patients thoroughly and those aspects were evaluated higher than in the author's study [7].

The patients' opinion about trust in their doctors were very positive. These results are far better than those obtained in 2001 by CBOS where 16% of people declared very high trust in their doctors, 64% quite high, 13% quite low, 1% very low and 5% could not describe their trust in a doctor [8].

Conclusions

Patients are generally satisfied with the services of out-patient clinics.

High and very high opinion about functioning of out-patient clinics is connected with:

1. high and very high opinion of registration desk work organisation,
2. high and very high opinion about relation with nurses and midwives,
3. high satisfaction with gynaecological care,
4. high opinion about gynaecologist's qualifications and trust in physician.

References

1. Opinie o stosunku do pacjentów w placówkach opieki zdrowotnej. Komunikat z badań. Warszawa: CBOS; 2001.
2. Eriksen LR. Patent satisfaction: an indicator of nursing care quality? Nurse Manage, 1987; 18(7): 31-5.
3. Cieślik P. Personel medyczny w oczach pacjentów. Służba Zdrowia, 2002; 5(8): 3101-2.
4. Kabacińska B. Satysfakcja pacjentów hospitalizowanych na oddziałach chirurgicznych i internistycznych w szpitalach województwa wielkopolskiego – analiza wybranych aspektów. Zdrowie i Zarządzanie. 2002; IV(3-4): 85-7.
5. Marcinkowski JT, Stachowska M, Maciejewski S. Priorytetowe znaczenie umiejętności komunikowania się z pacjentem w praktyce lekarskiej. Materiały pokonferencyjne Międzynarodowego Sympozjum Naukowego Dni Medycyny Społecznej i Zdrowia Publicznego „Między Profilaktyką a Medycyną Kliniczną". Poznań, 2003.
6. Informowanie pacjentów o stanie zdrowia i sposobie leczenia. Komunikat z badań. Warszawa: CBOS; 2001.
7. Hupert Z. Jakość w opiece medycznej koncepcja pomiaru, IMW. Lublin, 2003.
8. Opinie o błędach medycznych i zaufaniu do lekarzy. Komunikat z badań. Warszawa: CBOS; 2001.

Serum isoprostanes levels in patients after abdominal hysterectomy

Szymczyk G[1], Bełtowski J[2]*, Marciniak A[2], Kotarski J[1]

[1] 1st Department of Gynaecology, Medical University, Lublin, Poland
[2] Department of Pathophysiology, Medical University, Lublin, Poland

Abstract

Purpose: Reactive oxygen species (ROS), continuously generated in tissues, are involved in signal transduction under physiological conditions. The amount of ROS increases in response to surgical trauma. Isoprostanes are novel sensitive and specific markers of lipid peroxidation in vivo. Plasma concentration of isoprostanes increases in patients with various diseases associated with oxidative stress. In the present study we investigated the effect of abdominal hysterectomy on serum isoprostanes concentration.

Material and methods: The study was performed in 20 women (aged 45-63, average 50.3) who had undergone total abdominal hysterectomy with salpingooophorectomy, operated for benign diseases in the 1st Department of Gynaecology of Lublin Medical University. Isoprostanes were assayed by enzyme immunoassay (EIA) using 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA).

Results: Serum concentration of isoprostanes before the surgery had value 38.9±10.7 pg/ml and it decreased at 8, 24 and 96 h after the operation.

Conclusions: Measurement of serum isoprostanes in small group of patients after hysterectomy did not brought the clear answer if the assessment of isoprostanes levels is a valuable method for evaluation of oxidative stress after a surgery.

Key words: abdominal hysterectomy, isoprostanes, reactive oxygen species.

* CORRESPONDING AUTHOR:
Department of Pathophysiology
Medical University
ul. Jaczewskiego 8, 20-093 Lublin, Poland
Tel/Fax: +48 81 742 58 35 (Jerzy Bełtowski)

Received 22.11.2005 Accepted 12.04.2005

Introduction

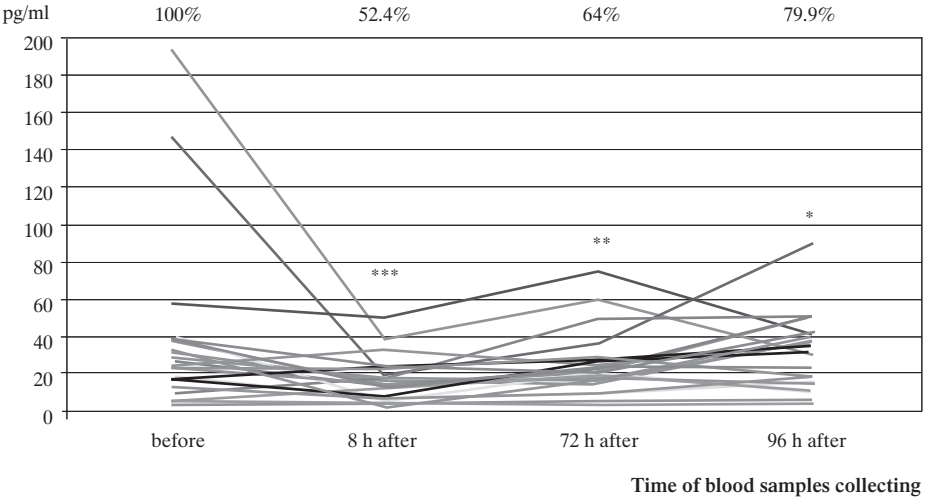
Reactive oxygen species (ROS), continuously generated in tissues, are involved in signal transduction under physiological conditions. Increased amount of ROS leads to damage of biologically relevant macromolecules including lipids, proteins and nucleic acids [1]. The amount of ROS increases in response to surgical trauma [2]. The level of lipid peroxidation products is often used as a marker of oxidative stress [1,3]. However, in most studies addressing the effect of surgery on oxidant antioxidant balance the nonspecific markers of lipid peroxidation such as thiobarbituric acid reactive substances (TBARS) or malonyldialdehyde (MDA) are measured. Thus we have recently demonstrated that the concentration of malonyldialdehyde + 4-hydroxyalkenals (MDA+4-HDA) as well as lipid hydroperoxides in serum have been increased after abdominal hysterectomy [4]. Isoprostanes are novel sensitive and specific markers of lipid peroxidation in vivo. These compounds are formed during ROS-mediated nonenzymatic peroxidation of arachidonic acid and other polyunsaturated fatty acids [5-9]. Plasma concentration of isoprostanes increases in patients with various diseases associated with oxidative stress [5,6,8].

Nevertheless, to our knowledge the level of isoprostanes after gynecological surgery has not been studied so far. In the present study we investigated the effect of abdominal hysterectomy on serum isoprostanes concentration to perform the evaluation of oxidative stress level in different methods of gynaecological operations in future.

Material and methods

The study was performed in 20 women (aged 45-63, average 50.3) who had undergone total abdominal hysterectomy with salpingooophorectomy, operated for benign diseases in the 1st Department of Gynaecology of Lublin Medical University. Average BMI of patients was 29.2. Peripheral blood samples were collected from patients before surgery as well as at 8, 24

Figure 1. **Serum isoprostanes levels**



* p<0.05; ** p<0.01; *** p<0.001 versus before the operation

and 96 hours after the operation. Blood samples were allowed to clot for 1 hour at a room temperature and centrifuged at 2000 x g for 10 minutes to separate serum. Serum samples were frozen and stored at -70°C until analysis.

Isoprostanes were assayed by enzyme immunoassay (EIA) using 8-isoprostane EIA kit (Cayman Chemical, Ann Arbor, MI, USA). Serum samples were mixed with ethanol and centrifuged to remove particulate matter. Ethanol was evaporated from the supernatant under a stream of nitrogen. Then, supernatant was acidified with acetate buffer to pH 4.0 and isoprostanes were extracted using C-18 SPE cartridges (Waters Corporation, Milford, MA). Cartridges were activated by rinsing with 5 ml methanol and 5 ml H_2O, and then the sample was passed through the cartridge. The cartridge was rinsed with 5 ml of water, dried, and rinsed with 5 ml of HPLC grade hexane. 8-isoprostanes were eluted with 5 ml ethyl acetate containing 1% methanol. The solvent was evaporated to dryness; the sample was dissolved in 450 µl of EIA buffer and used for the analysis. All samples were assayed in duplicate; before purification one set of samples was spiked with 8-isoprostane standard contained in the kit to correct for individual recovery. The recovery averaged 76%. The limit of the sensitivity of the assay was 5 pg/ml and the intraassay coefficient of variation was 8%. The nonparametric Wilcoxon's test was used to compare the data at different time points. The relationships between variables were evaluated by Spearman's rank correlation test. $p<0.05$ was considered significant. The Bioethical Committee at the Medical University in Lublin approved the investigation and all patients gave their informed consent to participate in it.

Results

Serum concentration of isoprostanes before the surgery had value 38.9±10.7 pg/ml and it decreased by 47.6% at 8 hours after the operation. Subsequently, isoprostane concentration progressively increased, however, it was still significantly reduced in comparison to preoperative values at 24 h (-36.0%) and at 96 h (-21.1%) after the operation (*Fig. 1*).

Table 1. **The characteristics of morphotic elements and biochemical parameters of patients' blood**

Morphotic blood elements		
Haemoglobin (g/dl)	Before operation	13.4
	24 h after the operation	12.2
Haematocrit (%)	Before operation	39.97
	24 h after the operation	36.44
Leukocyte count (K/µl)	Before operation	7.049
	24 h after the operation	10.41
Erythrocyte count (M/µl)	Before operation	4.707
	24 h after the operation	4.28
Thrombocyte count (K/µl)	Before operation	301.7
	24 h after the operation	278
Biochemical parameters of blood before operation		
Protein level (g/dl)	7.295	
Total cholesterol level (mg/dl)	213.2	
HDL level (mg/dl)	55.6	
LDL level (mg/dl)	133.5	
Triglicerides (mg/dl)	128.8	

The changes of blood parameters of patients before and after the operation are shown in *Tab. 1*. The granulocyte count increased by 47.7% after the surgery. The erythrocyte count decreased by 8.1%, thrombocyte count by 7.9% and haematocrit by 8.8% 24 hours after the operation. Patients were hospitalised for 5-8 days (average 6.6) after the operation.

Discussion

The generation of ROS increases after surgery as a result of tissue ischemia associated with anesthesia-induced hypotension as well as due to acute phase response which stimulates oxidative burst of phagocytes [3]. The increase of granulocyte count, observed after surgery, may suggest the induction of acute phase response, observed in our present and former investigations. Consistently with this, we have recently observed increase in plasma concentration of 'classical' lipid peroxidation products such as MDA+4-HDA and lipid hydroperoxides after abdominal hysterectomy [4]. Serum concentration of isoprostanes increases in patients with diseases associated with oxidative stress such as diabetes mellitus, hypercholesterolemia, hyperhomocysteinemia and connective tissue diseases [5-8]. Unexpectedly, in the present study we observed decrease in serum isoprostanes between 8 and 96 hours after surgery. The reason why MDA+4-HDA and isoprostanes change in the opposite directions is unclear at present. When we pass over the scattering of isoprostanes concentrations before the experiment we have to look for different explanation of this phenomenon. MDA and 4-HDA are low molecular weight compounds which can freely diffuse from tissues to plasma. It may be speculated that oxidative stress after surgery occurs mainly in injured tissues and most MDA originates in extravascular compartment. Thus, tissue oxidative stress will be only partially reflected by plasma lipid peroxidation markers. In contrast, isoprostanes are fatty acid derivatives and most of them is associated with triglycerides and phospholipids. Therefore, isoprostanes will very slowly exchange between tissue and plasma pools. Serum isoprostanes originate mainly during peroxidation of plasma lipoproteins and therefore may not adequately reflect the local oxidative stress, especially in the short run. Secondly, plasma lipoprotein level decreases after surgery as a result of hemodilution and acute phase response [10,11], which could lead to decrease in isoprostanes due to reduced availability of substrates for peroxidation. Finally, we have previously demonstrated that the activity of paraoxonase-1 (PON1) – an antioxidant enzyme contained in high-density lipoproteins – decreases following the surgery [12]. PON1 hydrolyses oxidised phospholipids and releases free isoprostanes [13,14]. Because in the method used by us mainly free non-estrified isoprostanes are detected, it is possible that reduced PON1 activity could contribute to fall in isoprostanes following the operation. Interestingly, two studies reported increase in plasma isoprostanes following coronary artery bypass grafting (CABG), however, the increase was short-lasting and observed only until less than 1 hour from the beginning of the operation [15,16]. Although we did not analysed blood samples obtained so early, one should take into account that it will be expected CABG causes much more marked oxidative stress.

Conclusion

In our initial studies we have observed decrease of serum isoprostanes levels after abdominal hysterectomy. These data suggest that measurement of serum isoprostanes in small group of patients after hysterectomy did not brought the clear answer if the assessment of isoprostanes levels is a valuable method for measurement of oxidative stress after operations.

Acknowledgement

The studies were funded by The Polish State Committee for Scientific Research grant 3PO5E 082 23.

References

1. Halliwell B. Free radicals, antioxidants, and human disease: curiosity, cause, or consequence? The Lancet, 1994; 344: 721-4.
2. Sane AS, Chokshi SA, Mishra VV, Barad DP, Shah VC, Nagpal S. Serum lipoperoxide levels in surgical stress of abdominal hysterectomy. Panminerva Medica, 1993; 35: 131-3.
3. Frei B, Stocker R, Ames B. Antioxidant defences and lipid peroxidation in human blood plasma. Proc Natl Acad Sci USA, 1988; 85: 9748-52.
4. Szymczyk G, Bełtowski J, Marciniak A, Kotarski J. Assessment of serum lipid peroxide levels and antioxidant status in females who had undergone total abdominal hysterectomy without closing of peritoneum. Gin Pol, 2003; 74: 1397-403.
5. Fam SS, Morrow JD. The isoprostanes: unique products of arachidonic acid oxidation – a review. Curr Med Chem, 2003; 10: 1723-40.
6. Cracowski JL, Durand T, Bessard G. Isoprostanes as a biomarker of lipid peroxidation in humans: physiology, pharmacology and clinical implications. Trends Pharmacol Sci, 2002; 23: 360-6.
7. Pratico D, Lawson JA, Rokach J, Fitzgerald GA. The isoprostanes in biology and medicine. Trends Endocrinol Metab, 2001; 12: 243-7.
8. Roberts LJ, Morrow JD. Measurement of F(2)-isoprostanes as an index of oxidative stress in vivo. Free Radic Biol Med, 2000; 28: 505-13.
9. Lawson JA, Rokach J, Fitzgerald GA. Isoprostanes: formation, analysis and use as indices of lipid peroxidation in vivo. J Biol Chem, 1999; 274: 24441-4.
10. Juvonen T, Kervinen K, Kairaluoma MI, Kesaniemi YA. Effect of cholecystectomy on plasma lipid and lipoprotein levels. Hepatogastroenterology, 1995; 42: 377-82.
11. Guvener M, Ucar I, Ozkan M, Dogan OF, Serter FT, Pasaoglu I. Effect of cardiopulmonary bypass on plasma levels of lipoprotein (a) in hypercholesterolemic patients. Jpn Heart J, 2001; 42: 563-74.
12. Szymczyk G, Bełtowski J, Maciejczyk-Pencuła M., Kotarski J. Assessment of paraoxonse activity in the early postoperative period after pelvic operations in women. Gin Pol, 2003; 74(Suppl. 1): 212.
13. Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN, Kuksis A, Connelly PW. Multiple substrates for paraoxonase-1 during oxidation of phosphatidylcholine by peroxynitrite. Biochem Biophys Res Commun, 2002; 290: 391-6.
14. Ahmed Z, Ravandi A, Maguire GF, Emili A, Draganov D, La Du BN, Kuksis A, Connelly PW. Apolipoprotein A-I promotes the formation of phosphatidylcholine core aldehydes that are hydrolyzed by paraoxonase (PON-1) during high density lipoprotein oxidation with a peroxynitrite donor. J Biol Chem, 2001; 276: 24473-81.
15. Ansley DM, Xia Z, Dhaliwal BS. The relationship between plasma free 15-F2t-isoprostane concentration and early postoperative cardiac depression following warm heart surgery. J Thorac Cardiovasc Surg, 2003; 126: 1222-3.
16. Ulus AT, Aksoyek A, Ozkan M, Katircioglu SF, Basu S. Cardiopulmonary bypass as a cause of free radical-induced oxidative stress and enhanced blood-borne isoprostanes in humans. Free Radic Biol Med, 2003; 34: 911-7.

Binding of α_1-antichymotrypsin to the surface of lymphocytes – preliminary study

Sobieska M*

Department of Physiotherapy, Rheumatology and Rehabilitation, Karol Marcinkowski University of Medical Sciences, Poznań, Poland

Abstract

Purpose: The paper presents preliminary results of investigation of binding of α_1-antichymotrypsin to the surface of peripheral blood lymphocytes.

Material and methods: Pooled serum samples from healthy individuals served as source of α_1-antichymotrypsin for isolation using chromatography. Binding of α_1-antichymotrypsin to the surface of peripheral blood lymphocytes was measured by flow cytometry.

Results: Even on native cells α_1-antichymotrypsin may be detected. After incubation with isolated preparation the percentage of positive lymphocytes increased.

Conclusions: The presence of α_1-antichymotrypsin on the surface of lymphocytes may imply its regulatory role during acute phase response and early immune response.

Key words: α_1-antichymotrypsin, lymphocytes, flow cytometry.

Introduction

α_1-antichymotrypsin (ACT) belongs to human serum α_1-globulins. It is a glycoprotein, MW of about 68,000 Da, with 22.7% of carbohydrate content. It is encoded on chromosome 14, region q31-q32.3, the same area as antitrypsin gene. Both proteins are highly homologous, suggesting shared evolution history [1]. Biologic function of ACT is not entirely clear: it is a protease inhibitor, mainly of chymotrypsin, katepsin G and several others (to a lesser extent), however, much lower serum concentrations imply that its function as an antiprotease is rather a minor one [2]. Its role as an acute phase reactant appears much more important. In contrast to antitrypsin and alpha2-macroglobulin the concentration of ACT increases within 6 hours during acute phase response, similarly to CRP and SAA, making ACT one of the first line of acute phase reactants. It may also be involved in local regulation of homeostasis (or balance between proteases-antiproteases), among others in lungs, the predominant site of synthesis, and in brain, where it is synthesized by astrocytes [3].

In healthy individuals the serum concentration of ACT is 0.35-0.45 g/L. It increases in acute and chronic infections (mainly bacterial), in trauma, particularly in cases with massive necrosis or if polytrauma is accompanied by cranio-cerebral trauma [4]. There were also several reports that ACT is able to bind chromatin, similarly to CRP. It could thus participate in removal of cellular debris, preventing autoantibodies formation [5]. No homozygous deficiency of ACT was ever described, in contrast, only heterozygous deficiency clinically presented as decreased serum concentration [6,7].

There is a discrepancy in descriptions of glycans on ACT molecules: three or four glycosylations sites, bi- or triantennary glycans, consisting of NacGlc, Gal and Sia. If the glycosyaltion profile was investigated using crossed affinity immunoelectrophoresis with Concanavalin A as ligand, following variants are observed: first migrating, clearly separated, weakly reactive with ConA variant A1, variants A2, A3 and A4 hardly separated, but showing increasing reactivity with ConA. In particularly acute conditions and often in children a fifth variant (A5), almost non-migrating due to very strong reaction with ConA appears. Acute inflammatory conditions cause dramatic increase in ACT concentration and a shift towards biantennary glycans (higher reactivity with ConA). Particularly expressed changes were observed for cranio-cerebral trauma and burns with massive necrosis [8-10]. It seems that concentration of ACT reflects not the size, but the severity of damage of the

* CORRESPONDING AUTHOR:
Department of Physiotherapy, Rheumatology and Rehabilitation
Karol Marcinkowski University of Medical Sciences
ul. 28. Czerwca 1956 roku 135/147
61-545 Poznań, Poland
e-mail: magdula2811@poczta.onet.pl (Magdalena Sobieska)

Received 11.04.2005 Accepted 14.07.2005

central nervous system [11]. The prolonged (longer that one week) presence of the altered glycosylation profile is highly unfavourable prognostic factor, especially in patients with cranio-cerebral trauma [4].

All these data suggest that antichymotrypsin may exert an unknown biological role influencing lymphocytes during early stages of acute phase response. Some data were also obtained from observation of ACT behaviour in children suffering from food allergy. Decreased ACT concentration was observed in symptomatic children, whereas normalization of serum concentration accompanied tolerance [12]. To check this it seemed necessary to show that antichymotrypsin is able to bind with the surface of lymphocytes.

Material and methods

Isolation of antichymotrypsin was performed using affinity chromatography: first simple ion exchange chromatography to remove albumin and majority of gammaglobulins, then on a column with coupled polyclonal anti-ACT antibody. Both columns were washed with 0.1 M phosphate buffer. ACT was isolated from serum pool from healthy persons, showing normal concentration and glycosylations profile of ACT. In this way a crude preparation was obtained, but the concentration of ACT was significantly higher in comparison to some trace proteins. The preparation was finally dialyzed against PBS (+4°C, overnight). The concentration of ACT was measured using rocket immunoelectrophoresis acc. to Laurell [13]. Determination of glycosylation profile of the preparation obtained was done using crossed affinity immunoelectrophoresis with ConA [14].

The very preliminary data was obtained from indirect staining. As primary antibody, DakoCytomation anti-ACT (Code number A0022) was used, as secondary antibody: anti-rabbit IgG, conjugated with FITC (DakoCytomation, Dennmark). As polyclonal antibody and indirect staining caused too many problems, and no conjugated anti-ACT antibody was available, it was decided to perform coupling of anti-ACT antibody with FITC, using Fluorotag Conjugation kit (FITC-1) from SIGMA, USA. First experiments were performed to detect ACT on the surface of peripheral blood cells, both native (without any procedure) and incubated first with ACT preparation. Peripheral blood samples from routine laboratory (drawn with addition of EDTA) were used for binding experiments.

Next set of experiments was performed to show the binding of ACT to CD3+ T lymphocytes and CD19+ B lymphocytes (in both cases antibodies from DakoCytomation, anti CD3, RPE-Cγ5, and anti-CD19, RPE-Cγ5, were used). DakoCytomation antibodies were used according to prescription: 5 microliters per 100 microliters of whole blood, and also 5 microliters of anti-ACT-FITC antibody was used. As previously, samples of native cells were stained in parallel with cells incubated with ACT preparation (mean concentration 100 mg/l, mixed 1:1 with whole blood, 1 hour in RT, then 3x washing with PBS).

Results

The obtained anti-ACT/FITC conjugate had the concentration of 0.5 g/L and the molar protein/FITC ratio of about 0.8. First experiments using this antibody confirmed the presence of ACT to various extents on the surface of native leukocytes. Preincubation of whole blood with ACT preparation caused increase in the percentage of ACT positive cells, to various extents. Cells were gated as lymphocytes, monocytes and granulocytes acc. to routine forward scattering (FW-SC) versus right scattering (RT-SC), and only the percentage of positive cells (FITC – green fluorescence) was analyzed in each gate. Following controls were used:

– incubation of blood cells with another rabbit antibody;
– incubation of blood cells with normal rabbit serum;
– incubation of blood cells with normal goat serum;
– incubation of blood cells with secondary anti-rabbit IgG^{FITC} antibody only;
– incubation of blood cells with primary unconjugated a-ACT antibody only.

In none of those controls the positive staining was present.

The results of the above measurement showed that even for limited number of experiments (n=7) the statistically significant difference was observed in t-test for dependent variables (p=0.000083 for lymphocytes, 0.000112 for monocytes and 0.02375 for granulocytes). Thus, ACT can be detected on the surface of peripheral blood cells and preincubation of blood cells with ACT preparation increased the percentage of ACT positive cells.

Next set of experiments (n=9) was performed to show the binding of ACT to CD3+T lymphocytes and CD19+B lymphocytes. The summary of results is presented on *Fig. 1* – double staining with anti-ACT (FITC) and anti-CD3 (PE), on the *Fig. 2* – double staining with anti-ACT (FITC) and anti-CD19 (PE).

Discussion

It was detected that ACT is present not only on the surface of monocytes that produce it, on the surface of granulocytes that may engulf it in phagocytosis, but also on the surface of lymphocytes. This feature was not described before. Additionally it was shown that preincubation of lymphocytes with crude ACT preparation increased the percentage of lymphocytes bearing ACT on their surface and also that the intensity of staining with anti-ACT antibody increased after incubation. The main binding was observed on CD3- and CD19-lymphocytes, presumably NK cells.

Preincubation mimics naturally occurring increase of ACT at concentration in peripheral blood due to trauma or burn. In early acute phase response several mechanisms related to cellular activity are inhibited and mainly the unspecific immunity is involved in first stages of the reaction, also during unspecific inflammation occurring in Alzheimer's disease [15]. Thus it is possible that ACT may exert a regulatory role during these early events, possibly acting as anti-apoptotic agent [16]. Higher incidence of ACT on the surface of NK than T lymphocytes may

Figure 1. **Double staining (mean results for 9 experiments) with anti-ACT (FITC) and anti-CD3 (PE) – percentage of positive lymphocytes, native versus incubated with ACT preparation (point = mean, frame = SD, range = 1.96 SD)**

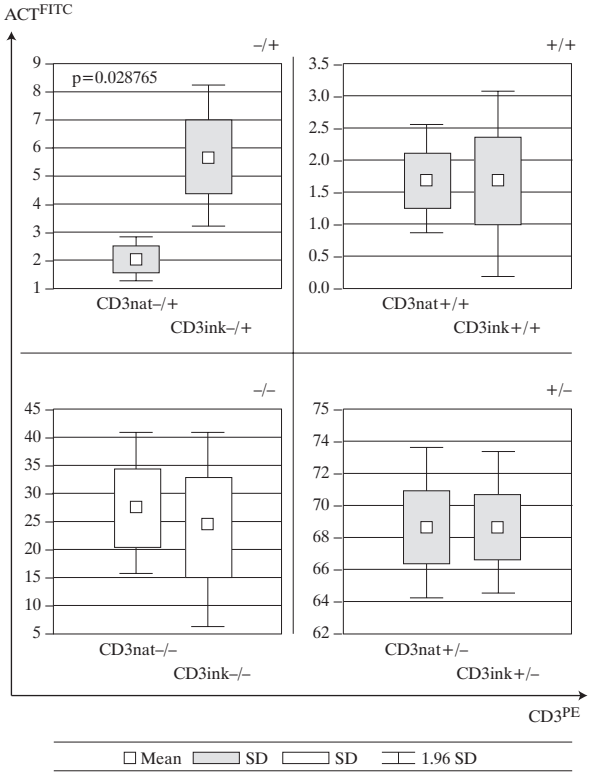
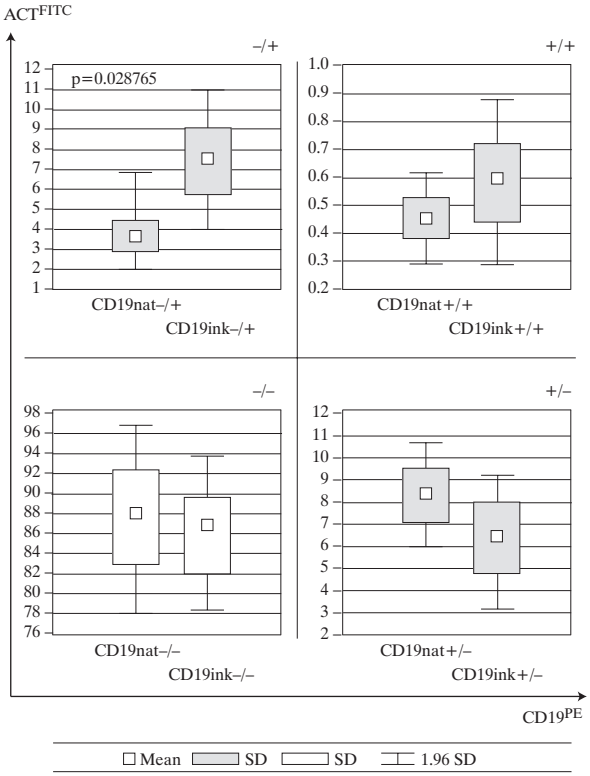


Figure 2. **Double staining (mean results for 9 experiments) with anti-ACT (FITC) and anti-CD19 (PE) – percentage of positive lymphocytes, native versus incubated with ACT preparation (point = mean, frame = SD, range = 1.96 SD)**



indicate its link with innate immunity. Weaker reaction with B lymphocytes might also point to the regulatory role of ACT in the specific response. It has to be investigated further what is the link between ACT presence on the surface and activity of particular lymphocytes subpopulations and whether there is any relationship between ACT glycosylations profile and its influence on lymphocytes. Further investigation will be performed to establish the binding particularly with NK cells.

Acknowledgement

Author wants to express gratitude to Mr. Artur Kostrzewa, MSc, for the excellent technical assistance with flow cytometry and to Professor Krzysztof Wiktorowicz for the support and discussion of results.

References

1. Vila N, Obach V, Revilla M, Oliva R, Chamorro A. α1-Antichymotrypsin Gene Polymorphism in Patients With Stroke. Stroke, 2000; 31: 2103-5.

2. Gooptu B, Hazes B, Chang W-SW, Dafforn TR, Carrell RW, Read RJ, Lomas DA. Inactive conformation of the serpin a1-antichymotrypsin indicates two-stage insertion of the reactive loop: Implications for inhibitory function and conformational disease. PNAS, 2000; 97(1): 67–72.

3. Ikari Y, Mulvihill E, Schwartz SM. α1-Proteinase inhibitor, α1-Antichymotrypsin, and α2-Macroglobulin are the antiapoptotic factors of vascular smooth muscle cells. J Biol Chem, 2001; 276(15), 11798-803.

4. Sobieska M, Mikstacki A, Wiktorowicz K. Zmiany stężeń cytokin i wybranych białek ostrej fazy pod wpływem urazów wielonarządowych. Nowiny Lekarskie, 1998; 67(4): 515-23.

5. Weaver VM, Carson CE, Walker PR, Chaly N, Lach B, Raymond Y, Brown DL Sikorska M. Degradation of nuclear matrix and DNA cleavage in apoptotic thymocytes. J Cell Science, 1996; 109: 45-56.

6. Lanzrein AS, Johnston CM, Perry VH, Jobst KA, King EM, Smith AD. Longitudinal study of inflammatory factors in serum, cerebrospinal fluid, and brain tissue in Alzheimer disease: interleukin-1beta, interleukin-6, interleukin-1 receptor antagonist, tumor necrosis factor alpha, the soluble tumor necrosis factor receptors I and II, and alpha1-antichymotrypsin. Alzheimer Dis Assoc Disord, 1998; 12: 215-27.

7. Licastro F, Pedrini S, Caputo L, Annoni G, Davis LJ, Ferri C, Casadei V, Grimaldi LM. Increased plasma levels of interleukin-1, interleukin-6 and alpha-1-antichymotrypsin in patients with Alzheimer's disease: peripheral inflammation or signals from the brain? J Neuroimmunol, 2000; 103: 97-102.

8. Machyńska-Bućko Z, Sobieska M. Ilościowe zmiany wybranych białek ostrej fazy u chorych z ciężkim oparzeniem. Nowiny Lekarskie, 1998; 67(12): 1254-62.

9. Kaźmierczak M, Sobieska M, Wiktorowicz K, Wysoki H. Changes of acute phase proteins glycosylation profile as a possible prognostic marker in myocardial infarction. Int J Cardiol, 1995; 49: 201-7.

10. Kaźmierczak E, Sobieska M, Kaźmierczak M, Mrozikiewicz A, Wiktorowicz K. Intense acute phase response in ischemic patients. Int J Cardiol, 1999; 68: 69-73.

11. Kołowrotkiewicz A, Sobieska M, Wołowicka L, Mikstacki A. Estimation of acid glycoprotein (AGP) and antichymotrypsin (ACT) in patients with polytrauma. In: 7th European Congress on Intensive Care

Medicine, Innsbruck, 14-17 June 1994. Int Care Med, 1994; 20(Suppl. 2): S84.

12. Springer E, Sobieska M, Wiktorowicz K. Measurements of acute phase proteins in food allergy and tolerance. XVII International Congress of Allergology and Clinical Immunology, 15-20 October 2000, Sydney, Australia. Abstract P-540.

13. Laurell, CB. Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies. Scand J Clin Invest, 1972; 124: 21-8.

14. Bøg-Hansen TC. Crossed immuno-affinoelectrophoresis: an analytical method to predict the result of affinity chromatography. Anal Biochem, 1973; 56: 480-3.

15. Zhanga S, Janciauskiene S. Multi-functional capability of proteins: α1-antichymotrypsin and the correlation with Alzheimer's disease. Journal of Alzheimer's Disease, 2002; 4: 115-22.

16. Robertson AS, Belorgey D, Lilley KS, Lomas DA, Gubb D, Dafforn TR. Characterization of the Necrotic Protein That Regulates the Toll-mediated Immune Response in Drosophila. J Biol Chem, 2003; 278(8): 6175-80.

The analysis of mortality from cardiovascular diseases in Pomeranian province

Bartosińska M*, Ejsmont J, Zaborski L, Zagożdżon P

Department of Hygiene and Epidemiology, Medical University of Gdańsk, Gdańsk, Poland

Absract

Purpose: Descriptive epidemiology characterises frequency of appearance of given event (here decease) in dependence of many factors concerning a person, region or time of existence of given salubrious phenomena. The source of information was the official death registry that provides complete records of all deaths that took place in Pomeranian province. This description of sanitary situation of people from particular area enables doing comparison between regions, facilitates researching etiological factors, planning work of medical workers and programming preventive rules. Cardiovascular diseases during last fifty years are the main reason of death of people from developed countries witch is also Poland.

Material and methods: The aim of this work is to find out differences in health condition between citizens of Pomeranian province and other people in Poland and countries of European Union.

Results: In Pomeranian province in 2002 the highest mortality from cardiovascular diseases was observed for Sztum county (587.5/100 000) and was 72.5% of all deaths in this region. Similarly, there was high mortality these reasons in Tczew county (442.1/100 000), Malbork county (406.9/100 000) and also in Tricity (424.8/100 000). The lowest mortality from cardiovascular diseases was observed in Gdańsk county (257.2/100 000) and was only 40% of all deaths in this region. Relatively low mortality was in Czluchow county (288.9/100 000). Frequency of death from cardiovascular diseases in Pomeranian province has become lower from year 2000 (361.0/100 000) to year 2002 (347.9/100 000). It was lower than in other parts of Poland (449.8/100 000 in year 2000) but higher than in countries of European Union (257.8/100 000 in year 2000).

Conclusions: Mortality from cardiovascular diseases has decreased during last few years. Also there are distinctions in this phenomena among regions of Pomeranian province, other parts of Poland and countries of European Union. From these reasons health care should be differentiated to address the differences in spatial patterns of risk observed.

Key words: epidemiology, mortality, cardiovascular diseases.

Introduction

Cardiovascular diseases are the most widespread reason of the decease and one of the most frequent reasons of disability in Poland. In 2001 died 363.2 thousand people and 173.8 thousand of them died because of cardiovascular diseases. That was 46.8% of all deaths. The mortality rate was 431.5, and in 2002 – 412.4 per 100 000 persons [1,2]. The mortality caused by cardiovascular diseases had risen in Poland since seventies to eighties. In the same time in countries of the former European Union the number of deaths because of cardiovascular diseases had decreased. In Poland from the beginning of nineties this tendency firstly had stopped and than, in 1992 had inverted [1]. It is thought that this occurrence was caused by no-medical conditions of state of health connected with changes in the diet quality. Complying with this diet was possible because of economical transformations in Poland [3,4]. However, in Poland mortality rate connected with cardiovascular diseases is one of the highest in all Europe and big differences in comparison with European Union countries had not decreased. Cardiovascular diseases are in Poland the main reason of the precocious mortality (which means less than 64 years old) and determine 37% of

* CORRESPONDING AUTHOR:
Department of Hygiene and Epidemiology
Medical University of Gdańsk
ul. Powstania Styczniowego 9b
81-519 Gdynia, Poland
Tel: +48 58 6998469; Fax: +48 58 699 8470
e-mail: mariabartosinska@amg.gda.pl (Maria Bartosińska)

Received 21.01.2005 Accepted 19.07.2005

Table 1. **Mortality in years 1999-2002 in Pomeranian province, Poland, Europe per 100 000 persons**

	Circulatory system disease					Ischaemic heart disease				
	1999	2000	2001	2002	Change 1999/2000 [%]	1999	2000	2001	2002	Change 1999/2000 [%]
Pomeranian province	381.5	361.0	371.1	347.9	- 8.8	b.d.	141.8	146.6	151.3	+ 6.6
Poland	469.0	444.0	431.5	412.4	- 12.1	147.9	141.0	133.5	125.5	- 15.1
Europe	266.7	257.8	244.7	238.5	- 10.6	103.2	97.4	94.2	b.d.	- 0.9

Table 2. **The general mortality rate connected with circulatory system diseases in counties of Pomeranian province**

Lp.	County	Number of deaths				Relation (2) to (1) in %
		(1) General mortality		(2) Circ. syst. diseases		
		total number	for 100 000 people	total number	for 100 000 people	
1	Bytów	563	749.9	227	302.2	40.3
2	Chojnice	677	750.7	334	370.1	49.3
3	Człuchów	423	740.8	165	288.9	39.0
4	Gdańsk	523	649.5	207	257.2	39.6
5	Kartuzy	687	660.2	314	301.7	45.7
6	Kościerzyna	493	750.7	252	383.6	51.1
7	Kwidzyn	623	779.8	281	351.7	45.1
8	Lębork	476	749.4	208	327.5	43.7
9	Malbork	562	890.4	257	406.9	45.7
10	Nowy Dwór Gdański	321	900.2	138	387.1	43.0
11	Puck	509	710.1	237	330.9	46.6
12	Słupsk	744	810.4	299	325.8	40.2
13	Starogard Gdański	991	820.7	446	369.3	45.0
14	Sztum	339	807.0	247	587.5	72.8
15	Tczew	996	889.5	495	442.1	49.7
16	Wejherowo	1377	789.8	511	293.0	37.1
	Pomeranian province	10780	812.0	4926	371.1	45.7
	Poland	364592	941.1	128426	331.5	45.9

all deaths for men and 17% for women [1]. The main reasons of decease in that group just like for all Polish people are cardiovascular diseases. World Health Organisation Regional Office shows that the level of precocious mortality caused by cardiovascular diseases was two and the half times higher in Poland than in EU countries.

Moreover, these big differences relating to the precocious mortality are observed only in reference to external reasons of men's mortality. It is anticipated that if in Poland present rate of reduction of the precocious mortality caused by cardiovascular diseases is going to be kept, it will achieve EU country's rate in 2018 (from 2001 in 17 years) [5].

Material and methods

The matter of this analysis was data concerning deaths from Pomeranian province. Data concerning deaths in particular counties, which had previously belonged to the Gdańsk province included five years period (since 1998 to 2002). Data from other counties of the Pomeranian province were possible to get only for three years period (since year 2000 to 2002). No standardised data, which was next taken to elaborate, had been received from Pomeranian Centre of the Health Protection. To analyse this data there was made in the Academy of Medicine in Gdańsk (in the Department of the Hygiene and Epidemiology) special computer program enabling quick choice of desirable parameters and moreover, the exact workout of chosen epidemiological coefficients and factors. It was necessary to use the publication of GUS (Principal Office of Statistic) relating to number of citizens in particular counties, during following years. In selection of data authors were using current symbols from International Statistical Classification of Diseases and Health Related Problem, Revision 10 – ICD 10.

Analysis was realised for deaths appointed with symbols:

- I 00 to I 99 – all deaths caused by cardiovascular diseases
- I 10 to I 15 – deaths because of ischaemic heart diseases
- I 21 to I 23 – deaths caused by infarct of heart muscle, repeated infarct of heart muscle and complications appearing during infarct of heart muscle.

The data about deceases in European Union and Poland come from the WHO database.

Results

Since 1999 to 2002 the mortality rate connected with cardiovascular diseases in the Pomeranian province had decreased about 8.8% (from 381.5 to 347.9/100 000 persons). However, the frequency of deaths connected with the ischaemic heart disease since 2000 to 2002 had increased about 6.6% (from 141.8 to 151.3/100 000 persons).

In Poland as well as in European Union countries (*Tab. 1*) in that period (since 1999 to 2001) the mortality rate connected with circulatory system diseases had decreased (in Poland about 12.1% and in Europe about 10.6%). Also the mortality rate connected with the ischaemic heart disease had decreased (since 1999 to 2001) in Poland about 15.1% and in Europe about 0.9%.

In *Tab. 2* the general mortality was compared with the mortality rate connected with cardiovascular diseases, in different counties of the Pomeranian province, in 2001. The highest mortality caused by cardiovascular diseases was observed in Sztum county (587.5/100 000 persons) and was 72.8% of all deaths. It

***Table 3*. Tendency in mortality connected with cardiovascular diseases in counties of Pomeranian province**

Lp.	County	Change of mortality rate					
		Circulatory system diseases totality		Ischaemic heart disease			
				Totality		Infarct of heart muscle	
		trend	[%]	trend	[%]	trend	[%]
1	Bytów	-	15.9	+	28.2	-	62.6
2	Chojnice	-	13.1	+	46.8	+	12.2
3	Człuchów	-	13.0	+	7.1	-	5.1
4	Gdańsk	+	7.8	-	1.3	-	6.3
5	Kartuzy	+	5.2	+	23.2	-	5.4
6	Kościerzyna	+	9.9	+	6.1	-	5.1
7	Kwidzyn	-	3.2	+	11.2	+	4.1
8	Lębork	+	1.1	+	61.8		0
9	Malbork	-	34.7	-	21.3	-	26.3
10	Nowy Dwór Gdański	+	28.8	+	49.6	+	25.4
11	Puck	-	10.2	-	11.3	-	1.2
12	Słupsk	-	5.3	+	17.2	+	18.8
13	Starogard Gdański	-	8.9	-	1.3	-	19.1
14	Sztum	+	10.1	+	9.4	+	10.2
15	Tczew	+	6.1	+	15.8	+	16.3
16	Wejherowo	+	4.3	+	14.2	+	14.2
	Pomeranian province	-	3.6	+	6.6	-	14.1
	Poland	-	7.1	-	10.9	-	15.1
	Europe	-	7.5	-	8.7		b.d.

***Table 4*. Relation between men's and women's mortality rate connected with cardiovascular diseases**

	Circulatory system diseases		
	Totality	Ischaemic heart disease	
		Totality	Infarct of heart muscle
Pomeranian province	100 / 111	100 / 94	100 / 61
Town	100 / 114	100 / 99	100 / 66
Village	100 / 104	100 / 83	100 / 51
Tricity	100 / 115	100 / 101	100 / 71

was also high in Tczew county (442.1/100000) and was 49.7% of the general mortality. A significant percentage of deaths caused by diseases of circulatory system in reference to the general mortality was observed in Kościerzyna county (51.1%) as well as in Chojnice county (49.3%). In Pomeranian province the coefficient of deaths caused by diseases of circulatory system was 45.7% of the general mortality when in whole Poland it was 45.9%.

Since 2000 to 2002 in 8 counties (from 16, which is 50.0%) frequency of decease because of cardiovascular diseases had decreased, while in 8 other counties had increased (*Tab. 3*). The highest decrease was observed in Malbork county (34.7%), and the highest increase in Nowy Dwór Gdański county (28.8%). In Pomeranian province the mortality rate connected with mentioned causes had decreased about 3.6 % and in EU countries about 7.5%. In the same period in most counties of the Pomeranian province mortality rate connected with ischaemic heart disease had increased. The highest increase was observed in Lębork county (61.8%), Nowy Dwór Gdański county (49.6%) and Chojnice county (46.8%). Also in whole Poland mortality rate from that reason had increased about 6.6% while in EU countries had decreased about 10.9%. In Malbork county mortality rate connected with ischaemic heart disease had decreased about 21.3%. Significant decrease of mortality rate connected with infarct of heart muscle was observed in Bytów county (62.2%), Malbork county (26.3%) and Starogard Gdański county (19.1%). However the highest increase was observed in Nowy Dwór Gdański county (25.4%) and Słupsk county (18.8%). In Poland the mortality rate connected with the infarct of heart muscle had decreased since 2000 to 2002 about 14.1% and in EU countries of about 15.1%.

In Pomeranian province in 2002 there was 100 men for 105 women. Among deceased because of cardiovascular diseases proportion between men and women was 100/111, because of ischaemic heart disease – 100/94 and infarct of heart muscle 100/61.

Among deceased inhabitants of cities in Pomeranian province that proportions were 100/114, 100/99, 100/66, in villages – 100/104, 100/83, 100/51, whereas in Tricity: 100/115, 100/101, 100/71 (*Tab. 4*).

Differences in age structure of people died because of cardiovascular diseases were not observed in towns as well as in villages (*Fig. 1*). However, there were some differences between mortality rate of men and women. Among men from younger age groups, proportion of decease is several times higher than for women. Only in groups of 70 and over 70 years old women outnumbered men (*Fig. 2*).

Discussion

Cardiovascular diseases from few dozens years are the most frequent reason of decease in Poland as well as in most countries. In 1970 the standardised mortality rate connected with cardiovascular diseases for men in age from 0 to 64 years old in Poland was similar to that in EU countries, while for women in the same age the mortality rate was lower in Poland than in EU countries. From that period to beginning of nineties the mortality rate had been increasing in Poland while in EU countries it had been decreasing. In Poland this unfavourable tendency in 1992 had inverted.

Results of our research confirm that the mortality rate connected with cardiovascular diseases in Pomeranian province, in Poland and in EU countries had been decreasing since 1999 to 2002. In Poland as well as in EU countries at the beginning of 21th century frequency of death because of ischaemic heart disease had decreased, however, in Pomeranian province since 2000 to 2002 it had increased about 6.6%. It requires further observation as well as urgent inclusion of preventive aids.

In particular counties of Pomeranian province there are differences in mortality rate connected with circulatory system diseases, as well as in the tendency of rate change since 2000 to 2002. Particularly disturbing is positive tendency of the mortal-

Figure 1. **Age structure and number of the people died because of circulatory system diseases in Pomeranian province in 2002 according to place of living**

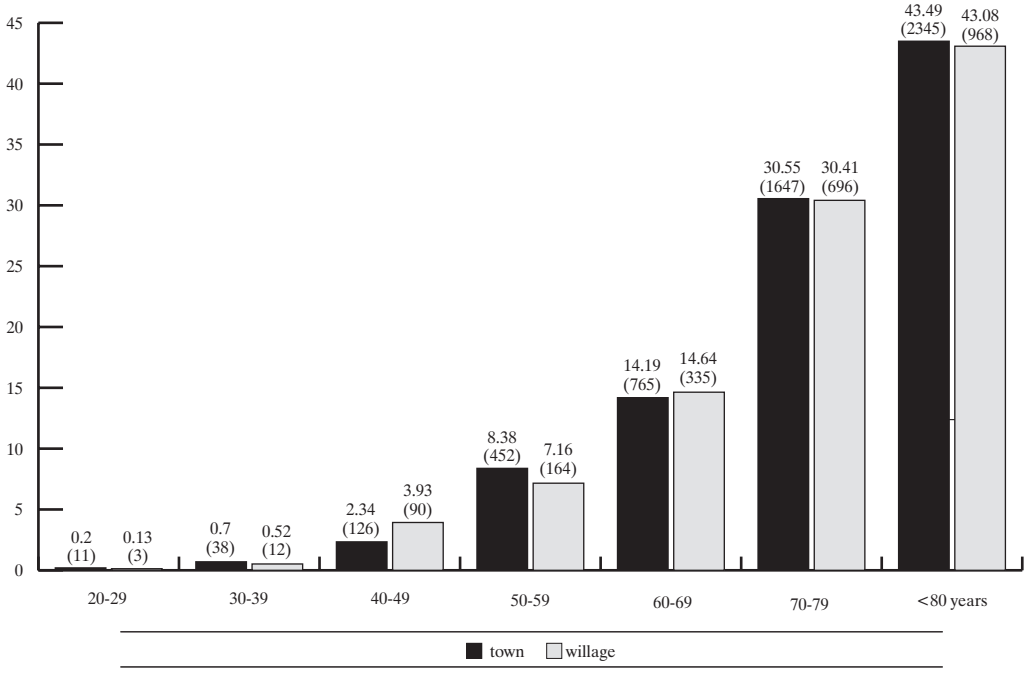
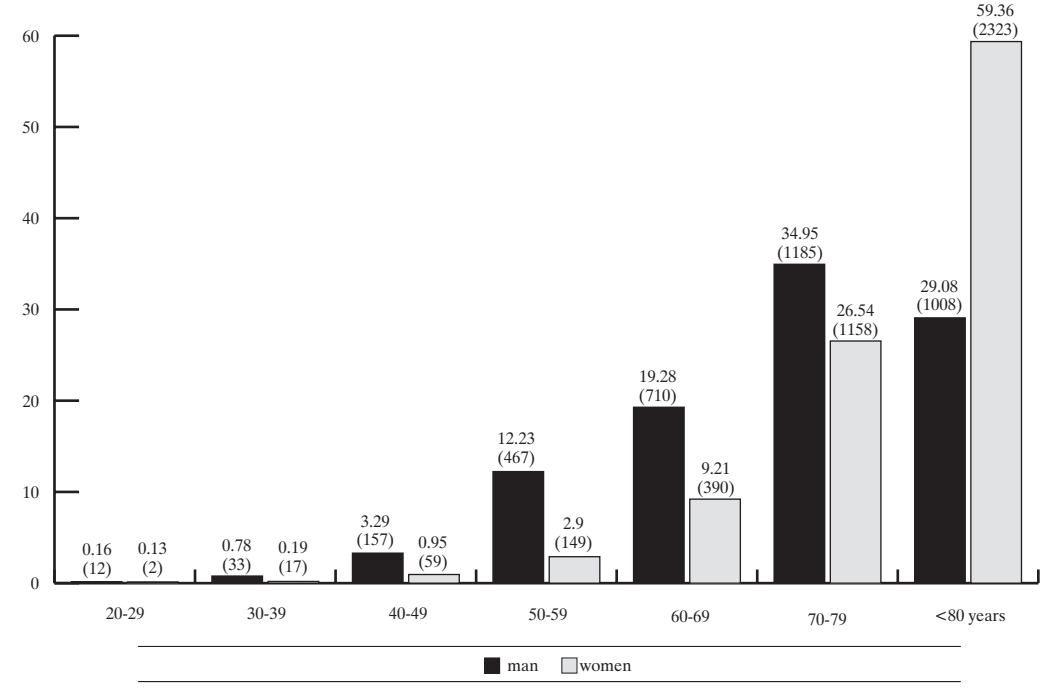


Figure 2. **Age structure and number of the people died because of circulatory system diseases in Pomeranian province in 2002 according to sex**



ity rate connected with cardiovascular diseases (including also mortality relative to ischaemic heart disease and infarct of heart muscle) in Nowy Dwór Gdański county, Sztum county and Wejherowo county.

Aetiology of circulatory system diseases is dependent to a large degree to environmental and social-economical factors as well as to pro-sanitary behaviours, while mortality depends to Health Care's quality.

Geographical variation of mortality from cardiovascular diseases could be attributable to regional differences in public health system policy. The differences in performance of health care services occurred mainly due to liberation in market of

health care services in transition period. The changes in health care services that would affect the cardiovascular mortality could be those related to emergency medicine, diagnostics procedures and prevention.

In Pomeranian province about 6.8% of employees in 2002 had been working in health damaging conditions. Pomeranian province in comparison with others polish provinces has 5th position in emission of dust pollution and 6th in emission of gas pollution. Number of medical advises was 5.5 per person during 2002 [6,7].

Among inhabitants of Pomeranian province in 2002 there were 100 men for 105 women. Comparison of men and women's mortality rate connected with circulatory system diseases (100/111) shows that it is higher for women. However, it is on the contrary for men if there are analysing mortality rate connected with ischaemic heart disease (100/94) and infarct of heart muscle (100/61).

It is necessary to find the reason of very high mortality rate connected with cardiovascular diseases in Sztum county (72.8% of all deaths, when normally it does not reach 50.0%).

References

1. Najważniejsze elementy sytuacji zdrowotnej Polski. Państwowy Zakład Higieny, Warszawa, 2002.
2. Pająk A. Zawał serca – zagrożenia i postępowanie. Długofalowa obserwacja populacji 280000 kobiet i mężczyzn – Projekt Pol-Monica. Kraków, Część I. Geneza i cele. The WHO Monica Project. Przegl Lek, 1996; 53(10): 703-6.
3. Labarthe DR. Epidemiology and prevention of cardiovascular diseases: A global challenge. Aspen Publishers, 1998.
4. Scheidt S. Changing mortality from coronary heart disease among smokers and no-smokers over 20-years interval. Prev Med, 1997; 26(4): 441-6.
5. Nolte E, McKee M. Population health in Europe: how much is attributable to health care? World Hosp Health Serv, 2004; 40(3): 12-4.
6. Demographic Yearbook of Poland, Warsaw, 2003.
7. Statistical Yearbook of the Regions-Poland, Central Statistical Office, Warsaw, 2003.

Evaluation of secretory mucin concentration of patients with squamous cell carcinoma oral cavity

Dziemiańczyk D*, Grabowska SZ, Balicki R

Department of Maxillofacial Surgery, Medical University of Białystok, Poland

Abstract

Purpose: **Secretory salivary mucins constitute a heterogenous group of glycoproteins, synthesized and secreted by submandibular, sublingual gland and small glands of oral mucosa. The most significant functions of mucins in case of oral cavity carcinoma are: participation in oral pellicle formation, lubrication and creation of heterotypic complexing.**

The aim of this study was to assess mucins concentration, and finally to establish the correlation between concentration of mucins in saliva and clinical advancement according to TNM.

Material and methods: **The research was conducted on mixed resting and stimulated saliva of patients with oral squamous cell carcinoma. Mucin's concentration was measured one day before, and thirty days after surgical procedure. The volume of saliva was volumetrically determined, quantitative evaluation of mucins was accomplished by PAS method.**

Results: **In comparison with K group, a significant decrease of mucins was found in resting and stimulated saliva of patients with carcinoma in all degrees of clinical advancement. Mean value of mucin in resting and stimulated saliva after surgical treatment were lowered. The degree of carcinoma clinical advancement correlated negatively with mucin concentration.**

Conclusions: **The decrease of mucin contained in saliva may be important in further evolution or progression of carcinoma. The results also suggest that saliva may be a significant diagnostic material in carcinoma research.**

* CORRESPONDING AUTHOR:
ul. Boboli 86/25
15-649 Białystok, Poland
Tel: +48 85 6634741; Fax: +4885 7468524
e-mail: dyrka2@wp.pl (Dorota Dziemiańczyk)

Received 31.05.2005 Accepted 27.07.2005

Key words: secretory mucins, saliva, oral cavity cancer.

Introduction

The oral mucosa is constantly exposed to the effect of damaging factors (physical, chemical, biological). In normal conditions, the effects of these factors are counteracted by many interconnected protective mechanisms of the oral cavity. They include: anatomical integrity of the mucosa, the presence of saliva, its constant flow, as well as protective and regenerative substances it contains.

The correct flow of saliva enables the formation of a protective layer covering the oral mucosa, limiting the penetration of potentially carcinogenic compounds into the epithelium. Mucins are among the agents participating in the formation of the architectural skeleton of the protective preepithelial layer in the oral mucosa, as well as maintaining correct concentrations of protective substances.

Mucins contained in human saliva represent a heterogenous group of glycoproteins synthesized and released by the submandibular and sublingual glands, and small glands of the oral mucosa [1,2]. Currently, two main groups of salivary mucins are identified: a) high-molecular MG1 mucins of molecular mass above 1 000 kDa, b) low-molecular MG2 mucins of molecular mass 200-300 kDa [3].

High viscosity of mucin, significantly affecting its separation from the other organic components of saliva, indirectly shows the ability of mucin molecules to form complex connections with other types of molecules. These connections are referred to as heterotypic complexes in which mucin molecules may selectively bond with the other organic substances in saliva such as: IgA, lysozyme and lipids [4]. This type of intermolecular interactions involves ionic and hydrogen chemical bonds, as well as hydrophobic interactions. Due to the formation of heterotypic complexes, salivary mucins may play the role of carrier of substances they are bound to. The shown ability of MG1 and MG2 to form heterotypic complexes may have an effect on the

Table 1. **Clinical stage**

Clinical stage	Number of patients	Percentage
S1	6	12.50 %
S2	8	16.70 %
S3	12	25.0%
S4	22	45.83%

Table 2. **Carcinoma location**

Location	Number of patients	Percentage
tongue	10	20.83 %
tongue + oral cavity floor	15	31.25 %
oral cavity floor	9	18.75 %
soft palate	5	10.42 %
buccal oral mucosa	6	12.50 %
inferior gingiva	3	6.25 %

increase in concentrations of protective substances in the layer of mucus covering the surface of the organs and tissues of the oral cavity [5].

Saliva and the protective agents it contains have been evaluated in patients with oral cancer in a few clinical trials involving small groups of subjects. The role of protective factors in saliva, including mucins, in the biology of the development of planoepithelial carcinoma of the oral cavity has not been determined so far.

Taking into account the above data, a study of resting and stimulated mixed saliva in patients with squamous cell carcinoma of the oral mucosa before and after surgery was undertaken. The purpose of the study was to determine mucin concentrations, and to establish correlation between its concentrations and clinical stage of tumour according to TNM classification.

Material and methods

The study material consisted of resting and stimulated mixed saliva from 48 patients with histopathologically verified planoepithelial carcinoma of the oral cavity. The age in the study population was 39 to 80 years (mean age 60 years). There were 39 men and 9 women among the patients.

To evaluate clinical stage of tumour, the four-degree scale according to TNM classification (fifth version) [6] was used:

I stage (S1) – T1, N0, M0
II stage (S2) – T2, N0, M0
III stage (S3) – T1, N1, M0 ; T2, N1, M0 ; T3, N0, M0 ; T3, N1, M0
IV stage (S4) – T4, N0, M0 ; T4, N1, M0 ; Each T, N, M1

The patients most often had tumours in clinical stage IV (45.83%), and less often in stage I (12.5 %) (*Tab. 1*).

The most frequent location of tumour was the tongue with the oral fundus (31.25%), then the tongue (20.83%), and then the oral cavity floor (18.75%) (*Tab. 2*).

The exclusion criteria were diseases in which saliva production is impaired (diabetes, Sjögren syndrome), or the use of pharmaceuticals affecting saliva production. All participating patients and all subjects in the control group were smokers (20-30 cigarettes a day on the average, for a period of about 20 years). The results were compared to the control group consisting of 25 healthy people (mean age 58 years).

Table 3. **Mucin concentration (mg/ml) in resting saliva of patients before surgical treatment. (S1–S4) and K group**

	K	S1	S2	S3	S4
mean value	0.88	0.79	0.68	0.52	0.43
SE	0.11	0.18	0.17	0.10	0.15
n	25	6	8	12	22
coefficient value (p)		$p > 0.05$	$p > 0.05$	<0.001 vs K	<0.001 vs K

In both patients and healthy subjects, saliva was taken using the spitting method, in 10-minute fractions (the first fractions were taken without secretory stimulation of the salivary glands, and the other fractions were taken upon saliva secretion stimulation by parafilm chewing). Until the time of assays, the material was stored at -80°C [7]. Saliva was taken from the patients 1 day before and 30 days after tumour excision surgery.

Data distribution was analysed with Shapiro-Wilk test. The subsequent hypotheses were tested using t-Student test, and the results were presented as arithmetic mean ±SE (standard error). The results for which the coefficient value was $p<0.05$ were considered statistically significant. To evaluate the mutual relationships between mean mucin concentrations and the clinical stage of tumour, Pearson linear correlation test was used. The correlation was considered complete for the coefficient values of $r\geq0.9$ [8].

A quantitative evaluation of mucin in saliva was performed based on the PAS method (periodic acid/Schiff reagent) described by Mantle et al. [9].

The subjects expressed written consent for saliva sampling, declaring informed participation in the clinical study. The above study was approved by the Bioethics Committee at the Medical University of Białystok (35/2000).

Results

In healthy people (K), mean mucin concentrations in resting saliva were at the level of 0.88 mg/ml. Compared to the K group, presurgery mean concentrations of mucin were reduced in the patients participating in the study (S1–S4). The lowest mucin concentrations were found in saliva of patients in clinical stage S4, and the highest – in stage S1. Compared to the control group, this reduction was only statistically significant in patients in disease stages S3, S4 ($p<0.001$) (*Tab. 3*).

In all patients, mean mucin concentrations in resting saliva became further reduced after surgical treatment. The observed differences in mucin concentrations in resting saliva of patients before and after tumour excision were only statistically significant in patients in stages S3, S4 ($p<0.05$) (*Fig. 1*).

Figure 1. **Mucin concentration in resting saliva of patients before and after surgical treatment**

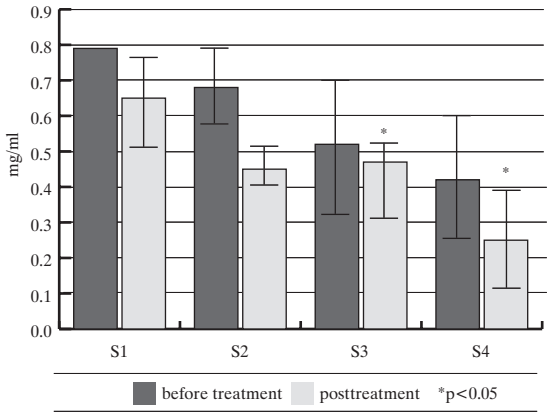


Figure 2. **Mucin concentration in resting and stimulating saliva of patients before surgical treatment and K group**

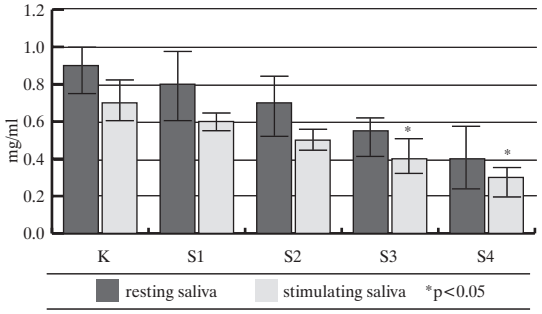
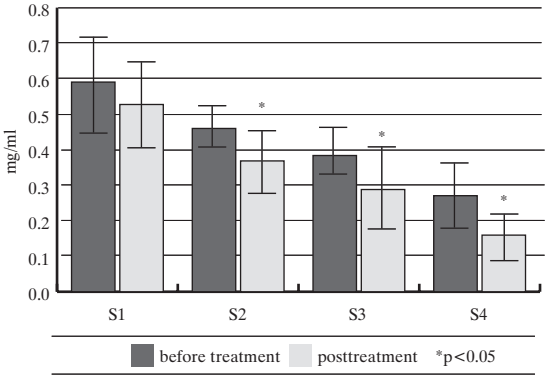


Figure 3. **Mucin concentration in stimulated saliva before and after surgical treatment**



Upon secretion stimulation in subjects from the K group, a reduction in saliva mucin concentrations was seen compared to concentrations achieved with resting secretion (0.71 mg/ml). The reduction was not significant ($p>0.05$). Also, in all patients (S1–S4), before surgery, a reduction in mucin concentrations in stimulated saliva was seen compared to the resting saliva concentrations. This glycoprotein reduction was statistically significant in patients in disease stages S3 and S4 ($p<0.05$) (*Fig. 2*).

In all patients (S1–S4), mean mucin concentrations in both stimulated and resting saliva were further reduced after surgical treatment.

The observed differences in mucin concentrations in stimulated saliva before and after tumour excision were statistically significant in patients in disease stages S2, S3, S4 ($p<0.05$) (*Fig. 3*).

Searching for mutual relationships between mean mucin concentration in resting and stimulated saliva in patients before and after surgery, and the clinical stage of tumour, a slight trend towards negative correlation was found $r=(-0.5)$.

Discussion

On the surface of mucosal epithelial cells, there are high-molecular glycoproteins referred to as mucins. Due to the variety of their functions and structure, they are divided into membrane mucins, forming part of the cellular membrane, and secretory mucins which are the primary component of mucus [10-12]. The significance of secretory mucins present in saliva in non-immune protective mechanisms has been well documented for the teeth [12,13]. However, little is known about the role of these glycoproteins in maintaining the integrity of the oral mucosa.

In the present study, an evaluation of mucins in resting and stimulated mixed saliva was performed in 48 patients with oral cancer in various clinical stages of tumour. The results were compared to a control group.

Before surgery, all patients had lower mucin concentrations in resting and stimulated saliva compared to the group of healthy subjects. The concentration values of this glycoprotein were reduced with the increase in clinical stage of tumour. It was also confirmed by the observed trend towards negative correlation. The results of the authors' own studies have shown that in patients with oral cancer, the ability of the salivary glands to secrete mucin is limited.

In the literature, there are no studies evaluating secretory mucin concentrations in saliva of patients with oral cancer.

The evaluation of minute secretion of saliva before surgery shows normal function of the salivary glands in the study subjects in qualitative aspect of produced and secreted saliva [14]. However, it does not rule out the possibility of impaired quality of saliva components produced. Eliasson et al. showed a reduction in mucins and other salivary components in smokers. Assessing the morphology of the palatine glands (mixed, prevalence of mucous cells), they showed a dilation of external ducts with excessive mucus retention, as well as atrophy of the acinic cells. They also observed the presence of mucus and inflammatory cell clusters in the interstitium. These authors link the reported changes with the vasoconstrictor effect of the products of tobacco smoking and the subsequent reduction in blood flow through the glands, and the change in qualitative composition of saliva produced, including a reduction in mucins [15].

The subjects in our study were long-term heavy smokers. Taking into account this fact and the results of the cited studies, it may be assumed that mucin reduction in patients with oral cancer may be caused by similar morphological changes in sub-

lingual and submandibular glands which are the main source of secretory mucins in saliva. However, in the group of the patients participating in the study, it is difficult to distinguish the results of using nicotine from the possible effect of tumour mass compressing the submandibular and/or sublingual glands. It should be emphasised that most tumours located in the fundus of the oral cavity and in the ventral surface of the tongue are in disease stages S3–S4. It is possible that mechanical effect of tumour by compression may also be a cause of local reduction in blood flow in the glands, and as a consequence, a reduction in secretory mucin concentrations.

It may therefore be assumed that as a result of reduction in mucin concentrations in saliva of patients, the mucosa may be more susceptible to the effect of damaging factors which, with long-term exposure, may lead to initiation, promotion and/or progression of the carcinogenesis process.

The question is whether the reduction in mucin concentrations in saliva of patients is primary or secondary to the neoplastic process? The studies carried out in the last ten years by various authors [2,4,16-18] emphasise that mucins which are the main component of the preepithelial protective layer of the mucosa not only represent a mechanical barrier but also a dynamic structure modelling the oral cavity environment.

Specific rheological properties of salivary mucins contribute to the formation of a thin layer covering all structures in the oral cavity. A strong affinity of mucins to the gastrointestinal, respiratory and genital epithelium has been accepted since long ago as the prerequisite for film formation on the oral surfaces. The stability of these interactions was described as various hydrophobic and ionic bonds between mucins and the surface of the mucosa [19-21].

Moreover, Słomiany et al. [17] showed that mucins in the area of the gastric mucosa bind to a specific membrane receptor. Similar receptors were also identified and described in the epithelial cells of the buccal mucosa. It was shown that the mucin-receptor bond requires presentation of oligosaccharide chains of mucins by breaking some β-glycosidic bonds inside the chains. It evidences the dynamic character of these interactions.

The studies of Murty et al. showed that the breakage of mucin-receptor bonds by bacterial glycosidases may lead to a loss of pre-epithelial barrier of the oral epithelium. When unprotected against the effects of exogenous factors (including carcinogens), the mucosa becomes susceptible to ulceration and further progression of lesions towards cancer [17,22]. It is an important observation in the aspect of carcinogenesis of the oral mucosa, because the factors determining the promotion and progression of cancer process may include chronic inflammation, as well as poor oral hygiene [23,24]. The presence of bacteria in the saliva, accompanying the described changes, may be associated with a loss or impairment of the mucin coating, and increased penetration of possible carcinogens into the epithelium.

Based on the morphological criterion, three compartments of protective action have been identified in the oral mucosa: preepithelial, epithelial, and postepithelial compartments.

Due to the fact that damaging factors act from the lumen of the gastrointestinal tract, the key importance is attached to the preepithelial barrier. The correct functioning of this barrier mostly relies on mucin and non-mucin proteins [25]. The protective effect of mucin depends on its ability to form an architectural skeleton inside the barrier, responsible for the inhibition of diffusion of damaging factors. As the barrier is highly hydrophobic and contains many phospholipids, it may bind other protective factors such as: epithelial growth factor (EGF), prostaglandins (PGE2) [2,4,18,26]. The thickness of the barrier is 0.05 to 0.1 mm, and direct dependence between its thickness and the protective properties was shown.

The ability to form heterotypic complexes with non-mucin proteins including sIgA and lysozyme [20], as well as EGF and PGE2 [16] also highly determines the functions of mucins in the oral cavity. A reduction in mucin concentrations in saliva of our patients may, therefore, be the cause of reduction in concentrations of many protective factors in the mucinous layer, and as a consequence, in the epithelium of the mucosa. The reduced protective potential of the epithelium in patients with S3–S4 stage may therefore exacerbate the existing lesions in the tumour but also in other parts of the mucosa, and in the upper gastrointestinal tract. Recent studies have also shown that mucins, apart from forming the described mechanical protective layer and the formation of heterotypic complexes, may also modulate/regulate intramembranous mechanisms such as regulation of intracellular calcium levels, related to the function of various growth factor receptors [27]. The results of studies performed in the latest years suggest that disorders of so-called calcium transmitter system [22] underlie the "chemical oncogenesis" occurring in the oral cavity. Calcium ions belong to the group of so-called secondary cell transmitters and represent an important element regulating many functions of the cell. The secondary transmitter system multiplies the signal in the cell [28].

The study of Knaus et al. showed that the function of calcium channels depends on polyanionic molecules, e.g. heparin and GM1-ganglioside. Peppelenbosch et al. [29], Słomiany et al. [17] found that the process of phosphorylation of these channels is a response to the effect of growth factors. Later studies of Słomiany et al. of calcium channels in the buccal mucosa showed that mucins (both low- and high-molecular) may also modulate their activity in the soft tissues of the oral cavity. Subsequently, it was found that the acidic fractions of mucins have an inhibitory effect against calcium channels. It was shown that this effect was related to the presentation of sialic acid and ester sulphone groups in the oligosaccharide chains of mucins [17,30].

Słomiany et al. [17] presented the inhibitory effect of mucins against calcium channels using the example of EGF. EGF, by binding to a membrane receptor connected with a calcium channel, causes its phosphorylation, activation of tyrosine kinase, leading to calcium channel opening and an increase in calcium ion concentrations in the intracellular environment. In these conditions, EGF bound to salivary mucins does not bind to membrane receptors; as a result, a reduction in calcium ion concentrations in the intracellular environment occurs.

The possibility of calcium channel activity modulation by mucins is, therefore, another property contributing to the multifunctionality of salivary mucins in the aspect of oral mucosa protection.

At present, many authors agree that disorders of transmission of signal received by EGFR have a significant effect on the basic function of the cell controlled by this receptor, i.e. differ-

entiation, maturation, proliferation, adhesion, migration and apoptosis inhibition [31,32]. Sometimes the disorders of the pathways of signal transmission from EGFR to the cell nucleus are also manifested as promotion of cancer transformation and tumour proliferation, an increase in invasiveness of its cells and cell survival, as well as in the form of neoangiogenesis.

Taking into consideration these data from my studies, a reduction in mucin concentrations in patients with oral cancer may directly affect the functioning of various membrane receptors of growth factors with tyrosine kinase activity, thus promoting the carcinogenesis process in the entire area of carcinogenesis.

In our studies, mucin concentrations in patients after surgery were also lower than in the group of healthy subjects. These differences were significant for patients with disease stages 3 and 4. However, an analysis of mucin concentrations in resting and stimulated saliva in the group of patients before and after surgery showed a significant reduction of this glycoprotein after tumour excision.

In physiological conditions, salivary mucins are synthesized by the salivary cells of the sublingual glands (60% of mucous cells) and submandibular gland (5% of mucous cells), as well as small salivary glands located in the palatine, buccal and labial mucosa. On the contrary, the parotid glands represent the type of serous glands and the mucous cells are rarely seen in their structure [33]. It has been shown in both an animal model and in humans that these glands do not participate in the production of salivary mucins.

Taking into account the above data, further reduction in mucins in both fractions of the saliva after surgery, seen in the group of patients, may be related with the removal of submandibular and sublingual glands during surgery.

The reduction in mucin concentrations in the saliva in patients with cancer due to impairment of the preepithelial barrier may have an effect of the progression of tumour growth, and further reduction in their levels after surgery may be responsible for the occurrence of local relapse. As resting saliva represents the oral environment for a significant part of the day (14 to 16 h) [34], mucin concentrations in this fraction of saliva seems particularly important.

To recapitulate, it should be concluded that a reduction in secretory mucins in both fractions of saliva in patients with oral cancer, and a trend towards negative correlation with the clinical stage of disease, may indicate functional impairment of the preepithelial barrier of the mucosa in these patients. However, the issue requires further investigation.

Acknowledgement

I thank Professor S. Z. Grabowska and Dr R. Balicki for a critical review of this manuscript and G. Pakiela for a great help.

References

1. Cohen RE, Aguirre A, Neiders ME, Levine MJ, Jones PC, Reddy MS, Haar JG. Immunohistochemistry of high molecular-weight human salivary mucin. Arch Oral Biol, 1990; 35: 127-36.

2. Levine MJ, Reddy MS, Tabak LA, Loomis RE, Bergey EJ, Jones PC, Cohen RE, Stinson MW, Al-Hashimi I. Structural aspects of salivary glycoproteins. J Dent Res, 1987; 66(2): 436-41.

3. Kawagishi S, Fahim REF, Wong KH, Bennick A. Purification and characterization of subunits of a high molecular weight human salivary mucin. Arch Oral Biol, 1990; 45: 265-72.

4. Iontcheva I, Oppenheim FG, Troxler RF. Human salivary mucin MG1 selectively forms heterotypic complexes with amylase, proline-rich proteins, statherin, and histatins. J Dent Res, 1997; 76: 734-43.

5. Słomiany BL, Murty VLN, Piotrowski J, Słomiany A. Salivary mucins in oral mucosal defense. Gen Pharmac, 1996; 27(5): 761-71.

6. Sobin L, Wiitekind C, editors. TNM Classification of Malignant Tumors. New York: Wiley-Liss; 1997.

7. Marcinkiewicz M, Markowski AR, Kreczko S. Metody pobierania śliny. Diagn Lab, 1998; 34: 115-23.

8. Stanisz A. Przystępny kurs statystyki w oparciu o program Statistica pl na przykładach z medycyny. Kraków: StatSoft Polska; 2001.

9. Mantle M, Allen A. A colorimetric assay for glycoprotein based on the Periodic Acid/Schiff stain. Biochem Soc Transac, 1978; 6: 607-9.

10. Paszkiewicz-Gadek A, Gindzieński A. Mucyna MUC1 i jej znaczenie w procesach nowotworzenia. Postępy biochemii, 2000; 46(4): 342-50.

11. Paszkiewicz-Gadek A, Porowska H. Mucyny ludzkie przewodu pokarmowego jako produkty genów MUC. Post Hig Med Dośw, 2000; 54(2): 183-98.

12. Zalewska A, Borzym M, Marcinkiewicz M, Zwierz K. Glikoproteiny śliny ludzkiej. Magazyn Stomatologiczny, 1999; 10: 28-35.

13. Piotrowski J, Czajkowski A, Murty VLN, Słomiany A. Identification of human salivary protease activity toward mucin: differences with caries. Biochemistry International, 1992; 28(5): 939-94.

14. Balicki R, Grabowska SZ, Citko A. Salivary epidermal growth factor in oral cavity cancer. Oral Oncol, 2005; 41: 48-55.

15. Eliasson L, Heyden G, Landahl S, Steen B. Effects of tabacco and diuretics on human palatal salivary glands. J Oral Pathol Med, 1991; 20: 126-9.

16. Sarosiek J, McCallum RW. Do salivary organic components play a protective role in health and disease of the esophageal mucosa? Digestion, 1995; 56(Suppl. 1): 32-7.

17. Słomiany BL, Fekete Z, Murty VLN, Słomiany A. Modulation of buccal mucosal calcium channel activity by salivary mucins. J Phys Pharm, 1993; 44(3): 199-211.

18. Słomiany A, Tamura S, Grzelińska E, Piotrowski J, Słomiany BL. Mucin complexes: characterization of the "link" component of submandibular mucus glycoprotein. Int J Biochem, 1992; 24(6): 1003-15.

19. Allen A, Flemstrom G, Garner A, Kivilaakso E. Gastroduodenal mucosal protection. Physiol Rev, 1993; 73: 823-57.

20. Biesbrock AR, Reddy MS, Levine MJ. Interaction of salivary mucin-secretory Immunoglobulin A complex with mucosal pathogens. Infection and Immunity, 1991; 59(10): 3492-7.

21. Bradway SD, Bergey EJ, Scannapieco FA, Ramasubbu N, Zawacki S, Levine MJ. Formation of salivary-mucosal pellicle: the role of transglutaminase. Biochem J, 1992; 284: 557-64.

22. Madej JA. Etiologia i patogeneza nowotworów. Medicapress; 1999.

23. Kryst L. Wczesne wykrywanie i rozpoznawanie nowotworów jamy ustnej. Czasopismo Stomatologiczne, 1994; 47: 307-9.

24. Kryst L. Chirurgia głowy i szyi. PZWL, Warszawa; 1996.

25. Kongara KR, Soffer EE. Saliva and esophageal protection. Am J Gastroenterol, 1999; 94(6): 1446-52.

26. Dziemiańczyk D, Marcinkiewicz M, Grabowska SZ, Czyżewska E. Mucyny ślinowe – skład biochemiczny i ich wpływ na biologiczne funkcje śliny w środowisku jamy ustnej – przegląd piśmiennictwa. Czas Stomat, 2000; 11: 715-20.

27. Słomiany A, Nishikawa H, Słomiany BL. Screening and modulation of extracellular signals by mucous barrier. Serum glycosylphosphatidylinositol phospholipase D (GPI-PLD) releases protective mucus barrier from oral mucosa. J Phys Pharmacol, 2002; 53(1): 21-38.

28. Bańkowski E. Biochemia. Urban&Partner, Wrocław; 2004.

29. Peppelenbosch MP, Tertoden LGF, deLaat SW. Epidermal growth factor-activated calcium and potassium channels. J Biol Chem, 1991; 266: 19938-44.

30. Słomiany BL, Murty VLN, Słomiany A. Structural features of carbohydrate chains in human salivary mucins. Int J Biochem, 1993; 25: 256-65.

31. Baselga J. Why the Epidermal Growth Factor Receptor? The rationale for Cancer Therapy. Oncologist, 2002; 7(Suppl. 4): 2-8.

32. Christensen ME. The EGF receptor system in head and neck carcinomas and normal tissues. Dan Med Bull, 1998; 45(2): 121-34.

33. Ostrowski K. Histologia, PZWL, Warszawa, 1988.

34. Sreebny LM. Saliva in health and disease: an appraisal and update. Int Dental J, 2000; 50: 140-61.

Low dose rofecoxib, inflammation and prostacyclin synthesis in acute coronary syndromes

Kuklińska AM[1]*, Musiał WJ[1], Kamiński KA[1], Kralisz P[2], Modrzejewski W[1], Sobkowicz B[1], Dobrzycki S[2], Stec S[3], Wojtkowska I[1]

[1] Department of Cardiology, Medical University of Białystok, Białystok, Poland
[2] Department of Invasive Cardiology, Medical University of Białystok, Białystok, Poland
[3] Department of Cardiology, Postgraduate Medical School, Grochowski Hospital, Warsaw, Poland

Abstract

Purpose: To assess the influence of low dose rofecoxib on inflammatory mediators and prostacyclin synthesis in patients with acute coronary syndromes (ACS) in a short-term follow up.

Material and methods: Twenty nine patients with ACS without ST elevation were randomized to simvastatin alone or together with low dose rofecoxib. Serum levels of interleukin 6 (IL-6), 6-keto-PGF-1α – stable product of prostacyclin (PGI2) and hs-C-reactive protein (hs-CRP) were assessed on enrollment and after 30-day follow up.

Results: Combination of rofecoxib with statin significantly decreased levels of hs-CRP after one month therapy (5.21 mg/l ± 4.12 vs 2.11 mg/l ± 2.1; p=0.0092). This effect was not evident in a group on statin alone (3.95 mg/l ± 3.33 vs 2.48 mg/l ± 2.39; p=0.31). 6-keto-PGF-1α increased not significantly in both groups. IL-6 concentration has not changed during follow up.

Conclusions: Low dose of selective COX-2 inhibitor exerts significant anti-inflammatory effect and does not diminish PGI2 synthesis in study group of patients with ACS.

Key words: acute coronary syndromes (ACS), inflammation, simvastatin, cyclooxygenase-2 inhibitors, prostacyclin.

* CORRESPONDING AUTHOR:
Department of Cardiology,
Medical University of Białystok
ul. M. Skłodowskiej-Curie 24a
15-276 Białystok, Poland
Tel: +48 85 7468656; Fax: +48 85 7468604
e-mail: kuklinska@poczta.onet.pl (Agnieszka Małgorzata Kuklińska)

Received 05.08.2005 Accepted 05.08.2005

Introduction

Cytokines involved in atherosclerosis together with hypoxia and tissue damage are the main factors to activate cyclooxygenase-2 (COX-2) [1]. COX-2 contributes to vascular prostacyclin (PGI2) synthesis, which prevents local thrombosis. Selective COX-2 inhibitors – coxibs, like rofecoxib, in opposite to nonspecific inhibitors (NSAIDs), reduce inflammation without significant gastrointestinal side effects [2]. HMG-CoA reductase inhibitors – statins have a strong lipid lowering effect and increase HDL levels. They also reduce C-reactive protein (CRP) levels [3]. Simvastatin in approved doses increases HDL-induced PGI2 release through COX-2 dependent mechanisms in vascular smooth muscle and endothelial cells [4]. This indicates their not only lipid-lowering and anti-inflammatory effect, but also endothelial protection.

Recent observations demonstrate that selective COX-2 inhibition via suppression of PGI2 biosynthesis shift the haemostatic balance toward a prothrombotic state [5,6]. At the end of September 2004, Merck Sharp & Dohme has withdrawn rofecoxib (Vioxx) from the market worldwide allegedly in response to the results of the APPROVe (Adenomatous Polyp Prevention On Vioxx) Trial [7]. In this placebo-controlled trial for colon cancer progression, its use in a dose of 25 mg/day after 18 months of therapy was associated with significant increased incidence of thromboembolic events. Whether this effect is dose-dependent remains an open question. At the time of Vioxx withdrawal some reports have shown that various doses of rofecoxib could have different effects on endothelial function, inflammatory cytokines and finally on cardiovascular events [8-10]. Besides it was postulated that rofecoxib added to statin may increase beneficial anti-inflammatory effect in patients after PCI [11].

We wanted to assess the anti-inflammatory effect of a low dose rofecoxib (12.5 mg/day) together with statin in a group of patients with ACS and the influence of the drugs combination on PGI2 synthesis, in short-term follow up.

Table 1. **Baseline clinical and biochemical characteristics of ACS patients**

	Simvastatin n=14	Simvastatin + rofecoxib n=15	p
Sex			
Male/ Female	9 (64.4)/ 5 (35.6)	5 (33.4)/ 10 (66.5)	0.1953
Age, years			
Mean	59	64	
Range	(50-74)	(49-78)	0.248
History			
IHD*	6 (42)	7 (46)	0.867
Smoking	7 (50)	4 (26)	0.3622
Hypertension	10 (71)	12 (80)	0.9165
Diabetes	3 (21)	4 (26)	0.9165
Dyslipidemia	5 (35)	2 (13)	0.3304
At admission:			
TIMI Risk Score (points)	3.4 (2-6)	3.3 (2-5)	0.965
Cholesterol (mg/dl)			
total	184±36.6	185±23.2	0.905
LDL	115±35.7	112±28.9	0.769
HDL	38±7.2	40±11.3	0.595
Triglicerides (mg/dl)	148±68.6	137±55.4	0.649
Troponin I (ng/ml) (range)	4.2 (0.0-45.0)	2.74 (0.01-10.9)	0.555

* previous: MI, angiographically proven IHD, PCI and/or CABG
Values are n (%) unless otherwise indicated

Table 2. **Pharmacological and invasive treatment in both groups of ACS patients**

	Simvastatin + rofecoxib group n=15	Simvastatin group n=14	p
Before admission:			
aspirin	6 (40)	7 (50)	1.0
β-blocker	4 (26)	4 (28)	0.763
ACE-I	4 (26)	5 (35)	0.908
At 30 day follow up:			
β-blocker	13 (86)	13 (92)	0.949
ACE-I	13 (86)	10 (71)	0.579
PCI (admission to 30 day follow up)	11 (73)	8 (57)	0.599
PCI in TIMI>4 points	6 (40)	4 (28)	0.79

Values are n (%) unless otherwise indicated

Material and methods

We have undertaken a study to investigate whether low dose of rofecoxib together with added *de novo* simvastatin has greater influence on inflammatory markers in patients with acute coronary syndromes (ACS) and what is the effect of both drugs on PGI2 synthesis. During this study we assessed the safety profile of the drugs.

The study had ethical committee agreement and written informed consent was obtained from each patient before enrollment. Study protocol was accomplished before rofecoxib withdrawal from the market worldwide.

Twenty-nine consecutive patients fulfilling the entry criteria (mean age 61.7±11.2 years) admitted to our Department because of ACS without ST segment elevation were included to the study. TIMI Risk Score was assessed in every patient at admission. Inclusion criteria were chest pain within previous month (IIB Braunwald class) or within the last 48 hours (IIIB Braunwald class) associated with ST segment depression (≥0.5 mm), T wave inversion or no ischemic changes in ECG. Exclusion criteria were persistent ST segment elevation, use of any cholesterol-lowering agent in the preceding month, acute myocardial infarction or revascularization procedures within the preceding month, any inflammatory disease or treatment with anti-inflammatory drugs, use of anticoagulant therapy, malignancy or contraindication to the study drugs.

The patients were randomized to simvastatin (20 mg daily) alone or together with rofecoxib (12.5 mg daily). All patients received acetylsalicylic acid (ASA; max 150 mg daily). Other medication was given according to acknowledged indications. Invasive treatment was preferred when TIMI Risk Score was over 4 points.

Medical history, lipid profile and serum levels of IL-6 (ELISA kits, Quantikine R&D Systems), hs-CRP (nephelometry, Dade Behring) and serum 6-keto-PGF-1α – a stable product of PGI2 (ELISA kits, R&D Systems) were assessed in both groups on enrollment and after 30-day follow up. We also controlled the renal, liver and skeletal muscle function (creatinine, transaminases, creatine kinase) in both groups during study.

Fasting blood samples were drawn, centrifuged (3000 rpm, 5 minutes). The serum was divided into aliquots and kept frozen at -20°C until analysis. According to the method, indomethacin (10 μg/ml) was added to samples in which 6-keto-PGF-1α was measured.

Data are expressed as mean ± standard deviation (SD). Categorical variables are presented as actual number of patients with relative frequencies given in brackets. These variables were assessed with Chi-square test. Mann-Whitney U test was used for comparison of non-categorical variables between groups and Wilcoxon matched pair test was used for comparison of two measurements within one group. A p value of less then 0.05 was considered as statistically significant.

Results

Fourteen patients were enrolled to simvastatin group and fifteen received both simvastatin and rofecoxib. There were neither clinical nor biochemical baseline differences of the patients in both groups (*Tab. 1*). Mean TIMI Risk Score for the whole group was 3.4 points-intermediate risk group.

During whole study all patients received ASA – max 150 mg daily. There were no significant treatment differences in both groups with regard to pharmacological and invasive treatment (*Tab. 2*).

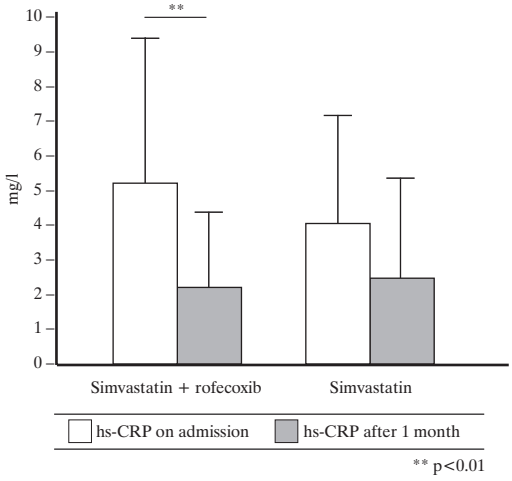
There were no differences in IL-6 levels on admission and its change during follow-up in both groups (Wilcoxon and Mann-Whitney tests) (*Tab. 3*).

Table 3. **IL-6 and 6-keto-PGF-1α values in both groups**

	Interleukin 6 on enrollment (pg/ml)	Interleukin 6 after follow up (pg/ml)	p	6-ketoPGF-1α on enrollment (pg/ml)	6-ketoPGF-1α after follow up (pg/ml)	p
Simvastatin	8.33 (1.3-9.6)	7.6 (3.8-9.2)	0.972	279.1 (209.1-342.1)	329.4 (93.8-1040)	0.345
Simvastatin+ rofecoxib	10.8 (1.2-14.9)	9.5 (7.5-10.4)	0.975	356.2 (207.4-768.3)	603.9 (294.7-1604)	0.109

Data are given as median value and interquartile range. Wilcoxon matched pair test was used for statistical analysis

Figure 1. **The effect of combination of low dose rofecoxib with simvastatin or simvastatin alone on serum concentration of CRP in ACS patients. Data are presented as mean ± standard deviation**



Admission levels of 6-keto-PGF-1α did not differ between groups. Although not significant, the levels similarly increased during therapy (*Tab. 3*).

There were no differences in hs-CRP concentrations at admission in both groups (3.95 mg/l ± 3.33 vs 5.21 mg/l ± 4.12; p=0.369). This result was confirmed in Mann-Whitney test. Rofecoxib with statin significantly decreased levels of hs-CRP during one month follow up. Such trend was not statistically significant in patients who took statin alone. (*Fig. 1*)

There were neither differences in side effects, nor in biochemical markers of renal, liver and sceletal muscle function between groups (data not shown).

Discussion

This is one of few studies to demonstrate that a selective COX-2 inhibitor together with standard therapy with added *de novo* statin reduces markers of inflammation.

High levels of inflammatory mediators, like IL-6 and CRP are found in patients with ACS [12]. It has been shown that high CRP levels are associated with endothelial dysfunction [13]. Treatment with statins reduces levels of inflammatory mediators indicating their anti-inflammatory effect [3]. Besides this, they are capable of modulating cell signaling and vascular function [4,14]. Recently it has been reported that simvastatin induces PGI2 release through COX-2-dependent mechanisms in vascular smooth muscle cells (VSMC) and endothelial cells. This indicates another effect of statins – improvement of endothelial function [4].

We wanted to check what would be the effect of low dose (12.5 mg daily) rofecoxib together with de novo added simvastatin not only on inflammatory cytokines, but also on PGI2 synthesis. We showed that simvastatin alone does not significantly lower inflammatory biomarker profiles (hs-CRP, IL-6) during one month treatment. Although not significantly simvastatin still increases PGI2 synthesis, which is consistent with its endothelial protection.

Coxibs, selective blockers of COX-2 isoform, have two main effects: lowering cellular-derived eicosanoides, which indicate their anti-inflammatory effect and decrease PGI2 synthesis-thus could impair endothelial function and disorder a thrombotic balance [5,15]. There are some data regarding the use of coxibs in patients with atherosclerosis. Their effect depends not only on the type of coxib used, but also, especially according to rofecoxib, seems to be dose-dependent. The dose of 25 mg daily used in APPROVe Trial was the one who lead Merck to withdraw rofecoxib from the market. According to APPROVe Trial rofecoxib in a dose of 25 mg daily, after 18 months of therapy doubled the risk of a myocardial infarction compared with placebo [7]. Ray et al. [8] in a meta-analysis (almost 400 thousand patients) showed that users of high dose rofecoxib, >25 mg daily (lowest approved daily dose is 12.5 mg), were 1.7 times more likely to have cardiac events (acute myocardial infarction and/or cardiac death) than non-users; among new users this rate increased to 1.93. There was no evidence of raised events risk rate among users of rofecoxib at doses of 25 mg daily or less or among users of other NSAIDs (including celecoxib). In another study rofecoxib in a dose of 25 mg/day had no favorable and adverse effects not only on endothelial function, but also on vascular inflammation (measured as hs-CRP, soluble intercellular adhesion molecule-1 and soluble IL-6 receptor levels) in patients with angiographically proven coronary artery disease (CAD) [10]. Solomon et al. showed that rofecoxib in doses >25 mg was associated with higher risk of acute myocardial infarction than dosages ≤25 mg [9].

The aim of our study was to test the low dose of rofecoxib in ACS patients. The completion of a statin-free study group was

difficult and was stopped in the moment of rofecoxib withdrawal from the market worldwide. Our data suggest that a low dose rofecoxib added to simvastatin does not decrease PGI2 levels during therapy. Perhaps the explanation is not only a dose of rofecoxib added to simvastatin, but also the finding that COX-2 blockade still allows COX-1 to produce PGI2 [16]. Still because of rofecoxib withdrawal we could not continue our study. Our small study pilot group did not allow us to evaluate clinical effects of the drugs. However we showed that the combination of the drugs has an important influence on hs-CRP levels and does not change IL-6 levels. Although Monakier et al. [17] showed that rofecoxib in a dose of 25 mg per day lowers both CRP and IL-6 levels, after three months of therapy the effect persisted only for CRP, but not for IL-6. This reveals the new point of activity of selective COX-2 inhibitors and their possible greater role in inhibiting liver CRP synthesis than in inflammatory cells-derived cytokines. Similar effect was observed in patients receiving statins, which lowered CRP levels and did not changed IL-6 [18]. On the other hand in REVERSAL Trial intensive lipid-lowering treatment with atorvastatin reduced progression of atherosclerosis, which was compliant with the greater reduction in C-reactive protein, independently of atherogenic lipoproteins [19].

Conclusions

Our study supports the hypothesis that low dose of selective COX-2 inhibitor added to standard therapy with statin in ACS patients has a significant anti-inflammatory effect and does not diminish prostacyclin synthesis.

Acknowledgement

This paper was financed by grant from Medical University in Białystok, Poland, No 3-53845. Preliminary data were published on ESC Congress in Munich, 2004.

Special thanks to Irena Słomińska and Elżbieta Kulikowska for excellent technical assistance and to Lucyna Traciecka who was involved in patient sampling. The authors are grateful to Jolanta Tobolczyk MSc PhD, from Department of Paediatric Allergology Medical University in Białystok for laboratory work.

References

1. Schönbeck U, Sukhova GK, Graber P, Coulter S, Libby P. Augmented expression of cyclooxygenase-2 in human atherosclerotic lesions. Am J Pathol, 1999; 155: 1281-91.

2. Bombardier C, Laine L, Reicin A, Shapiro D, Burgos Vargas R, Davis B, Day R. for the VIGOR Study Group. Comparison of upper gastrointestinal toxicity of rofecoxib and naproxen in patients with rheumatoid arthritis. N Engl J Med, 2000; 343: 1520-8.

3. Wierzbicki AS, Poston R, Ferro A. The lipid and non-lipid effects of statins. Pharmacology and Therapeutics, 2003; 99: 95-112.

4. Martinez-Gonzalez J, Escudero I, Badimon L. Simvastatin potentiates PGI2 release induced by HDL in human VSMC: effect on COX-2 up-regulation and MAPK signalling pathways activated by HDL. Atherosclerosis, 2004; 174: 305-13.

5. Catella-Lawson F, Crofford LJ. Cyclooxygenase inhibition and thrombogenicity. Am J Med, 2001; 110(Suppl. 3A): 28S-32S.

6. Mukherjee D, Nissen SE, Topol EJ. Risk of cardiovascular events associated with selective COX-2 inhibitors. JAMA, 2001; 286: 954-9.

7. Bresalier RS, Sandler RS, Quan H, Bolognese JA, Stat M, Oxenius B. For the adenomatous polyp prevention on vioxx (APPROVe) trial investigators. N Engl J Med, 2005; 352: 1-11.

8. Ray WA, Stein CM, Daugherty JR, Hall K, Arbogast PG, Griffin MR. COX-2 selective non-steroidal anti-inflammatory drugs and risk of serious coronary disease. Lancet, 2002; 360: 1071-3.

9. Solomon DH, Schneeweiss S, Glynn RJ, Kiyota Y, Levin R, Mogun H, Avorn J. Relationship between selective cyclooxygenase-2 inhibitors and acute myocardial infarction in older adults. Circulation, 2004; 109: 2068-73.

10. Title LM, Giddens K, McInerney MM, McQueen MJ, Nassar BA. Effect of cyclooxygenase-2 inhibition with rofecoxib on endothelial dysfunction and inflammatory markers in patients with coronary artery disease. J Am Coll Cardiol, 2003; 42: 1747-53.

11. Dudek D, Heba G, Legutko J. More pronounced decrease of inflammatory markers with combination of statins and COX-2 inhibitors following acute coronary syndromes. European Heart J, 2001; 22 (Suppl.): 1302.

12. Libby P, Ridker PM, Maseri A. Inflammation and atherosclerosis. Circulation, 2002; 105: 1135-43.

13. Fichtlscherer S, Rosenberger G, Walter DH, Breuer S, Dimmeler S, Zeiher AM. Elevated C- reactive protein levels and impaired endothelial vasoreactivity in patients with coronary artery disease. Circulation, 2000; 102: 1000-6.

14. Dichtl W, Dulak J, Frick M, Alber HF, Schwarzacher SP, Ares MPS, Nilsson J, Pachinger O, Weidinger F. HMG-CoA reductase inhibitors regulate inflammatory transcription factors in human endothelial and vascular smooth muscle cells. Arterioscler Thromb Vasc Biol, 2003; 23: 58-63.

15. Pitt B, Pepine C, Willerson JT. Cyclooxygenase-2 inhibition and cardiovascular events. Circulation, 2002; 106: 167-9.

16. Belton O, Byrne D, Kearney D, Leahy A, Fitzgerald DJ. Cyclooxygenase-1 and -2-dependent prostacyclin formation in patients with atherosclerosis. Circulation, 2000; 102: 840- 5.

17. Monakier D, Mates M, Klutstein MW, Balkin JA, Rudensky B, Meerkin D, Tzivoni D. Rofecoxib, a COX-2 inhibitor, lowers C-reactive protein and interleukin-6 levels in patients with acute coronary syndromes. Chest, 2004; 125: 1610-5.

18. März W, Winkler K, Nauck M, Böhm BO, Winkelmann BR. Effects of statins on C-reactive protein and interleukin-6 (The Ludwigshafen risk and cardiovascular health study). Am J Cardiol, 2003; 92: 305-8.

19. Nissen SE, Tuzcu EM, Schoenhagen P, Brown BG, Ganz P, Vogel RA, Crowe T, Howard G, Cooper CJ, Brodie B, Grines CL, DeMaria AN; REVERSAL Investigators. Effect of intensive compared with moderate lipid-lowering therapy on progression of coronary atherosclerosis. JAMA, 2004; 291: 1071-80.

Index of Authors

A

Abe H 101
Ablameyko S 284
Adamczak M 50
Amato G 216
Andrzejewska A 230

B

Bühl A 212
Balicki R 334
Banachowicz W 314
Baranowicz-Gąszczyk I 252
Bartosińska M 329
Barzdžiukas V 201
Bełtowski J 322
Bednarek-Skublewska A 252, 307
Belli CMC 216
Benetis R 45, 244
Blusiewicz K 188
Bojar I 319
Borys D 161
Buczko W 126
Bukin M 204
Butkiewicz A 293
Buzun L 166

C

Chabannaya TA 183
Chrostek L 220
Chukov SZ 183
Chyczewska E 197
Chyczewski L 179
Coatrieux JL 132
Cudowska B 261, 268
Czekalski S 122

D

Dąbrowski A 230
Długosz JW 230
Dobrzycki S 339
Dryglewska M 307
Dymicka-Piekarska V 293
Dziemiańczyk D 334

E

Egawa S 101
Eickhoff A 155, 212
Eickhoff JC 155
Ejsmont J 329
Esposito P 216

F

Fabre-Guillevin E 97
Fukuyama S 101

G

Głuszek S 224
Gajewska E 297
Gallucci F 216
Gendviliene V 241, 244
Glebov A 247
Górska M 148, 151
Grabowska SZ 334
Greenhalf W 73
Grygorczuk S 173
Grzegorzewska AE 314
Gugała K 148
Gurskaite H 241

H

Hartmann D 155
Haurylik LL 279
Hermanowska-Szpakowicz T 173
Hirnle T 161, 166
Hisada S 142
Howes NR 73

J

Jabłońska E 204
Jackowski R 166
Jakob H 37
Jakobs R 155, 212
Jaroszewicz J 179
Jóźwiak L 252
Jurevicius J 244

K

Kądziela-Olech H 302
Kaczmarski M 257, 261, 268, 274
Kamiński KA 339
Kanaporis G 244
Kargol A 237
Kargol M 237
Karpińska M 237
Kemona H 293
Kiersnowska-Rogowska B 204
Kinalska I 151
Kitszel A 208
Klachko NM 279
Kobayashi M 142
Kondrusik M 173
Kosicki M 161
Kostrzewa A 289
Kotarski J 322
Kotelevets SM 183
Kowalczuk O 179
Kozłowski M 193
Krüger CM 106
Krętowski A 148, 151
Kralisz P 339
Krawczuk-Rybak M 208
Książek A 252, 307
Kuklińska AM 339
Kurapkienė S 201

L

Löffler W 212
Lasota M 274
Leander M 314
Lehours P 54
Lenartowska L 161
Lialikau SA 279
Lieber CS 7
Lisowska A 166
Lisowski P 166

Ł

Łapiński TW 179

M

Mégraud F 54
Małyszko J 126, 166
Małyszko JS 126, 166
Macianskiene R 244
Majdan M 307
Majewska A 311
Manyś-Kubacka K 289
Marciniak A 322
Martisiene I 241
Matowicka-Karna J 293
Matsuno S 101
Matykiewicz J 224
Mikami Y 101
Mirończuk K 151
Modrzejewski W 339
Mojs E 297
Morisset J 21
Motoi F 101
Musiał WJ 339
Muszyńska-Rosłan K 208
Muszytowski M 311
Myśliwiec M 126, 166

N

Naumnik W 197
Nedzved A 284
Neoptolemos JP 73
Nowak K 230
Nowak M 161

O

Oczeretko E 284
Okruszko A 148, 151
Omura N 101
Ossolińska M 197
Ostrowski T 319

Ö

Öberg K 62

P

Pancewicz S 173
Parfieńczyk A 204
Pasechnikov VD 183
Pasquali C 85
Paunksnis A 201
Pedrazzoli S 85
Piedbois P 97
Piotrowska-Jastrzębska J 302
Popowska-Drojecka J 311
Prajs P 161
Prokopowicz D 179
Przestalski M 237
Pużewska W 204

R

Raff R 91
Rau BM 106
Restani P 268
Riemann JF 155, 212
Ritz E 50
Rogowski F 204
Rogowski M 237
Rolle A 193
Russo R 216
Rydzewska G 188
Rydzewski A 188

S

Samborski W 279, 289, 297
Sawicka-Żukowska M 208
Schilling D 155
Schilling MK 106
Schwanitz G 91
Sereika V 201
Shimizu K 142
Shiratori K 142
Siedlecki J 237
Skeberdis VA 244
Sobiło-Jarek L 311
Sobieska M 279, 289, 297, 325
Sobkowicz B 161, 339
Sperti C 85
Stec S 339
Sultan-Amar V 97
Sunamura M 101
Szmitkowski M 220
Szymczyk G 322

Ś

Świdzińska E 197
Świerzbińska R 173

T

Takeda K 101
Tanaka M 69
Thielmann M 37
Tomaszewski W 220
Trojan S 197

U

Uścinowicz M 257
Uomo G 116, 216

V

Vaičaitienė R 201
Vitone LJ 73

W

Wasilewska J 274
Wawrusiewicz-Kurylonek N 148, 151
Wdowiak L 319
Weickert U 155, 212
Wołczyński S 208
Wojtkowska I 339
Wrabec K 161
Wróblewski E 230

Z

Zablockaite D 241, 244
Zaborski L 329
Zagożdżon P 329
Zajkowska J 173
Zinchuk V 247
Zvárová J 138

Index of key words

Symbols

α_1-antichymotrypsin 325
5-fluorouracil 97

A

abdominal hysterectomy 322
acceptance 212
acute coronary syndromes 37
acute coronary syndromes (ACS) 339
acute lymphoblastic leukaemia 208
acute myocardial infarction 161
acute pancreatitis 101, 106, 116
acute phase proteins 279
ADHD 302
adiponectin 148
advanced colorectal cancer 97
alcoholic hepatitis 7
ambulatory blood pressure monitoring 252
amino acid-based formula 274
ammonia 188
angiography 101
anti-cytokine approaches 106
antibiotic prophylaxis 116
antibiotic treatment 116
apoptosis 179
artery calcification 311
atopic eczema/dermatitis syndrome 274, 262
atopy patch test 262
atrophy 183
A16V 73

B

B-cell chronic lymphocytic leukemia 204
bactericidal activity 293
benign biliary stricture 155
bevacizumab 97
birch-apple syndrome 268
blood 247
blood morphology 314
blood platelets 293
body mass index 208
breast feeding 302

C

cardiac troponin-I 311
cardiovascular diseases 122, 329
cardiovascular risk 50
cationic trypsinogen gene (PRSS1) 73
Ca19-9 73
cell cycle abnormalities 91
cell membrane 237
cerulein acute pancreatitis 230
cetuximab 97
CGH 91
chemokines 173
children 208, 302
Chlamydia trachomatis infection 307
cholecystokinin 21
cholecystokinin (CCK-8) 257
cholecystokinin receptors 21
choroidal melanoma 289
chromoendoscopy 183
chronic pancreatitis 142
chronic renal failure 126
civil and military medicine 201
composite karyotype 91
congestive heart failure 45
Continuous Regional Arterial Infusion (CRAI) therapy 101
contraction force 244
contrast enhanced CT 101
coronary artery 45
coronary artery bypass grafting 37
coronary artery bypass grafting (CABG) 166
coronary artery disease (CAD), haemostasis 166
coronary heart disease 151
cow's milk protein 274
cross-reactivity 268
CT 73
cyclooxygenase-2 inhibitors 339
cytochrome P450 (CYP2E1) 7
cytokines 106
cytoplasm 237

D

diabetes 69
diabetes type 1 151
diabetic nephropathy 122
diagnostic 284
diagnostic laparoscopy 212
dialysis 252
diurnal rhythm 252

E

e-health 138
e-learning 138
early diagnosis 69
ECDL 138
electronic health record 138
endostatin 197
epidemiology 329
ERCP 73
Esposito P 216
ET-1 230
ET-2 230
ET-3 230
ethanol 220
EUROPAC 73
EUS 73
exon 3 148
extensively hydrolyzed formula 274

F

failing human myocardium 241
flow cytometry 289, 325
fluoroquinolones 116
food allergy 262
force-frequency relationship 241
free wall cardiac rupture 161
functional abdominal pain 257

G

gastrin 21
gastrin-17 183
gastritis 188
gastrointestinal neuroendoctrine tumours (GEP-NET's) 62
green tea 220
growth 208
growth factors 208

H

H. pylori 54
HBsAg carriers 179
HBV-DNA levels 179
Helicobacter pylori 183, 188
hemodialysis 188, 307, 311
hemostasis 126
hereditary pancreatitis 73
herpes 284
highly selective vagotomy 224
human atrium 244
human red blood cells 237
hyperactivity 302
hyperosmosis 244

I

image processing 284
imipenem 116
immunoblotting 268
inflammation 339
interleukin 18 151
intestinal metaplasia 183
intraductal papillary mucinous neoplasm 69
iodine provision 279
irinotecan 97
iron 314
isoprostanes 322
isoproterenol 241

K

K-ras 73
Kt/V 314

L

L-type calcium current 244
laser surgery 193
leptin 208
lipid transfer protein 268
lipopolysaccharide 247
liver ALDH 220
long-term stenting 155
lung carcinoma 197
lung metastasectomy 193
Lyme borreliosis 173
lymphadenectomy 85
lymphocyte subpopulations 289
lymphocytes 325
lymphoma 54

M

macroalbuminuria 122
MALT 54
medical guidelines 138
medical information 201
meningitis 173
meropenem 116
meta- and interphase-FISH 91
microalbuminuria 50, 122
micronucleus formation 91
modelling 132
mortality 122, 329
multilevel descriptions 132
multimodal data 132
multipolar spindle apparatus 91
multiscale processing 132

N

nafamostat mesilate 101
nervous system 284
NF-κB 142, 230
nitric oxide 247
nonalcoholic steatohepatitis (NASH) 7
noradrenaline 252
nosocomial infections 216
N29I 73

O

obstetric and gynaecological services 319
oral cavity cancer 334
oral food challenge 262
outcomes 37
oxaliplatin 97
oxidative stress 247

P

pancreas 21
pancreas morphology 224
pancreas ultrastructure 224
pancreatic cancer 69, 73, 85
pancreatic necrosis 116
pancreaticoduodenectomy 85
patient's satisfaction 319
pepsinogen I 183
percutaneous transhepatic drainage 155
perioperative management 37
pH 314
phagocytic activity 293
plasma 237
polyenylphosphatidylcholine (PPC) 7
polykaryotic cells 91
polymorphism 148
PPARγ 142
prostacyclin 339
protease inhibitor 101
psychological and physiotherapeutic diagnosis 297
PTH 314
pyrrolidine dithiocarbamate 230
pyruvate 241
p16 73
p53 73

Q

quality assessment 319
questionnaire 212

R

rabbit 224
rats 230
reactive oxygen species 322
recombinant allergens 268
reduced glomerular filtration rate 50
rehabilitation methods 297
renal osteodystrophy 307
risk factor 302
R122H 73

S

S-adenosylmethionine (SAMe) 7
salbutamol 244
saliva 334
secondary screening 73
secretory mucins 334
sepsis 216
sFas 204
sFasL 204
simvastatin 339
skin-prick tests 262
soluble Intercellular Adhesion Molecule 1 151
soluble TNF-related apoptosis-inducing ligand 204
somatostatin 62
specific IgE 262
Staphylococcus aureus 216
surgical treatment 45

T

T lymphocytes with TCR 289
targeted therapy 97
teleconsultations 201
telemedicine 201
thiazolidinedione derivatives 142
thoracic surgery 193
thrombin activatable fibrinolysis inhibitor (TAFI) 166
thrombolytic therapy 161
thyroid hormones 279
TNF-α 142
transport equations 237
trichinellosis 293
truncal vagotomy 224
trypsinogen 230
tumor cytogenetics 91
tumor growth *in vitro* 91
type 2 diabetes 148

U

urea 188

W

water exchange 237
West Syndrome 297

Instructions for Authors

Scope

Roczniki Akademii Medycznej w Białymstoku (Annales Academiae Medicae Bialostocensis) is an international, scientific journal, indexed in Index Medicus/Medline, Chemical Abstracts and Index Copernicus, that publishes full-length original articles on live science, preclinical and clinical medicine and related disciplines, current review articles, reports on current research, case studies and special reports.

Roczniki Akademii Medycznej w Białymstoku is issued once a year in printed form, and in electronic form at *http://www.roczniki-amb.com*

Editorial Policy and General Information

Submission of Manuscripts

Three copies of the manuscript (in English), including the electronic file on floppy disk should be submitted to: Jacek Niklinski, MD, Editor-in-Chief, Editorial Office, Medical University of Bialystok, ul. Kilinskiego 1, 15-089 Bialystok, Poland (e-Mail: niklinsj@amb.edu.pl)

The manuscript must be accompanied by a written statement of corresponding author that no similar paper has been or will be submitted for publication elsewhere as well as all authors listed on a manuscript have agreed to its submission.

Upon acceptance of the manuscript, the author(s) will be asked to transfer the copyright to the publisher.

Review Process

All submitted manuscripts are subject to review by independent experts for scientific evaluation. When a decision is reached, a decision letter will be sent to the authors, including the comments of the referee(s).

Conflict of Interest

Authors are requested to provide a statement concerning any commercial associations or patent licenses that might result in a conflict of interest with the work presented in the submitted paper. This may be placed in the Acknowledgements sections.

Permissions

Materials copied from other sources must be accompanied by a written statement from both the author and publisher giving permission to *Roczniki Akademii Medycznej w Białymstoku (Annales Academiae Medicae Bialostocensis)* for reproduction. It is the author's responsibility to ensure that such permissions are obtained.

Preparation of Manuscripts

Articles should be written in English (either British or American spelling).

The format of the research article should be as follows:

Title page, includes the title, full names of authors and their affiliations, corresponding author contact information (address, fax, e-mail).

Abstract page and key words. Abstract, of no more than 250 words should be provided on a separate page, organized in structural form, and should consists of four paragraphs: Purpose, Material/Methods, Results, Conclusions. Key words (3-6) or short phrases should be added to the bottom of the abstract page. Use terms from the Medical Subject Headings list from Index Medicus.

Text should be arranged in the following manner: Introduction, Material and Methods, Results, Discussion, Acknowledgments, References

References should be numbered and listed subsequently as they are cited. All authors should be listed. The references should be cited in the text as numbers in square brackets.

Examples:

1. Harbour JW, Lai SL, Whang-Peng J, Gazdar AF, Minna JD, Kaye FJ. Abnormalities in structure and expression of the human retinoblastoma gene in SCLC. Science, 1988; 241: 353-7.
2. Niklinski J, Claassen G, Meyers C, Gregory MA, Allegra CJ, Kaye FJ, Hann SR, Zajac-Kaye M. Disruption of Myc-tubulin interaction by hyperphosphorylation of c-Myc during mitosis or by constitutive hyperphosphorylation of mutant c-Myc in Burkitt's lymphoma. Mol Cell Biol 2000, 20, 5276-84.
3. DeVita VTJ., Hellman S, Rosenberg SA. Cancer: Principles and Practice of Oncology. 4th ed. Philadelphia: J.B. Lippincott Co.; 1993.
4. Norman IJ, Redfern SJ, editors. Mental health care for elderly people. New York: Churchill Livingstone; 1996
5. Phillips SJ, Whisnant JR. Hypertension and stroke. In: Laragh JH, Brenner BM, editors. Hypertension: pathophysiology, diagnosis, and management. 2nd ed. New York: Raven Press; 1995, p. 465-78.

Tables should be typed on separate sheet of paper and provide a title for each. Tables should be numbered in arabic numerals. Vertical rules should be used. The position of each table should be indicated in the margin of the manuscript.

Figures should be submitted as black and white prints on glossy paper and have as much contrast as possible. They should be numbered in Arabic numerals in order of appearance in the text, where they are referred to as *Fig. 1*, *Fig. 2*, etc. Figure legends with descriptive titles should be provided on separate pages. All color photographs can be reproduced in full color, however the extra costs of color reproduction will be charged to the author(s).

General

Units of measurements. Authors must express measurements in Systeme International (SI) units.

Abbreviation. Except for units of measurement, only standard abbreviations are acceptable