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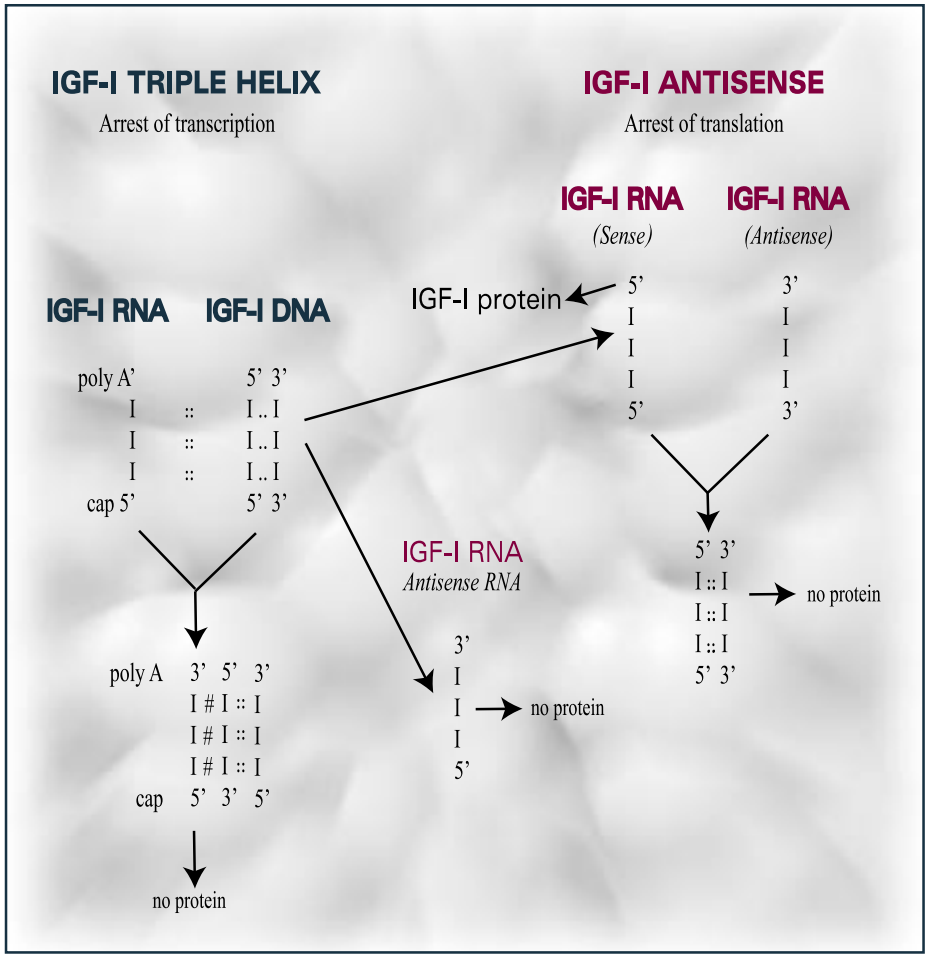

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Cover Illustration: Schema of IGF-I antisense and IGF-I triple helix IGF-I technology. In antisense technology the end result is the inhibition of IGF-I mRNA (sense RNA) activity by binding to the antisense RNA (red colour). In IGF-I triple helix technology the oligopurine third strand forms RNA-DNA triple helix with IGF-I gene (blue colour).
Hoogsteen hydrogen bonds; :: Watson-Crick bonds.

Chemoprevention of lung cancer: from concept to reality

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Abstract

Among all cancer, lung cancer is the leading cause of cancer death in the western world. The poor lung cancer survival figures argue powerfully for new approaches to control this disease such as chemoprevention, that has been defined as the use of agents that reverse, suppress or prevent lung carcinogenesis.

Over 80% of lung cancers are attributed to tobacco and carcinogens from cigarette smoke form the unquestionable link between nicotine addiction and lung cancer.

Confoundingly epidemiological studies show that not more than 15% of heavy smokers will ultimately develop lung cancer. The fact that 85% of heavy smokers will not develop lung cancer points to differences in susceptibility. Dietary and genetically determined factors seem to play an important role in modulating individual susceptibility and are closely linked to the chemoprevention approach.

Developments in tumor biology and chest radiology have given rise to optimism regarding to earlier diagnosis and improved possibilities for the management of preneoplastic – and early invasive lesions. The current article summarizes the chemoprevention efforts in lung cancer during the last two decades and identifies novel strategies for chemoprevention studies in high-risk individuals based on the developments in molecular targeted therapies and developments in response evaluation by using intermediate biomarker endpoints.

Key words: lung cancer, chemoprevention, carcinogenesis.

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Introduction

Lung cancer is the leading cause of cancer death in the western world. Despite several decades of intensive research in the thoracic oncology area, the prognosis for the patients with lung cancer is still poor, and less than 15% of the patients survive 5 years after the primary diagnosis. This grim outlook for lung cancer patients is mainly due to the lack of measure for early diagnosis and insufficient treatment possibilities for systemic disease. More than 2/3 of the patients present with regional or metastatic disease at time of primary diagnosis.

Chemoprevention, defined as the use of naturally occurring or synthetic agents to prevent, inhibit or reverse the process of carcinogenesis, is a relatively new approach to cancer prevention that has a precedence in other areas of medicine such as cardiology, in which cholesterol–lowering and platelet anti-aggregating agents are administered to prevent coronary heart disease in individuals with elevated risk. This concept has been studied in lung cancer but so far without significant success as is summarized below. However recent developments in biology and radiology have given new possibilities for early detection of preneoplastic and early neoplastic lesions. Along with these developments new avenues for chemoprevention are emerging [1]. It is within the scope of this article to describe the basis of chemoprevention and to point to the future perspectives based on recent developments in biology, bronchoscopic techniques and radiology. One concept of these new strategies is to already consider the carcinogenic process as a disease and clinical manifest lung cancer as the 'end stage' of the disease.

Tobacco carcinogenesis

Cigarette smoke contains a mixture of carcinogens, including a small dose of polycyclic aromatic hydrocarbons (PAH's) and 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) among other lung carcinogens, tumor promoters, and co-carcinogens [2].

Carcinogens such as PAH's and NNK require metabolic

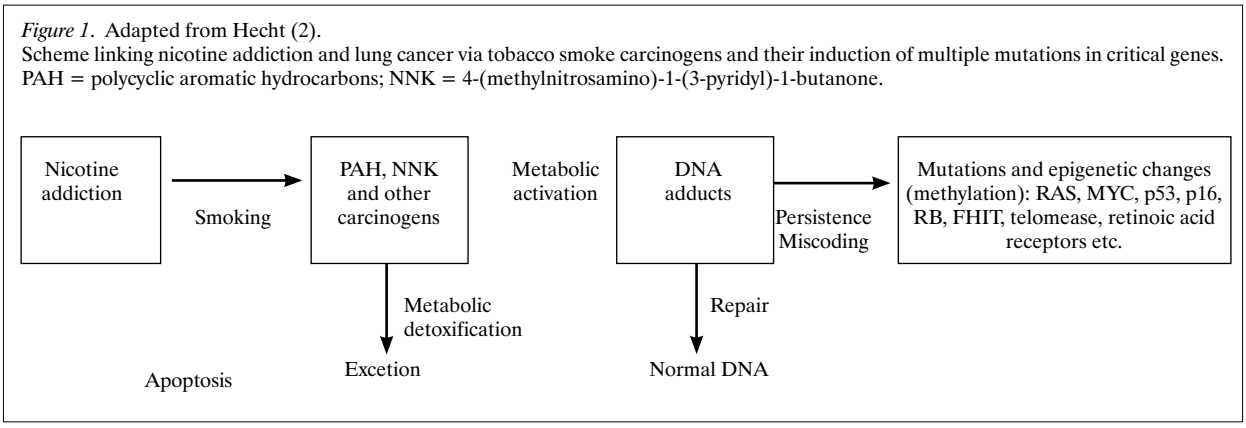

Figure 1. Adapted from Hecht (2).
Scheme linking nicotine addiction and lung cancer via tobacco smoke carcinogens and their induction of multiple mutations in critical genes. PAH = polycyclic aromatic hydrocarbons; NNK = 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

activation to exert carcinogenic effects. The activation pathways are competing with detoxification pathways and the balance between activation and detoxification is assumed to affect the cancer risk. The genes for the cytochrome P-450 carcinogen metabolizing enzymes (activation) have been found polymorphic and also the glutathione transferases (detoxification enzymes) are polymorphic. Approximately 40-50% of the human population has a so-called null genotype, that has a modest association with lung cancer [3].

Successful metabolic activation will lead to the formation of DNA adducts, which are carcinogen metabolites bound covalently to DNA. If DNA adducts escape cellular repair mechanisms and persist, they may cause miscoding, resulting in a permanent mutation [4,5] (*Fig. 1*).

In recent case-control studies defective repair of genetic damage and increased sensitivity to mutagens have been associated with increased susceptibility to lung cancer [6]. Also the DNA excision repair pathway has been suggested to influence the individual susceptibility for lung cancer [7]. From molecular studies it is known that genetic alterations (scars) persist after the cessation of tobacco use [8]. In this respect it is important to mention the fact that ex-smokers carry an elevated risk of lung cancer for many years and that most of those who now develop lung cancer in the US are ex-smokers [9,10].

In the last 10-20 years our understanding of the molecular and biological basis of lung cancer has significantly expanded. However, we must not forget that traditional epidemiological studies have provided reliable indicators of host susceptibility to tobacco carcinogenesis.

A number of case-control studies have suggested that females are more susceptible to the carcinogenic effects of tobacco smoke than men [11,12]. This observation has been confirmed by the analysis of smoking habits in a population of lung and head & neck cancer patients, that took part in a study aiming at preventing second primary tumors, revealing significantly less exposure to tobacco (pack-years) before occurrence of the first cancer in females in comparison with males [13]. It is not surprising that smoking has been found to interact synergistically with a family history of lung cancer [14,15].

Diet

The relationship between diet and lung cancer has been extensively explored in many ecologic and case-control studies and there are many leads to support an association between a high intake of fruits and vegetables and a reduced risk of lung cancer [16]. Much effort has been expended to identify the specific components of these foods that may be responsible for the lower lung cancer risk. In the majority of studies, attention has focused on the pro-vitamin A carotenoids, particularly beta-carotene because of their antioxidant properties and the importance of vitamin A in cell growth and differentiation.

More recently other micronutrients have been identified as having the potential to decrease lung cancer risk, including vitamin E, selenium, isothiocyanates, allyl sulphur compounds and green tea polyphenols. While Vitamin E (a term that refers to eight natural compounds, including the tocopherols) failed to lower the lung cancer risk in a large randomized (ATBC) trial a protective effect of selenium was found in a Skin Cancer Prevention Trial [17].

Isothiocyanates are non-nutrient compounds in cruciferous vegetables that can influence P-450 enzyme levels and enhance detoxification. A recent series of newly diagnosed lung cancer cases had significantly lower isothiocyanate intake when compared with controls. In this study also glutathione-S-transferase (GST) (null) genotype and smoking were associated with increased lung cancer risk, suggesting that smokers with low intake of isothiocyanates and a null GST genotype carry an extra risk [18]. Allyl sulphur compounds that may induce apoptosis, and green tea polyphenols have also been mentioned as potential preventive agents for NSCLC [20,21].

In addition attention has focused on cooking practices. Increased lung cancer risk has been noted as a consequence of high intake of heterocyclic amines, which are produced when meats are cooked at high temperatures [21].

Overall, the epidemiological data support the hypothesis that a different intake of dietary compounds could modulate the risk of lung cancer. The confirmation of this hypothesis, however, can only be provided by carefully designed prospective trials that balance for smoking behavior and ideally also take diet-gene interactions into account as in the recent isothiocyanate study [18,22].

Field Cancerization and Multi-step Carcinogenesis

The rationale for chemoprevention is based on two principles of tumor biology common to most epithelial can-

cers: field cancerization and multi-step carcinogenesis. Field cancerization was first described by Slaughter et al in 1953, who identified epithelial hyperplasia hyperkeratinization, atypia and also carcinoma in situ in grossly normal appearing epithelium adjacent to cancers of the oral cavity [23]. These histologic changes throughout the oral epithelium suggested that the development of malignancy in the aerodigestive tract is not a random event but rather the result of diffuse changes that are present throughout the epithelium. The existence of diffuse premalignant changes in the aerodigestive tract confirms the observation that patients who survive a first cancer in this region are subsequently susceptible to the development of a second primary tumor [24].

Specific genetic alterations seem to be involved. For example, it has been shown that highly specific deletions in the short arm of chromosome 3 occur during hyperplasia, the earliest stage on the way to lung cancer [25]. Similar evidence exists for deletions of the short arm of chromosome 9 and 17 [26]. One of the genes on chromosome 3 is the fragile histidine triad gene. Similarly to the p53 gene on chromosome 17 this gene is thought to have a tumor-suppressor function [27].

Although the series of events eventually leading to the development of lung cancer are not yet completely elucidated, it is generally accepted that lung cancer develops in a stepwise fashion as the result of multiple genetic events. Suppressing one or more of the pre-invasive steps may impede the development of cancer.

Retinoids

Due to their ability to regulate cell proliferation and differentiation retinoids represent a potentially useful class of drugs in the chemoprevention field. In multiple animal model systems at different organ sites and with a variety of inducing carcinogens retinoids were found to be preventive [28].

A randomized phase II study with cis-retinoic acid in heavy smokers suggested however that smoking cessation was more important than the actual prevention with retinoids [29].

Several randomized clinical chemoprevention trials have been completed in the 1990s. Among these the Alpha Tocopherol Beta Carotene (ATBC) and the Beta Carotene and Retinol Efficacy (CARET) trials [30,31]. The ATBC trial accrued men, all heavy smokers and tested the effects of dietary supplementation of beta-carotene and alpha-tocopherol. Against all expectations this trial did not show any protective effect from either alpha-tocopherol or beta-carotene. On the contrary, beta-carotene was associated with a statistically significant increase (18%) in lung cancer incidence and mortality (8%). The detrimental effect of beta-carotene was confirmed by the CARET study, also involving smokers. CARET revealed a 28% higher rate of lung cancer and 17% higher overall death rate in those participants taking beta-carotene. The findings of ATBC and CARET have clearly been a shock to the prevention community but they have shown the unquestionable importance of large-scale controlled (randomized) studies. Since the detrimental effect of beta-carotene was seen primarily in smokers, it has been hypothesised that cigarette smoke in the lungs, which is highly oxidizing, may interact with beta-carotene, yielding unstable by-products that could have pro-oxidant activity. A recent experimental study in ferrets exposed to cigarette smoke and beta-carotene suggests that such an interaction exists [32]. Smokers should therefore avoid beta-carotene supplementation and the importance of smoking cessation is once more emphasized. An explanation for the lack of preventive effects of retinoids in the human situation is provided by the observation that the nuclear retinoid receptor beta, which act as transcription factor, was found to be suppressed in preneoplastic bronchial lesions and NSCLC's [33]. Promoter methylation and allelic loss have been thought to be responsible for this suppression [34].

Recently a new class of retinoids has been identified that seems to be more effective in growth inhibition and induction of apoptosis of lung cancer cell lines [35]. This observation together with the favorable early clinical experience with bexarotene, a retinoid-X-receptor selective retinoid, in NSCLC patients [36] and the negative impact of loss of retinoic acid receptor beta in stage I NSCLC patients [37] indicates that the retinoid receptors will remain a target for lung cancer chemoprevention studies. We must however avoid the premature start of large and costly comparative chemoprevention trials i.e. before validation studies with intermediate markers of carcinogenesis have shown an unequival positive outcome.

Second Primary Tumors

One of the earliest studies with relevance to lung cancer was a trial in 103 patients with previous head & neck cancer in which the effect of 13-cis-retinoic acid on recurrence and second primary cancer was studied [38]. The incidence of second primary tumors was significantly lower in the treatment arm (4% versus 24%). The second trial involved 307 patients with early stage lung cancer, randomized after complete surgical resection to receive either retinyl palmitate or no further treatment [39]. Also in this study there was a significant difference in the frequency of second primaries (12% versus 21%).

The large EUROSCAN study designed to assess the effects of retinyl palmitate and N-acetylcysteine (an aminothiol precursor of reduced glutathione) in almost 2600 patients with early stage head and neck cancer or lung cancer following treatment with curative intent, did not confirm the positive outcomes of the previous studies with 13-cis-retinoic acid and retinyl palmitate. In contrast to expectations this study did not show any benefit for a 2-year supplementation with retinyl palmitate and/or N-acetylcysteine in a population at risk for second primary tumors and tumor recurrences in the upper and lower airways [40].

A similar result was recently obtained in the US NCI intergroup trial with 13-cis-retinoic acid to prevent second primary tumors (SPT) in Stage I NSCLC. 13-cis-retinoic acid did not impove the rate of SPT's or mortality [41]. Subgroup analyses suggested that 13-cis-retinoic acid might have been harmful in smoking patients and beneficial for those patients belonging to the category of never smokers. The EUROSCAN and the NCI intergroup trials underline the importance of large confirmatory trials. Agents long assumed to be helpful may be found to have no preventive effect at all, when tested in a rigorous way.

EUROSCAN also strengthened the importance of smoking cessation: participants who had permanently stopped smoking had a better survival than those who continued smoking.

Intermediate markers

When invasive cancer is taken as an endpoint, as has been done in the aforementioned studies, significant investments in time and money are needed. One approach to avoid the primary evaluation of potential chemopreventive agents in large clinical trials with cancer as primary endpoint is to use intermediate biological endpoints. This is often referred to as reversal of premalignancy. Potential intermediate markers include microscopic changes of the bronchial epithelium, such as metaplasia, or more specific markers, such as cytogenetic or molecular changes. In an effort to develop a more dependable outcome than a pathologic judgement on a single biopsy, the metaplasia index, a semiquantitative method to characterize the degree of metaplasia in a number of bronchial biopsies, was introduced [42].

However, differences in study population might reflect differences in the level of metaplastic/dysplastic changes in the bronchial mucosa, which needs to be taken into account if morphology is used as intermediate marker. In a study from MD Anderson most of the high-risk patients had metaplasia, but very few patients had dysplasia [43], while in a study population of the University of Colorado Cancer center about 55% of the high-risk population had moderate dysplasia or worse at bronchoscopy [44]. In the Colorado study fluorescence bronchoscopy (LIFE) was shown to improve the detection of preneoplastic bronchial lesions when compared with white light bronchoscopy. Essential is a reproducible classification of (i.e. minimal interobserver variation) of these lesions. Hirsch et al. undertook an interactive study on the Internet that revealed very little interobserver variability among pathologists involved in this area [45]. Another issue is the reversibility of preneoplastic changes. There is increasing evidence that metaplasia is a spontaneous reversible condition whereas dysplasia is not [46,47]. Consequently metaplasia may not be a reliable intermediate marker to study the efficacy of chemopreventive measures. More data from longitudinal analyses are needed to validate the prognostic implication of subtle epithelial changes in the airways. Based on new technologies such as microchip gene array or protein analysis, new markers are under development, but so far it is too early to predict whether these markers will indeed be useful as intermediate endpoints for chemoprevention studies.

New Classes of Chemopreventive Agents

Advances in molecular biology has led to a better understanding and knowledge about important pathways necessary for cancer development, and antibodies and small molecules have been developed to target specific proteins and block several important signaling pathways. Some of these molecules have attracted attention as potential chemopreventive agents opening a new era of therapeutic possibilities.

The identification of the *ErbB*-family (Epidermal Growth Factor EGFR, Her-2/*neu*, HER-3 and HER-4) as a family of receptors has been especially important. These receptors activate, after ligand binding, a cascade of biological- and physiologically reactions eventually leading to cell proliferation and apoptosis. These signaling pathways can be blocked at different levels. Preliminary studies of bronchial preneoplasia have demonstrated significant expression by immunohistochemistry of EGFR and HER-2 [48] and a chemopreventive treatment approach with antibodies or small molecules blocking these pathways seems to be an attractive approach (49). Likewise, activation of the EGFR involves *RAS* activation, which again requires a farnesylation. Thus, treatment with a farnesyl transferase inhibitor (FTI) might block this signaling pathway. In fact in the US a group of centers (Lung Cancer and Biomarker and Chemoprevention Consortium) are currently studying the chemopreventive effects of a combination of an EGFR inhibitor and FTI in a prospective randomized study in persons at risk for lung cancer with reversal of morphological changes and Ki-67 modulation as the primary and secondary endpoint.

The prostacyclin and the eucosonoid pathways are important for tumor function and progression. There are several ways to block prostaglandin synthesis, and COX-inhibitors are under clinical investigation as chemopreventive agents [49,50]. In addition several other molecular targets have been identified and the therapeutic potential is expanding rapidly. Today's challenge is to find the most optimal targeted therapy (or combination of therapies) given the fact that certain requirements for feasibility and low level of toxicity are needed for "healthy" populations at risk for cancer. Reflecting on all the new trials that are needed it cannot be underlined enough how important the need is for reliable intermediate biomarkers and robust models for response evaluation.

Conclusions

The understanding of the molecular and the biological mechanisms of lung cancer development has significantly expanded over the last 20 years. Multiple genetic and biological events have been identified that are considered essential for the carcinogenic process leading to the final malignant phenotype. Some of these lesions have been found closely associated with tobacco smoke exposure, and it has become increasingly clear that there is a wide variation in individual susceptibility to lung cancer. Valid risk markers are now appearing on the horizon, and more recently several novel agents with chemopreventive potential have been identified including molecules that block cellular receptors and important signaling pathways.

It is anticipated that after a period with increasing insight into lung carcinogenesis but without appreciable benefits of the chemoprevention approach in randomized trials, the road is now being paved for more successful studies in high-risk individuals. Current challenges are the identification of the most optimal high-risk population for chemoprevention studies and the validation of intermediate markers.

Considering the continuing lung cancer epidemic worldwide it is clear that we must continue our effort to strengthen the fundamental strategy of avoidance the exposure of carcino-

gens in parallel with the development of additional approaches such as chemoprevention.

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Epidemiology of viral hepatitis in the Mediterranean Basin

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Abstract

The prevalence of viral hepatitis is high and remains a serious public health challenge throughout the world. New molecular biology techniques provided a better understanding of the viruses over the last decades. Novel therapeutic options seem to be promising but preventing measures including donor screening, immunization against hepatitis A virus (HAV) and hepatitis B virus (HBV), universal use of disposable syringes and implementation of better hygienic conditions play a major role in the control of viral hepatitis. The Mediterranean basin has special demographic and socioeconomic features. We reviewed in this article the seroepidemiological features of viral hepatitis in this particular region.

Improving general conditions led to a tendency to be infected in older ages with HAV. Hepatitis B and C virus still remain to be the major causes of chronic hepatitis. The seroprevalence of hepatitis D virus, which was once endemic in the Mediterranean region seem to decrease nowadays whereas hepatitis E virus is still prevalent in some areas. Other viruses such as hepatitis G virus (HGV), TT virus (TTV) and SEN virus do not seem to be a major problem and their clinical importance remains to be determined in further studies.

Key words: epidemiology, hepatitis virus, Mediterranean.

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Introduction

Hippocrates was the first to use the term "epidemic jaundice" to describe a disease resembling outbreaks of viral hepatitis in the fifth century B.C. in Greece [1]. Despite considerable advances in medical technology and attempts to find "the magic bullet" to cure the disease, viral hepatitis still remains a major public health problem with its worldwide high morbidity and mortality. With new technologies over the last decade, there have been enormous advances in the understanding of viruses; their antigenic and constitutional features with particular focus on gene analyses and nucleotide sequences of the viral DNA/RNA.

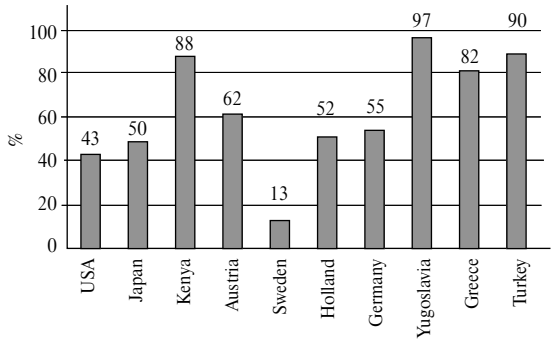
Development of sensitive assays, identification of new viral agents, safe and effective vaccines for hepatitis A virus (HAV) and hepatitis B virus (HBV) have been achieved. New nucleoside analogs have been found as effective alone or in combination with interferon in the therapy of viral hepatitis. Determination of viral genotypes and subtypes facilitated not only to track the epidemiological origin of these important pathogens but also to predict their future behavior and probability of response to antiviral treatment. Implementation of donor screening, effective inactivation of plasma derived products and widespread use of universal precautions vastly contributed to the prevention and control of viral hepatitis.

With its peculiar demographic and socioeconomic features, the Mediterranean basin has some specific significance in reference to seroepidemiology of viral hepatitis. In this article, we reviewed the present situation of the epidemiology of various forms of viral hepatitis in the Mediterranean basin.

Hepatitis A

HAV, an RNA virus classified in the Picorna virus family, is the cause of most common form of acute viral hepatitis in most parts of the world. The virus is strongly resistant to degradation by environmental conditions providing a very high rate of infectivity. Since the major mode of transmission is by person-to-

Figure 1. Seroprevalence of viral hepatitis A in various countries.



person contact via the fecal-oral route, the sanitation and the quality of water supply are highly related with the prevalence of infection. Nearly a third of patients acquire the infection from household or sexual contact with an infected person. Large families, poor education, inappropriate human-waste disposal system, crowded day-care centers and international travel have been linked to outbreaks and endemicity of HAV infection. Approximately, 50% of patients with hepatitis A do not have a recognized source of infection [2,3].

High-risk groups are cleaning personnel in hospitals, staff in day-care centers, pediatric nurses, IV drug users, homosexuals, patients with chronic liver disease, international travelers and consumers of high-risk foods (bivalve shellfish from stagnant water).

Epidemiology of HAV in the Mediterranean countries: The incidence of the virus varies with age, race, ethnicity and geographic region [4,5]. HAV is still endemic in the Mediterranean basin due to socio-economic, sanitary and hygienic conditions. *Fig. 1* shows seroprevalence of HAV in various countries including Mediterranean basin. Seroepidemiological studies of HAV were undertaken in various parts of Europe.

A multi center seroepidemiological study in 1977 showed, that anti-HAV seroprevalence rates were 95.3% in Israel and 96.9% in Yugoslavia [6]. Frösner et al. reported the rates of anti-HAV seropositivity as 75% in France and 82% in Greece in 1979 [7]. Other studies showed the rates as 95.9% (people >40 years old) in Crete [8], 59.1% in Spain [9], almost 10% in Northern and 40% in Southern regions of Italy [3,10]. In Egypt, anti-HAV seroprevalence was found to be higher than 95%, even in the youngest age group (in children <5 year old) [11]. Ungan et al. [12] notified the seropositivity as 68% in Western and 80% in Eastern regions of Turkey in 1998. Hepatitis A is mostly an asymptomatic infection of childhood. However, improving social conditions made a trend of getting infected in older ages resulting in an increase in morbidity and mortality of the disease in this specific age group. Anti-HAV positivity was 54.5% between the ages 3 to 10 and 100% over the age of 40 in Turkey [13]. By the age of 18, 30 to 40% of the Jewish population had anti-HAV antibodies in a study from Green et al. [14].

Prevention and vaccination strategies: HAV is vaccine preventable and the protective effect of the vaccine is 95% to 100% in healthy subjects [15]. A single dose of HAV vaccine was shown to provide 100 % of seroprotection within 2 weeks.

Table 1. Indications for HAV vaccine.

Travelers to endemic areas for more than 3 months
Military, diplomatic personnel
Patients with CLD (chronic liver disease)
Hemophiliacs receiving Factor Concentrates
IVDU (intravenous drug users)
Laboratory staff working with the virus
Homosexuals
Nursery home staff
Food handlers with poor hygiene

Active immunization can be done with inactivated (formaline), live attenuated or combined vaccines. There is no known contraindication and very few side effects related to HAV vaccine have been described. Combined HBV and HAV vaccine was found superior to monovalent vaccines regarding the tolerability, early antibody response, safety and cost [16]. Indications for active vaccination are summarized in *Tab. 1*.

Current recommendation is to give the vaccine at 12-24 months of age as two doses 6 months apart. In high endemicity area antibody test is recommended. Since universal vaccination for HBV is implemented in many countries both vaccines can be given in the same program. Cost-benefit analysis of outbreaks showed that HAV vaccination without screening anti-HAV antibodies is a cost-effective approach in the regions where anti-HAV rates are lower than 45% [17].

Hepatitis A vaccine may provide a protection of up to 30 years. Post-exposure administration of polyvalent immune globulin within 2 weeks of the exposure is recommended after suspicious contacts of household and sex partners. Passive immunization with Immune serum globulin should be applied at the doses of 0.02-0.06 ml/kg i.m., 2 weeks before and 2 weeks after exposure to virus and also to travelers to endemic areas, during epidemics of AVH, after a suspicious sexual intercourse and to consumers of high-risk foods for HAV. Current data support immediate immunization of young individuals against HAV in cases of dubious contacts with active HAV infection without screening for anti-HAV in the Mediterranean countries.

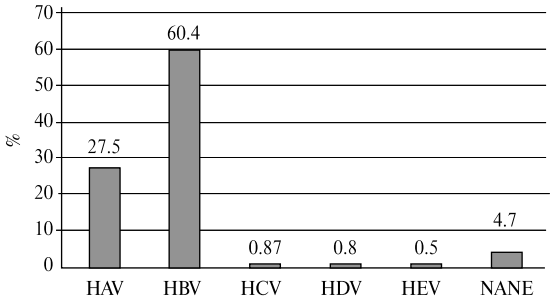
Hepatitis B

HBV, a DNA virus classified in the Hepadna virus family, is a devastating global healthcare issue. Around 1 million people die each year from HBV infection, making it the 9th leading cause of death worldwide [18].

Perinatal, vertical and horizontal transmissions occur during the birth or childhood in highly endemic populations such as Asians, whereas sexual, parenteral and horizontal transmissions are the major route of acquisition in low endemicity areas. In about 1/3 of cases the source of the infection is unknown. Seven genotypes of HBV were reported in the literature. Genotypes A and D are common in Western Europe whereas Genotype C is common in Asia [19]. Whether these genotypes have a role in the development and progression of chronic liver disease (CLD) or hepatocellular carcinoma (HCC) is still under investigation.

Epidemiology of HBV in the Mediterranean Countries: Hepatitis B infection has a wide range of seroprevalence in

Figure 2. Etiology of acute viral hepatitis in Turkey (pooled data of 4471 cases) [26].



HAV: Hepatitis A virus, HBV: Hepatitis B virus, HCV: Hepatitis C virus, HDV: Hepatitis D virus, HEV: Hepatitis E virus, NANE: Non-A-non-E virus.

Table 2. Preexposure application of HBV vaccines.

Health care personnel working with infected tissues or blood
Staff at institutions for disabled people
Hemophiliacs
Hemodialysis patients
Close family contacts of HBV infected individuals

Table 3. Postexposure application of HBV vaccines.

Infants born to HBsAg positive mothers or to mothers who had acute hepatitis B during pregnancy
Healthcare workers who had needle stick injury from a HBV positive patient
Sexual partners of individuals with HBV infection

the Mediterranean countries. In seroepidemiological studies, markers of hepatitis B infection (anti-HBs, anti-HBc or HBsAg) were observed in 11.7% (58/497) in healthy Spanish population [9]. HBsAg and anti-HBc prevalences were 3.7% and 34.2% in Italy respectively [20]. In Turkey, HBsAg prevalence was found as 1% in 1.377.688 blood donors while seroprevalence of HBV (HBsAg and/or anti-HBs) was between 25-60% [21]. Seroprevalence study of Greek warship personnel showed the rates of 1.1% in HBsAg and 17.7% in anti-HBs [22]. In another study from Greece, HBs Ag seroprevalence was found to be lower than 1% in the groups of military recruits, military personnel and family donors [23]. Roumeliotou et al. [24] found an increased prevalence of HBsAg carriers (6.9%) and HBV serologic markers (77.1%) among intravenous drug addicts. In a well-defined area in rural Crete, the levels of HBsAg and anti-HBs were reported as 1.2% and 24.57% respectively [8].

HBsAg positivity among Turkish children between the ages 2 to 12 was found to be 4.9% [25], while in adult age it was 7.1% together with 21.9% of anti-HBs seropositivity [21]. In other surveys from Turkey HBsAg seropositivity in various risk groups was found as 10% among healthcare staff, 40-60% among HCC patients, 40-80% in patients with chronic liver disease and 8.6% among pregnant woman [26]. *Fig. 2* shows etiology of acute viral hepatitis in Turkey [26].

HBe Ag negative mutants were reported most prevalent in Mediterranean countries as compared to Northern European countries and US. Funk et al. [27] reviewed that the median prevalence of HBe Ag negative chronic hepatitis was 33% in the Mediterranean basin and these patients had precore stop codon variants rate of 92%. The studies with the HBe Ag-negative phenotype in Turkish patients with chronic hepatitis B showed precore stop codon mutations (G to A change at nucleotide 1896) in 85% (29/34) but not core promoter mutation [28]. Actually, it is not clear if precore mutations directly cause detrimental effect on the course of chronic HBV infection. HBe Ag-negative patients who have 1896 stop codon mutation may subsequently develop precore translation initiation mutations, which seems to be related with enhanced HBV replication and severe liver disease [29].

Prevention and vaccination strategies: HBV infection can be prevented in non-infected individuals by vaccination with HBV vaccine. Series of 3 injections at 0, 1 and 6 months of vaccination is effective in over 90% of recipients [30]. The prevalence of HBsAg in general population has declined from 13.9 positive donations in 1986 per 10000 to 5.3 in 1991 in France [31] and 13.4% in 1978 to 3.7% in 1997 in Italy after the universal vaccination program [20]. European Consensus Group on Hepatitis B Immunity stated that booster vaccination after a successful primary vaccination series in healthy subjects is not required, because of the maintenance of HBsAg-specific memory expresses protection to clinically breakthrough infection even in the absence of detectable antibodies [32]. Only in the group of immune compromised individuals (hemodialysis, chronic renal failure, HIV positive patients, healthcare workers, travelers and others) booster doses are recommended Universal immunization can be the only strategy that will reduce the disease burden of HBV, as well as cirrhosis and hepatocellular carcinoma. The study of Chang et al. [33] has shown a significant decrease in the incidence of HCC among those who were vaccinated against HBV.

More than 116 countries included HBV vaccine into national immunization program since 1997. With regard to hepatitis vaccination in Spain and Italy, the coverage rate of immunization program increased to 90% and over [3]. Due to the successful vaccination programs most countries are in a transition from the high endemicity to the low endemicity status. *Tab. 2* and *3* show pre and post exposure prophylaxis in HBV infection.

Hepatitis B immune globulin is effective for passive immunization of the newborns to HBsAg-positive mothers, and in the individuals who had parenteral exposure and sexual contact with infected individuals.

Hepatitis C

Hepatitis C virus (HCV) is an RNA virus classified in the Flaviviridae virus family. Worldwide, one hundred and seventy millions of individuals worldwide are infected with HCV with an incidence rate of 3%.

Epidemiology of HCV in the Mediterranean countries: HCV has at least six genotypes from 1 to 6 and more than 80 subtypes. Genotypes 1a and 1b are the most common genotypes in Western Europe whilst genotype 4 is most prevalent in Egypt

(91.6%) [34]. In a study from Northern Spain, anti-HCV positivity was 1.6% and 80% of the patients had genotype 1b [35]. In Italy, 2 a/c genotype occurred in 60.9% of cases and HCV 1b genotype was found to be an independent risk factor for the development of cirrhosis [36]. HCV major genotypes in blood donors in France were 1b (34.3%), 3a (24%), 1a (19.5%), and 2 (11.4%) [37]. In the study of Diamantis et al. [38] genotype 3a was most prevalent in Greek patients (68% of all patients) and there was no significant difference regarding the genotypes among patients with different risk factors. Seroepidemiological studies from Turkey reported that the major genotype was 1b with a frequency of 70-98% [39].

World Health Organization estimated a prevalence rate for HCV infection of 4.6% in Eastern Mediterranean in 1999. Egypt had the largest scale of HCV infection prevalence ranging from 6% to 28% [30]. The prevalence rates reported from other Mediterranean countries were 2.9% (ages 20-61) [40], and 0.87% (ages 30-69) [41] in Italy; 0.6% (ages 6-75) in Spain [9], 1.8% (ages 12-55) in Turkey [42], 1.25% in Greek islands [43], 10.9% in Crete [8], 0.55-0.66 % in Israel [44] and 2.2% in the Gaza Strip of Israel [45].

In Egypt, history of antischistosomal injection therapy was found as a risk factor and was reported in 19% of anti-HCV positive individuals. Intravenous drug abuse is the most common risk factor identified with the rates of 71.1% in Spain [46], 67.5% in Italy [47] and 57% in Turkey [48]. The seropositivity rates among hemodialysis patients are 19.1% in Spain [49], 13.3% in Italy [50] and 14.4% in Turkey [49]. Transmission of HCV with blood or clotting factors diminished after implementation of routine donor screening and high seropositivity rates in hemophiliac patients were reported from various countries such as 63.9% in Spain [46], 100% in France [51] and 42% in Turkey [52].

Perinatal and intrafamilial transmission is uncommon in HCV as well as sexual transmission. In an epidemiological study from Turkey, Mert et al. [53] examined the spouses of the HCV carriers and the rate of seropositivity in this group was 0.64% (1/152). Nevertheless persons with multiple sexual partners were shown to be at greater risk for HCV transmission [54].

Prevention and vaccination strategies: There is no vaccine available for HCV and post exposure immune globulin prophylaxis is not effective to prevent it. In Mediterranean countries, screening for anti-HCV in blood / blood products has diminished the spread of HCV, but IV drug use, surgical interventions, dental treatment and hemodialysis still constitute a major route of transmission.

Hepatitis D

Hepatitis D virus (HDV) is a defective RNA virus that requires HBV to become infectious. Worldwide, 15 million people are infected with HDV which accounts for 5% of patients with HBV infection. Hepatitis D is endemic particularly in the Mediterranean basin. In other parts of the world, HDV infection occurs among intravenous drug addicts and persons who receive multiple blood transfusions. There are three genotypes of HDV. Genotype I predominates in Italy, Turkey and most of the other parts of the world. Genotype II is less aggressive while genotype III is one of the most aggressive ones. Italy and Eastern Europe are areas of highest prevalence for HDV [30]. Furthermore, IV drug addicts have higher prevalence of HDV in Greece and Italy [24,55].

Table 4. Peculiarities of HDV epidemiology.

Hepatitis Delta is declining in Europe / elsewhere
Precautions / vaccination for HBV are the major reasons for this decrease
Genotypes are important in determining the type of disease
Genotype I: Italy, Greece, Turkey and Africa
Genotype II: Japan, Taiwan-less aggressive
Genotype III: Peru-severe disease

Although HDV needs HBV for replication, the geographic distribution of HDV does not exactly match that of HBV distribution. Either as a superinfection or coinfection, hepatitis D is associated with more severe liver disease. In Southern Italy, HDV infection is high, appearing to have a more benign clinical course. In the absence of HBsAg, screening of blood donors for HDV is unnecessary. Sexual partners of the drug users with HDV infection were found to be infected in a rate of 33% [56] (*Tab. 4*).

The prevalence of HDV infection is decreasing due to HBV prevention programs and improvement in socio-economic conditions (*Tab. 4*). However for the individuals the prevention of HDV transmission depends on the behavior modifications and improvement of hygienic conditions.

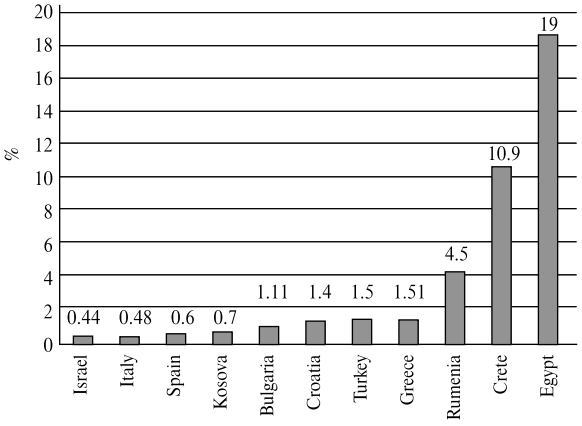
Hepatitis E

Hepatitis E virus (HEV), an RNA virus classified in the Caliciviridae virus family, is the most common cause of epidemic, enterally transmitted hepatitis. Ingestion of fecally contaminated water is the most common way, of transmission, which occurs most frequently during the rainy season that leads to outbreaks in endemic areas. Although the transmission is by the fecal-oral route, there is a low rate of clinical illness among household contacts of infected patients; an unexpected finding that may be related to the instability of the virus in the environment.

HEV accounts for as many as 50% of the cases of acute sporadic hepatitis in adults and children in some endemic areas [57]. Acute HEV infection is characterized by fluctuating aminotransferase levels and the overall case-fatality rate of 0.5% to 4%. However, pregnant women, especially when infected during the third trimester, have up to a 25% risk of mortality associated with acute HEV infection. HEV does not appear to cause chronic liver disease [58].

The seroprevalence of anti-HEV in Egypt is among the highest rate reported throughout the world, which exceeded 60% in the first decade of life, peaked at 76% in the second decade and remained above 60% until the eighth decade. In the Nile Delta, the seroprevalence of anti-HEV was 17.2% and increased by age [59]. Çetinkaya et al. [60] reported anti-HEV seropositivity as 7.6% in blood donors in Turkey. It is sporadic (2.1%) in Europe. In a survey covering 5 regions of Turkey the

Figure 3. Epidemiology of viral hepatitis C in various countries [76,77].



prevalence rate of anti-HEV in the general population was found to be 5.9%. The prevalence rates reported from other countries were 1.1% in Netherlands [61], 1.7% in Italy [62], 7.3% in Germany [63]. Immunoprophylaxis for HEV is not available and proper sanitation is the most important way of prevention. Boiling the water before consumption in doubtful situations appears to be effective in inactivating HEV.

Other viruses

GB virus / Hepatitis G

GB virus (GBV-C) and hepatitis G virus (HGV) are the minor variants of the same RNA virus in the Flaviviridae family. The virus can be transmitted parenterally, orally, vertically or by sexual intercourse. Clinical manifestations of HGV are not known, and the virus can persist in serum for many years. In Italy, high rates of GBV-C seropositivity of GBV-C were reported among blood donors (12.6%), hemodialysis patients (22%) and intravenous drug users (39%) [64]. In Greece, GBV-C/HGV RNA was found in 8.6%, 26.5%, 37.6% and 10% of patients with chronic hepatitis B, chronic hepatitis C, hemodialysis patients and normal subjects, respectively [65]. In Southern Italy GBV-C/HGV seroprevalence was 41.04% in HIV positive patients and 6.54% in healthy individuals [66]. In Egypt the prevalence was found to be 30% in chronic hemodialysis patients, 14% in patients with chronic hepatitis C, 12.2% in blood donors, 11.1% in chronic non-B non-C hepatitis, and 6.6% in health care personnel [67]. The contribution of HGV infection to chronic liver disease, HCC or other diseases of unknown origin is unclear. An association was investigated between HGV and non-Hodgkin lymphoma or GI cancer. Studies in various groups of diseases such as non-Hodgkin lymphoma [68], Behçet's disease [69] and ankylosing spondylitis [70] in Turkey have shown an associated a prevalence rate of 30%, 7.1% and 38.8% in HGV, respectively. More data is needed to achieve firm conclusions about the epidemiological and clinical importance of GBV-C/HGV infection.

TT virus

Hepatitis TT virus, or TTV is a parenterally transmitted DNA virus which has high prevalence among healthy population and chronic hepatitis patients. The virus can be transmitted both parenterally and orally. Seroprevalence of TTV in blood donors in France, Spain and Turkey were found as 2%, 14%, and 9.6-31% respectively [71]. In Turkey Avsar et al. [72] found TTV seropositivity of 43.5% in intravenous drug users and 20% in blood donors. In the study of Erensoy et al. [73] TTV DNA was found in 75% of hemodialysis patients, 80% of fulminant hepatitis patients, 61% of thalassemia patients and in 51.6% of volunteer blood donors. Among the sequenced isolates 35.9% belonged to genotype 1 and 64.1% belonged to genotype 2. In Egypt, the prevalence of TTV DNA did not differ among patients with chronic hepatitis B (46%), chronic hepatitis C (31%), schistosomal liver disease (36%) and blood donors (29%) [74]. TTV seems to be a worldwide pathogen with undetermined pathogenicity.

SEN virus

SEN virus, a DNA virus in the family of Circoviridae was discovered as a potential hepatitis virus, found in the blood of a HIV virus infected illicit drug user in 1999, in Italy. SEN virus can be transmitted parenterally. In a study coinfection of HCV with at least one of SEN D and H viruses correlated inversely with the effectiveness of the treatment with interferon and ribavirin [75]. The clinical data for this virus is far from being satisfactory and further studies are progressing to determine its role in liver disease.

Conclusions

The fact of globalization has made the propagation of diseases like viral hepatitis relatively easy throughout the world. Europe is a lively continent where thousands of people move to seek a job, to find new opportunities or simply for traveling purposes. Moreover wars, natural disasters or political instabilities make the trend for immigration even more justifiable. These events facilitate the spread of hepatitis viruses throughout the Mediterranean basin. The present cumulative data on the epidemiology of viral hepatitis C in various countries of this area is represented in *Fig. 3* [76,77].

At the other end of the spectrum morbidity and mortality rates related to viral hepatitis are progressively decreasing in this region of Europe. Better hygienic measures, widespread use of disposable syringes, definition of high risk groups, universal screening of blood donors and products for HBV and HCV, vaccination for HAV and particularly universal vaccination for HBV infection, demographic and behavioral factors may play a role in the reduced prevalence of viral hepatitides. Hepatitis E is a minor cause of viral hepatitis in the Mediterranean. Clinical importance of the other viruses remains to be determined by further studies.

The challenges to be faced in the next millennium involve the introduction of new and more antigenic vaccines for hepatitides as well as the development of safe and effective therapies for CLD. Mediterranean basin, well known for its exciting history, legends, natural beauties and healthy diet should get rid of the long standing assault of viral hepatitis and regain in the future its reputation as a healthy region, seat for eternal peace and felicity.

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IGF-I triple helix gene therapy of rat and human gliomas

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Abstract

Purpose: IGF-I anti-gene technology was applied in treatment of rat and human gliomas using IGF-I triple helix approach.

Material and methods: CNS-1 rat glioma cell and primary human glioblastoma cell lines established from surgically removed glioblastomas multiforme were transfected in vitro with IGF-I antisense (pMT-Anti-IGF-I) or IGF-I triple helix (pMT-AG-TH) expression vectors. The transfected cells were examined for immunogenicity (immunocytochemistry and flow cytometry analysis) and apoptosis phenomena (electron microscopy). $3x10^6$ transfected cells were inoculated subcutaneously either into transgenic Lewis rats or in patients with glioblastoma. The peripheral blood lymphocytes (PBL) derived from "vaccinated" patients were immunophenotyped for the set of CD antigens (CD4, CD8 etc).

Results: Using immunocytochemistry and Northern blot techniques, the transfected "antisense" and "triple-helix" cells showed total inhibition of IGF. Transfected cultures were positively stained either for both MHC-I and B7 antigens – 60% of cloned lines, or for MHC-I only – 40% of cloned lines. Moreover "triple helix" cells as compared to "antisense" cells showed slightly higher expression of MHC-I or B7. Transfected cells also showed the feature of apoptosis in 60%-70% of cells. In in vivo experiments with rats bearing tumors, the injection of "triple helix" cells expressing both MHC-I and B7 interrupted tumor growth in 80% of cases. In contrast, transfected cells expressing only MHC-I stopped development in 30% of tumors. In five patients with surgically resected glioblastoma who were inoculated with "triple helix" cells, PBL showed an increased percentage of CD4+CD25+ and CD8+CD11b- cells, following two vaccinations.

Conclusions: The anti-tumor effectiveness of IGF-I anti-gene technology may be related to both MHC-I and B7 expression in cells used for therapy. The IGF-I anti-gene therapy of human glioblastoma multiforme increases immune response of treated patients.

Key words: glioma, IGF-I, triple helix, immuno-gene therapy.

Introduction

Insulin-like growth factor-I, IGF-I, is a 70-amino acid polypeptide [1-4] involved in the growth and tissue differentiation [3,5-7]. IGF-I and the related polipeptides IGFs are expressed in many tissues, including brain, where they are involved in proliferation of neuronal and glial precursors [8]. Deregulated expression of IGF-I, is associated with growth as well as with pathology of different diseases, including tumors [5,7,9,10]. IGF-I and -II are expressed at high levels in some nervous system-derived tumors, e.g., astrocytomas and meningiomas [11] or tumor cell lines [12,13]. In contrast, the block of IGF-I synthesis, induces apoptotic and immunogenic phenomena [14].

Classically, in order to assign a functional role of a particular gene product, one relies on naturally occurring mutants that fail to express a particular gene in normal fashion. The in vitro generation of cellular mutants has limited applicability due to the diploid nature of most genes and to the lack of adequate mutant selection. Our experimental approach in this study centers on the use of IGF-I RNA-DNA triple helix [15,16] presumed to stop the transcription of the IGF-I gene and to produce phenocopies of a null mutation for IGF-I. Triple helix strategy [17,18] and comparatively used antisense strategy [19-21] bypasses inherent limitations of functional studies dependent upon natural mutant cells or artificially mutagenized cells [22].

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According to antisense concept, a nucleotide sequence is expressed in cells that complementary to a portion of the native RNA transcript (target sequence) of the gene of interest. Binding of the antisense disrupts the function of the target sequence either by blocking its function, promoting its degradation or by altering the structure of the target sequence.

Antisense strategy – i.e. the experimental inhibition of gene expression by introducing a source of nucleotide sequences complementary to a given endogenous mRNA has been applied successfully to a growing number of genes in cultured cells. Growth of transformed human astrocytes was inhibited by antisense to basic fibroblast growth factor [23]. Antisense inhibition of glial S 100 protein altered cell proliferation and cytoskeletal organization [24].

However, the antisense approach has frequently been complicated by incomplete inhibition of gene expression [25]. Ineffective antisense RNA-mediated inhibition of gene expression, is probably a consequence of insufficient antisense RNA levels and inadequate suppression of the encoded protein product due to integrating vectors. These vectors used for most antisense experiments to date, integrate randomly into the host chromosome and are subject to local regulatory effects exerted by neighboring sequences at sites of integration. Hence, transfected cells may at times express only limited amounts of antisense RNA.

We have applied the antisense strategy by employing a self-amplifying episomal vector that replicates to high copy numbers extra-chromosomally [26]. It was showed that such a vector can be used in human T-cell clones for effective (>95%) inhibition of the T lymphocyte surface molecule CD8 [27]. The utility of episome-based expression vectors for the effective inhibition of cellular RNA expression has been subsequently confirmed by others, e.g., N-myc in primitive human neuroectodermal cell lines [28].

Johnson et al. [29] and Kiess et al. [12] previously reported that the rat glioma cell line C6 expresses high levels of IGF-I. IGF-I expression is enhanced in these tumor cells when they are grown in serum-free medium.

We have used C6 glioma cell lines as model in which to study antisense and triple helix strategies of treatement and to define a role for IGF-I in the tumorigenesis of gliomas that involves evasion of immune surveillance. IGF-I transfectants that express antisense RNA to IGF-I were shown to elicit a curative anti-tumor immune response with tumor regression at sites distant from the sites of injection of the transfectants [10,15]. We previously showed that C6 rat glioma cells expressed MHC-I [30,31] and B7 [32-34] antigens when transfected with vectors producing IGF-I antisense RNA or inducing IGF-I triple helix RNA-DNA [35,36]. Moreover, the cells lost tumorigenicity and were able to induce a T-cell mediated immune response in syngeneic animals against both themselves and the non transfected tumorigenic parental cells [6,10]. Using the IGF-I antisense and triple helix approaches we recently demonstrated that transfected C6 cells become pro-apoptotic [36]. Unfortunately, the syngeneic model of rat DBIX permitting to study C6 cells in vivo is not available. For this reason, one of the aims of the present experiment was to verify the results we obtained in the syngeneic model of Lewis rat/CNS-1 glioma cancer (see *Fig. 1*), and to determine whether or not IGF-I triple-helix blockade of IGF-I syntheses changes phenotype of transfected CNS-1 cells. Moreover, we sought to determine if the change in immunogenicity is accompanied by apoptosis in this model system and if the expression of both antigens, MHC-1 and B7 in transfected cells used for injection is necessary to stop the growth of establish rat tumors. The injection of human immunogenic glioma cells should be followed by cellular immune response shown in the patients' blood. The experiment described here using a rat glioma syngeneic model permitted us to prepare human "vaccine" and introduce a Phase 1 clinical trial (treatment of five patients with glioblastoma multiforme).

Material and methods

Cell culture

The CNS-1 rat cell line was used for experiments. The cell line was offered by Dr William Hickey of the Dartmouth Medical School, Hanover, NH, USA. The cell line was cultivated in the laboratory of INSERM by Dr M. Sanson (Salpetrier Hospital, Paris) and then subcloned. Cells were cultured in DMEM (GIBCO-BRL) supplemented with 10 % FCS, 2 mM glutamine, 100 U/ml penicillin and 100 microg/ml streptomycin, at 37°C and 5% CO_2. Hygromycin B (Boehringer Mannheim) at a concentration of 0.05 mg/ml was added 48 hours after transfection to select for transfected cells. After one week, concentration of hygromycin B was changed to 0.15 mg/ml and maintained with each change of fresh medium over the next 3-4 month.

Primary cell cultures of human glioma were derived from tumors of 5 glioblastoma multiforme patients during surgical resection in the University Hospital of Bydgoszcz, Poland. Cell lines were established according to the technique described earlier [36-39].

Histology

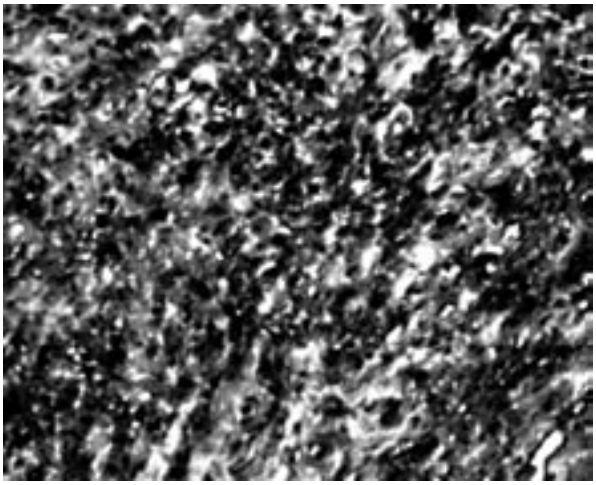
Male Lewis rats, 7 week-old male, were provided from CERJ, Lyon, France. Rats were inoculated subcutaneusly with 5 mln cloned CNS-1 cells expressing IGF-I. The removed tumors were fixed in 4% para-formaldehyde, and paraffin embedded sections were stained for IGF-I by immunoperoxidase technique (Vectastain ABC kit, Vector Laboratories, Burlingame, CA, USA) using polyclonal antibodies to IGF-I (Valbiotech, Paris, France) (*Fig. 1*).

Plasmids

The vector pMT-EP [6,26] was prepared in The School of Medicine, Case Western Reserve University (Joseph Ilan Laboratory) (*Fig. 2*). IGF-I "antisense" and "triple helix" technology was used (*Fig. 3*) to construct episome based plasmids expressing either IGF-I RNA antisense, pMT-Anti IGF-I [26], or IGF-I triple helix inducing vector, pMT-AG TH [15]. The pMT-EP is under control of the metallothionein, MT-I, inducible promotor. The casette contains the Epstein-Barr Virus origin of replication and the gene encoding nuclear antigen I which together drive extrachromosomal replication. Down stream of the insertion site is a poly A termination signal followed by the hygromycin B and ampilicin resistance genes (*Fig. 2*).

In pMT-AG triple helix the cassette consists of a 23 bp DNA fragment cloned into the vector pMT-EP which tran-

Figure 1. Histology. IGF-I immunoperoxidase staining of CNS1 rat glioma [55] resulting from subcutaneous injection of cloned cells into Lewis rat (counterstaining with hematoxyline). (X 200)



scribes a third RNA strand forming a triple helix structure within the target region of the human IGF-I gene, between its transcription and translation initiation sites. The vector pMT-EP containing cDNA expressing IGF-II antisense RNA as insert was used in control experiments [6].

Transfection

Cells, 60-80% confluent, were transfected in 6-well plates in a ratio of 1 ug of plasmid per 3-4 x 10^5 cells. The FuGENE 6 Transfection Reagent (Boehringer Mannheim) was used according to the supplier's instructions (3 μl of Reagent per 1 ug DNA). To determine the efficiency of transfection, the process was carried out using pMT-EP construct containing lac-Z as a reporter gene. Cell cultures were washed in PBS and incubated at 37°C in the presence of the staining solution which contained 5 mM $K_3Fe(CN)_6$, 2 mM $MgCl_2$, 0.8 mg/ml X-gal made in PBS.

Hygromycin B (Boehringer Mannheim) at a concentration of 0.05 mg/ml was added 48 hours after transfection to select for transfected cells. After one week, concentration of hygromycin B was changed to 0.10 mg/ml and maintained with each change of fresh medium over the next 3-4 month.

Parental, non transfected cells and transfected cells were verified for IGF-I presence using antibodies to IGF-I by immunoperoxidase technique (*Fig. 4*).

Northern blot

Content of IGF-I antisense RNA was determined in 50% confluent cell cultures. Cells were deprived of serum and cultured overnight in DMEM containing 0.1% BSA; 60μM $ZnSO_4$ (Sigma) was then added for 5 hours to induce the MTI promoter. The cells were then prepared for Northern blot as described earlier [37]. Labeling of rat and human IGF-I cDNA and chicken beta actin cDNA and hybridizations were done according to Maniatis and procedures previously described [37]; the 770 bp human IGF-I cDNA and 500 bp rat IGF-I cDNA used as probes were a gift from J. Ilan (CWRU,

Figure 2. Diagrammatic representation of steps employed to construct the episomal vector pMT/EP used for preparation of IGF-I antisense and triple helix expression vectors.

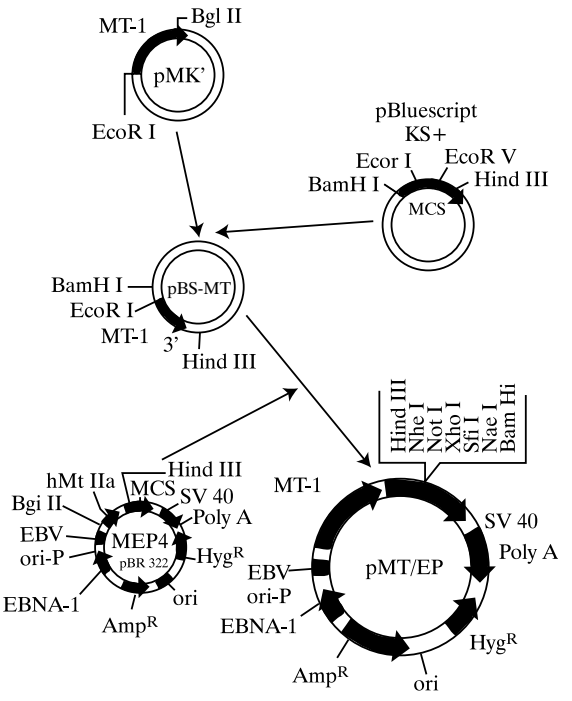
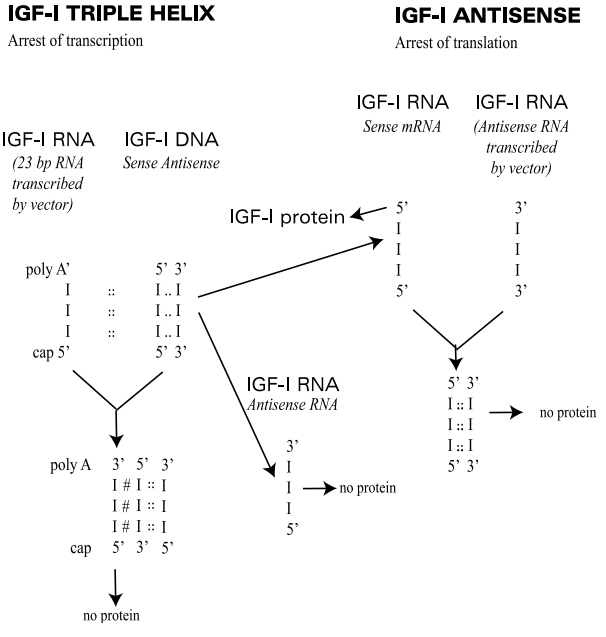
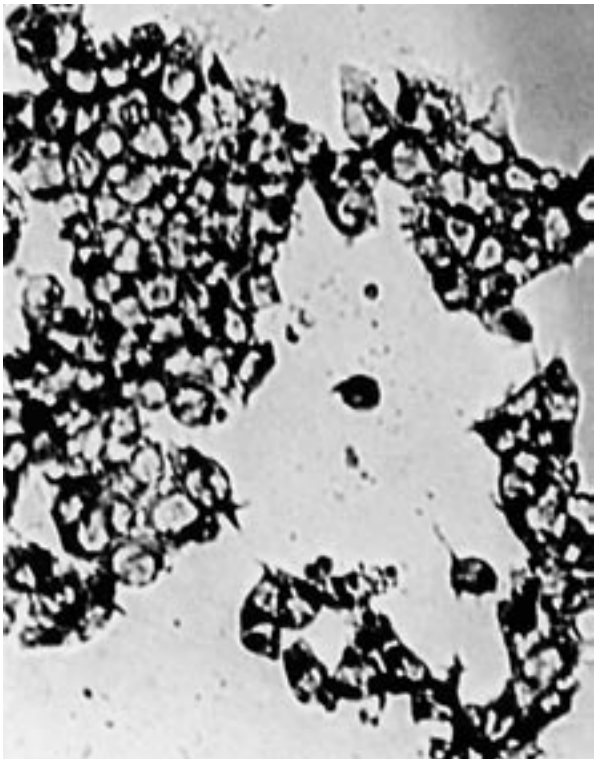


Figure 3. Schema of IGF-I antisense and IGF-I triple helix IGF-I technology. In antisense technology the end result is the inhibition of IGF-I mRNA (sense RNA) activity by binding to the antisense RNA [15]. In IGF-I triple helix technology the oligopurine third strand (23 bp) prepared by PCR forms RNA-DNA triple helix with IGF-I gene [16,36].



Homopurine triple helix is formed in 'in vitro' experiment (oligopurine strand poly A 3' GAG…GAG 5' cap) and DNA of IGF- I). We admit that similar mechanism occurs in 'in vivo' studies. # Hoogsteen hydrogen bonds; :: Watson-Crick bonds.

Figure 4. IGF-I immunoperoxidase staining of CNS-1 rat glioma cells. These cells after transfection with IGF-I antisense or IGF-I triple helix expression vectors lose their capacity to synthetize IGF-I (see also *Fig. 5*) (X 200).



Cleveland). The Northern blot was used also to verify expression of IGF-I in solid glioblastomas.

Immunocytochemical stainning

Localization of IGF-I and other antigens was done by immunofluorescence technique [6,10]. Cells were fixed in 4% methanol. Antibodies to rat MHC-I (OX-18) (from Valbiotech, Paris, France) and antibodies to mouse MHC-I (5 F1.3, anti Kb; from ATCC, Bethesda, MD, USA) gave the same staining. To stain B-7, a fusion protein CTLA4-Ig was used (Bristol Myers Squib, Seattle, WA, USA) [35].

Flow cytometric analysis

Cells were washed in PBS and incubated (30 min., 4^0C) with saturated amounts of monoclonal antibodies, labeling rat or human MHC-I (HLA ABC), MHC-II, CD80 and CD86 antigens (Becton Dickinson Pharmingen, direct immunostaining). Paraformaldehyde-fixed cells were collected (10.000 events per sample) in FACScan BD cytometer. Data were presented as percentage of positive cells. The human or rat glioma cells examined by flow cytometry were tested in two parallel groups of samples: one group of non irradiated cells; another group after irradiation with ^{60}Co, 5000 rads.

Electron microscopy

Cells were fixed in 1% glutaraldehyde and 4% formaldehyde in 0.l M phosphate buffer, pH 7.2. The fixed cells were then rinsed in 0.1 M phosphate buffer (3x) followed by a post-fixation in phosphate – buffered 1% OsO_4. After rinsing in distilled water for 20 min, the cells were stained with 2% uranyl acetate for 40 min at 60°C. They were then rinsed in distilled water (3x), dehydrated in progressive concentration of ethanol and 100% propylene oxide and embedded in Epon resin. 80-nm sections were cut, placed on 200 mesh copper grids, and contrasted with lead citrate and uranyl acetate. Photomicrographs were taken with a Philips EM-2000 electron microscope operating at 80 kV.

In vivo experiment

For determination of tumorigenicity 5 mln CNS-1 rat cells were injected subcutaneously into Lewis rats. Animals were separated into groups of eight. Experimental sets were injected with: a) parental cells; b) IGF-I «triple-helix» transfected cells expressing MHC-I; c) IGF-I «triple-helix» transfected cells expressing both MHC-I and B-7 molecules.

Vaccination of glioblastoma patients

Human glioma cell lines were transfected with the pMT–AG TH plasmid vector. Clones of transfected cells (down-regulated for IGF-I and expressing MHC-I and B-7 molecules) were selected two months after day of transfection. Before injection, cells were irradiated as described previously and 5 mln cells were injected subcutaneously into the left arm of operated glioblastoma patients. Blood was collected before the first vaccination, and again 3 weeks after the first and the second injection. Peripheral blood lymphocyte (PBL) typing was performed using mouse monoclonal antibodies directed against the superficial cell antigens (*Tab. 1*).

Results

Parental rat glioma CNS-1 line was subcloned using cloning cylinders. The subcloned cell line expressing IGF-I (*Fig. 1*) was used for in vitro transfection and for in vivo injection experiments. Endogenous IGF-I was down-regulated, when CNS-1 cells were stably transfected with either IGF-I “antisense” or “triple helix” vector and grown in presence of Zn^{2+}, in order to activate the MT-I promoter. Transfected cells examined by northern blot analysis show the IGF-I RNA in “antisense” orientation (*Fig. 5*). The RNA of non-transfected cells is distributed in 7.5 kb and l.0 kb bands corresponding to the different processing stages of endogenous IGF-I RNA (lane a). The RNA of pMT-Anti IGF-I transfected cells shows only an abundant 1. 0 kb band (lane b). As expected, RNA of pMT-AG triple helix transfected cells shows no band when rat IGF-I cDNA is used as probe (lane c). The internal control to this experiment was the beta actine bands (*Fig. 5*).

IGF-I «triple helix» or «antisense» cells were up-regulated in expression of MHC-I and B-7 molecules as measured by flow cytometry and immunocytochemical staining (*Fig. 6A, B*). Transfection with either pMT-EP «empty vector» or with vector expressing IGF-II antisense RNA [6] in place of IGF-I antisense RNA, produced no increase in MHC-I or B-7 molecules. The transfected cultures were positively stained either for both MHC-I and B-7 antigens (in 60% of cloned lines), or for MHC-I (only in 40% of cloned lines). The data show that trans-

Table 1. Peripheral blood lymphocyte (PBL) typing. Monoclonal antibodies (MoAb) used for direct immunofluorescence. FL1 and FL2 indicate standard fluorescence channels of flow cytometer, FITC, fluoresceine isothiocyanate, PE, phycoerythrin.

Sample no	FL 1 MoAb FITC conjugated	FL 2 MoAb PE conjugated	Notes
1	CD45	CD14	PBL gate defining, contamination of monocytes
2	CD4	CD8	CD4/CD8 ratio
3	CD3	CD16+CD56	T & NK cells
4, 5	CD25	CD4 (CD8)	Activated Th (Tc) cells – Th (Tc) cells positive with interleukine-2 receptor
6, 7	CD45RO	CD4(CD8)	Th (Tc) memory cells
8	CD19	CD5	B cell subsets (T dependent and T independent B cells)
9	CD8	CD11 b	Tc & Ts cells
10	control antibody (IgG-1)	control antibody (IgG-2a)	Negative isotype control

Table 2. Expression of IGF-I, MHC-I and B-7 in "antisense" and "triple helix" transfected cells*.

Cells	Rat CNS-1 glioma			Human primary glioma		
	IGF-I	MHC-I	B-7	IGF-I	MHC-I	B-7
Non transfected	++	<0.5	<0.5	++	<0.5	<0.5
IGF-I antisense	--	12.5	18.0	--	9.0	11.0
IGF-I triple helix	--	14.5	19.5	--	10.0	12.5
IGF-II antisense	++	<0.5	<0.5	++	<0.5	<0.5

* Cells were analyzed by immunocytochemistry for IGF-I (ABC method with immunoperoxidase staining), and by flow cytometry (FACScan Becton Dickinson) for MHC-I and B7. The data of flow cytometry are presented as percent change in value of fluorescence relative to fluorescence in control non transfected cells. The average values of three experiments are shown; the increase in MHC-I and B7 expression in antisense and triple helix transfected cells are significant at the $P<0.01$ level (Wilcoxon's signed rank test).

fection with pMT-Anti IGF-I and pMT-AG triple helix vectors induced a significant increase in expression of MHC-I and B-7 (*Tab. 2*). The "triple helix" rat and human cells as compared to "antisense" cells showed slightly higher expression of MHC-I or B7: i.e. transfection of rat glioma with either the pMT-Anti IGF-I or pMT-AG triple helix vector induced mean increases of 12,5% and 14,5% in the expression of MHC-I, respectively.

Apoptosis induced morphological changes in pMT-Anti-IGF-I and pMT-AG triple-helix transfected cells were detected using electron microscopy (*Fig. 7A,B*). The apoptotic changes occurred within 5-6 hours after incubation of IGF-I «triple-helix» or «antisense» transfected cells in presence of 55-60 μM $ZnSO_4$. Apoptosis was also analyzed in culture cell population using the May-Grünwald-Giemsa and Hematoxylin/Eosine stainings. Apoptosis was detected in approximately 70% of the IGF-I antisense and triple helix transfected cells. In contrast, evidence of apoptosis was observed only in 3-5% of the non-transfected cells. These results, confirmed in three separate experiments, are in agreement with electron micrographies images of transfected IGF-I «antisense» and «triple-helix» cells.

When 5 mln of CNS cells were injected subcutaneously into Lewis rats, tumor was observed in 6-10 days. In contrast, no tumors were observed in animals injected subcutaneously with

Figure 5. Northern blot analysis of rat CNS-1 glioma cell line: lane a – parental cells showing two bands; lane b – IGF-I antisense transfected cells showing one band; lane c – IGF-I triple helix transfected cells presenting negative blocks. Beta-actin was used as the control (lowest band).

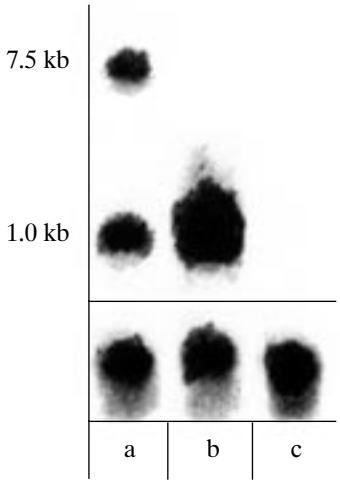


Figure 6. Immunofluorescence localization of MHC-I *A)* and B-7 *B)* in IGF-I "triple helix" transfected rat glioma cells using polyclonal antibodies (X400).

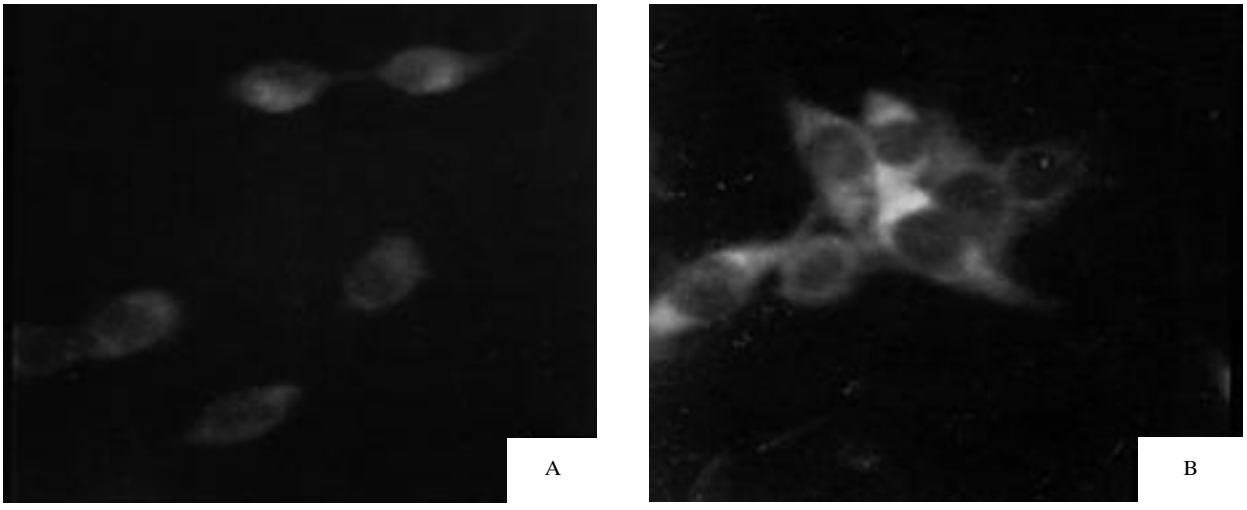
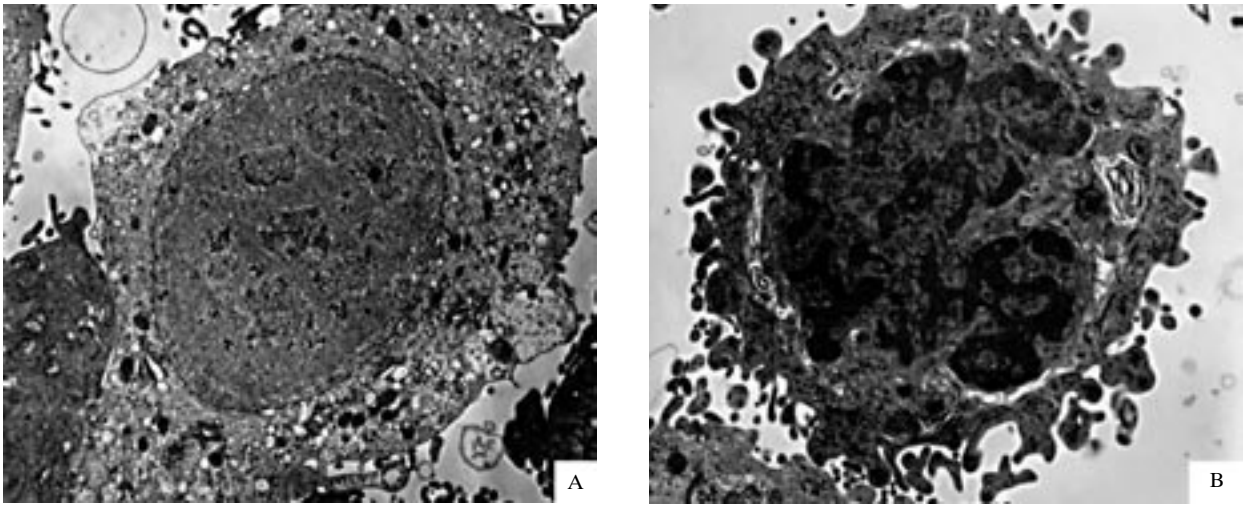


Figure 7. Ultrastructure characteristics of CNS-1 rat glioma cells: (a) parental non-transfected cells; (b) IGF-I triple-helix transfected cells. a) Parental cell with a nucleus which is uniform and well separated from cytoplasm. b) Transfected cell has characteristics of apoptotic cell with distorted nucleus and condensed electron dense chromatin (original magnification X7000).



5×10^6 CNS-1 cells stable transfected with IGF-I «triple-helix» vector, and expressing either MHC-I or both MHC-I and B-7. All eight of these mice remained tumor free for 8-10 months. The efficiency of triple helix approach to suppress CNS-1 rat glioma established tumors was 80% using the transfected cells expressing both MHC-I and B-7, and 30% using the transfected cells expressing only MHC-I (*Fig. 8*).

Human glioma cell cultures established from five glioblastoma multiforme cancers demonstrated morphological characteristics similar to those previously described [36]. Each cell line was subcloned to obtain IGF-I positive clones the percent of IGF-I positive cells in the human cell lines ranged from 50 to 70%). Analysis of RNA of human glioma cells four weeks after transfection was described earlier [36].

The phenotypic changes in peripheral blood lymphocytes (PBL) of the vaccinated glioblastoma patients were as presented in the *Tab. 3*. In the group of five glioma patients, each of those injected with autologous cells presenting both MHC-I and B-7 (*Fig. 9*), the significant changes were observed mostly after the first vaccination. There was increase in CD 8 percentage with characteristic switching from CD8+CD11b+ to CD8+CD11b-phenotype. Additionally, there was an increased percent of lymphocytes positive for the superficial interleukine-2 receptor (CD25).

Discussion

These data demonstrate that in IGF-I «triple helix» transfected CNS-1 rat glioma and in primary human glioma cells, changes in immunogenic properties and apoptosis occur. These properties were used for selection of human glioma cells that were used in IGF-I triple-helix immunogene therapy.

In the triple helix strategies, the oligonucleotides are

Figure 8. Survival curves of Lewis rats inoculated subcutaneously with CNS-1 rat glioma cells nad injected above the hind leg with 5000000 CNS-1 cells. The following groups of animals (every group, n=8) were considered according to the type of injection: group 1, PBS; group 2, IGF-I "triple helix" cells expressing only MHC-I; group 3, IGF-I "triple helix" cells expressing both MHC-I and B-7. 0 point = 5 days after primary inoculation of CNS-1 cells. The % survival was significant at the $p<0.01$ level. (1.0 corresponds to 100%).

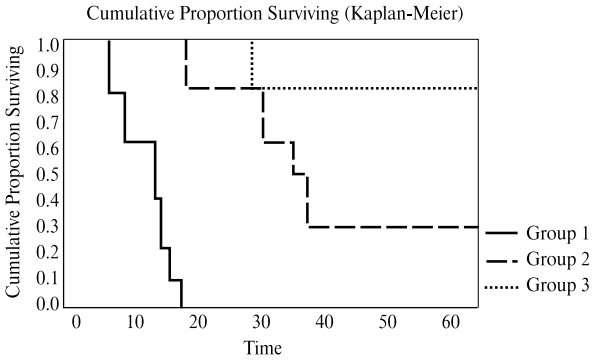
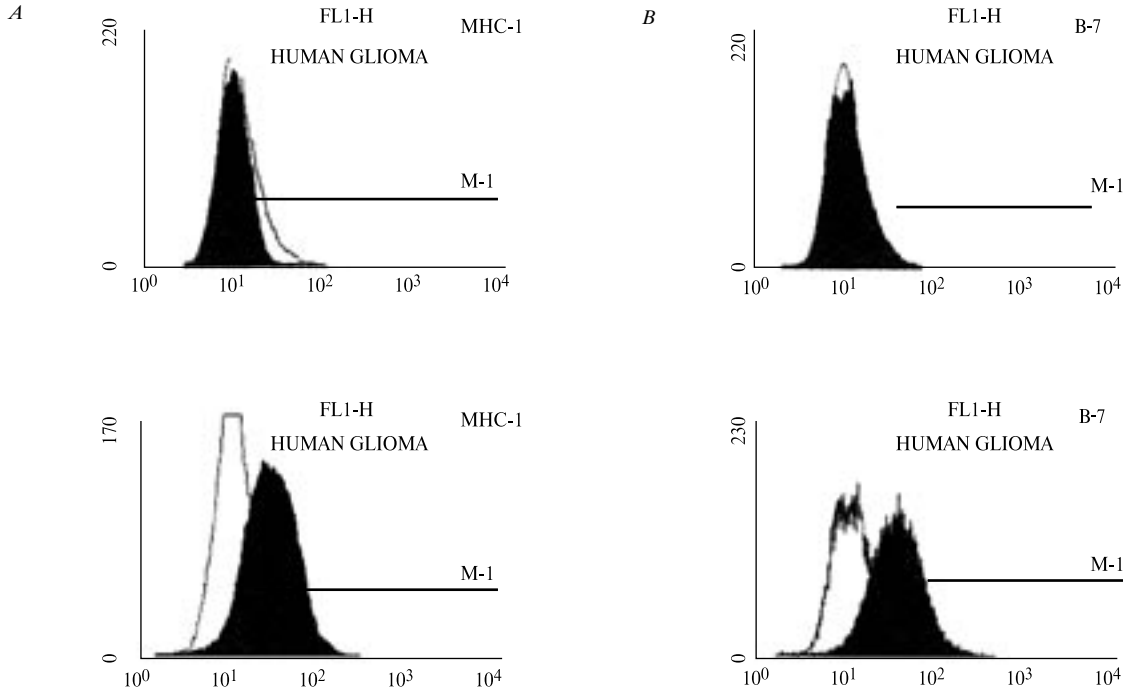


Figure 9. Flow cytometry analysis (FACScan Becton Dickinson). Expression of MHC-I (*A*) and B-7 (*B*) in primary human glioblastoma cell line. *A*) and *B*) upper panels: non transfected cells; lower panels: transfected cells (upregulation of MHC-I and B-7). The glioblastoma cell line presented here corresponds to the patient "P" of *Tab. 3*.



targeted to double stranded DNA containing polypurine-polypyrimidine sequences that readily form triple helixes. The results of the triple helix strategy presented in this study show that an RNA strand containing a 23-nucleotide (nt) oligopurine sequence [15,17,38] may be capable of forming triple-helix structures with an oligopurine-oligopyrimidine sequence of the IGF-I gene as well in cultured rat C6 glioma [15] as in CNS-1 rat glioma. Although we cannot exclude other mechanisms [15], triple helix formation remains the most plausible possibility for the inhibition of IGF-I gene expression. The suppression in IGF-I RNA level (*Fig. 5*) is accompanied by a reduction of cellular IGF-I. This suggests that the RNA strand, which forms the triple helix (*Fig. 3*), has inhibited gene transcription in glioma cells.

The induction of IGF-I triple-helix forming structure, similarly to IGF-I antisense approach, was followed by enhanced expression of MHC-I and B-7 (*Tab. 2*), and loss of in vivo tumorigenicity. Moreover, our previous studies on the IGF-I antisense strategy demonstrated an extensive lymphocytic CD8 positive infiltrate 4-5 days after injection of transfected glioma, teratocarcinoma and hepatoma in the respective animal bearing tumor model systems [6,10,16]. Recently it was demonstrated that a subject with glioblastoma multiforme was recruited for an ex vivo IGF-I antisense gene therapy protocol [39]. After subcutaneous injection, the subject developed peritumor necrosis; the tissue section bordering the necrotic tumor tissue showed the infiltration of lymphocytes consisted of both CD4 and CD8 T-cells. In the results presented here concerning peripheral blood lymphocytes (PBL) of glioblastoma patients treated by IGF-I triple helix immuno-gene therapy the eminent changes were observed after vaccinations (*Tab. 3*). There was increase in percentage of CD 8+ lyphocytes with characteristic switching from CD8+CD11b+ to CD8+CD11b- phenotype. This alteration may reflect the enhanced activation of cyto-

Table 3. IGF-I triple helix gene therapy of glioblastoma patients. Expression of CD molecules in peripheral blood lymphocytes (PBL) derived from "vaccinated " persons*.

Molecule	Before vaccination					After 1st vaccination					After 2nd vaccination				
	P	Q	R	S	T	P	Q	R	S	T	P	Q	R	S	T
CD3	84	71	78	66	80	88	77	79	71	90	85	84	79	71	91
CD4	34	32	42	42	56	41	23	47	46	45	37	40	47	39	48
CD8	54	46	34	30	27	53	61	36	32	45	50	48	40	42	44
CD4/CD8	0.63	0.70	0.62	1.4	2.07	0,77	0.38	0.85	1.44	1.0	0.74	0.83	1.18	0.93	1.09
CD19	3	7	10	9	4	3	2	12	17	2	2	6	11	9	2
CD3-(16+56)+	9	11	9	23	10	6	15	10	14	6	11	9	12	22	7
CD25	11	9	13	18	15	20	17	19	27	26	33	24	21	24	27
CD4+25+	4.8	6.5	9.4	12.4	10.4	8.1	9.5	13	11	14.1	19	17	15	16.5	18
CD8+25+	3.4	0.9	2.0	1.9	3.2	3.6	4.0	3.7	10	1.7	5.2	1.0	2.9	0.8	1.7
CD4+45RO+	nt	nt	nt	41	26	12	nt	nt	40	32	22	24	26	36	30
CD8+45RO+	nt	nt	nt	18	6	17	nt	nt	13	25	14	18	17	19	22
CD8+11b+	26	43	21	20	14	27	46	21	15	19	20	31	24	26	27
CD8+11b-	28	3	13	10	13	26	15	15	17	26	30	17	16	16	17
CD5+19-	81	76	75	67	78	79	77	77	60	89	84	79	77	65	88
CD19+5+	1	0.7	2.1	1.1	0.7	1.1	0.7	2.9	3.2	0.6	0,7	0.4	4	5.7	1

* Five cases indicated by P, Q, R, S, T; nt = not tested. PBL were analyzed by flow cytometry (FACScan Becton Dickinson). Double direct immunotyping with pairs of monoclonal antibodies conjugated with FITC and PE, respectively. Lymphocyte gate was defined according to the CD45 backgating. The data are expressed as percent of positive cells as compared to the isotype control.

toxic T-cells in blood. Additionally, an increased percentage of lymphocytes positive for superficial interleukine-2 receptor (CD25) was found, especially in the context of CD4 molecules. Generally, this has been raported as a specific features of activation for both Th and Tc populations in our results. This finding is compatible with the previous observation of T lymphocytes in IGF-I antisense gene therapy of gliomas [6,10,39].

The simulateous increase in the presence and role of B-7 and MHC-I antigens in the induction of T-cell immunity against tumors has been extensively investigated [26,33,34]. In the current paper we demonstrate that the injection of IGF-I triple helix transfected cells presenting both MHC-I and B-7 molecules stopped effectively the established rat glioma tumors. This was not the case for cells expressing MHC-I only (*Fig. 8*). In in vivo experiments using IGF-I «antisense» vector in place of the IGF-I «triple-helix» vector gave similar results; data not shown.

In this context, antigenic peptides presented by class I MHC molecules were necessary but, in general, not sufficient to stimulate T cell response. In the absence of B-7 molecule, MHC-peptide complexes could selectively inactivate T-cells [40]. (Although "triple helix" cells as compared to "antisense" cells show slightly higher expression of MHC-I and B7 there are no qualitive immunogenic and apoptotic differences between IGF I "antisense" and "triple helix" approaches.)

The costimulatory molecule B-7 of antigen presenting cells (APCs) bound to the counter-receptor CD28 and/or CTLA4 expressed on the T-cells [41-43]. In our work, the absence of IGF-I synthesis in «triple-helix» transfected cells, could lead to a higher level of IGF-I receptor and hence to greater tyrosine kinase content; IGF-I and IGF-II present in fetal calf serum of culture medium, as well as intracellular IGF-II can act via the type I receptor [37]. There is a relation between the signal transduction pathway of tyrosine kinase and induction of B-7 molecules: enhancement in B-7 co-stimulation through a cAMP mechanism linked to tyrosine kinase of the CD 28 receptor has been previously reported [44]. Similar signaling through the tyrosine kinase activity of the IGF-I receptor shows that: tyrosine kinase activates IRS-1 (Insulin receptor substrate-1), and then IRS-1 activates PI3K (phosphatidilinositol 3 kinase) [45,46]. This mechanism could be considered in the cytokine induced B7-1 expression demonstrated in fetal human microglia in culture [47].

Using CNS-1 glioma cells we have confirmed the relation between the immunogenicity and apoptosis found in IGF-I transfected cells [36]. The appearance of apoptotic cells demonstrated that cell death by apoptosis for «triple-helix» cells, is directly related to the suppression of IGF-I expression. IGF-I can block the apoptosis pathway leading to a proliferation of the cell population that is normally programmed to die [14,48]. On the other hand, when function of IGF-I receptor (IGF-I-R) is depressed, certain cancer cells i.e. glioma cells undergo massive apoptosis [49]. It was concluded that IGF-I-R activated by its ligand plays a very protective role in programmed cell death, and that this protection is even more striking in vivo than in vitro [50]. Apoptosis could play a specific role in our strategies; the phenotypic modifications due to apoptosis may explain the recognition of the transfected cells by the immune system like tumor-specific immunity mediated by CD8+T described earlier by us [10,37]. Apoptotic cells, in the context of MHC-I,

are recognized by dendritic cells activating lymphocytes T-CD8 [51,52]. B-7 molecules can be included in this mechanism, because both MHC-I and B-7 molecules are necessary for T cell activation [32,33].

The IGF-I antisense approach for treatement of human gliomas and hepatomas was recently published [39,53,54]. The phenotypic changes in PBL found in IGF-I triple helix gene therapy of patients with glioblastoma multiforme, and establishment of the immune characteristics necessary to produce an effective vaccine, constitute the start point for a Phase II clinical protocol of triple-helix cellular therapy for cancers highly expressing IGF-I.

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Growth hormone treatment in pituitary insufficiency: selected cases of children with craniopharyngioma and medulloblastoma

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Abstract

Purpose: The work concerns the substitution treatment with growth hormone (GH) in hypopituitary children, including cases that occurred in the course of tumor disease, craniopharyngioma (CP) and medulloblastoma (MB).

Material and methods: The studied population concerned 117 children who presented either somatotropic or polyhormonal pituitary insufficiency (the average age was 12.6 years for girls and 13.6 years for boys). The diagnosis of somatotropic pituitary insufficiency (SPI) was based on insulin and clonidin stimulation tests evaluating GH reserve of hypophysis. The computer tomography (CT) and nuclear magnetic resonanse (NMR) examinations were carried out before GH substitution in all children. The tumors (four CP cases and one case of MB) were all found in boys and they were treated with surgery and/or radiotherapy. All studied children, including CP and MB operated patients were treated with human GH (hGH) – Genotropin 16 IU, administered in subcutaneous injections. The daily dose was calculated as 0.5 IU/kg/week.

Results: The annual increase of children height before GH therapy was about 3.2 cm. In the first year of GH therapy the difference in children growth between the CP/MB group as compared with the rest of patients was less than 1.0 cm: 9.4 and 10.2 cm/year, resp. During the second year of hormone substitution the growth became slower: average values were 8.2 cm and 7.4 cm/year, resp. In CP and MB patients the height increase calculated as SDS values was significant (2.7 and 1.0 resp.). Control NMR examination performed in CP/MB patients treated with surgery with subsequent hGH therapy did not demonstrate any recurrence of tumor.

Conclusions: After two years of hGH therapy the final heigth of hypopituitary children, including CP patients, nearly reached the values observed in healthy children. GH therapy did not induce a recurrence of neoplasm in CP and MB patients.

Key words: growth hormone, pituitary insufficiency, craniopharyngioma, medulloblastoma.

Introduction

The growth retardation and deficit in height uncorresponding to the age constitute physical as well as psychological problems for children and young adults [1,2] commonly leading to negative social and professional effects [3,4]. The problem becomes particularly important if the underlying tumor disease occurs [4].

In the current paper, we studied population composed of hypopituitary children. The insufficiency of the anterior pituitary gland was treated with human growth hormone [5,6]. During the treatment, the CT scans showed in some children the presence of tumor, either CP [7,8] or MB [9,10]. CP is usually localized in the suprasellar region. It can also occur, especially in children, as intrasellar lesion [8]. CP is related to the multiple hormonal deficiency known as hipopituitarism [7]. Medulloblastoma is an invasive embryonal tumor of the cerebellum with an inherent tendency to disseminate via cerebrospinal fluid (CSF) pathways [9,10].

The detected tumors appeared as the reason of existing hypopituitarism. We have analyzed the effects of hGH treatment in the tumors bearing children population.

According to literature [11-13], hGH substitution should be ordered in all cases with apparent GH deficiency regardless of the child's height. Our CP and MB patients showed GH deficiency, thus they were all treated with hGH. In addition, the question if the children suffered from pituitary insufficiency

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combined with neoplasm disease should or should not be treated with GH, was largely discussed in the study.

The physiological mechanism of GH action is closely related to insulin-like growth factor-1 (IGF-1) effects [6,14,15]. IGF-I is known as one of the principal factors responsible for tumor initiation and proliferation [16-19]. For this reason, in CP and MB cases mentioned above, the existing tumors were eliminated by surgery followed by radio- and chemiotherapy, just before GH therapy. However, we cannot completely eliminate the risk of tumor reccurence, especially after treatment with hGH.

Material and methods

The population of 117 children (33 girls and 84 boys) was studied. The age was 4.6-18.1 years (the average age was 13.6 years for boys and 12.6 years for girls). All children presented isolated somatotropic pituitary insufficiency (SPI, n=79) or polyhormonal pituitary insufficiency (n=38).

In all studied children, head CT (Siemens Somatom HiQ) examination was carried out before GH substitution. In any case of CT image interpretation problems the NMR (Siemens Magnetom Impact 1 Tesla) examination was additionally performed. The children with recognised tumor disease were submitted to the surgery with subsequent pathological examination.

The diagnosis of SPI was based on two different stimulation tests which evaluated GH reserve of anterior pituitary gland [20,21]: 1) insulin (I.V, 0.15 U/kg of body mass) and 2) oral clonidine (150 μg/m^2 of body area) were used as stimuli. The timing for blood collection to test GH levels was following: 0, 30, 60, 90, 120 min (both tests) and then 150 and 180 (clonidine test) [20-22].

The hormone serum levels were quantified by radioimmunoassays for: GH, TSH, free T4 (Microparticle Enzyme Immunoassay, MEIA, Abbott Laboratories) and cortizol (RIA, Radioimmunoassay, Biochem Immuno Systems). The LH and FSH anterior pituitary secretion was estimated by LH-RH test [20,21] and their levels were quantified with use of the appropriate kit (MEIA, Abbott Laboratories). The serum IGF-1 level was detected before and after surgery by radioimmunoassay.

The patients were treated with subcutaneous injections of hGH, Genotropin 16 IU (Pharmacia-UpJohn), using GenotropinPen 16 IU. Human GH was administered every night just before sleeping. The dose was calculated as 0.5 IU/kg/week.

Growth was measured by Holtain standiometer, and growth deficit was assessed by height standard deviation score (SDS). The growth termination was defined as annual growth less than 2 cm and by knitting of long bones epiphysial plates (bone X rays of undominating upper extremity). The bone age was evaluated by Gruelich-Pyle method [23].

Results

Mean metric age (MA), at the diagnosis of pituitary insufficiency and GH therapy involvement was 13 years (12.6 years in girls and one year more, i.e. 13.6 years in boys).

Table 1. Pretreatment characteristics of the children/youth group with somatotropic or polyhormonal pituitary insufficiency, included in the study.

	n	MA	BA	Growth retardation (MA-BA)
Girls	33	12.6	8.3	-4.3
Boys	84	13.6	9.7	-3.9
Average	-	13.0	9.0	-4.1
Together	117			

MA, metric age; BA, bone age

At the diagnosis bone age (BA) was remarkably delayed as compared with MA. The average retardation was of about 4 years and was more evident in girls (-4.3y). BA deficit in boys was -3.9y, the difference is not significant. The particular data was summarized in the *Tab. 1*.

The average annual growth rate measured during the last half year before treatment amounted 3.2 cm and was a little higher for boys then for girls (3.4 cm and 2.9 cm, resp.). Prepubertal female patients grew distinctly slower than girls with puberty signs. This difference was practically absent for boys (*Tab. 2*).

During the first six months after GH involvement, growth was visibly accelerated and reached 10.4 cm/y for boys and 10.5 cm/y for girls, on average. In the second half-year the growth rate in both sex slightly decreased, the average values were 10.0 cm/y for girls and 9.9 cm/y for boys. The difference in growth rate dependent on prebubertal vs pubertal age observed in the first year of treatment was considered as not significant. In the consecutive years the growth rate became retarded, particularly for girls (*Tab. 2*).

In 6 children, the CT examination revealed the eminent anatomic lesions responsible for altered hormone secretion [24]. In other 8 cases, CT scans showed the pathological image of hypophysis and other central nervous system areas and were completed then by NMR. In 6 cases of this group the presence of tumor was recognised.

The NMR detected tumors were removed by surgical treatment. The subsequent tumor pathology demonstrated the presence of neoplasm in 5 cases (in the last child the lesion was not neoplasm).

The final diagnosis established in five children, all boys, was one case of medulloblastoma (MB) desmoplasticum, that infiltrated the chamber IV, and four cases of craniopharyngioma (CP), with both, supra- and intrasellar localization. The boy with MP (4 years old) was treated with surgery followed by radio- and chemiotherapy. For two boys with CP (14 years old both), the surgery was completed by subsequent radiotherapy. For the other two boys the surgical treatment was sufficient enough.

The data concerning the serum level of secreted hormones are presented in the *Tab. 3*. The deficient GH secretion was demonstrated in all patients. The baseline IGF-I level in children with tumors was increased and it has strongly declined after surgery, just below the age normal value limit. The sec-

Table 2. The growth rate of hypopituitary patients before the hGH substitution and during the first two years of the treatment.

Sex	Prepuberty growth rate (cm)				Growth rate during puberty (cm)			
	before hGH therapy	The time of hGH therapy			Before hGH therapy	The time of hGH therapy		
		0–6 months	6–12 months	2nd year		0–6 months	6–12 months	2 nd year
Girls	2.4	10.8	10.1	7.2	3.4	10.1	9.9	6.2
Boys	3.4	10.6	9.6	7.8	3.4	10.2	10.3	8.6

hGH, human growth hormone

Table 3. Hormonal disturbances in CP/MB children included in the study.

No	Diagnosis	SPI	HT	GD	CPI	ACI
1.	CP	+	+	+	+	+
2.	CP	+	+	+	+	+
3.	CP	+	+	+	-	+
4.	CP	+	+	-	-	-
5.	MB	+	+	-	-	-

SPI, somatotropic pituitary insufficiency; HT, hypothyreosis; GD, gonadotropin deficiency, CDI, central diabetes insipidus; ACI, adrenal cortex insufficiency

Table 4. The growth of CP and MB patients treated with GH after surgery.

No	Diagnosis	Growth before hGH therapy (cm)	Growth during hGH therapy (cm)			
			0-3 months #	3-6 months #	6-12 months #	2nd year
1	CP	3.0	8.1	9.0	8.0	7.3
2	CP	3.6	9.45	9.35	9.0	7.4
3	CP	4.2	10.9	9.7	10.1	7.5
4	MB	1.8	9.4	9.0	9.2	--

CP craniopharyngioma; MB medulloblastoma; hGH human growth hormone.
Results recalculated as annual growth

ondary hypothyreosis was recognized in all these patients, additionally in two CP boys the central diabetes insipidus appeared. The adequate substitution treatment (with thyroid hormones and Adiuretin, resp.) was introduced. In three children with CP the gonadothropin deficiency was found. For these patients, the need of hydrocortisone replacement therapy occured.

The children with CP were treated with GH during 1-1.7 years after surgery. In one CP boy the parents did not agree to treat their child with hGH (so the study concerned only three CP patients). In the case of MB the GH treatment started 8 years after surgery.

The data on the growth of GH treated children were presented in *Tab. 4*. The children grew relatively faster in the first year of the substitution therapy, especially at its beginning, than during the second year of treatment (about 9.4 cm/year and 7.4 cm/year, resp.).

Actually two patients after craniopharyngioma radical surgical treatment has already finished their GH treatment; the boys are 166 and 178 cm tall. Their growth SDS values increased during the treatment from -4.1 to -0.52 and from -1.6 to +0.1, resp. The boy with removed medulloblastoma with pretreatment SDS value of -3.4, responded to the replacement therapy with relatively rapid growth: his SDS value was -2.41 after one year of treatment (*Tab. 2*).

Control NMR examinations carried out in all GH treated patients did not reveal any recurrence of neoplasia.

The growth data of all studied groups treated with hGH, i.e. CP/MB group vs the group of patients free of tumor disease (non-tumor, NT), were presented in *Tab. 5*.

Discussion

The comparison of GH treatment of non-tumor and CP/MB tumor patients, both with SPI was limited in our study to the male sex; the girls were not taken into consideration. By the way, the maturation did not affect the growth results obtained in boys before and during GH therapy, especially during the first year of treatment.

On the other side, the height increase in CP/MB patients, as compared with the non-tumor (NT) children did not show any difference before GH treatment. On the contrary, the dif-

Table 5. The growth of CP/MB patients and "non-tumor" male patients treated with hGH after surgery.

No	Diagnosis	Anual growth before hGH therapy (cm)		Growth during hGH therapy (cm)					
				0-6 months #		6-12 months #		2nd year	
1	CP	3.0		8.6		8.0		7.3	
2	CP	3.6	3.6*	9.4	9.4*	9.0	9.0*	7.4	7.4*
3	CP	4.2		10.3		10.1		7.5	
4	MB	1.8		9.4		9.2		–	
5	NT	3.4		10.4*		9.8*		8.2*	

CP craniopharyngioma; MB medulloblastoma; NT non-tumor patients; hGH human growth hormone
* mean values
# Results recalculated as anual growth

ference seemed to appear during the first year of substitution therapy, especially in the first six months of GH treatment, i.e. the NT children were growing relatively faster; however, the maximal growth difference found during the first six months of therapy did not exceed 1.0 cm. Before GH therapy the growth rate in CP patients, as compared to NT group, was higher and statistically significant. Later, during GH treatment, the rate of growth became similar in both groups. These observations showed that the appearance of craniopharyngioma as the cause of pituitary insufficiency in children did not affect considerably the results of GH therapy.

GH deficiency is present in 72% of CP children, however the reduced height in CP children is observed after surgery only in 53% of cases [25]. Due to literature data, in some operated cases the children with prior GH deficiency developed hyperphagia and obesity. They grew as "healthy" children and revealed the increase in insulin secretion, the phenomenon that can explain the normal IGF-1 level [7,12,26]. However, some authors [12,13] suggest that these children, despite their normal growth, should be treated with GH because of metabolic alterations.

CP patients treated with surgery and radiotherapy may even present the exacerbation of the prior metabolic disturbances [27]. Any way, the study presented by de Vile and al. [28] showed that surgery increased the extent of existing panpituitarism from 71% to 75% only. According to other researchers, the SPI was observed in 90-100%, hypothyreosis in 64% and diabetes insipidus in 13% of CP patients in the preoperative period [29,30]. However, according to the cited authors, the surgery enhanced the frequency of diabetes two fold and additionally caused gonadotropin deficiency in all patients.

Similarly to our results concerning the presence of SPI and gonadotropin deficiency after surgery in CP patients, some authors observed SPI in 100% and gonadotropic deficiency in 50 % of treated cases (in our results 60%). Moreover they found hypothyreosis in 62.5% and corticotropic insufficiency in 75% of cases [8]. In our study corticotropic insufficiency occured only in 60% of treated patients, however, the diapedes insipidus was more frequent (50%).

We admit in principle the GH substitution after CP or MB surgery. However, after this type of therapy we can not exclude the risk of tumor reccurence. The study performed in England by Swerdlow et al. [31] showed that among 100 adult CP patients after surgery followed by hGH therapy, only in one person a new neoplastic proliferation appeared and it was finally stopped during treatment [32]. In another study that included 180 English children with CNS tumors treated with surgery, radiotherapy and subsequent hGH substitution, no new tumor process was observed. Kanev et al. [33] confirmed the low risk rate showed by Swerdlow.

On the other side, Uchino [11] has showed, that among 25 children treated with hGH, the neoplastic disease has reappeared in four patients – two of CP, one of astrocytoma and one of germinoma. In cells of craniopharyngioma, the author demonstrated the presence of GH receptor, thus he explained the possible increased risk of neoplastic growth as the undiserable side effect in this type of therapy.

Kranzinger [34] has described the 14 year old girl with CP, treated by surgery, radiotherapy and GH replacement. Four years later an astrocytoma occurred. Other twelve similar cases were also described [34]. However, it seems quite possible that the astrocytoma proliferation was caused rather by radiotherapy than by GH treatment. Moreover, the reappearance of neoplasia was found generally in patients treated only with surgery and without subsequent radiotherapy [35].

According to the published data concerning 422 children with CP treated with surgery and hGH substitution (Kabi International Growth Study, KIGS Database) [12], the neoplastic process reappears in 13.6% of cases, in a average four years after surgery and two years after hGH therapy. Nevertheless, hGH therapy seems to be useful for evident reason of child growth and GH-related metabolic mechanisms [12]. Moshang et al. [35] demonstrated that in studied population of 1262 cases only 6.6% of children with CNS tumors treated by surgery and hGH replacement showed the tumor "come back", commonly in glioma and medullobastoma cases; the authors suggest that the risk of CP and other CNS tumors reapparance is much lower in children treated with both surgery and hGH than with surgery alone [36]. For example, Cowell and Dietsch [Cowell], have published data of patients treated with hGH showing that CP and other CNS tumors reappeared only in 3.8% and 2.2% cases, respectively (and the leukemia apperance rate in the same group was 1.1%). The authors conclude that neoplastic reccurence frequency is identical in hGH treated and untreated patients [37].

The most important effect of hGH therapy in CP children was the growth acceleration observed in this group, the result which could be compared to the parallel effects of the same

therapy applied to children with idiopathic GH deficit [12,35]. Our results obtained in CP patients have to be compared with those of Price et al. [12,35] and Hogeveen et al. [38]. In the study of Hogeveen the two years of hGH therapy diminished the GH deficit up to SDS value -0.9. In the studies of Price et al. [12,35] the growth of treated CP children reached the values not far from the average values of healthy population: -0.71 SDS. Price and Jonnson [12] consider that hGH therapy allows in CP patients to increase SDS of growth up to 1.5. In our study in CP patients treated by hGH, growth deficit assessed by height standard deviation score (SDS) was reduced in two cases up to -0.52 and +0.1. SDS of growth increased as much as 2.7 (statistically $p<0.001$) and it was the reason for the final height of our CP patients after two years of hGH therapy which approached the values characteristic for "normal" children.

On the contrary, according to Price et al. [39] the effect of hGH therapy in other CNS tumors was less spectacular than in CP patients. By the way, other researchers consider that in 20--30% of hGH treated CP children the growth is more delayed than in healthy population. However, this observation concerns mainly the treated girls [28,40]. Herbert et al. [40] were even less successful, as they found the positive results only in 44% of CP cases. Moreover, the patients' growth was less evident if radiotherapy was used in CP and other CNS tumors [28]. Kanev et al. [33] suggest that the approach to the "normal" growth in hGH therapy cases depends on the start point of the treatment: the earliest the treatment is started, the better the effect are. Price et al. [35,39] have also observed that the deficit of final height correlates with the growth deficit before treatment and the moment of hGH therapy onset.

As to the case of our medulloblastoma patient treated with hGH, despite the fact that radiotherapy was also used, his growth was practically identical with the one of our CP treated patients. After one year of hGH therapy, height SDS rose from -3.4 μp to -2.4, so the increase in height observed in this boy (1.0 of SDS values) was statistically significant ($p<0.001$). The last observation is to be compared with similar growth acceleration events observed after one year of hGH therapy in CP patients studied by Hogeveen [38]. Nevertheless, as it was mentioned above, Price considers that the positive results obtained in CP patients do not concern other CNS tumors [39].

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Serum DNA as a tool for cancer patient management

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Abstract

Purpose: Genetic analysis has shown that cell-free circulating DNA in plasma or serum of cancer patients shares similar genetic alterations to those described in the corresponding tumor. One of the most important alterations involved in carcinogenesis is aberrant promoter methylation. The interest in this field has grown due to the implementation of the methylation-specific PCR (MSP) assay. The main objective of this study is to analyze the methylation status of different genes in tumor and serum DNA obtained at the time of surgery in two different tumor models (glioblastoma [GBM] and non-small-cell lung cancer [NSCLC]) and their relationship to clinico-pathological characteristics and response to chemotherapy.

Material and methods: Using MSP assay, we assessed the methylation status of MGMT, RASSF1A, p16, DAPK, TMS-1 in tumor and serum DNA obtained at time of surgery or stereotactic biopsy from 28 GBM patients and from 51 NSCLC patients.

Results: In GBM patients, the prevalence of MGMT, p16, DAPK, and RASSF1A promoter methylation was 38.1%, 66.7%, 52.4%, 57.1%, respectively, in glioma tissue, and 39.3%, 53.6%, 34.3%, 50%, respectively, in serum. A high correlation between methylation in tumor and serum (Spearman test p=0.0001) was observed. In NSCLC patients, RASSF1A, DAPK and TMS-1 were methylated in 34%, 45% and 35% tumors, respectively, and in 34%, 40% and 34% serum, respectively. A good correlation was found between alterations found in tumor and serum (Spearman test p=0.0001).

Conclusions: The study of serum or plasma DNA has opened new roads for translational research and new strategies for molecular diagnosis. Due to the similarities of alterations found in serum DNA and primary tumor, we can use this tool to calculate the risk of local or distant recurrence and its relationship with survival and its value in patient follow-up to evaluate response to therapy.

Key words: serum DNA, tumor DNA, aberrant methylation, NSCLC, GBM.

Introduction

Free DNA can be detected in different body fluids, e.g.: urine, synovial fluid, pancreatic duct secretions, sputum, and serum/plasma. This DNA circulating in serum/plasma is found in small amounts in healthy controls, but in cancer patients higher concentrations of DNA are present. The presence of nucleic acids (not only DNA but also RNA) in plasma or serum of cancer patients has been recognized since the 1970s [1,2], when Leon et al. reported the presence of DNA in the serum of patients with diverse neoplastic diseases. In this study they correlated the amount of circulating DNA with stage and response to treatment and they observed that the DNA levels in patients with metastatic disease were higher compared to those with non-metastatic disease. In 1989, Stroun et al., using a technique based on decreased strand stability of cancer cell DNA, recognized that part of the plasma DNA had the origin in cancer cells [3]. Five years later, Sorensen et al. reported the detection of tumor derived mutated k-ras DNA sequences in human blood from pancreatic cancer patients [4], and in 1994 Vasioukhin et al. reported N-ras mutations in plasma samples from patients with myelodysplastic syndrome [5]. Recently, an analysis published by Sozzi et al. [6] , they evaluated whether the amount of circulating DNA in plasma could discriminate between lung cancer patients and healthy individuals. In this work, they also

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determined the positive association between plasma levels and clinical progression. Moreover to date, several studies have reported the presence of free DNA concentrations in the serum of cancer patients, and molecular studies have provided evidence that this DNA has a tumoral origin. Thus, Chen et al. [7] analyzed the presence of microsatellite instability and loss of heterozygosity (LOH) in plasma DNA isolated from breast cancer patients and they found identical alterations in the corresponding plasma samples and tumor specimens. Anker et al. [8] commented in their review the detection of an increased concentration of DNA in plasma of cancer patients and how different molecular biological techniques, such as point mutation of the ras gene and the analysis of microsatellite instability, were used to establish the origin of this circulating tumoral DNA. De Kok et al. [9] investigated the fact that increased cell-free DNA in cancer patients was derived from tumor DNA as they describe the same point mutation, corresponding to the K-ras mutation in the primary tumor in the serum DNA. Furthermore, microsatellite instability in cell-free DNA has also been reported in lung cancer [10], breast carcinoma [11] in melanoma [12] and in head and neck squamous cell carcinomas [13]. All these studies suggest that tumor DNA is released into the circulation and is enriched in plasma and serum, on average, 3-4 times the amount of free DNA, compared to normal controls.

In addition to the molecular genetic alterations described above, transcriptional silencing of tumor suppressor genes by aberrant promoter methylation is a common feature in human cancer [14] and can contribute to oncogenesis. Hypermethylation of normally unmethylated CpG islands in the promoter region of many genes, including p16, p15, E-Cadherin, VHL, and hMLH1, correlate with loss of transcription in human tumors [15,16]. One interesting case that shows the importance of the analysis of aberrant methylation is the study performed by Tang et al. where they analyzed the hypermethylation of DAPK in patients with pathological stage I NSCLC who had undergone curative surgery. They found that patients whose tumors showed hypermethylation had significantly poorer overall survival compared to those without any alteration [17]. In cancer patients, aberrant promoter DNA methylation has been observed in a number of genes including several well-characterized candidate tumor suppressor genes including p16 [18] involved in cell cycle regulation, RAR-beta [19] involved in cell differentiation, RASSF1A [20] involved in signal transduction, FHIT [21], APC [22] also involved in aberrant signal transduction and TMS-1 gene [23] involved in apoptosis. Esteller et al. [24] reported a study in lung cancer patients that combined the analysis of aberrant promoter methylation of tumor suppressor genes in serum DNA comparing to tumor paired DNA. They found that aberrant promoter methylation of at least one of the genes p16, MGMT, GSTP1, and DAPK was identified in 68% of NSCLC patients [24]. The same alterations were found in serum/plasma of 71% of patients who presented methylated DNA in primary tumor [24]. These experiments were carried out using MSP, which is a sensitive and specific technique for methylation analysis. In this technique, DNA was amplified using primer pair sets in order to distinguish between methylated and non-methylated DNA by taking advantage of sequence differences resulting from sodium-bisulfite treatment, where unmethylated Cytosine are converted to Uracil, and methylated Cytosine remains as a cytosine [25].

However, one interesting conclusion from most of these studies is the fact that it is possible to find alterations in serum/plasma in the vast majority of cancer patients. This finding opened a lot of possibilities for the use of this serum/plasma as a tool for the detection of potential molecular markers to avoid invasive tools in order to obtain tumoral tissue [24,26]. To date, most of the studies published in the literature have analyzed the pattern of methylation only in serum/plasma or tissue, but in general they don't correlate gene promoter methylation status between serum and tumor DNA obtained at the time of surgery or biopsy. For this reason, the goal of our study is to try to answer two important questions. Firstly, are the alterations that appear in tumor DNA the same as the ones found in serum/plasma DNA? Secondly, do these alterations have a good correlation between tumor and serum DNA? To answer these questions, in the present study we examine the presence of genetic abnormalities in primary tumor and paired serum DNA (obtained at time of surgery) in two different tumor models (GBM and NSCLC), and their relationship with clinico-pathological characteristics and response to chemotherapy.

Using the sensitive MSP technique, we analyzed the methylation patterns of genes involved in apoptotic signalling molecules and cell cytoskeleton (TMS-1, DAPK) and in DNA repair and cell cycle control (RASSF1A), in non-microdisected tumor specimens and matching serum DNA from 51 resected NSCLC [27]. Our objective was to correlate the methylation status in paired tumor and serum samples, and to examine the impact of their aberrant methylation on survival. We also collected tumor and serum from 28 GBM patients treated prospectively with surgery, radiotherapy, and chemotherapy with BCNU or Temozolamide plus cisplatin [28]. Our objective was to confirm the presence of circulating serum DNA in these patients; to examine the concordance between methylation of MGMT (related with DNA repair capacity), p16 (cell cycle control), DAPK (apoptosis) and RASSF1A (ras signalling) in tumor and serum DNA; and finally, to correlate the methylation status of these genes with response and time to progression in GBM patients.

The study of serum or plasma DNA has opened up numerous areas of investigation and new possibilities for molecular diagnosis. It is very important to understand the study of these new areas of analysis due to the similarities of alterations found in serum DNA and primary tumor, the value as a prognosis tool to calculate the risk of local or distant recurrence and its relationship with survival; its value for patient follow-up or to evaluate response to therapy, and finally the possibility to use it as an early diagnostic tool in an asymptomatic high-risk population [29].

Material and methods

Patients. In this study we have analyzed methylation status in four different genes in tumor and paired serum DNA samples from 28 GBM patients and 51 NSCLC patients treated at the University Hospital Germans Trias i Pujol.

Table 1. PCR primer sequences, annealing temperatures and PCR length products.

Gene/ Marker	Forward primer	Reverse primer	MT (^{0}C)	Size (Bp)
MGMT M	5´-TTT CGA CGT TCG TAG GTT TTC GC-3´	5´-GCA CTC TTC CGA AAA CGA AAC G-3´	65	81
MGMT U	5´-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3´	5´-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3´	63	93
P16 M	5´-TTA TTA GAG GGT GGG GCG GAT CGC GTG C-3´	5´-ACC CGA CCC CGA ACC GCG ACC GTA A-3´	65	150
P16 U	5´-TTA TTA GAG GGT GGG GTG GAT TGT-3´	5´-CAA CCC CAA ACC ACA ACC ATA A-3´	62	151
RASSF-1 M	5´-CGC GTT TAG TTT CGT TTT CG-3´	5´-AAC TTT AAA CGC TAA CAA ACG C-3´	63	165
RASSF-1 U	5´-TTT GTG AGA GTG TGT TTA GTT TTG-3´	5´-CCC AAT TAA ACC CAT ACT TCA-3´	55	200
TMS-1M	5´-TTG TAG CGG GGT GAG CGG C´-3´	5´-AAC GTC CAT AAA CAA CAA CGC G-3´	65	96
TMS-1U	5´-GGT TGT AGT GGG GTG AGT GGT-3´	5´-CAA AAC ATC CAT AAA CAA CAA CAC A-3´	65	98
DAPK-M	5´-GGA TAG TCG GAT CGA GTT AAC GTC -3´	5´-CCC TCC CAA CAG CGC A-3´	64	98
DAPK-U	5´-GGA GGA TAG TTG GAT TGA GTT AAT GTT-3´	5´-CAA ATC CCT CCC AAA CAC CA A-3´	60	108

M: methylated-specific primers; U: unmethylated-specific primers; MT: melting temperature

In GBM, DNA was obtained from 21 tumors and 28 serum samples that were collected at time of surgery. After pathologic review, 27 patients had confirmed GBM and one had gliosarcoma. The median age was 59 years (range, 30 to 76 years), 16 were men and 12 were women, Karnofsky performance status was over 70% in 16 patients and 70% in 12 patients. Twenty one patients underwent partial resection with measurable residual disease observed in post-operative brain magnetic resonance imaging (MRI), 2 patients had radical surgery and the remaining 5 patients, stereotactic biopsy. Frozen glioma tissue was available only for 21/28 patients. Sixteen patients were treated with Temozolamide 200 mg/m^2/day for 5 consecutive days plus cisplatin 100 mg/m^2 on day 1, every 28 days for 3 cycles before radiotherapy 60 Gy over the primary tumor and edema; 8 patients were treated with BCNU 200-250 mg/m^2 every 6 weeks and radiotherapy. One patient received only Temozolamide plus radiotherapy, and 3 patients received no treatment due to early progression.

In NSCLC, DNA was obtained from 51 tumors and the corresponding paired serum samples in patients who had undergone curative surgery between October 1998 and September 1999. Seven patients were stage I tumors (T1-2, N0), 14 patients had stage II (T1-2, N1), 24 patients had stage IIIA (T1-3, N2), and 6 patients had stage IV. According to histological criteria, 26 patients were classified as squamous cell carcinoma, 15 as adenocarcinoma and 10 as large cell undifferentiated carcinoma. Patients were enrolled on the basis of the availability of fresh-frozen stored tissue.

All these studies were approved by the Clinical Research Ethics Committee, and all patients gave informed consent for obtaining the blood and tumor samples and for the gene methylation assays.

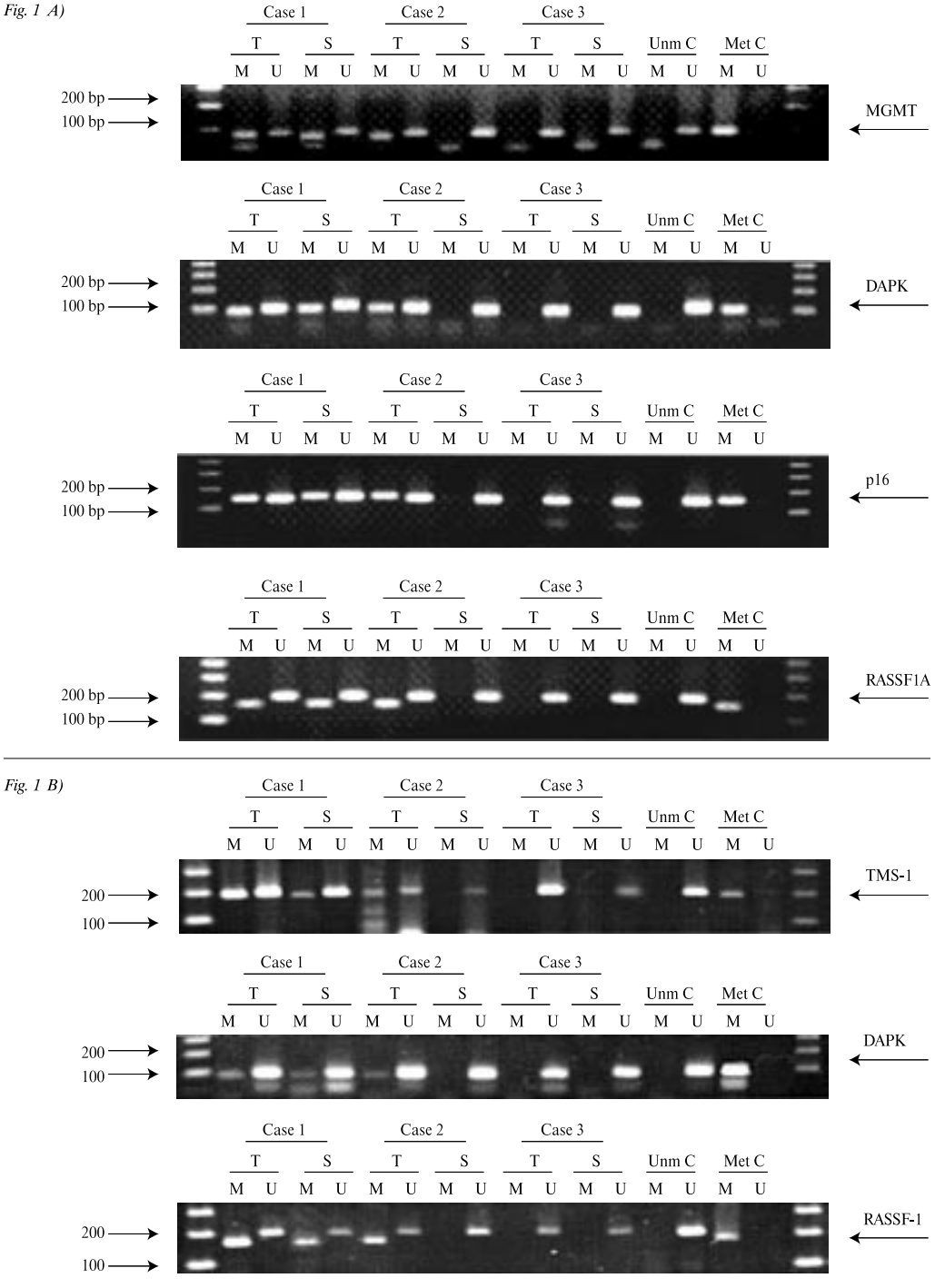
Sample collection and DNA extraction from tumor and serum DNA

We collected freshly resected surgical specimens and biopsies and paired peripheral venous blood drawn in the operating theater. Tumor samples were immediately snap-frozen in liquid nitrogen, and stored at -80^{0}C until DNA extraction was performed. Serum samples were collected before surgery or at the same time of surgery.

Five to ten mL of venous blood from each patient was withdrawn into vacutainer tubes containing SST gel and clot activator (Becton Dickinson, Plymouth, United Kingdom). Serum was isolated after centrifugation at 2500 rpm for 10 min and stored at -20^{0}C until use. Genomic DNA was obtained from 25 mg of each tissue sample, using DNAeasy Tissue kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. Serum DNA was extracted using the QIAmp Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions.

MSP. DNA methylation patterns in the CpG islands of MGMT (GenBank accession No X_61657), p16 (GenBank accession No X_94154), RASSF1a (GenBank No. XM_011780), TMS-1 (GenBank accession no AF184072), and DAPK (GenBank accession No NM_004938) were determined by MSP. Primers are described in *Tab. 1*. Briefly, up to 1μg of genomic DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega), again treated with

Figure 1 A) Methylation-specific PCR of RASSF1A, TMS-1 and DAPK in primary resected NSCLC and paired serum DNA. *B)* MSP of MGMT, p16, DAPK and RASSF1A in Glioblastoma patients (GBM) comparing tumoral tissue vs. serum. Five 1μ of bisulfite-modified DNA was amplified by PCR using primers that were specific for methylated or unmethylated sequences of each of these genes. Pt: patient sample; T: tumor; S: serum; M: methylated; U: unmethylated; Unm C: unmethylated control (lymphocytes); Met C: methylated control (SW-48 for MGMT gene, modified placental for DAPK gene, HT-29 for p16 gene, MOLT-4 for RASSF1A gene and SKBR-3 for TMS-1 gene).



NaOH, precipitated with ethanol, and resuspend in water. Controls without DNA were performed for each set of PCR. Each PCR products (15μL) was loaded onto a 2% agarose gel, stained with ethidium bromide, and visualized under UV illumination. DNA from peripheral blood lymphocytes and placenta treated with SssI Methyltransferase (New England Biolabs) was used as a positive control for unmethylated (lymphocytes) and methylated PCR reaction (placenta). In addition, all of the amplification assays included positive control for methylated form; thus the MOLT-4 cell line as a methylated control for RASSF1A, the SW-48 cell line is used as a positive control for MGMT methylation and finally, the cell line SKBR3 is used for TMS-1 methylated control (*Fig. 1*).

Results

The results obtained for GBM and NSCLC patients were analyzed separately and are described in *Tab. 2*.

Epigenetic alterations in Serum DNA and Tumor DNA in GBM patients

Methylation status for patients was studied in DNA extracted from 28 serum samples and 21 available paired glioma samples. Methylation status did not differ according to age, gender or treatment approach.

The prevalence of MGMT, p16, DAPK, and RASSF1A promoter methylation was 8/21 (38.1%), 14/21 (66.7%), 11/21 (52.4%), 12/21 (57.1%), respectively, in glioma tissue, and 11/28 (39.3%), 15/27 (53.6%), 9/26 (34.3%), 13/26 (50%), respectively, in serum.

We found that the concordance between alterations in tumor versus serum DNA was 17/21 (80%) for MGMT, 17/20 (85%) for p16, 17/21 (80%) for DAPK and 16/21 (76%) for RASSF1A. *Tab. 3* summarizes the concordance between methylation status of these genes in tumor and serum. All correlation coefficients were highly significant (Spearman correlation <0.05).

We only found 1 patient without any alteration in the four genes analyzed. The rest of the populations have at least one alteration in either tumor or serum DNA. We observed that methylation status did not differ according to age, gender, or treatment approach.

Overall, complete or partial response, or stable disease was observed in 15 patients (60%) whereas 10 patients had progressive disease. When patients were divided into those with MGMT methylation (either in tumor or serum) and those without MGMT methylation, response defined as no progression was noted in 10 of 11 (90,9%) of methylated patients as opposed to 5 of 14 (35, 7%) unmethylated patients (Fisher's exact test P=0.01). In addition, when response was broken down by methylation status in serum only, response plus stable disease was seen in 8 of 8 (100%) patients with methylated bands (Fisher's exact test p=0.008), but for tumor tissue alone, the differences in response were not significant.

When patients were broken down by treatment group, in the 16 patients treated with Temozolamide plus Cisplatin, no significant correlation between MGMT methylation status and response was observed, whereas in BCNU-treated patients, a significant difference was observed in favour of those with methylated MGMT. No correlation was observed between response and p16, DAPK or RASSF1A methylation in tumor and serum (data not shown).

Finally, for the analysis of methylation status and time to progression we observed that for patients who present aberrant methylation in MGMT in either tumor or serum, time to progression was 29.9 weeks (95% CI, 24.3 to 35.4). Time to progression for patients without MGMT methylation was 15.7 weeks (95% CI, 14.3 to 17.2) (log-rank test P=0.006) (*Fig. 2*). Neither p16, DAPK nor RASSF1A methylation correlated with time to progression.

Table 2. A) Summary of methylation patterns in matching tumor and serum DNA in NSCLC

	Tumor DNA/Serum DNA				
Patient No	RASSF1A	DAPK	TMS-1	Stage(TNM)	Histology
Pt#1	M/M	U/U	U/U	IB (T2N0M0)	AC
Pt#2	U/U	U/M	U/U	IA (T1N0M0)	SCC
Pt#3	M/M	U/U	M/M	IIB (T3N0M0)	AC
Pt#4	U/U	M/M	M/M	IIIA (T1N2M0)	LCC
Pt#5	M/M	M/U	U/U	IA (T1N0M0)	AC
Pt#6	U/U	U/U	M/M	IIIA (T2N2M0)	SCC
Pt#7	U/U	M/M	U/U	IIA (T2N1M0)	SCC
Pt#8	U/U	M/M	U/U	IIB (T3N0M0)	AC
Pt#9	U/U	M/U	U/M	IB (T2N0M0)	SCC
Pt#10	U/U	U/U	U/U	IIB (T2N1M0)	SCC
Pt#11	M/M	U/U	M/M	IV (T3N0M0)	AC
Pt#12	M/M	U/U	M/M	IIIA (T3N2M0)	LCC
Pt#13	U/U	U/U	U/U	IIIA (T3N2M0)	LCC
Pt#14	U/U	U/U	M/U	IIB (T3N0M0)	SCC
Pt#15	M/U	M/M	M/M	IIIA (T3N2M0)	ACC
Pt#16	U/U	U/U	U/U	IV (T3N2M1)	SCC
Pt#17	U/U	U/M	U/M	IIIA (T1N2M0)	SCC
Pt#18	M/U	M/U	U/U	IIIA (T2N2M0)	SCC
Pt#19	U/U	M/U	M/M	IB (T2N0M0)	SCC
Pt#20	U/U	M/U	M/M	IA (T1N0M0)	SCC
Pt#22	M/M	M/U	M/U	IB (T2N0M0)	AC
Pt#23	U/U	M/U	U/M	IIB(T2N1M0)	SCC
Pt#24	U/U	M/U	M/M	IB (T2N0M0)	SCC
Pt#25	U/U	U/U	U/U	IIB (T2N1M0)	LC
Pt#26	U/U	M/U	U/M	IIIA (T3N1M0)	SCC
Pt#27	U/U	U/U	U/U	IB (T2N0M0)	LC
Pt#28	U/M	M/M	U/U	IB (T2N0M0)	AC
Pt#29	M/M	M/U	U/U	IB (T2N0M0)	LC
Pt#30	U/U	U/U	U/U	IIIA (T3N1M0)	SCC
Pt#31	U/U	U/U	M/U	IV (T2N1M0)	SCC
Pt#32	U/U	U/U	U/U	IIIA (T2N2M0)	AC
Pt#33	U/M	U/U	U/U	IIIA (T2N2M0)	SCC
Pt#34	U/M	U/U	M/U	IB (T2N0M0)	AC
Pt#35	M/M	U/M	M/M	IIIA (T2N2M0)	AC
Pt#36	M/U	U/U	M/U	IB (T2N0M0)	SCC
Pt#37	U/M	U/M	U/U	IIB (T3N0M0)	LCC
Pt#38	U/U	M/M	M/M	IIIA (T3N1M0)	LCC
Pt#39	U/U	M/U	U/U	IV (T2N0M1)	SCC
Pt#40	U/U	U/U	U/U	IA (T1N0M0)	LCC
Pt#41	M/M	U/M	U/U	IB (T2N0M0)	SCC
Pt#42	U/M	M/M	U/U	IV (T1N1M1)	AC
Pt#43	U/U	U/U	U/U	IIIA (T3N1M0)	SCC
Pt#44	M/M	M/M	U/M	IB (T2N0M0)	SCC
Pt#45	M/U	M/M	U/U	IV (T3N0M1)	LCC
Pt#46	M/M	M/M	U/U	IIIA (T2N2M0)	SCC
Pt#47	U/U	U/U	U/U	IIA (T1N1M0)	AC
Pt#48	U/U	U/U	U/M	IIIA (T2N2M0)	AC
Pt#49	U/U	U/M	M/M	IIIA (T3N2M0)	AC
Pt#50	M/U	M/M	U/U	IIA (T1N1M0)	SCC
Pt#51	M/M	U/M	U/U	IIIA (T2N2M0)	LCC

AC: Adenocarcinoma; SCC: Squamous Cell Carcinoma; LCC: Large Cell Carcinoma

Table 2. B) Summary of methylation patterns in matching tumor and serum DNA in Glioblastoma Multiforme

	Tumor DNA/Serum DNA			
Patient No.	MGMT	P16	RASSF1A	DAPK
Pt#1	M/U	M/M	U/U	M/U
Pt#2	M/M	M/M	M/U	U/U
Pt#3	U/U	M/M	U/U	M/M
Pt#4	-/M	-/M	-/M	-/U
Pt#5	-/M	-/U	-/U	-/U
Pt#6	U/U	M/M	U/U	M/M
Pt#7	-/U	-/M	-/U	-/U
Pt#8	U/U	M/M	U/M	U/U
Pt#9	U/U	M/M	M/M	M/M
Pt#10	U/U	U/U	M/M	U/U
Pt#11	-/M	-/U	-/U	-/U
Pt#12	U/U	U/U	U/U	U/U
Pt#13	U/U	M/M	M/M	U/U
Pt#14	-/M	-/M	-/M	-/U
Pt#15	U/U	M/M	M/M	M/M
Pt#16	U/M	U/U	M/M	U/U
Pt#17	M/M	-/M	U/U	M/M
Pt#18	M/U	M/M	U/U	U/U
Pt#19	M/M	U/U	M/U	M/M
Pt#20	-/M	-/U	-/M	U/M
Pt#21	M/M	M/M	M/M	M/M
Pt#22	U/U	M/U	M/M	U/U
Pt#23	M/U	U/U	U/U	M/U
Pt#24	-/U	-/U	-/U	-/U
Pt#25	U/U	M/U	U/M	M/U
Pt#26	U/U	M/M	U/U	U/U
Pt#27	M/M	U/M	M/M	M/M
Pt#28	U/U	M/M	M/U	M/U

Theses tables represent alterations in tumoral tissue DNA vs. serum DNA, (T/S). U for Unmethylated gene and M for Methylated gene. –/S indicates Tumor DNA not available.

Table 3. Concordance between methylation of MGMT, p16, DAPK and RASSF1A in tumor and serum DNA from glioblastoma patients.

Markers	Assessment in TISSUE	Assessment in SERUM		Spearman Correlation
		Unmethylated No (%)	Methylated No (%)	
MGMT	Unmethylated	12 (57.1)	3 (14.3)	0.005
	Methylated	1 (4.8)	5 (23.8)	
DAPK	Unmethylated	10 (47.6)	3 (14.3)	0.00001
	Methylated	–	8 (38.1)	
p16	Unmethylated	6 (28.6)	2 (9.5)	0.00001
	Methylated	1 (4.8)	12 (57.1)	
RASSF1A	Unmethylated	8 (38.1)	2 (9.5)	0.00001
	Methylated	1 (4.8)	10 (47.6)	

Figure 2. Time to progression according to MGMT methylation pattern in serum or tissue.

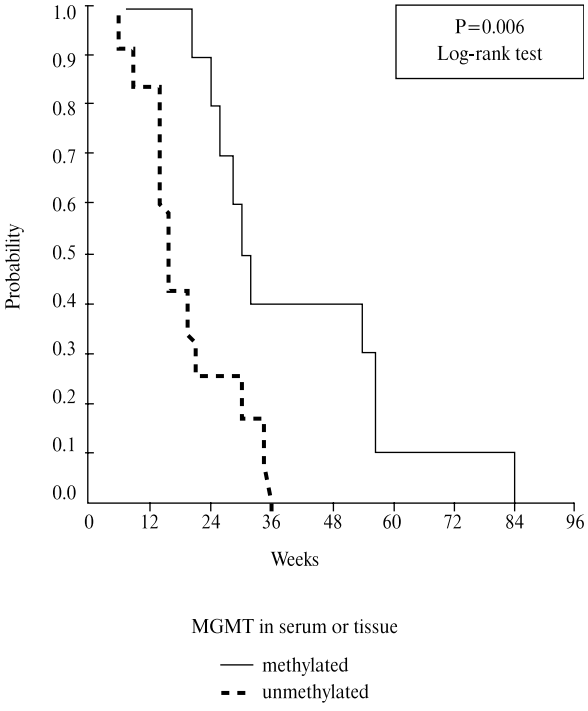


Table 4. Methylation patterns of RASSF1A, DAPK, and TMS-1 found in tumor and serum DNA from NSCLC patients.

Markers	Assessment in TISSUE	Assessment in SERUM		Spearman Correlation
		Unmethylated No (%)	Methylated No (%)	
TMS1	Unmethylated	27 (54)	6 (12)	0.0001
	Methylated	5 (10)	12 (24)	
DAPK	Unmethylated	19 (39.6)	9 (18.7)	0.05
	Methylated	8 (16.7)	12 (25)	
RASSF1A	Unmethylated	28 (56)	5 (10)	0.0001
	Methylated	5 (10)	12 (24)	

Gene promoter Hypermethylation Profiles in NSCLC patients

The analysis for methylation status in NSCLC was developed in DNA extracted from 51 patients (49 male smokers and two female non-smokers). Neither gender, age nor stages were associated with the promoter methylation status of the genes.

The prevalence of RASSF1A, DAPK and TMS-1 methylation was 17/51 (34%), 23/51 (45%) and 18/51 (35%), respectively, in tumor tissue, and 17/51 (34%), 20/51 (40%) and 17/51 (34%), respectively, in serum (*Tab. 4*).

For RASSF1A gene we found that 41/51 (80%) of patients had the same alteration in tumor and serum DNA; for DAPK was 33/51 (65%); and for TMS-1 was 40/51 (78%). In other words, a good correlation was found between alterations found in tumor and serum (Spearman correlation <0.05).

Overall, 74.5 % of patients had at least one genetic alteration in tumor, and 76.5 % had at least one in serum. Eighty-two percent of patients had at least one alteration in either tumor or serum. Stage I NSCLC patients had at least two genetic alterations in either tumor or serum in 87.5 % of cases.

We observed no relationship between the number of genetic alterations and tumor size. We found no correlation between methylation status of RASSF1A, TMS-1 or DAPK, either in tumor or serum, and survival.

Discussion

The most important of the studies published in the literature that analyze aberrant methylation have been performed in DNA isolated from tumor tissue, and few studies on aberrant DNA methylation have been done in cell free circulating serum DNA [24,26]. In addition, these studies do not analyze the correlation of gene promoter methylation between serum and tumor DNA at time of surgery or biopsy. For these reasons, we have assessed methylation of MGMT, p16, DAPK and RASSF1A, TMS-1 in tumor and matching serum of GBM patients and NSCLC patients, and we have found that the concordance between methylation of these genes in tumor and serum DNA is highly significant.

Furthermore, we demonstrate, for the first time, that in GBM patients it is possible to detect aberrant promoter hypermethylation in serum DNA and that these alterations have the origin in the tumoral tissue. As we know, when malignant gliomas are transplanted into subcutaneous tissues, the glioma cells grow locally but do not metastasize, indicating that primary brain tumors are fundamentally different from tumors originating elsewhere [30]. This was one of the reasons that it is particularly intriguing to screen methylation in serum of GBM patients. The only previous research along these lines was the seminal work by Leon et al. [1] examining the concentration of free serum DNA in several tumors including four gliomas. Another reason for analyzing MGMT status was the study performed by Esteller et al. [31], where they found an increased responsiveness to BCNU and cisplatin in MGMT-deficient anaplastic astrocytomas and GBM. In our study, MGMT methylation, especially in serum DNA, was significantly associated with response and time to progression in all patients, as had previously been suggested [32,33]. However, when patients were broken down by treatment group, no significant correlation between MGMT methylation and response was observed in patients treated with Temozolamide plus Cisplatin, suggesting a role for Cisplatin, as previous in vitro experience suggests that Cisplatin may enhance the activity of Temozolamide by the inhibition of MGMT [34]. Although there are other DNA repair pathways that are important in chemoresistance, the examination of these factors involved in these pathways requires primary tumor tissue, whereas the MSP assay of MGMT methylation in serum DNA can be easily performed in samples obtained by non-invasive tools. The importance of this study lies in the findings that serum MGMT methylation predicted response and time to progression in BCNU-treated GBM patients and that, moreover, serum DNA is a feasible alternative source for testing methylation.

We also analyzed aberrant promoter methylation in tumoral and serum DNA from NSCLC patients, obtained at the time of surgery. The prevalence of RASSF1A and DAPK methylation found in the study was along the same lines as that previously reported [17,10,35]. In this study, we have detected a similar frequency of methylation of all three genes in the paired serum DNA and a good correlation was found between alterations found in tumor and serum (Spearman test $P=0.0001$) (*Tab. 4*).

For DAPK gene there are previous studies that also detected this gene methylated in serum DNA from NSCLC patients [14]. Although DAPK methylation was found in 59/135 (44%) of resected NSCLC patients and correlated significantly with worse survival [35], in another study, DAPK methylation was not significantly associated with worse survival [17]. In our study, no differences in survival were observed according to DAPK methylation status. A growing list of reports indicates that methylation in serum DNA correlates with methylation in primary tumors, including DAPK in gastric carcinoma [36].

For the first time, TMS-1 has been examined in lung cancer and found in 18/50 (35%) primary tumors, which concurs with the frequency described in breast cancer [23] .We have found TMS-1 methylated in 35% of primary tumors and 34% of serum samples, similar to the 40% found in breast cancer [23]. The good correlation between tumor and serum methylation status makes the examination of TMS-1 in serum DNA particularly interesting since it has been postulated that methylation-mediated silencing of TMS-1 could confer resistance to cytotoxic drugs [23].

Finally, RASSF1A methylation in either tumor or serum did not correlate with survival. The importance in this study is the fact that the high concordance of methylation patterns in tumor and serum DNA makes it particularly attractive to use peripheral blood for methylation assessment.

In summary, in this report we demonstrate the feasibility of finding the presence of alterations in tumor DNA and in serum DNA and the possible origin of these free circulating DNA. This evidence is supported by the fact that a good correlation exists between methylation in serum and primary tumor tissue. Moreover, the molecular techniques most commonly used to detect the neoplastic DNA in serum/plasma DNA are based on PCR assays. These PCR techniques can detect very few copies in tumor DNA containing a specific alteration among an excess background of normal DNA; for this reason, we decided to use the MSP protocol, which is a highly sensitive technique that allows approaching 1 methylated gene copy in 1000 unmethylated copies in dilution experiments [25]. The results obtained in different cancers open a new research area indicating that serum/plasma DNA could be used for the development of non-invasive tests for cancer patients in diagnosis, prognosis and follow-up. Since promoter methylation of different genes has been shown to be related to chemotherapy response or survival, the analysis in serum or plasma might be a useful tool for tailoring chemotherapy.

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Importance of nitric oxide (NO) and adenosine in the mechanism of gastric preconditioning induced by short ischemia

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Abstract

Purpose: Gastric mucosa subjected to repeated brief episodes of ischemia exhibits an increased resistance to damage caused by a subsequent prolonged ischemic insult and this is called gastric preconditioning. In this study, L-NNA, a non-selective NO-synthase inhibitor, and aminoguanidine, a relative inhibitor of inducible NO-synthase (iNOS), were applied prior to short ischemia (occlusion of celiac artery 1-5 times for 5 min) followed by a subsequent exposure to 0.5 h of ischemia and 3 h of reperfusion (I/R).

Material and methods: Male Wistar rats were used in all studies.

Results: Short ischemia reduced significantly I/R-induced lesions while raising significantly the GBF and luminal NO content. These effects were attenuated by L-NNA and aminoguanidine and restored by addition of L-arginine and SNAP to L-NNA and aminoguanidine. Pretreatment of with adenosine (10 mg/kg i.p.) significantly reduced I/R lesions and accompanying fall in the GBF induced by I/R. These protective and hyperemic effects of standard preconditioning and adenosine were significantly attenuated by pretreatment with 8-phenyl theophylline (SPT, 10 mg/kg i.g.), an antagonist of adenosine A_1 and A_2 receptors.

Conclusions: We conclude that gastric ischemic preconditioning is considered as one of the major protective mechanism in the stomach that involves key vasodilatory mediators such as NO and adenosine.

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Key words: gastric preconditioning, ischemia/reperfusion, gastric blood flow, prostaglandins, cyclooxygenase, nitric oxide, adenosine, adaptive cytoprotection.

Introduction

Preconditioning to ischemic tolerance is a phenomenon in which brief episodes of a subtoxic insult induce a robust protection against deleterious effect of subsequent, prolonged, lethal ischemia [1]. These protective effects of ischemia preconditioning were first described in the heart by Murry and coworkers [2]. Since that time, preconditioning has been shown to reduce the extent of myocardial infarct size as well as the damage of skeletal muscle, brain, kidney and intestine induced by subsequent exposure to prolonged severe ischemia/reperfusion in a variety of species [3-6] but the mechanism of this protection remains unknown.

Cardioprotective effect of ischemia preconditioning is well documented because repeated short episodes of ischemia due to the temporal occlusion of coronary vessels were shown to prevent lethal cell injury of the myocardium against the damage induced by long-term severe ischemia/reperfusion [7]. In another report, ischemic preconditioning of rat mesenteric venules led to enhance bioavailability of nitric oxide (NO) and abolished oxidant production as well as attenuated the leukocyte adhesion and emigration in the mesentery [8]. This was the first evidence that ischemia preconditioning can also exists in the gut, at least in part, preventing the mesenteric microvascular barrier dysfunction and inactivation of NO in the intestine. Recently, we have shown that brief episodes of ischemia of the stomach afforded protection against the gastric mucosal damage induced by prolonged ischemia/reperfusion insult by multifactorial mechanism involving endogenous prostaglandins, NO, adenosine and neuropeptides released from sensory afferent neurons [9]. This protection by ischemic preconditioning of gastric mucosa mimicked that exerted by

certain mild irritants such 20% ethanol, 5% NaCl or 5 mM taurocholate against the damage induced by these agents applied intragastrically in much larger necrotizing concentrations and called adaptive cytoprotection [10-12]. The protective action of mild irritants has been attributed to the activity of endogenous PG but besides PG the importance of other protective factors such as NO, nonprotein sulfhydryl compounds and sensory nerves were implicated in this phenomenon [13-17].

The mechanism of ischemic preconditioning remains unclear but adenosine, which is produced during the ischemic preconditioning was proposed to act as an initiator of this preconditioning in different organs such as heart and liver. Adenosine was effective in attenuating the injury caused by severe ischemia/reperfusion and this protection induced by ischemic preconditioning can be reversed by adenosine receptor antagonists [3,18]. The question remains whether adenosine mimics the protective effect of ischemia preconditioning in the stomach and can exert protection against gastric lesions caused by prolonged ischemia/reperfusion and if so which adenosine receptors are involved in this response of preconditioning.

This study was designed to determine the effect of the ischemia preconditioning in the stomach on gastric lesions induced by ischemia/reperfusion (I/R) and to elucidate the role of GBF, NO release into the gastric lumen and adenosine in the gastric mucosa subjected to ischemia preconditioning with or without prolonged I/R.

Material and Methods

Male Wistar rats weighing 180-220 g were used in all studies. Rats were fasted 18 h before the experiment but they had free access to the drinking water.

Production of gastric lesions induced by ischemia-reperfusion I/R erosions were produced in 120 rats by the method originally proposed by [19]. Briefly, under pentobarbital anesthesia (50 mg/kg i.p.), the abdomen was opened, the celiac artery identified and clamped with a small device for 30 min followed by removal of the clamp to obtain reperfusion. We attempted to determine the effect of various time periods of gastric ischemic preconditioning on the lesions induced by regular I/R. For this purpose, rats were preconditioned with single episode of gastric preconditioning ranging from 37 up to 300 s before the exposure to 30 min of ischemia followed by 3 h of reperfusion. The second goal was to test whether the increasing number of short ischemic episodes affects the lesions induced by I/R. For this purpose gastric mucosa was pretreated with 1 to 5 episodes of short ischemia (5 min each) before the exposure to regular I/R. In addition, short ischemia (occlusion of celiac artery for 5 min 1-5 times – ischemia preconditioning) was applied 30 min before subsequent exposure to longer 30 min of ischemia (also induced by clamping of celiac artery) followed by 3 h of reperfusion (I/R). Involvement of NO in the protective effect of gastric preconditioning.

The implication of NO in the effect of gastric preconditioning on damage induced by ischemia/reperfusion was determined by three ways: 1) by the use of N^G-nitro-L-arginine (L-NNA) applied i.p. in a dose of 20 mg/kg to suppress nonspecifically the activity of NOS [21] and aminoguanidine (10 mg/kg i.p.), a relative specific inhibitor of iNOS [21] to inhibit iNOS activity; 2) by the indirect measurement of NOS product i.e. NO in gastric lumen [22]; and 3) by addition to L-NNA of L-arginine, a substrate for NOS or D-arginine, which is not a substrate for NO [23]. The rats with gastric lesions induced by regular ischemia/reperfusion were pretreated either with: 1) sham operation or standard ischemic preconditioning (occlusion of celiac artery twice for 5 min) alone; 2) L-NNA (20 mg/kg i.g.) and aminoguanidine (10 mg/kg i.p.) with or without the preconditioning; 3) L-arginine (200 mg/kg i.g.) plus L-NNA (20 mg/kg i.g.) combined with the preconditioning; 4) D-arginine (200 mg/kg i.g.) plus L-NNA (20 mg/kg i.g.) combined with the preconditioning, and finally; 5) SNAP (5 mg/kg i.g.) plus aminoguanidine (10 mg/kg i.p.) combined with the preconditioning.

The luminal concentration of NO was quantified indirectly as nitrate (NO_3^-) and nitrite (NO_2^-) levels in the gastric contents using the nitrate/nitrite kit purchased from Cayman Lab, Michigan, USA as described in details before [24]. This method is based on the Griess reaction and generation of chromophore absorbing at 595 nm, according to the original procedure reported previously [25]. Since NO released by epithelial cells into the gastric lumen is quickly transformed into NO_3^- and NO_2^- [22], the sum of these products of NOS gives an index of production of NO by the enzyme in the gastric mucosa. In order to determine NOx, the gastric content was aspirated just before the removal of the stomach following the i.g. injection of 1 ml of saline to wash out the luminal content. After centrifugation for 10 min at 3000 rpm, the samples were mixed with Griess reagent from the commercially available kit. In all tests including gastric preconditioning with or without the combination with L-NNA and L-arginine or D-arginine and aminoguanidine with or without SNAP, the GBF was measured in the oxyntic mucosa in each group of animals in similar manner as described below and expressed as the percent control value recorded in vehicle-treated gastric mucosa.

Implication of adenosine in ischemic gastric preconditioning

The involvement of adenosine in the mediating of the effect of preconditioning on the gastric mucosa was determined by two ways: 1) by pretreatment with 8-p-sulphophenyl theophylline (SPT) at a dose (10 mg/kg i.g.), that was reported to inhibit adenosine receptors and to attenuate the effect of ischemic preconditioning on the heart infarct size [26], and 2) by the application of exogenous adenosine (10 mg/kg i.g.) to check whether pretreatment with exogenous adenosine can protect the gastric mucosa lesions induced by regular I/R.

Measurement of gastric blood flow (GBF)

At the termination of experiment, the gastric blood flow (GBF) was measured by H_2-gas clearance technique. Rats were lightly anesthetized with ether, the abdomen was opened and the stomach was exposed. The GBF was measured in the oxyntic gland area of the stomach by means of local H_2-gas clearance method using an electrolytic regional blood flow meter (Biomedical Science, Model RBF-2, Japan) as described previously [27]. The measurements were calculated in three areas of the mucosa and the mean absolute values

Figure 1. Mean area of gastric lesions (columns) and gastric blood flow (GBF) (lines) in the gastric mucosa of rats pretreated with sham (control) or gastric preconditioning (IP) lasting from 37 up to 300 s and then exposed to 30 min of ischemia followed by 3 h of reperfusion. Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control animals.

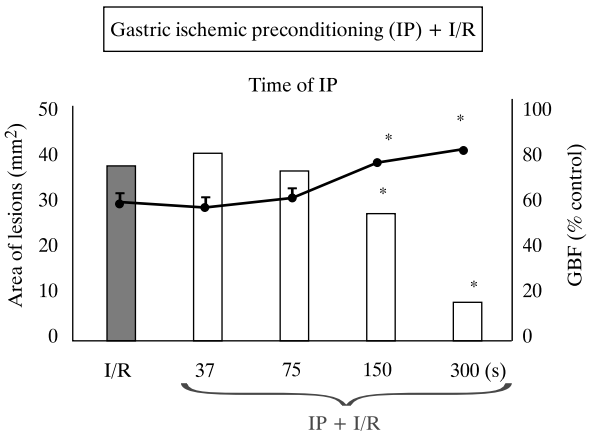
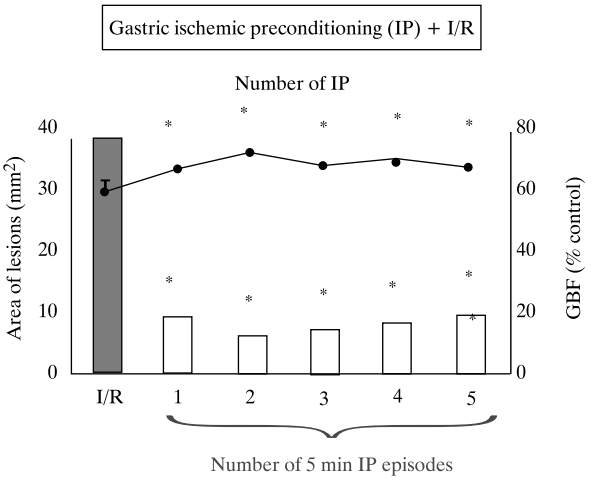


Figure 2. Mean area of gastric lesions (columns) and gastric blood flow (GBF) (lines) in the gastric mucosa of rats pretreated with sham (control) and various numbers of 5 min ischemic episodes. Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control animals.



(ml/100 g-min) of these measurements were calculated and expressed as percent changes from those recorded in control animals treated with vehicle.

Statistical analysis

Results are expressed as means ± SEM. The significance of the difference between means was evaluated using analysis of variance followed by Duncan's test with a level of confidence at $P<0.05$.

Results

Effect of short ischemic episodes on the gastric lesions induced by I/R insult and the accompanying changes in the GBF.

Fig. 1 shows the effects of various time duration of single short ischemic episode lasting from 37 s up to 300 s on gastric lesions and accompanying changes in the gastric blood flow induced by regular ischemia/reperfusion. Ischemic episodes shorter than 75 s failed to influence significantly the area of ischemia/reperfusion-induced gastric lesions and to affect the gastric blood flow. With prolongation of ischemic episodes up to 150 s or 300 s applied before regular ischemia/reperfusion a significant reduction in the area of acute gastric lesions and a significant rise of the gastric blood flow were observed. The short ischemia of 300 s (5 min), that caused reduction of ischemia/reperfusion lesions by about 80% was used as a standard ischemic preconditioning and used in subsequent studies.

In order to select how many episodes of short ischemia is necessary to afford the maximal protective action against the damage evoked by prolonged I/R, we tested the effect of various numbers of standard (5 min) ischemic episodes ranging from 1 to 5 on the area of gastric erosions induced by regular ischemia/reperfusion. *Fig. 2* shows that single standard (5 min) preconditioning episode reduced the area of I/R erosions by about 67%. Increase in number of standard ischemic episodes to two (2x5 min occlusion) did not result in any further significant reduction in lesion area caused by regular ischemia/reperfusion. The GBF in the intact stomach averaged 53 ± 6 (taken as 100%) and this was significantly reduced (by about 40%) at the end of 3 h of reperfusion that followed 30 min of ischemia (*Fig. 2*). A single standard ischemic episode increased significantly the gastric blood flow by about 25% as compared to that recorded in sham-operated controls exposed to regular ischemia/reperfusion. Exposure of the gastric mucosa to 2-5 ischemic episodes produced similar rise in the gastric blood flow but this increase was not significantly different than that obtained in animals with single ischemic episode (*Fig. 2*).

Effect of L-NNA and aminoguanidine on gastric lesions, gastric blood flow and NO production in gastric mucosa exposed to ischemia/reperfusion with or without gastric preconditioning. *Fig. 3* shows the results of tests with standard preconditioning with or without addition of L-NNA or the combination of L-NNA plus L-arginine or D-arginine on the area of gastric luminal contents of NO_3^-/NO_2^- and GBF. The pretreatment with short ischemia resulted in usual attenuation of lesion area and an increase in GBF and produced a significant rise in luminal contents NO_3^-/NO_2^-. L-NNA applied i.p. in a dose of 20 mg/kg, aggravated significantly the lesions induced ischemia/reperfusion and decreased the gastric blood flow and luminal release of NO degradation products as compared to those in vehicle-treated animals. Such treatment with L-NNA abolished the decrease in ischemia/reperfusion lesions, the rise in gastric blood flow and the production of NOx into gastric lumen recorded in animals subjected to gastric preconditioning applied before I/R (*Fig. 3*). Addition of L-arginine but not D-arginine to the combination of L-NNA and short ischemic restored the protective effect, the rise in gastric blood flow and luminal NO_3^-/NO_2^- content to the levels observed in rats pretreated with short ischemia (*Fig. 3*). As shown in *Tab. 1*, pretreatment with aminoguanidine by itself failed to affect gastric

Figure 3. Effect of standard ischemic preconditioning (IP) with or without pretreatment with N^G-nitro-L-arginine (L-NNA 20 mg/kg i.p.) applied with or without the combination with L-arginine (L-Arg, 200 mg/kg i.g.) or D-arginine (D-Arg, 200 mg/kg i.g.) on the area of gastric lesions (columns) and accompanying changes in the gastric luminal NO concentration (lines) induced by the exposure to regular ischemia/reperfusion (I/R). Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control gastric mucosa. Cross indicates a significant change as compared with the value obtained in rats without treatment with L-NNA. Double cross indicates a significant change as compared to the value obtained in rats without L-Arg administration.

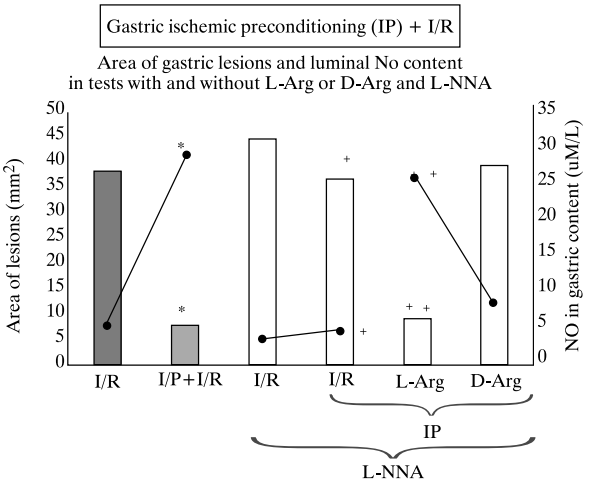
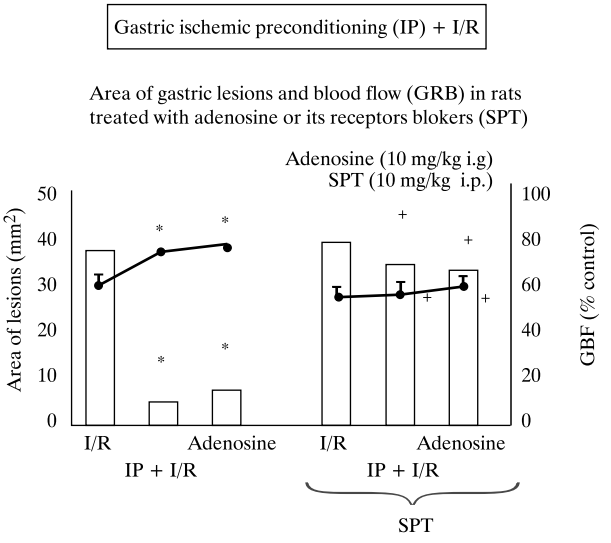


Figure 4. Effect of standard ischemic preconditioning (IP) and adenosine (10 mg/kg i.g.) applied alone or combined with 8-p-sulphophenyl theophylline (SPT; 10 mg/kg i.p.) on the area of gastric lesions (columns) and accompanying changes in the GBF (lines) induced by the exposure to standard ischemia/reperfusion. Results are mean ± SEM of 6-8 rats. Asterisk indicates a significant change as compared with the value obtained in sham-control gastric mucosa. Cross indicates a significant change as compared with the value obtained in rats without treatment with SPT.



lesions induced by I/R but significantly attenuated the decrease in the area of I/R-induced gastric lesions and accompanying rise in the GBF caused by gastric preconditioning. Addition of SNAP, a NO-donor to aminoguanidine, restored the protective and hyperemic effects of short ischemia against prolonged I/R (*Tab. 1*).

Effect of exogenous adenosine and suppression of adenosine receptors by 8-phenyl theophylline on the gastric lesions induced by ischemia/reperfusion with or without short ischemia. Pretreatment with adenosine (10 mg/kg i.g.) attenuated significantly the lesions induced by regular ischemia/reperfusion and increased the gastric blood flow with the extent similar to that observed with standard preconditioning (*Fig. 4*). A non-selective antagonist of adenosine receptors, 8-p-sulphophenyl theophylline (10 mg/kg i.g.), which by itself failed to influence the area of gastric lesions and accompanying increase in the gastric blood flow, reduced significantly the protection and rise in the gastric blood flow caused by both, gastric preconditioning or pretreatment with exogenous adenosine against lesions induced by ischemia/reperfusion (*Fig. 4*).

Discussion

This study shows that the preconditioning of the gastric mucosa with short episodes of ischemia in the stomach exerts significant protection against lesions caused by longer exposure to regular I/R and documented that this protection may involve NO. Furthermore, we found that the protective and hyperemic effects of preconditioning against ischemia/reperfusion were antagonized by 8-p-sulphophenyl theophylline (SPT), an antagonist of adenosine receptors and that exogenous adenosine attenuated significantly gastric lesions induced by I/R with the extent similar to that observed after standard ischemic preconditioning suggesting that adenosine may also contribute to the beneficial effect of gastric preconditioning in the stomach.

The phenomenon of preconditioning was described originally in various organs including heart, lungs, liver and intestine [1,2,4-6,8,28], resulting in the limitation of the mucosal damage evoked by the I/R. We demonstrated recently that gastric preconditioning may represent an important gastroprotective mechanism against the damage induced by severe I/R as well as it can exhibit protective action against the damage caused by various necrotizing substances including 100% ethanol, 25% NaCl and 80 mM taurocholate in the stomach [9].

In this study, we attempted to determine the possible mechanism of gastric preconditioning with the major focus on the role of endogenous NO and adenosine as potential mediators of this protection in the rat stomach. Previous studies revealed that NO released from vascular endothelium sensory afferent nerves or gastric epithelium is essential for the gastroprotection and ulcer healing [17,20,22,23,27,29]. Furthermore, non-steroidal anti-inflammatory drugs (NSAIDs) that contain NO moiety are believed to counteract the vasoconstriction effects of conventional NSAIDs therefore limiting the gastrotoxicity and ulcerogenic effects of NSAID-therapy [30]. We have shown previously that administration of suppression of NO-synthase activity with NO-inhibitors abolished the gastroprotective activity of capsaicin in the stomach and delayed healing of chronic gastric ulcers [17] but the question which enzyme

Table 1. Effect of standard ischemic preconditioning (IP) without or with the pretreatment with aminoguanidine or SNAP (5 mg/kg i.g) added to aminoguanidine on the area of gastric lesions induced by ischemia/reperfusion (I/R) and the changes in the GBF. Results are mean ± SEM of 8-10 rats. Asterisk indicates a significant change as compared to the value obtained in vehicle-treated gastric mucosa. Cross indicates a significant change as compared to the value obtained in gastric mucosa exposed to I/R. Double cross indicates a significant change as compared to the respective value obtained in gastric mucosa pretreated with aminoguanidine.

Type of test	Mean lesion area (mm^2)	GBF (% Control)
I/R	44 ± 3	49 ± 4
IP + I/R	6 ± 0,4*	72 ± 5*
Aminoguanidine +I/R	48 ± 5	47 ± 3
Aminoguanidine +IP + I/R	32 ± 6+	63 ± 6+
SNAP + aminoguanidine + IP + I/R	10 ± 2,5++	68 ± 5++

becomes a source of NO in the protective response of transient short ischemia against I/R-induced gastric lesions has not been fully clarified.

Preconditioning refers to a phenomenon in which a tissue is rendered resistant to the deleterious effects of severe and prolonged ischemia followed by reperfusion by previous exposures to brief periods of vascular occlusion [28]. Previous studies revealed that the mechanism of gastric preconditioning is multifactorial in nature and may depend upon several mediators including prostaglandin derived from cyclooxygenase-1 and cyclooxygenase-2 activity and CGRP released from sensory afferent nerve endings [9,31,32]. These mediators play a key role in the mechanism of this protection possibly by causing vasodilatation and enhancing the gastric blood flow. Our present report is in keeping with this hypothesis because the protection and accompanying rise in the gastric blood flow induced by gastric preconditioning were significantly attenuated by non-selective suppression of NO-synthase activity by L-NNA and by relative inhibitor of iNOS, aminoguanidine. The involvement of NO is supported by the fact that the concurrent treatment with L-arginine to provide a substrate for NO synthase and with SNAP, a potent donor of NO, added to L-NNA and aminoguanidine, respectively, restored the protective and hyperemic activity of gastric preconditioning against I/R injury. It is known that NO and various vasodilatatory neuropeptides such as CGRP are released from sensory nerves and functional ablation of sensory nerves with capsaicin was shown to attenuate both, protection and gastric hyperemia induced by gastric preconditioning indicating that NO derived from afferent nerves could be also considered as an important mediator of protection induced by short ischemia [32].

We also tested in this report the hypothesis, suggested by others in hepatic preconditioning [18], that adenosine plays a crucial role in the mechanism of gastric preconditioning. Indeed, the beneficial effects of preconditioning were blocked, at least in part, by the administration of non-selective adenosine receptor antagonist SPT. Furthermore, the pretreatment with adenosine in non-preconditioned animals resembled the protective effect of preconditioning against the I/R gastric injury. These observations are consistent with the hypothesis that locally released adenosine during preconditioning might trigger the gastroprotection afforded by preconditioning via activation of adenosine receptors.

As mentioned, the ischemic preconditioning has been best studied in coronary artery disease, where transient ischemia was found to reduce the degree of infarction following severe and sustained ischemia. Stefano et al. [33] postulated that short ischemia mimics the cell response to normal dips in ATP levels caused by metabolic demands; the action involving NO originating from cNOS and resulting in temporary down-regulation of the cell excitatory state. This suggests that the major enzyme involved in the short-term gastric preconditioning appears to be cNOS rather than iNOS. This notion is in keeping with the original findings in heart that ischemic preconditioning elicited a biphasic response in cardiac NOS activity induced by transient and late preconditioning, namely, an immediate activation of cNOS and late upregulation of iNOS. This upregulation of cNOS together with downregulation of transcription factor, NFkB, were postulated to play a major role in the short ischemia-induced protection against the damage caused by the next severe insult. We did not look in this study which NOS isofrom is involved in gastric preconditioning but it is rationale to consider a distinctive role of NOS isoforms in gastric preconditioning, with cNOS serving as the trigger of protection by transient ischemia preconditioning and with the iNOS as the possible mediator of late preconditioning.

In summary, we found that the gastroprotection afforded by gastric preconditioning is accompanied by the rise in the gastric blood flow probably due to enhanced production of NO and activation of adenosine receptors in the gastric mucosa. Both these effects occurring after preconditioning were significantly attenuted in rats with suppressed NO synthase activity by L-NNA and in those with the blockade of adenosine receptors by SPT. The important role of NO is further supported by the finding that addition to L-NNA of L-arginine, the substrate for NOS activity, but not D-arginine, restored the gastroprotection against I/R, luminal release of NO and the hyperemia evoked by gastric preconditioning. Similar reversal of protective effects of gastric preconditioning against I/R was observed in rats with capsaicin-induced deactivation of sensory nerves [32] suggesting that NO, adenosine and sensory nerves cooperate in the beneficial effects of short ischemic episodes against severe lesions caused by prolonged ischemia/reperfusion.

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Diethylnitrosamine may induce esophageal dysplasia after local intramural administration

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Abstract

Purpose: To assess the possibility of promoting esophageal squamos cell carcinoma after direct administration of diethylnitrosamine (DEN) into the wall of the esophagus.

Material and methods: Adult male Wistar rats weighing 250-300 g were used in the studies. Via laparotomy, solution of DEN (at the volume of 0.1 ml) was injected directly into the esophageal wall. Animals were divided into 3 groups: CONTROL group – injected with saline, DEN1 group – injected with DEN 100 mg, DEN2 group – injected twice with DEN at the dose of 100 mg with 7 days interval (total dose of 200 mg).

Results: Microscopic evaluation after 180 days revealed signs of esophagitis in 20% and 30% subjects in DEN1 and DEN2 group respectively. In 30% of animals from DEN1 and 50% animals from DEN2 group, low-grade dysplasia was recognized. The difference between DEN2 and control animals was statistically significant with $p<0.03$. Neither high-grade dysplasia nor invasive carcinoma were found in both experimental groups. None of the liver specimens showed the evidence of pathology.

Conclusions: These initial results may indicate the possibility of development of premalignant lesions after local administration of carcinogen into esophageal wall. Observed changes were limited exclusively to esophagus which became the "target organ" in this model.

Key words: diethylnitrosamine, esophageal cancer, intramural administration.

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Introduction

In 1961 nitrosamines were first recognised as the most potent carcinogens responsible for the induction of esophageal squamous cell carcinoma (SCC) [1]. Since that time several experimental studies as well as researches on human subjects have confirmed the crucial role of nitrosamines in carcinogenic processes in the esophagus. Several nitrosamines, including N-nitrosomethylbenzylamine (NMBA), have been isolated and identified in the diets and gastric juice collected from subjects in regions of high incidence of esophageal SCC. The detection of O^6-methylguanine in the DNA of normal esophageal tissue taken from esophageal cancer patients further indicates to the role of methylating nitrosamines in the development of esophageal cancer [2,3]. Contaminated food often contains nitrates, nitrites and secondary and tertiary amines, which act as precursors for nitrosamine formation *in vivo*. Under acidic conditions *N*-nitroso compounds can easily be formed in the stomach by the reaction of nitrites and amines [4]. Diethylonitrosamine (DEN) is one of the most powerful nitrosamines for experimentally induced esophageal cancer [5]. Systemic administration: oral (p.o.), intraperitoneal (i.p.), subcutaneous (s.c.) of DEN can also result in tumorigenesis in the liver and tracheobronchial tree. Experimental procedures include long-term, repeated administration of low doses of carcinogen, and usually require prolonged observation until malignancy occur. The purpose of the present study was to assess the possibility of promoting esophageal SCC in rats after direct administration of DEN into the wall of the esophagus.

Material and methods

Animals and experimental conditions

The study was conducted in accordance with the Declaration of Helsinki and the Guiding Principles for the Care and Use of Laboratory Animals, approved by Lublin University Ethical Committee (approval 23/2000). Adult male Wistar rats weighing 250-300 g were used in the studies. Animals were

housed in standard, laboratory conditions, with an alternating 12-h light–dark cycle, and room temperature maintained at 22±1°C. All animals had free access to rodent chow and water. Groups of five animals were allocated to plastic cages covered with a metal grid and bedded with sawdust. The cages were cleaned every two days.

Experimental solution

Diethylonitrosamine (DEN) was supplied by Sigma Chemicals, St. Louis, MO, USA (cat. no. 0756, $C_4H_{10}N_2O$, density 950 mg/ml, molecular weight 102.1).

Surgical procedure

Before surgery, the animals were starved overnight and water was discontinued on the morning of surgery. Rats were anasthetized with an intraperitoneal injection of xylazine hydrochloride (12 mg/kg), and ketamine (75 mg/kg). Laparotomy was performed via short (2.5-3 cm) upper midline abdominal incision. Abdominal part of the esophagus was identified and isolated. Then solution of DEN (at the volume of 0.1 ml) was injected directly into the esophageal wall. Water was permitted when animals awoke and food was provided on the next day.

Experimental groups

A total of 30 animals were randomly divided into 3 experimental groups. According to the individual regimens, they were submitted to: group 1-10 animals injected with 0.1 ml of 0.9% NaCl solution (CONTROL), group 2-10 animals injected with DEN at the single dose of 100 mg into the esophageal wall (DEN1), group 3-10 animals injected twice with DEN at the dose of 100 mg with 7 days interval (total dose of 200 mg) (DEN2).

Collection of organs

180 days after the procedure animals were killed by ethyl ether inhalation in the glass chamber. Esophagus and stomach were dissected as a single block and fixed by the ends on the cork slide.

Macroscopic and microscopic analysis

The organ block was opened longitudinally on its anterior surface. Esophageal length (cm), and widest diameter (mm) were measured. The mucosa was stained with toluidine blue and assessed in order to reveal the presence of pathological lesions. For histopathological analysis the organs were fixed in 10% buffered formalin, sectioned transversely into about 2 mm-wide serial slices (9-12/case), processed routinely and embedded in paraffin blocks. Then, 5 μm sections were stained with hematoxylin and eosin (H&E) and examined in a blinded fashion by the pathologist. Examination included identification of the following findings: normal histology, esophagitis, low-grade dysplasia, high-grade dysplasia, invasive carcinoma. All histopathological changes were classified according to the reports of Robbins and Cotran [6] and Kruel et al. [7]. Normal esophagus: one wing of basal cells located between the border of the epithelium and submucosa, with egg-shaped or round nuclei; a corneal tract that occupies one-third to one-half of the epithelium; muscularis mucosae not defined; some rudimentary cells present; lamina, if present, usually contains few and sparse mononuclear inflammatory cells. Esophagitis: the epithelial papillae project one-third to two-thirds of the epithelial thickness and/or inflammatory infiltrate in the lower third of the epithelial thickness. Low-grade dysplasia: alterations of basal cell layers to one-third of the epithelial thickness, with pleomorphic nuclei; mitotic nuclei visible in lower third of the epithelium, outside the basal layer. High-grade dysplasia: dysplastic alterations occur throughout the epithelial thickness which is composed of markedly abnormal cells; nuclear polarity is lost; cytoplasmatic maturation occurs in most parts of the epithelial layer. Invasive carcinoma: submucosal invasion by squamous neoplastic epithelium, containing cells exhibiting a markedly increased nucleus to cytoplasm ratio and with keratinized cytoplasm, and large, grossly granulated and hyperchromatic nuclei, irregularly distributed; malignant cells are encountered below the lamina, with unquestionable stromal invasion.

Statistical analysis

All quantitative variables were compared with analysis of variance. Fisher's exact probability test was applied to assess qualitative variables. P values lower than 0.05 were considered as statistically significant.

Results

Macroscopic analysis

In the group of 30 animals included in the study, no losses were noted within the observation period. No significant differences in the esophageal lenght and widest diameter between all groups were noted. In 1 subject from control group, 1 from DEN 1, and 3 from DEN 2 group macroscopic signs of esophagitis (erythema, mucosal breaks) were observed. The differences in the prevalence were not statistically significant. In both experimental groups grossly visible esophageal tumors were not observed in the area injected with DEN (*Tab. 1*).

Microscopic analysis

In control group histopathological examination revealed normal esophageal wall (*Fig. 1*) in all subjects exept one in which mild esophagitis was found. Similary, signs of esophagitis were found in 2 (20%) and 3 (30%) subjects in DEN1 and DEN2 group respectively. In 3 (30%) animals from DEN1 and 5 (50%) animals from DEN2 group, low-grade dysplasia was recognized (*Fig. 2*). The difference between DEN2 and control animals was statistically significant with $p<0.03$. Neither high-grade dysplasia nor invasive carcinoma were found in both experimental groups (*Tab. 2*). None of the liver specimens showed the evidence of pathology.

Discussion

Nitrosamines as promotors of carcinogenesis have been investigated for almost 6-7 last decades. Their possible mechanism of action in inducing cancer development is DNA alkylation, as has been shown by Harris et al. in human tissues [8]. Most of the nitrosamines administered systemically

Table 1. Macroscopic analysis.

	CONTROL	DEN1	DEN2	P (ANOVA)
Esophageal length (cm)	7.5±0.17	7.63±0.21	7.63±0.23	n.s.
Widest diameter (mm)	5.5±0.84	6.3±0.94	5.6±0.84	n.s.
	CONTROL	DEN 1	DEN 2	P (Fisher's Exact Test)
Inflammation (%)	10	10	30	n.s.
Tumors (%)	0	0	0	n.s.

Table 2. Microscopic analysis.

	CONTROL	DEN1	DEN2	P (Fisher's exact test)
				Control vs. DEN1 n.s.
Normal (%)	90	50	20	Control vs. DEN2 $p < 0.0055$
				DEN1 vs. DEN2 n.s.
Esophagitis (%)	10	20	30	n.s.
				Control vs. DEN1 n.s.
Low-grade dysplasia (%)	0	30	50	Control vs. DEN2 $p < 0.03$
				DEN1 vs. DEN2 n.s.
High-grade dysplasia (%)	0	0	0	n.s.
Invasive carcinoma (%)	0	0	0	n.s.

Figure 1. Normal esophageal mucosa and submucosa in rat from control group (H&E, x200).

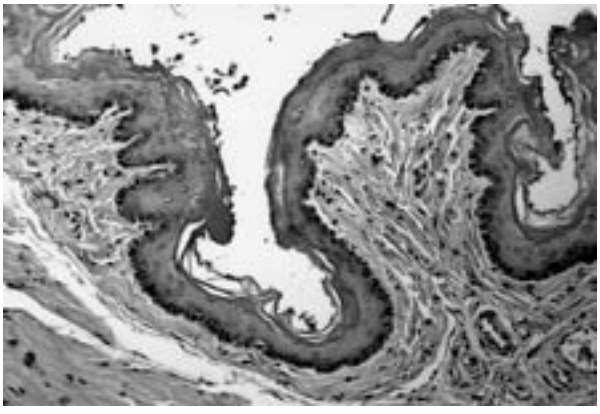
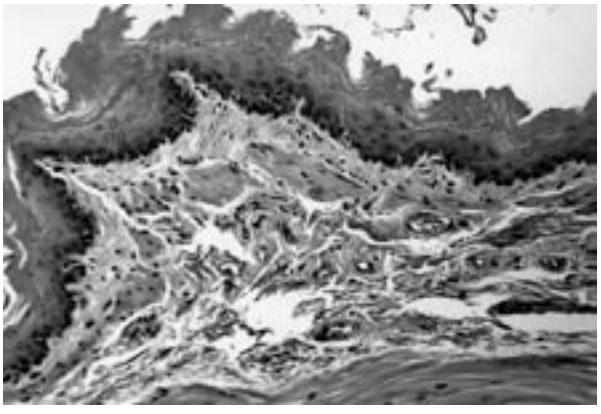


Figure 2. Esophageal squamous epithelial low-grade dysplasia in rat from DEN1 group (H&E, x200).



(p.o., s.c., i.p.) present specifity to particular organs e.g. liver, kidney lungs [9,10]. Thus, different nitrosamines are employed as the promotors of cancer in many, experimental models of carcinogenesis in several organs. DEN (compound used in the present study) produces esophageal and kidney tumors in rats in addition to tumors in the main target organ, the liver [11]. It is possbile that extrahepatic tumors need higher exposures to DEN, probably because low doses are subject to first pass clearance in the liver [12]. Most experimental procedures aiming the induction of esophageal cancer usually require administration of diluted carcinogen at low concentrations for long time to avoid or minimize the adverse tumors in other organs. Long-lasting observation periods of the experiments as well as their unrepeatable, inconsistent results make all those procedures complicated and unusable. Thus, the purpose of the present study was to assess the possibility of promoting esophageal SCC by delivering the specific, carcinogen directly to the esophageal wall. Such manner of administration would allow to obtain the direct action of the carcinogen on tissue of the esophagus, and to eliminate the adverse influence in other organs. Moreover, it would help to resolve the problem of poor diffusion (flux) of DEN into the esophagus that has been noted in Haorah research study [13]. DEN doses (100 mg and 200 mg in DEN1 and DEN2 group respectively) were established, as promoting the carinogenesis, basing on previous literature data. Given weekly i.p. as injections of 80 mg/kg for 10 weeks (total dose of 800 mg/kg), the incidence of esophageal tumors in BD6 rats was 35% [14]. Total dose of DEN per animal which has been administered in the present study in DEN2 group appeared to be equivalent. However, conditions and methods established in this study did not allow to induce either invasive carcinoma or even high-grade dysplasia, the incidence of low-grade dysplasia in esophageal specimens from DEN2 group reached 50%. These initial results may indicate the possibility of development of premalignant lesions after local administration of carcinogen into the esophagus. No pathological changes were noted in

liver. Observed changes were limited exclusively to esophagus which became the "target organ" in this model. Potential significance of these results seems to be very interesting in relation to future research. Strong esophageal cancer promotor e.g. DEN or NMBA, given directly into the esophagus, under another experimental conditions e.g. co-administered with ethanol, could result in esophageal tumors development with no other lesions. Obtaining the "pure" esophageal cancer model would allow to understand more about biology of this disease and investigate new methods of treatment or chemoprevention.

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The experimental distention of dissected bile duct for the restoration of its continuity in dogs using a device of own construction

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Abstract

Purpose: The segmental resection of constricted bile duct and end-to-end biliary anastomosis could be an attractive alternative in the treatment of benign biliary tract stricture. The aim of this study was to restore the anatomical integrity of the hepatic-common bile duct after an artificially produced defect while maintaining the large duodenal papilla, using microsurgical technique.

Material and methods: The experiments were carried out on 25 mongrel dogs. The common bile duct was ligated in all of the animals during laparotomy, as a model of bile duct obstruction in humans. Relaparatomy was performed 3 days after the initial operation. The segment of bile duct, 4 cm in length was resected together with the ligature. The continuous bile flow into the duodenum was assured by a polyvinyl catheter introduced into both ends of dissected bile duct. The proximal end of the hepatic-common bile duct was fixed to a device constructed by us for the distention of the bile duct (DDBD). The anterior part of the device was exteriorized through a separate fistula and fixed to the abdominal wall. The hepatic-common bile duct distention was gradually continued during 18 days, by pulling out the mobile part of the device. After 18 days the device was removed and the distended proximal end of the hepatic-common bile duct was anastomosed end-to-end with its distal end. The sequels of this procedure were observed for up to 6 months.

Results: The hepatic-common bile duct was distended 4 cm within 18 days. The histopathological examination has shown partial damage of the duct framework due to the distention and tension. However the patency of the duct was preserved and the recovery of normal structures were observed after the device was removed and anastomosis fashioned.

Conclusion: This method, developed by us, offers the possibility of restoring the integrity of injured extrahepatic bile ducts, allowing effective treatment of benign biliary strictures.

Key words: bile ducts, biliary strictures, distention, biliary anastomosis.

Introduction

The incidence of benign strictures of the biliary tract has increased over the past decade. A number of factors with a higher risk of injury, including iatrogenic complications after laparoscopic cholecystectomy, have been identified [1]. It is necessary to diagnose and treat even low-grade biliary obstruction because of the severe consequences for the patients, like ascending cholangitis, sepsis, jaundice, hepatic cirrhosis or bile stones formation [2]. The most commonly employed surgical procedure to bypass the stricture is Roux-en-Y choledocho- or hepatico-jejunostomy, but sometimes there is a need for reinterventions for anastomotic stricture [3]. The role of endoscopic treatment by repeat balloon distention or stenting have increased, but morbidity occurrs more frequently in patients treated with endoscopic procedures, than those treated surgically [5,6]. The choice of surgical technique depends on the location and extension of the stricture [7].

This is a big challenge for surgeons: is there a possibility to resect the constricted part of bile duct and to create end-to-end biliary anastomosis for reconstruction of biliary tree? At least for some selected patients, it would be a permanent solution to their problem.

The duct-to-duct anastomosis becomes a valid alternative to standard hepatico-jejunostomy in living donor liver transplantation [8,9]. However, bile leakage occurrs in up to 27% of

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Figure 1. Extrahepatic bile ducts in a dog. A: hepatic ducts. B: common bile duct. C: cystic duct. D: common hepatic duct. The levels of common bile duct ligation and the place of segmental resection are depicted.

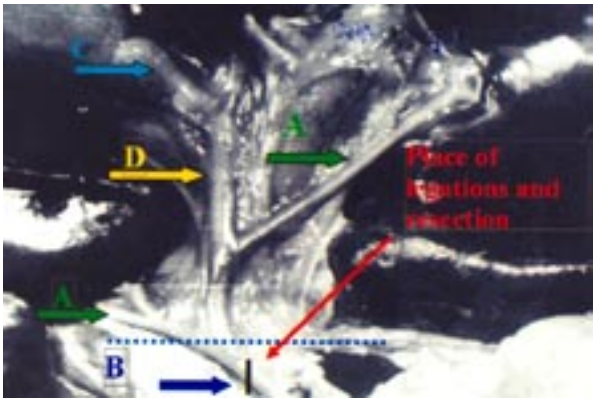
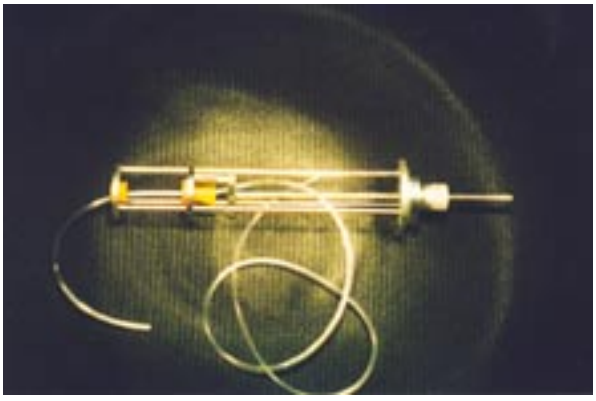


Figure 2. The device for the distention of the bile duct (the description in the text).



liver transplant patients after biliary reconstruction, even when longitudinal stress is absent [10]. In order to optimize end-to--end ductal anastomosis, different approaches to interpose the resected part of the bile duct and to preserve a tight junction of both ends of the reconstructed bile duct have been used in experimental studies. Autologous vein graft in rats aided by a plastic stent, using microsurgical technique have been successfully used to repair segmental common bile defect [11]. In another study, bilio-biliary anastomosis was performed in dogs, using Gore-Tex vascular graft prior to resection of the stenotic area with consecutive release of cholestasis [12]. In both of these studies, the observation period was 12 weeks and 3 months respectively; the later effects are not known.

Our original idea was to assess the possibility to repair a bile duct stricture, by resection of the involved area, and adaptation of the dissected duct to end-to-end biliary anastomosis by gradual distention of the hepatico-choledochal part of the biliary tree.

Therefore the purpose of our study was to restore bile duct integrity, after an artificially produced defect, corresponding to the removed stricture of this duct, by the distention of the dissected bile duct, using temporarily implanted device of our own construction in dogs.

Material and methods

The experiments were carried out on 25 mongrel dogs of both sexes, 12-23 kg of body weight. The animals were housed in standard laboratory conditions under 12 hr day-and-night cycles, with a pelleted laboratory diet and water ad libitum. Care was provided according to current guidelines for the use of laboratory animals and experiments were performed according to the Helsinki Declaration.

The choice of experimental animals

The dog's liver is comprised of 6 lobes. Extra hepatic bile ducts are represented by hepatic ducts, cystic duct, common hepatic duct and common bile duct (*Fig. 1*). The gall bladder is pyriform, well developed and located on the liver middle lobe. The length of the gall bladder varies from 3.5 cm to 7.0 cm. The width varies from 2.5 to 4.5 cm. The length of the cystic duct varies from 1.5 to 3.5 cm. Six hepatic ducts come out of the liver lobes. Four of them are connected between each other and the cystic duct to form the common hepatic duct. However, it is necessary to note that two hepatic ducts are connected with the common hepatic duct below the cystic duct to create the common bile duct. Therefore, the part of the bile tree from the outlet of the cystic duct to the outlet of the common bile duct into the duodenum is called hepatico-common bile duct (ductus hepaticocholedochus). The length of the common bile duct can reach up to 6 cm and the diameter varies from 1.5 to 2.5 mm. Despite these differences, the common bile duct in dogs could be a satisfactory model of the common bile duct in humans to create biliary end-to-end anastomosis, after segmental resection of the stricture.

Construction of the device for the distention of the bile duct (DDBD)

Our DDBD consisted of a metal part, 12 cm in length and 2 cm in width, presented in *Fig. 2*, and a polyvinyl catheter 15 cm in length and 2 mm in width on the inside, passing through two small dacron rings (orange), for the fixation of the resected proximal end of the common bile duct between the two rings. The second, distal ring was connected with a metal axis, passing through a bigger metal ring with 4 small holes for the fixation of it to the anterior wall of the abdomen by sutures, after exteriorization of it by the fistula, made in the abdominal wall by separate incision (*Fig. 3*). After positioning the device, the external end of the axis could be gradually pulled out about 4 cm. The procedure of extension could be regulated according to the scale located on the axis.

The experimental procedure

The abdominal cavity was opened under sterile conditions under ethyl-ether ventilated general anesthesia. The common bile duct was ligated in all of the dogs, 2.5 cm above its outflow into the duodenum and the wall of the abdomen was sutured. The relaparotomy was performed 3 days after the initial operation. The segment of ligated common bile duct, 4 cm in length was resected, together with the ligature, close to the duodenum. The shorter end of the polyvinyl catheter of DDBD was introduced into the ductus hepaticocholedochus for the bile drainage. The other, longer end of the catheter was

inserted into the distal part of the common bile duct and fixed with ligature, to assure free bile flow into the alimentary tract. The remaining, proximal part of the common bile duct, cannulated with a catheter was fixed between the rings of the device, with its wall inside them, using medical glue (MK-7) (Russia). The device was positioned in the cover with latex and fixed to the anterior abdominal wall after exteriorization of its metal ring. The proximal part of the common bile duct was gradually distended between the rings (about 2 mm daily) during 18 days. The polyvinyl catheter and DDBD were removed 18 days after the initial surgery during another relaparotomy and the distended proximal part of the hepatic-common bile duct was anastomosed end-to-end with the distal end of the common bile duct. Microsurgery technique was used for end-to-end biliary anastomosis. The microsurgical procedures were performed under a microscope (Zeiss, Germany) using 7/0 sutures (Ethicon). The resected ends of the duct were positioned closely to each other and the posterior wall was sewn up first by separate sutures. The sutures were made as closely as possible in order to avoid any leakage of bile.

All of the animals were observed under the standard vivarium conditions and sacrificed in general surgical anesthesia at different interval after the first operation. In 8 dogs, the experiment has been finished on the 3, 5, 10 and 18 days after the second operation (2 dogs in each point of time). The dynamic changes after distention were evaluated by histology, after 20, 30, 50, 80, 120, 150 days (2 dogs in each interval) and after 180 days (3 dogs) following the second operation.

Roentgen graphic examinations

The completeness of common bile duct ligation and the patency of biliary anastomosis were controlled by Roentgen graphic examination, using i.v. contrast medium (60% solution of Triombrastum, Russia).

Biochemical assays

The presence of cholestasis was evaluated by routine laboratory estimation of the bilirubin level and AST activity in the venous blood plasma.

Histopathological examination

At autopsy, the specimens of distended and anastomosed bile ducts, and the livers were taken for histopathological examinations, which included the staining with haematoxyline and eosine (H&E) and according to van Gieson. The preparates were evaluated at x60 and x90 original magnification.

Statistical analysis. The results are descriptive, therefore the statistical analysis is not applicable.

Results

Postoperative Complications and Mortality

None of the animals suffered postoperative complications, nor died within the first 15 days after surgery. One dog died after 26 days because of bile peritonitis and another one 62 days after the surgery because of mechanical jaundice. The cause of bile peritonitis was the necrosis of common bile duct wall after biliary anastomosis. The cholestasis in other dog evolved because of technical problems in microsurgery. They caused the obliteration of common bile duct lumen in its anastomosed part.

Figure 3. External part of the device for the distention, fixed to the anterior wall of the dog abdomen after fashioning of fistula.

Figure 4. Dilatation of the ductus hepaticocholedochus in the dog, after its ligation (roentgenogram).

Roentgen graphic findings

Roentgenograms of ligated – duct animals have shown a complete obstruction of common bile duct (*Fig. 4*). After positioning of the DDBD, the bile freely flowed into the duodenum (*Fig. 5*). Consecutively, after fashioning of biliary anastomosis, all survived animals showed no signs of its stenosis. The contrast medium also flowed freely into the duodenum in each case. Dilatation of the bile ducts proximal to the anastomosis was not observed in any animal (*Fig. 6*).

Biochemical parameters

The postoperative estimations have shown the evidence of cholestasis within the first 18 days after the first surgery. Maximal level of bilirubin reached 18.8 μmol/L (normal 0.9--10.6 μmol/L) and maximal activity of AST achieved 80.5 U/L (normal 8.9-48.5 U/L). The cause was probably the polyvinyl catheter inserted into the ductus hepaticocholedochus. The parameters of cholestasis were normalized after removing of the catheter and fashioning of biliary anastomosis. All esti-

Figure 5. The roentgenogram of the device for the distention of bile duct in working position with the proximal end of ductus hepaticocholedochus fixed to the device.

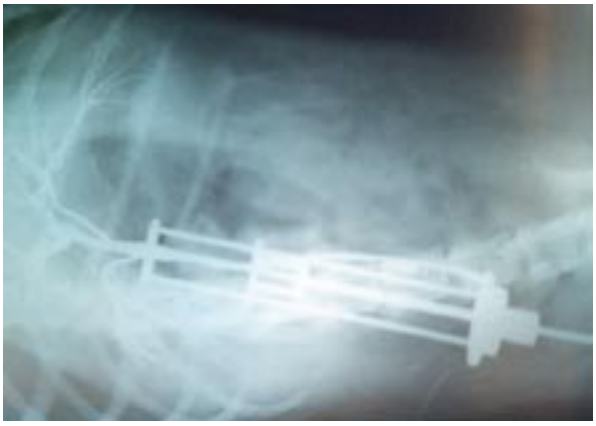
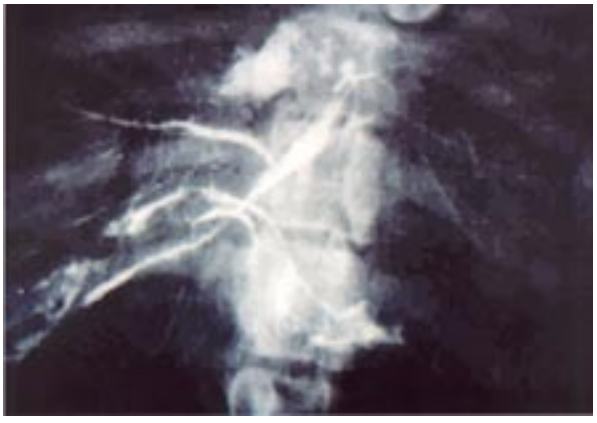


Figure 6. The roentgenogram of the dog biliary tree after fashioning of biliary end-to-end anastomosis. Free bile flow into the duodenum is evident.



mated biochemical parameters have been normalized within a month and remained within normal range throughout the whole period of the observation.

Macroscopic findings

At the autopsy, the presence of connective tissue capsule around the distention device was observed. The color of common bile duct anastomosis was normal. No signs of hyperemia or ischemia of proximal end of ductus hepaticocholedochus, nor changes of its diameter were observed. The bile flowed freely into the duodenum via the catheter. In the dogs after the device removing and biliary anastomosis fashioning, at the autopsy 20, 30, 50, 80, 120 days later, the reconstructed ductus hepaticocholedochus was not increased in diameter and the bile free passage into the alimentary tract was observed.

Histopathological changes

Histological examinations, performed consecutively during the ductus hepaticocholedochus distention have shown sclerotisation of mucosal layer. The muscular fibers following axis of ductus hepaticocholedochus have been evidently affected by the distention and tension. The loosening and swelling of muscular fibers were observed 3 days after the second operation. The local accumulation of young fibroblasts was noted beginning 5 days after the second operation. Analogical picture was seen in the dense connective tissue of biliary anastomosis. Newly formed fibers were distributed regularly. They were more densely packed and straightened. The wall of biliary anastomosis was thickened due to accumulation of newly formed tissue within 18 days. The results of histological examination indicate on partial destroying of the ductus hepaticocholedochus connective tissue, due to the distention and tension. The recovering of common hepatic duct and common bile duct mucosa was observed after removing of the device and bilio-biliary anastomosis creation. The area of anastomosis was revealed as not distinctly delineated. The liver biopsy performed at the same time has shown normal parenchyma in all animals, without signs of cholangitis, cholestasis, or biliary cirrhosis.

Discussion

Our results indicate that the adaptation of ductus hepaticocholedochus in dogs (corresponding to common bile duct in humans) after its partial resection (resembling resection of its stricture) by gradual distention during 18 days, using the device for the bile duct distention (DDBD) of our own construction is suitable procedure to fashion biliary, end-to-end anastomosis by microsurgery technique. The created anastomosis was patent up to 180 days of observation. Only 2 of 25 dogs died during experiments, but the causes of their fatal outcome seems to be avoidable. The remodeling of bile ducts walls, as observed in histological examination, seems to be permanent, assuring the long-lasting effect, however even longer period of observation is necessary to draw final conclusions on the affectivity of this method. The histological feature of the livers was normal, suggesting that the method is safe for the integrity of this organ.

Among many factors determining the choice of optimal surgical method of the benign biliary lesions management, the level and length of the strictures are prominent [7]. The creation of biliary, end-to-end anastomosis is possible very rare in the case of stenosis, when the significant part of the bile duct is intact, easily mobilized without blood flow disturbance. The choledochoduodeno- and choledochojejunostomy are methods of choice in the cases of low strictures. The resection of the stricture and bilio-biliary anastomosis creation are indicated for some middle (circular) strictures. In cases of total or subtotal strictures, most authors prefer to perform the hepaticojejunostomy with a long Roux-en-Y loop [13-16].

The results of biliary-enteric anastomoses are relatively good, however they do not restore the anatomical integrity of bile ducts nor preserve the function of Oddi sphincter. Therefore, more interesting for clinicians are the methods, which give the possibility to remove a stricture, to restore anatomical integrity of extra-hepatic bile ducts and to maintain a function of Oddi sphincter. Different authors tried to solve this problem using different allo- and xeno-transplants or prosthesis made of several synthetic materials for the restoration of bile duct

continuity [11,12]. The results of experimental and clinical application of the above-mentioned grafts were more or less unsatisfactory.

In our previous studies we evaluated the common bile duct reconstruction, after similar segmental resection, by venous auto-graft or by Gore-Tex vascular graft in dogs. We have found, that venous auto-graft might not be suggested for the common bile duct repair because of frequent necrosis of the graft wall, absence of the graft epithelization, the graft wall inflammation and imbibition by bile. As to Gore-Tex vascular prosthesis, the performed investigations have shown that the inflammation developed around the graft on the 2-3 days after the grafting. The pores of Gore-Tex graft were filled by elements of young connective tissue on the 3-4 day after implantation. The formation of the connective tissue capsule around the graft was observed 5-7 days after the operation. At the same time, the graft rejection from newly formed capsule was observed and the space between graft and newly formed capsule was filled by fresh connective tissue. The graft moved spontaneously into the alimentary tract in 20 of 30 animals. In 10 dogs, the graft was retained in the capsule that lead to the obturation of reconstructed common bile duct after 30-40 days. It is noteworthy, that repaired common bile duct has been functioning well during 6 months in the animals, in which the Gore-Tex graft has moved spontaneously into the alimentary tract [17,18].

This encouraging, however not fully satisfactory results of these studies, directed our attention to the possibility of adaptation of common bile duct after resection of its constricted part to end-to-end anastomosis by gradual mechanical distension, using the device for the distention of bile duct (DDBD) of our own construction. Received results have proved, that our method, after further development, could be useful in clinical practice for the restoration of anatomical integrity of bile ducts after the stricture resection in spite of its level and extent.

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The role of adenosine receptors for pancreatic blood flow in caerulein-induced acute pancreatitis

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Abstract

Purpose: The aim of our study was to evaluate the possible influence of adenosine receptors agonists and antagonists on pancreatic blood flow (PBF) and the development of acute pancreatitis (AP) in rats.

Material and methods: Ninety male Wistar rats were subdivided into ten equal groups, nine rats in each. The study was carried out in two stages. In the first one the first group (control) received i.v. saline infusion for 12 hours. The groups 2-5 (the first stage) received i.v. caerulein infusion as in the first group, but with pretreatment with: in the second group – DPCPX (A_1 receptor antagonist), in the third group – CGS 21680 (A_2 receptor agonist), in the fourth group – ZM 241385 (A_2 receptor antagonist), in the fifth group – IB-MECA (A_3 receptor agonist). In the second stage the first group received i.v. caerulein infusion at the dose of 5 μg/kg/h for 12 hours. The groups 2-5 (the second stage) received i.v. caerulein infusion as in the first group, but with pretreatment with: in the second group – DPCPX (A_1 receptor antagonist), in the third group – CGS 21660 (A_2 receptor agonist), in the fourth group – ZM 241385 (A_2 receptor antagonist), in the fifth group – IB-MECA (A_3 receptor agonist). Pancreatic blood flow was measured by laser Doppler flowmetry. Pancreatic inflammation was evaluated by serum α-amylase activity, pancreatic weight and histological changes in the pancreatic tissue.

Results: We observed a significant attenuation of serum α-amylase activity increase (19.1 ± 2.8 kIU/L vs 30.12 ± 2.64 kIU/L), pancreatic weight (expressed as percentage of rat's body weight – 0.85 ± 0.16% vs 1.25 ± 0.14%), and improvement of PBF (79.8 ± 6.1% vs 60.1 ± 3.6%), a reduced degree of pancreatic tissue damage (oedema, leukocyte infiltration, vacuolisation of acinar cells) in the third group (CGS 21680 + caerulein) compared with the first group in the second stage (only caerulein infusion). Neither agonists nor antagonists exerterd any appreciable effects on measured parameters in healthy rats.

Conclusions: Pretreatment with A_2 receptors agonist seems to be protective against the damage to the pancreas during the course of caerulein-induced acute pancreatitis in rats. This effect could be due to improvement of pancreatic blood flow. This finding could have some therapeutic implications.

Key words: adenosine receptors, pancreatic blood flow, acute pancreatitis, caerulein, rats.

Introduction

Adenosine is a potent, endogenous proinflammatory factor released by cells under metabolically unfavorable conditions (hypoxia, ischaemia) [1]. Reperfusion of ischaemic and hypoxaemic tissues results in locally increased permeability of vessels and organ oedema associated with neutrophil accumulation in microcirculation. The interaction of neutrophils and endothelial cells, on the other hand, leads to microcirculation impairment. Adenosine is involved in the inflammatory reactions by its action on neutrophils and A_2 receptors of the endothelium inhibiting chemotaxia, phagocytosis and the adhesion of activated leukocytes to the endothelium, decreasing the oxygen free radical generation and thrombocyte aggregation and activity [2].

Acute pancreatitis leads to hypoxia – the secondary phenomenon to decreased blood supply of this organ. This, in turn, results in the dysfunction of intracellular organelles, which may activate lysosomal and digestive enzymes to initiate the autodigestion and tissue damage within the pancreas. Leukocytes become activated and the expression of adhesive molecules is higher, which enables the margination and adhesion of

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activated leucocytes to the endothelium resulting in their diapedesis into the inflammatory focus. Activated leukocytes are the source of proinflammatory cytokines and oxygen free radicals, which intensify the inflammatory process. Li et al. [3] reported that adenosine and its analogues were likely to partially prevent the activation of the above-mentioned processes during ischaemia in acute pancreatitis.

Adenosine receptors have been found in blood vessels (the smooth muscles and endothelium) and in membranes of cells involved in the cascade of inflammatory processes, i.e. in the neutrophils, basophils, lymphocytes, mastocytes, macrophages and thrombocytes [1]. They mediate the influence of adenosine and its analogues on vasodilatation and modulation of the inflammatory process in acute pancreatitis.

The aim of the study was to determine the influence of substances acting on the adenosine receptors in the development of experimental caerulein-induced acute pancreatitis. The severity of the inflammatory process was assessed on the basis of the serum α-amylase activity, pancreatic weight and intensity of histopathological changes in the pancreatic tissue.

Material and methods

The experiments were carried out on 90 male Wistar rats, weighting 250-300 g, subdivided into 10 equal groups, 9 rats in each. The animals were kept in individual cages; they were fed with standard laboratory chow diet and fasted overnight before the experiment with free access to water. The care was provided and experiments were performed according to current guidelines for the use of experimental animals. The study was carried out in two stages. The first stage was to evaluate the effects of substances stimulating or blocking adenosine receptors on normal pancreas; during the second one we assessed the action of these substances in the course of caerulein-induced acute pancreatitis.

In the first stage, the rats were randomly divided into 5 groups: group 1 – controls receiving only intravenous (i.v.) infusion of 0.15 mmol/L NaCl during 12 hours and groups 2-5, in which the animals were injected intraperitoneally with the substances examined in the following doses: group 2 – DPCPX (A_1 receptor antagonist) 1 mg/kg; group 3 – CGS 21680 (A_2 receptor agonist) 1 mg/kg; group 4 – ZM 241385 (A_2 receptor antagonist) 3 mg/kg; and group 5 – IB MECA (A_3 receptor agonist) 0.5 mg/kg.

In the second stage, the animals were also divided into 5 groups: group 1, in which acute pancreatitis was induced according to the method of Lampel and Kern [4] by a 12 h i.v. infusion of caerulein at the supramaximal dose of 5 μg/kg/h, and groups 2-5 in which identical caerulein infusion was proceeded by intraperitoneal (i.p.) injections of the substances studied in the following doses: group 2 – DPCPX (A_1 receptor antagonist) 1mg/kg; group 3 – CGS 21680 (A_2 receptor agonist) 1mg/kg; group 4 – ZM 241385 (A_2 receptor antagonist) 3 mg/kg; and group 5 – IB MECA (A_3 receptor agonist) 0.5 mg/kg; all substances and the doses as in the first stage.

After the 12 h infusion with saline or caerulein, the rats were anaesthetized with ethyl ether and weighted. Then, the peritoneal cavity was opened and blood samples collected to determine serum α-amylase activities. The pancreas was excised, weighted, the tissue samples were fixed in formaline solution. The ratios of pancreas/body weight were calculated. The α-amylase activity was expressed in IU/L [5]. After the 12 h infusion with saline or caerulein in part of animals the pancreatic blood flow was measured by laser Doppler flowmetry [6] and expressed as percent of pancreatic blood flow of the control group.

Pancreatic inflammation activity was evaluated by serum α-amylase levels, pancreatic weight and histological changes in the pancreatic tissue. The histopathological parameters were evaluated in the descriptive manner. The extension of oedema, degree of leucocyte infiltration and pancreatic cell vacuolization were assessed.

Statistical analysis. The results were presented as mean values ± standard deviation (SD) and the differences were compared using the Student's t-test for unpaired data and Cochran-Cox tests. The differences were considered statistically significant when $p<0.05$.

Results

The rats which received the substances examined before the i.v. infusion of 0.15 mmol/L NaCl, showed no statistically significant changes in the pancreatic weight and serum α-amylase activity as compared to the controls (*Tab. 1*). Moreover, the histological features of the pancreases from the above groups and controls were normal.

The 12 h i.v. caerulein infusion induced acute pancreatitis. The pancreas was enlarged, oedematous and about a twofold increase in its weight was observed. There was almost an 8-fold increase in serum α-amylase activity (*Tab. 1*). Histopathological examination showed interlobular and marked intralobular oedema in all of the animals. Perivascular leucocyte infiltration was observed and in some animals the features of diffuse infiltration were present. The majority of acinar cells showed marked vacuolisation of their cytoplasm (*Tab. 1*).

The i.p. administration of the A_2 receptor agonist (CGS 21680, group 3) before inducing acute pancreatitis, reduced the organ injury. It decreased pancreatic ischaemia caused by caerulein administration, which was observed during laser Doppler flowmetry measurements (*Tab. 1*). The A_2 receptor agonist statistically significantly decreased the pancreatic weight gain, as compared to the control groups. Similar observations concerned the serum α-amylase activity; which was statistically significantly lower after the CGS 21680 administration (*Tab. 1*). The histopathological picture revealed markedly decreased oedema, limited to the interlobular. The leukocyte infiltration of the pancreatic tissue was almost completely reduced. Vacuolization of cytoplasm involved a significantly lower number of acinar cells and as compared to histopathological picture in caerulein-infused animals, its intensity was substantially reduced.

The i.p. administration of the A_2 receptor antagonist (ZM 241385, group 4) before induction of acute pancreatitis enhanced the pancreas damage. The pancreatic oedema was massive and pancreatic weight gain was higher. An increase in the serum α-amylase activity was statistically significant (*Tab. 1*).

Table 1. The role of adenosine receptors agonists and antagonists administration on serum α-amylase activity, pancreatic blood flow and pancreatic weight.

Group	Serum α-amylase (kIU/L)	Pancreatic blood flow** [%]	Pancreatic weight* [%]
I. THE FIRST STAGE			
1. Control	4.15 ± 0.70	100.0 ± 2.2	0.45 ± 0.07
2. DPCPX	1.95 ± 0.50	99.3 ± 4.6	0.46 ± 0.06
3. CGS 21680	2.02 ± 0.58	100.0 ± 5.1	0.45 ± 0.08
4. ZM 241385	1.89 ± 0.65	98.7 ± 4.1	0.43 ± 0.07
5. IB-MECA	2.05 ± 0.65	100.0 ± 4.7	0.43 ± 0.07
II. THE SECOND STAGE			
1. CAERULEIN	30.12 ± 2.64^{A}	60.1 ± 3.6	1.25 ± 0.14^{A}
2. DPCPX+CAERULEIN	30.83 ± 2.55	60.4 ± 5.5	1.28 ± 0.20
3. CGS 21680+CAERULEIN	19.10 ± 2.80^{AB}	79.8 ± 6.1	0.85 ± 0.16^{AB}
4. ZM 241385+CAERULEIN	40.12 ± 2.25^{AB}	55.3 ± 5.2	1.46 ± 0.15^{AB}
5. IB-MECA+CAERULEIN	30.22 ± 2.91	64.5 ± 4.3	1.25 ± 0.21

* pancreatic weight is expressed as percentage of rat's body weight
** pancreatic blood flow as percentage of pancreatic blood flow in control group
^{A} $p<0.05$ compared with control group
^{B} $p<0.05$ compared to the value from the group where caerulein was given alone

The histopathological picture showed marked enhancement of inter- and intralobular oedema, intensive perivascular leucocyte infiltration with the features of diffuse infiltration in the majority of rats in this group. Vacuolization involved all acinar cells and showed high intensity in comparison to the histopathological findings in the caerulein-infused animals.

The A_1 receptor blockade (DPCPX, group 2) as well as A_3 receptor stimulation (IB MECA, group 5) in the animals with induced acute pancreatitis did not affect the pancreatic weight, serum α-amylase activity (*Tab. 1*) or histopathological picture of the pancreas.

Discussion

The majority of the studies, carried out recently, concern the relation between the cascade of events in acute pancreatitis and factors which may modulate it. One of such factors is adenosine, an important physiological modulator of the inflammatory process in its various stages which affects adenosine receptors located on many biological targets [1]. The aim of the present study was to determine the effects of adenosine receptor agonists and antagonists on the development of caerulein-induced acute pancreatitis in rats.

Our study revealed, that the adenosine A_2 receptor stimulation before the induction of acute pancreatitis reduced pancreas damage, while the A_2 receptor blockade enhanced the caerulein-induced injury. The protective action of adenosine in acute pancreatitis has not been fully explained. It seems that it induces the vasodilatation, prevents activation of the transcription factor – NF-κB, responsible for the synthesis of proinflammatory interleukins and adhesive molecules, stimulates the synthesis of antiinflammatory cytokins, inhibits chemotaxis and adhesion of leukocytes to the endothelium, decreases aggregation or activation of thrombocytes and inhibits the production of reactive exygen metabolites by the activated leukocytes.

Reduced damage to the pancreas and decreased serum α-amylase activity observed after the A_2 receptor agonist administration may result from its vasodilating effects preventing a decrease in the pancreatic blood flow in acute pancreatitis. In caerulein-induced acute pancreatitis, a decrease in local blood flow was observed, even by 50% of the baseline value [7].

Adenosine and its analogues may be important mediators of vasodilatation, especially in conditions of impaired oxygen supply. The A_2 receptor located in the smooth muscle cells of blood vessel walls participates in this action. Its participation in the vascular tone regulation was also confirmed by the studies concerning the A_2 receptor antagonist. It has been shown, that ZM 241385 eliminated vasodilatation responses induced by adenosine and caused increased activation of stimulated neutrophils [8,9]. Many authors observed improved organ microcirculation after the administration of adenosine or its analogues.

Vasodilatation and improved blood flow in the pancreas caused by adenosine or its analogues partly prevents the development of pancreatic inflammatory changes. Fenton et al. [10] found, that adenosine acts as a potent vasodilator not only by its effects on smooth muscles. Their findings indicate that it also stimulated the NO production in the arterial endothelium. The protective effects of NO in acute pancreatitis result from its properties.

It is generally accepted that NO is a cellular transmitter. It may diffuse through membranes and affect adjacent cells. This mechanism is thought to be the main mechanism of NO actions in the tissues. NO is a biological signaling molecule, which may also affect main proteins or signal pathways in apoptosis [11]. Its deficiency favours neutrophil accumulation, accompanied by an increase in the number of dead acinar cells in the pancreas. On the other hand, Tsukahara et al. [12] showed the cytotoxic effects of NO, generated in pulmonary macrophages in acute pancreatitis. There are some findings, which do not confirm direct toxic effects of NO (per se), but suggest that NO

plays a permissive role for toxicity induced by glutamic acid. It is increasingly accepted that in ischaemia, NO is likely to be both protective and cytotoxic in action in a dose-dependent manner.

After supramaximal caerulein stimulation, the pancreas shows increased activation of the transcription factor, NF-κB. This factor regulates transcription of proinflammatory cytokines: IL-1, IL-6, IL-8, TNFα, adhesive molecules: ICAM-1, VCAM, E-selectine and P-selectine. These observations indicate that this factor may play an important role in initiating and spreading of the inflammatory process. The studies performed by Li et al. [3] show that adenosine and its analogues prevent the activation of NF-κB transcription in ischaemia.

In acute pancreatitis, numerous proinflammatory cytokines are released (TNFα, IL-1, IL-6, IL-8) whose concentration is related to the severity of the disease and multiorgan complications [13,14]. The antiinflammatory effects of adenosine also exerts an influence on cytokin release. Le Moine et al. [15] showed that adenosine increased secretion of interleukin-10 (IL-10) by monocytes and thus participated in inhibiting the TNFα release, and determined that adenosine inhibited TNFα and IL-6 expression in macrophages. Schmid et al. [16] showed that the IL-1-therapy decreased caerulein-induced pancreatitis. The administration of IL-10 after inducing acute pancreatitis led to a significantly decreased level of mRNA for TFNα and TFNα protein in the pancreas. The mechanism of action of adenosine described above is particularly relevant in the severe phase of acute pancreatitis when the balance between cytokines and their antagonists is disturbed and the disease becomes destructive for remote organs. Some authors suggest that anticytokine treatment is effective in experimental models of acute pancreatitis. However, it should be stressed that the protective effects can be observed only when cytokines are blocked before the failure of remote organs and peak cytokin concentrations have occurred.

In the pathogenesis of acute pancreatitis, the role of platelet activating factor (PAF) has also been confirmed. PAF, similarly to IL-1 and TNFα, was shown to be responsible for multi-organ damage. PAF receptors have been found in the endothelial cells of the pancreatic vessels, pancreatic follicular cells, macrophages, monocytes and neutrophils [17]. Exogenous adenosine or its analogues decreased arachidonic acid release and synthesis of leucotrien B_4 (LTB4), occurring after neutrophil stimulation by PAF. This effect was achieved through A_2 receptors. Adenosine and its analogues reduced the production of reactive oxygen metabolites in the PAF activated neutrophils and the adhesion of those cells to the endothelium.

The phagocyte stimulation of granulocytes observed in acute pancreatitis results in increased production of oxygen reactive forms. The impairment of blood flow leads to a decrease in endogenous oxygen free radical utilization and this reduces tissue antioxidative capacity, which, in turn, results in oxidative stress. Oxygen free radicals through peroxidation of structural lipids in the cell membranes damage the walls of capillaries and follicular cells increasing their permeability. This intensifies the enzyme release and the inflammatory process advances. Bullough [2] showed that through A_2 receptors adenosine inhibited the oxygen free radical formation by stimulated leucocytes, decreased their phagocytosis and adhesion to the endothelial cells. These findings indicate that adenosine may have an important function in protecting the organism against oxidative stress.

Conclusions

The administration of adenosine A_2 receptor agonists reduces the severity of pancreatic damage in caerulein-induced acute pancreatitis in rats. This action is likely to result from the modulating effects of the above-described substances on various stages of the cascade of inflammatory responses and pancreatic ischaemia. This study, however, did not show any effects of the A_1 receptor agonist and A_3 receptor antagonist on the inflammatory process in the presented experimental model of acute pancreatitis.

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Chymotrypsin-like activity in rat tissues in experimental acute pancreatitis

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Abstract

Purpose: Increase in intracellular chymotrypsin activity was reported during acute pancreatitis. Beside chymotrypsin, there are at least two enzymes with chymotrypsin-like activity: proteasome and lysosomal cathepsin A. Until now it is not known whether and to what extent they contribute to increases in chymotrypsin activity in acute pancreatitis. Our aim was to study organ chymotrypsin-like activities during experimental acute pancreatitis.

Material and methods: Rat cerulein model of acute pancreatitis was used. The chymotrypsin-like activities were assessed in pancreas, liver, lung, heart, spleen and kidney using highly selective synthetic substrates of the proteasome and the cathepsin A, at neutral and acidic pH. Determinations after addition of selective inhibitor were also performed.

Results: During acute pancreatitis we found in the pancreas an increase only in neutral chymotrypsin-like activity, as compared to the control animals. In other organs neutral chymotrypsin-like activity did not increase, and in kidney it even decreased. There were no changes in acidic chymotrypsin-like activity in any of organs studied. The studies using the inhibitor of the proteasome showed that the neutral chymotrypsin-like activity in the pancreas of the rats with acute pancreatitis should not be attributed to the proteasome activity, but rather to the chymotrypsin.

Conclusions: Our results did not confirm any significant contribution of proteasome or cathepsin A to increased chymotrypsin-like activity in acute pancreatitis. We showed a decrease in neutral chymotrypsin-like activity of proteasome in the kidney, but the significance of this finding remains to be established.

Key words: chymotrypsin-like proteases, acute pancreatitis, cerulein, rats.

Introduction

Acute pancreatitis is associated with an increase in the activity of pancreatic enzymes in the pancreas and body fluids, as well as multi organ dysfunction [1]. The pathomechanism of the disease remains unclear. Some authors [2-6] reported premature intracellular activation of trypsinogen and chymotrypsinogen as a result of instability of membranes of cell organelles, comprising also lysosomal membranes. In cerulein pancreatitis [2] subcellular fractions of secretory granules and vacuoles showed significant amounts of free chymotrypsin activities. However, there are at least two enzymes other than chymotrypsin, that exhibit chymotrypsin-like activity: proteasome and lysosomal cathepsin A. Until now it has not been reported whether and to what extent they contribute to increases in chymotrypsin activity in acute pancreatitis.

The proteasome is a large, multicatalytic proteinase complex (MPC). It exhibits at least 5 proteolytic activities responsible for the degradation of numerous cellular proteins including those mediating inflammatory and neoplastic conditions [7]. The chymotrypsin-like enzyme, which cleaves peptide bonds next to large hydrophobic residues, is the major component of the proteasome. It has been reported that the potent selective inhibitors of this enzyme such as microbial products lactacystin/ /β-lactone and epoxomicin and synthetic peptide analogues are apparently effective anti-tumor and anti-inflammatory agents [8]. Moreover, it was shown that proteasome activity may be affected by acute pancreatitis [9,10].

Cathepsin A (EC 3.4.16.1) is a multicatalytic lysosomal serine protease. At acidic pH it exhibits mainly the activity of carboxypeptidase. However, at neutral pH it acts predominantly

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as amidase. It preferentially hydrolyzes N-blocked hydrophobic peptides having phenylalanine and other hydrophobic amino acids at C-terminus, both synthetic (Cbz-Phe-Ala) and naturally occurring (angiotensin I, endothelin-1, substance P or bradykinin) [11]. The main function of this enzyme is a co-operation with other cathepsins, mainly cathepsin D, in protein degradation within the lysosomes. However, it is also postulated that after secretion from the lysosomes, cathepsin A may play an important role in local metabolism of bioactive peptides [12]. Beside catalytic function, cathepsin A was found to protect some enzymes from intralysosomal proteolysis by forming a multienzyme complex, especially with β-galactosidase and neuraminidase [11]. Earlier studies have shown that carboxypeptidase activity of cathepsin A is strongly inhibited by cell-permeable proteasome inhibitor lactacystin/β-lactone [13,14], and its amidase activity is inhibited by a potent antihypertensive agent ebelactone B [12]. The activity of lysosomal cathepsin A increases in muscular dystrophy [12] and neoplasms of the skin [13].

In acute pancreatitis proteolytic enzymes play a crucial role by inducing pancreas autodigestion, production of cytokines stimulating systemic inflammatory response syndrome (SIRS) and in consequence developement of multi organ failure (MOF). The importance of proteasome in the disease has recently been raised and for technical reasons its activity had to be measured together with cathepsin A. As cerulein acute pancreatitis in rats is a good equivalent of an oedematous form of the disease in humans, in the present study we investigated whether there is a change in the neutral and acidic chymotrypsin-like activities in the organs of rats with cerulein acute pancreatitis.

Material and methods

Chemicals

Cbz-Phe-Ala, ninhydrin reagent solution, Bradford reagent were purchased from Sigma Chemical Co, St. Louis, M, USA; Suc-Leu-Leu-Val-Tyr-AMC, lactacystin/β-lactone were purchased from Affinity research Product, UK.

Acute pancreatitis

Acute pancreatitis was induced in male Wistar rats weighing 180-200 g as described previously [15]. In brief, two intraperitoneal injections of 40 micrograms/kg of body weight of cerulein (analogue of cholecystokinin) were given at interval of 1 h. Control animals were injected similarily with 0.9% NaCl in the same volume. All animals were kept in single cages in 12 hours light/darkness cycle and were fed standard laboratory diet and water ad libitum. 24 hours after the first injection animals were sacrificed under anesthetic (intraperitoneal pentobarbital 40 mg/kg of body weight) after collection of blood and studied organs. The blood was taken from the right ventricle. 7.2 ml of blood was poured into a tube containing 0.8 ml of 3.8% sodium citrate. Immediately after sampling, blood was centrifuged at 3.500 rpm for 10 minutes at 4°C. The plasma was separated and stored at -70°C until it was assayed later. The following rat organs were taken for analyses: pancreas, liver, spleen, kidney, lung and heart. All organs were stored at -70°C until processing. To confirm changes typical of acute pancreatitis, amylase activity was determined in plasma, a portion of pancreas was examined histologically and pancreas wet/dry ratio was measured. The experiment was conducted in accordance with permission of the local ethical committee.

Amylase

Plasma amylase activity was measured using a quantitative colorimetric assay (Phadebas Amylase Test, Pharmacia & Upjohn, Sweden).

Histologic examination

A 3-5 mm^3 portion of pancreas head was fixed in 10% neutral formaldehyde solution and subsequently embedded in paraffin. Sections were cut at 5 μm thickness and stained with hematoxylin and eosin. Assessment comprised edema, inflammatory infiltration, fat necrosis, parenchymal necrosis and hemorrhage.

Pancreatic edema

Pancreas water content was assessed by the tissue wet/dry ratio. Pieces of pancreas were weighed immediately after harvest to obtain the wet weight and again after desiccation to obtain the dry weight. Desiccation was achieved in an oven at 120°C for 24 hours.

Preparation of tissue homogenates

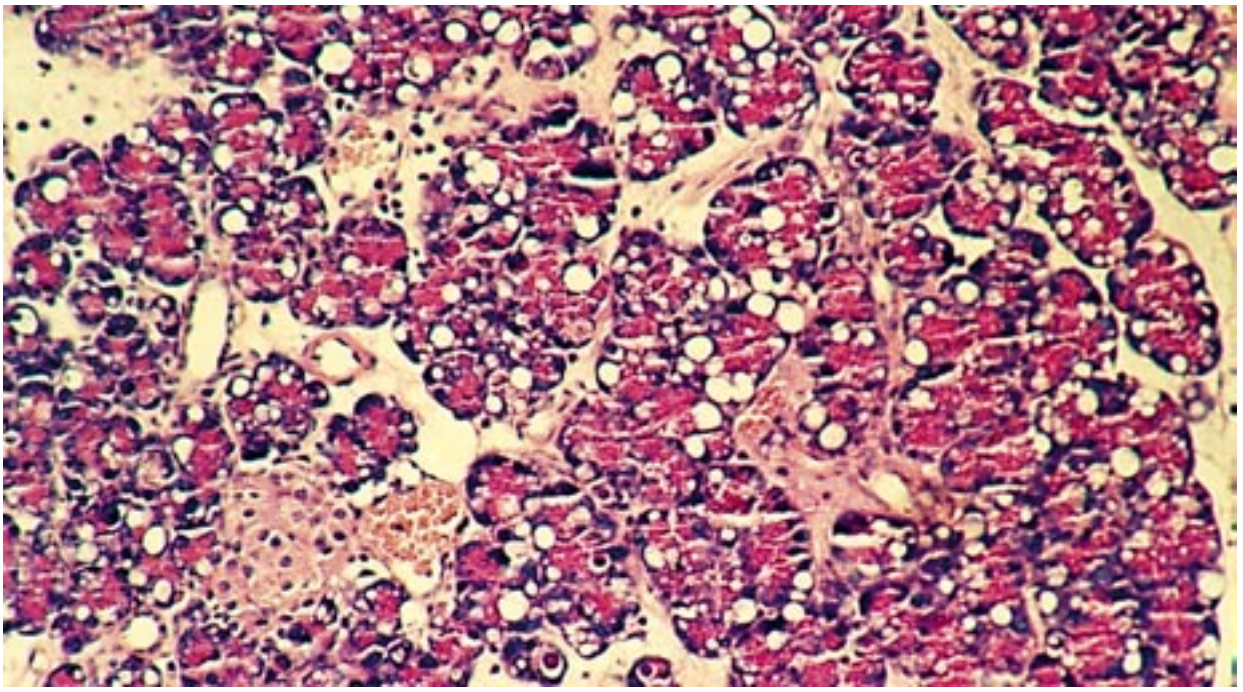
10% homogenates (w/v) were prepared using a knife homogenizer Combest X-120 (3x30 sec, 0°C) in 50 mmol/L Tris/HCl buffer at pH 8,0 containing 10% glycerol for assessment of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis [16] or in 50 mmol/L sodium acetate buffer at pH 5.5 containing 0.1 mol/L NaCl, 0.1 mol/L sucrose and Triton X-100 (0.5%) for assessment of Cbz-Phe-Ala hydrolysis. During processing tissues were cooled with ice. The homogenates were then centrifuged at 15000 g for 30 minutes at a temperature +4°C, and the supernatants were used for experiments.

Biochemical Assays

The neutral chymotrypsin-like activity was assayed at pH 8.0 using Suc-Leu-Leu-Val-Tyr-AMC according to Farout et al. [16]. Incubation mixture (total volume of 200 μL) contained homogenate (10-100 μg of protein), 40 μmol/L of substrate and 50 mmol/L Tris/HCl/1mM DTT buffer at pH 8.0. After 30 minutes of incubation at 37°C, the reaction was stopped by adding 800 μL of 100 mM monochloracetic acid in 30 mM of sodium acetate, and the samples were centrifuged to obtain supernatants. The amount of AMC released from the substrate was determined spectrofluorometrically (Ex 370/Em 420). One unit of the neutral chymotrypsin-like activity was designated to 1 μmol of liberated AMC during 1 minute. Specific activity was expressed as units/mg of protein.

The acidic chymotrypsin-like activity was assayed at pH 5.5 using Cbz-Phe-Ala as described by Ostrowska et al. [14]. Briefly, the reaction mixtures (500 μL) contained homogenate (100-200 μg of protein), 5 mmol/L substrate and 50 mmol/L sodium acetate buffer at pH 5.5 containing 0.1 mmol/L NaCl, 0.1 mmol/L sucrose and 1 mmol/L EDTA. After 30 minutes of incubation at 37°C the reaction was stopped by the addition

Figure 1. Histology of pancreata of rats with oedematous acute pancreatitis 24 hours after the first injection of cerulein (H&E, original magnification x200).



of 500 µL of 10% TCA, and the amount of liberated alanine was determined by ninhydrin method [17]. One unit (U) of the activity was defined as the µmoles of alanine released per minute. Specific activity was expressed in units/mg of protein.

Lactacystin/β-lactone in a standard dose of 10 µmol/L inhibits Suc-Leu-Leu-Val-Tyr-AMC and Cbz-Phe-Ala hydrolysis in cell lysates by up to 90% [13,14]. The inhibitor was immediately added to the supernatants and reactions were started by the addition of the synthetic substrate.

All buffers contained the inhibitors of cysteine, aspartyl and metalloproteinases (2.5 mmol/L pepstatin, 1 mmol/L EDTA, 1 mmol/L EGTA and 50 nmol/L E64) [16].

Protein determination

The protein concentration in supernatants was determined by the method of Bradford [18] using Sigma reagent with bovine serum albumin as a standard.

Statistical analysis

All groups were tested with Kruskal-Wallis Anova test and inter-groups comparisons were made with Mann Whitney test. The threshold of significance was $p<0.05$.

Results

Histologic assessment (*Fig. 1*), amylase activity measurement in plasma and wet/dry weight ratio (*Tab. 1*) have confirmed the presence of eclematous acute pancreatitis in studied animals. Significant increase in plasma amylase activity was seen in the rats with acute pancreatitis 24 hours after the onset of disease, as compared to the control group ($p<0.05$) (*Tab. 1*). Also pancreas wet/dry ratio was significantly greater in rats with acute pancreatitis than in the control group ($p<0.05$) (*Tab. 1*).

Table 1. Amylase activity in plasma and wet/dry ratio of pancreas samples.

Group:	Control	Acute pancreatitis – 24h
Amylase (IU)	523 ± 81	1581 ± 207 *
Wet/dry ratio	2.13 ± 0.11	6.31 ± 0.72 *

Data represent the mean ± SEM; *-$p<0.05$; **$p<0.01$ vs. control

A comparison of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis at pH 8.0 and Cbz-Phe-Ala hydrolysis at pH 5.5 in rat organs from healthy and acute pancreatitis rats is presented in *Tab. 2*. Suc-Leu-Leu-Val-Tyr-AMC was hydrolyzed with the highest rate in the pancreas among all organs studied. This chymotrypsin-like activity increased about 3-fold in the pancreas of rats with experimental acute pancreatitis (25.2 µmol/min/mg) as compared to the control animals (8.4 µmol/min/mg). In contrast, the hydrolysis of Cbz-Phe-Ala at pH 5.5 in the pancreas of acute pancreatitis rats was low (0.78 µmol/min/mg) and did not differ from this activity in the pancreas of healthy rats (0.88 µmol/min/mg). In the kidney we found a decrease in chymotrypsin-like activity assayed using Suc-Leu-Leu-Val-Tyr-AMC at pH 8.0 after 24 hours of induction of acute pancreatitis. In other organs, there were virtually no differences in comparison to the control group. The acidic chymotrypsin-like activity did not

Table 2. Comparison of Suc-Leu-Leu-Val-Tyr-AMC hydrolysis at pH 8.0 and Cbz-Phe-Ala hydrolysis at pH 5.5 in organs of control rats and 24 hours after induction of acute pancreatitis.

Organ	Suc-LLVT-AMC hydrolysis at pH 8.0μmol/min/mg	Suc-LLVT-AMC hydrolysis at pH 8.0μmol/min/mg	Cbz-Phe-Ala hydrolysis at pH 5.5μmol/min/mg	Cbz-Phe-Ala hydrolysis at pH 5.5μmol/min/mg
	Control	acute pancreatitis	Control	acute pancreatitis
Pancreas	8.4±1.4	25.2±6.3 **	0.88±0.1	0.78±0.08
Kidney	1.1±0.13	0.74±0.021 **	16. 2±2.8	12.6±1.8
Liver	0.1 ± 0.024	0.09±0.03	3.5±0.2	2.6±0.1
Spleen	0.028±0.002	0.02±0.06	2.4±0.6	2.1±0.4
Lung	0.025±0.004	0.022±0.008	1.64±0.44	1.54±0.5
Heart	0.029±0.0024	0.032 ±0.009	0.88±0.15	0.72±0.5

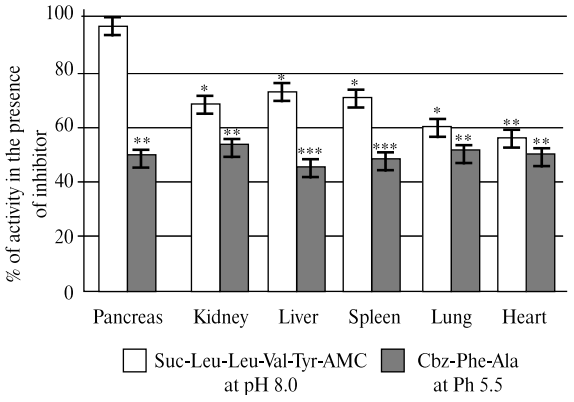
Mean values from 3 measurements for each organ of rats (n=5)±standard deviation; Statistically signi Figure description; ** p<0.01

change in any of organs studied during acute pancreatitis, as compared to the control group.

The studies using 10μM lactacystin/β-lactone, a potent inhibitor of the proteasome, showed that Suc-Leu-Leu-Val--Tyr-AMC hydrolysis was not inhibited in the pancreas (*Fig. 2*). In other organs of acute pancreatitis rats, lactacystin/β-lactone decreased the neutral chymotrypsin-like activity by about 30-40% indicating that also other proteases were involved in Suc-Leu-Leu-Val-Tyr-AMC hydrolysis.

In contrast, 10μM lactacystin/β-lactone decreased the hydrolysis of Cbz-Phe-Ala at pH 5.5 by about 50-60% in all organs studied, indicating that cathepsin A was mainly responsible for this activity.

Figure 2. Effect of lactacystin/β-lactone on Suc-Leu-Leu-Val-Tyr--AMC hydrolysis at pH8.0 and Cbz-Phe-Ala hydrolysis at pH5.5 in the organs of rat with experimental acute pancreatitis 24 hours after its induction. Relative chymotrypsin-like activities are shown as % of the activity in the absence of the inhibitor.



Mean values from 3 measurements for each organ of rats (n=5); *p<0.05; **p<0.01; ***p<0.001.

Discussion

Activation of zymogens within the pancreatic acinar cell is an early feature of acute pancreatitis [19]. Activity of several enzymes has been studied so far [20,21]. It has been speculated that the intracellular activation of trypsinogen is an early step in the development of acute pancreatitis [15]. However, it has been reported that chymotrypsin caused marked cell damage at micromolar concentrations, similarily to lipase, whereas even milimolar concentrations of trypsin failed to cause significant damage. Is has been concluded that activation of trypsin might be the trigger to start the activation cascade in acute pancreatitis, however, trypsin itself is markedly less noxious to acinar cells when compared with other digestive enzymes. It has also been suggested that studies analyzing therapeutic effects of protease inhibitors should evaluate not only the inhibitory potential against trypsin but also that against other digestive enzymes [22].

In our study we have shown the highest neutral chymotrypsin-like activity in pancreas among all organs studied. This activity increased about 3-fold in the pancreas of rats with experimental acute pancreatitis as compared to the control animals. However, the studies with a potent inhibitor of the proteasome, showed that Suc-Leu-Leu-Val-Tyr-AMC hydrolysis was not inhibited in the pancreas. These results indicate that the neutral chymotrypsin-like activity in the pancreas should not be attributed to the proteasome activity, but rather to the chymotrypsin generated by abnormal activation of chymotrypsinogen by cellular proteases in the pancreas of rats with acute pancreatitis. In other organs of rats injected with cerulein, lactacystin/β-lactone decreased the Suc-Leu-Leu-Val-Tyr-AMC hydrolysis by about 30-40% indicating that also other proteases were involved in neutral chymotrypsin-like activity.

The acidic chymotrypsin-like activity, typical of cathepsin A, was low in the pancreas of rats with acute pancreatitis and did not differ from this activity in the pancreas of healthy rats. There were no changes in neutral nor acidic chymotrypsin-like activity in liver, heart, lung or spleen.

In the kidney the neutral chymotrypsin-like activity clearly decreased after 24 hours of induction of acute pancreatitis. The significant decrease in enzymatic chymotrypsin-like activity in the kidneys is of interest in view of frequent renal function impairment during acute pancreatitis [23]. Also other enzymatic activities have been reported to be reduced in the kidneys during acute pancreatitis. Lakowska et al. [24] have found a decrease in the activity of lysosomal cathepsin B, D, L. The authors have concluded that the monitoring of lysosomal

enzyme activity can be used to evaluate the impariment of kidney function in the course of experimentally induced acute pancreatitis.

The decrease in active enzymes content in the kidneys during acute pancreatitis is not fully explained. Hypothetically this may result from inactivation of proteases or their release to the bloodstream and/or urine because of cell membranes damage.

Acute renal failure during acute pancreatitis has been explained on the basis of hypovolemia and hypotension and a role of renin-angiotensin alterations in acute pancreatitis as mediators of renal failure was postulated [23]. Levy et al. have reported that decrement in renal perfusion found in experimental acute pancreatitis in dogs could be duplicated by the infusion of chymotrypsin into normal dogs. The authors speculate, that this was due to a decline in plasma volume, associated with a rising hematocrit and declining plasma protein concentration [25].

Conclusions

In conclusion, our results indicate that induction of acute pancreatitis in rats leads to a significant increase in the neutral chymotrypsin-like activity in pancreas during acute pancreatitis. However this should be attributed to activated chymotrypsin rather than proteasome, because of lack of inhibition of Suc-Leu-Leu-Val-Tyr-AMC by a potent proteasome inhibitor lactacystin/β-lactone at the concentration suppressing the proteasome activity in cell cultures. There were no changes in neutral chymotrypsin-like activity (typical of proteasome) in liver, heart, lung or spleen during acute pancreatitis. The decrease in neutral chymotrypsin-like activity of proteasome in kidney needs further exploration. The acidic chymotrypsin-like activity typical of cathepsin A was not altered in any of organs studied. To clarify the significance of the cytosolic proteasome in acute pancreatitis, immunochemical studies would be of value. So far, to our knowledge, no commercial immunochemical kit is available.

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Effect of different treatment methods on survival in patients with pancreatic cancer

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Abstract

Purpose: To assess the benefit of treatment modalities on the survival in patients with pancreatic cancer.

Material and methods: Eighty-five patients with pancreatic cancer were treated by surgery, radiotherapy, chemotherapy and combined therapy. The data was reviewed retrospectively and the benefit of various treatment methods to the median survival time of the patients was assessed.

Results: Median survival time of the patients diagnosed with local disease treated by radical resection and adjuvant treatment was 21.5 months; with radical resection only – 12.6 months (p=0.6). In patients with locally advanced disease and treated by radical resection and adjuvant therapies the median survival time was 12.1 months and by radical resection only 7.7 months (p=0.6). For patients treated by palliative surgery, chemotherapy and radiotherapy median survival was 8.8 months and by palliative surgery alone 1.8 month (p=0.015). 1-year actual survival of patients treated with radical resection and adjuvant therapies was 11.7%. For patients with radical resection only – 10.5%. 3-year actual survival for the same groups of patients was 3.5% and 2.3%, respectively. 1-year actual survival of patients treated by palliative methods was 2.3%.

Conclusions: Surgery with adjuvant treatment seems to be beneficial for pancreatic adenocarcinoma patients. In locally advanced and metastatic pancreatic cancer palliative chemotherapy or radiotherapy statistically significantly improved survival.

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Key words: pancreatic cancer, radical surgery, palliative surgery, adjuvant treatment, median survival.

Introduction

Pancreatic carcinoma is the fourth commonest cause of death worldwide, with over 40000 deaths/year in Europe. In Lithuania about 400 new pancreatic cancer cases are diagnosed a year and more than 50% of cases are diagnosed in the advanced stages [1,2]. Median survival of patients with pancreatic carcinoma is in the range of 7-12 months. Less than 15% of patients are operable at the time of diagnosis. Although patients undergo potentially curative surgery, the median survival is only 10-18 months and 5-year survival is 17-24% [3].

The role of surgery, radiotherapy and chemotherapy in the treatment of local, locally advanced and metastatic pancreatic cancer was analyzed in many studies. Radical surgery is a treatment of choice in local forms of pancreatic cancer, but in locally advanced tumors does not substantially improve survival. Most failures occur within 1-2 years of surgery, therefore there is a need for adjuvant therapy [4]. Patients diagnosed with local and locally advanced tumors in addition to surgery are treated with chemotherapy and radiotherapy.

Many chemotherapeutic agents have been used in the treatment of advanced pancreatic cancer, but only older agents such as 5-fluorouracil (5-FU), mitomycin, and new agent such as gemcitabine have shown effect [5]. In most studies radiation therapy only slightly improved local control of pancreatic cancer. A more promising treatment in advanced operable and inoperable pancreatic cancer was shown to be chemoradiation [6]. Using various modes of radiotherapy and different chemotherapeutic agents there is an improvement in survival. Chemoradiotherapy is a toxic treatment and, therefore, patients with a better performance status do better.

We report the results of the study that aimed to address the results of the treatment of pancreatic cancer patients at our institution from years 1998 to 2000; and to estimate the survival comparing different modes of treatment of pancreatic cancer.

Table 1. The survival of pancreatic cancer patients with local, locally advanced or metastatic disease treated by different treatment methods.

Treatment method	Stage	No of patients	Median survival (months)	p
Radical surgery and adjuvant treatment (Group 1)	I-II	8	21.5	0.6
Radical surgery and no adjuvant treatment (Group 2)		7	12.6	
Radical surgery and adjuvant treatment (Group 3)	III-IVA	9	12.1	0.6
Radical surgery and no adjuvant treatment (Group 4)		11	7.7	
Palliative surgery and radiotherapy, chemotherapy (Group 5)	III-IVB	5	8.8	0.015
Palliative surgery only (Group 6)		21	1.8	

The purpose of our work was to analyze median survival time, 1-year and 3-year actual survival rates of various groups of patients.

Material and methods

Eighty-five patients diagnosed with pancreatic cancer and treated between 1998 and 2000 at Kaunas Medical University Hospital were retrospectively analyzed.

Sixty-five of the patients were operated. In thirty nine cases radical pancreatoduodenectomy was performed. Remaining twenty six patients have undergone biliary bypass procedure.

Seventeen patients were treated with radiation. Radiation therapy was administered either after operation, after operation with adjuvant chemotherapy, or for palliative purposes. Radiation therapy was given primarily to patients with advanced adenocarcinoma of the pancreas in order to palliate symptoms. Positive tumor margins, residual tumor, involved nodes, perineural infiltration or direct extension of tumor were the main indication for radiotherapeutic management. External beam radiation therapy – 15 MV photons from linear accelerator, was used in the dose range from 40 (20 fractions) to 60 (30 fractions) Gy. The classical dose fractionation scheme was applied for the treatment of the patients. The four-field (anteroposterior – posteroanterior, right and left lateral) or three-field (anterior and wedged right and left lateral) techniques were used to irradiate pancreatic tumor or tumor bed and adjacent lymph nodes. The radiation fields and treatment volume were established with information from computed tomography (CT), surgical description and clips. If the tumor was in the head of the pancreas treatment volume was 2-3 cm larger than the tumor volume, including the primary tumor, adjacent pancreatic tissue, the superior head, the pancreaticoduodenal celiac axis, portal hepatic lymph nodes. CT based treatment planning was performed on 3D computerized treatment planning system.

According to the stage nineteen patients were treated with either 5-FU or gemcitabine in standard doses and schedules. Four to six courses of 5-FU were administered depending on the performance status of the patients. No WHO grade 3 or 4 toxicity was observed while giving 5-FU regimens. Patients treated with gemcitabine, usually, because of their performance status, received three to four courses of treatment. Chemotherapy was generally well tolerated. Toxicity was mild and mostly thrombocytopenia. There were no toxic deaths.

The patients were grouped according to the stage of the disease and type of operation; either radical surgery or palliative surgical procedure. There were six groups. In Group 1 patients were diagnosed with local disease (stage I-II) and treated by radical surgery and adjuvant treatment (radiotherapy and/or chemotherapy). Group 2 – patients were diagnosed with local disease and treated by radical surgery only. Group 3 – patients with locally advanced disease (stage III-IVA) and treated with radical surgery and adjuvant treatment (radiotherapy and/or chemotherapy). Group 4 – patients with locally advanced disease and treated by radical surgery only. Patients in Group 5 – were diagnosed with locally advanced or metastatic disease (III-IVB) and treated by palliative surgery, radiotherapy or chemotherapy. Group 6 patients were diagnosed with locally advanced or metastatic disease and treated by palliative surgery only (*Tab. 1*).

The primary endpoint for analysis was death. Survival was calculated from the date of resection until the date of death or censored at the latest follow up. Kaplan-Meier survival curves were drawn and Cox-Mantel test was used to assess differences between the groups.

Results

Mean age was 60 years. The main tumor location was the head of the pancreas; histology for all cases – ductal adenocarcinoma. The mean duration of symptoms until the diagnosis was 2.5 months. The main symptoms were pain and jaundice. 90 percent of patients indicated pain as the main symptom.

In patients who underwent radical resection node positive disease was diagnosed in 20 patients. Distant metastases were diagnosed in 18 patients treated by palliative operation.

The median survival time of patients in Group 1 was 21.5 months. The median survival time of patients in Group 2 was 12.6 months. The result was not statistically significant (p=0.6) (*Fig. 1 A*).

For patients in Group 3 the median survival time was 12.1 months. The median survival time for Group 4 was 7.7 months. No significant difference in survival curves was observed (p=0.6) (*Fig. 1 B*).

In Groups 5 and 6 the median survival time was 8.8 months

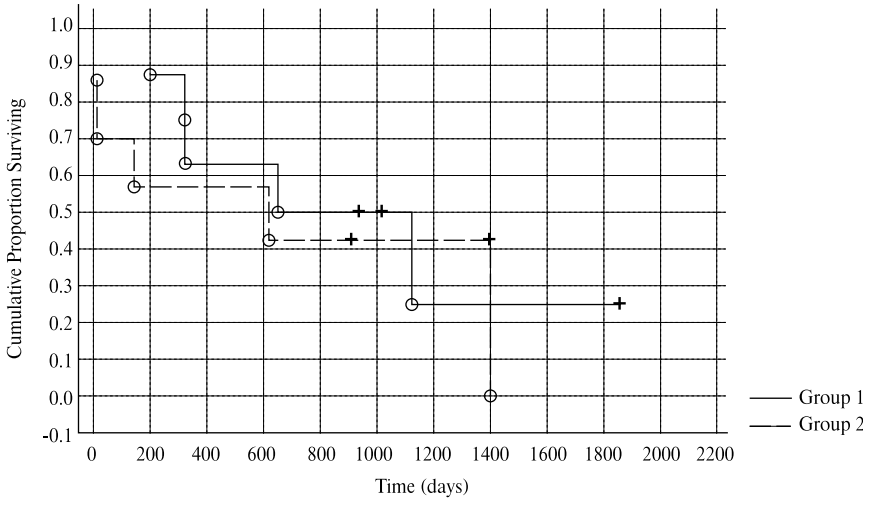

Figure 1 A. Kaplan-Meier estimates of median survival time in patients with local disease treated with radical surgery and adjuvant therapy (Group 1) and patients with local disease treated with radical resection only (Group 2) (p=0.6).

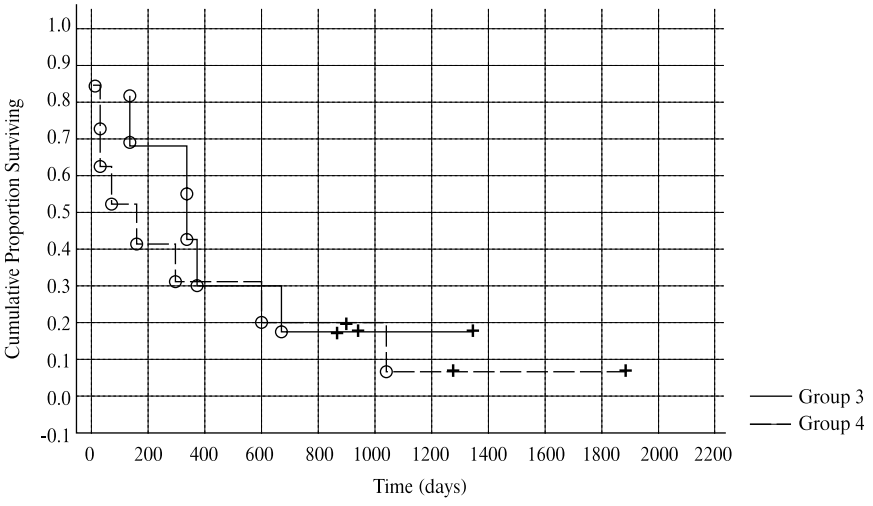

Figure 1 B. Kaplan-Meier estimates of median survival time in patients with locally advanced disease treated with radical surgery and adjuvant therapy (Group 3) and in patients with locally advanced disease treated by radical resection only (Group 4) (p=0.6).

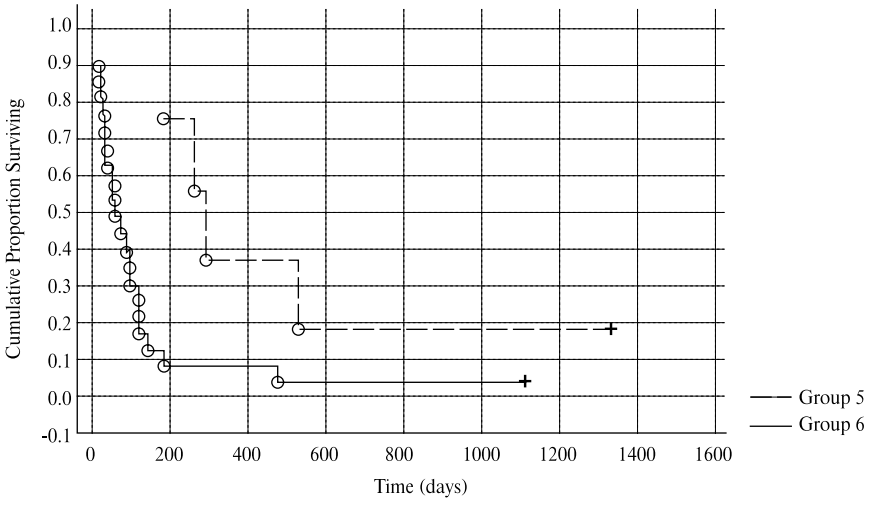

Figure 1 C. Kaplan-Meier estimates of median survival time in patients with locally advanced and metastatic disease treated by palliative surgery, radiation or chemotherapy methods (Group 5) and in patients with locally advanced and metastatic disease treated with palliative surgery only (Group 6) (p=0.015).

and 1.8 months respectively. The difference between the two groups was significant (p=0.015) (*Fig. 1 C*).

The 1-year actual survival of patients treated with radical resection and adjuvant therapy was 11.7%. For patients with radical resection only 1-year actual survival was 10.5%. A 3-year actual survival of the same groups of patients was 3.5% and 2.3% respectively.

The 1-year actual survival of patients treated by palliative methods (surgery, radiotherapy or chemotherapy) was 2.3%.

Discussion

The morbidity with pancreatic cancer is low but the results of the treatment are poor. We analyzed some demographic and clinical factors. Pancreatic cancer is a disease of elderly people. The mean age in our study was 60 years: 64 years for women and 59 years for men. In Lithuania there is a similar rate of pancreatic cancer diagnosis for men and women. In the world men are more often diagnosed with pancreatic cancer, but in

Europe the incidence of pancreatic cancer among women is increasing [7].

In our study tumor was mainly located in the head of the pancreas, and main symptoms were pain and jaundice. Symptoms, usually, appeared 2.5 months before the diagnosis.

The treatment of pancreatic cancer is problematic and results are poor. The disease is treated with combined treatment methods, but because of the advanced stage of the disease at diagnosis and poor performance status of the patients the treatment is complicated. The earlier the stage, the more radical treatment is possible and the results of treatment are better.

There are not many randomized multicenter studies on the effect of pancreatic cancer treatment modalities. The GITSG (Gastrointestinal Tumor Study Group) in 1983 proved the efficiency of the adjuvant treatment in pancreatic cancer. After surgery patients who were treated with radiotherapy and chemotherapy statistically significantly lived longer, comparing to the patients who were just operated. Median survival was 20 and 11 months respectively [8]. EORTC (The European Organization for Research and Treatment of Cancer) study 40891 analyzed also two groups of patients where one group of patients was operated only and the other group of patients was treated by operation and chemoradiotherapy. The median survival was 19 and 23.5 months, respectively [9]. In our study patients with local disease who were treated by radical surgery and adjuvant therapies lived 9 months longer, than patients with no adjuvant treatment. Patients diagnosed with locally advanced disease and treated by radical resection and adjuvant treatment lived 4 months longer than by radical resection only. The results are not statistically significant because of the small patient groups, but the addition of the adjuvant treatment in both cases improved survival.

Patients treated with palliative surgery, radiotherapy and/ /or chemotherapy survived longer (median survival 8.8 months), than patients who had undergone palliative surgical procedure only. The survival was 7 months longer and the difference was statistically significant. Similar results were described by the researchers from the Mayo Clinic (US). In the study patients with unresectable tumors were treated by external beam radiation therapy with or without chemotherapy and their median survival was 12.6 months [10]. The median survival time of patients treated by similar protocols at Tomas Jefferson Hospital (US) was 7.3 months [10].

In recent ESPAC-1 study the median survival time in patients treated with chemotherapy was significantly improved; 19.7 months, compared to 14 months in a group that did not received chemotherapy [11,12]. These and other studies show the need of new standardized protocols of pancreatic cancer treatment, especially for advanced pancreatic cancer.

In the world five year survival in different countries is about 3 to 5% [13,14]. In our study 1-year and 3-year actual survival rates in pancreatic carcinoma patients were low too. The best result was achieved when patients were treated with radical surgery and adjuvant treatment. 1-year survival was 10-11% of patients.

Conclusions

Surgery and adjuvant treatment seems to be beneficial for pancreatic adenocarcinoma patients.

In locally advanced and metastatic pancreatic cancer palliative chemotherapy or radiotherapy statistically significantly improved survival.

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Model of single left rat lung transplantation. Relation between surgical experience and outcomes

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Abstract

Purpose: The model of unilateral orthotropic left rat lung transplantation is well known and established experimental procedure. The author's personal learning curve of mastery process of this microsurgical procedure is presented.

Material and methods: During 18 months the author has performed 197 single left lung transplantations on the Thoracic Surgery Ward in University Hospital, Berne, Switzerland. There were 147 allogeneic and 50 isogeneic transplantations done. The allogeneic transplantations were carried out from Brown-Norway to Fischer F344 rats whereas isogeneic transplantations were done among Fischer F344 rats solely. Grafted lung was obtained from the intravenously anaesthetised, oxygen-ventilated donor. The implantation was carried out through left postero-lateral thoracotomy on the gas anaesthetised, respirator ventilated recipient. The anastomoses of the vessels were done using the cuff technique, bronchi were sutured using continuous running over-and-over suture. Recipients were sacrificed on day 5 post-transplant. All recipients were divided into four consecutive groups. Warm ischaemia time and presence of perioperative pure technical complications were observed.

Results: We observed time dependent decline of complications number of consecutive recipient groups, respectively 20, 5, 4, 1. The warm ischaemia time in minutes decreased from 35.6±5.4 in group I through 26.7±4.4 in group II, 24.8±2.3 in group III to 22.0±3.1 in group IV.

Conclusions: Continuous training of the procedure shortens the average warm ischaemia time and reduces the number of complications. This tedious microsurgical procedure is possible to master by the surgeon.

Key words: learning curve, rat lung transplantation, warm ischaemia time.

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Introduction

Lung transplantation is an established method to treat some chronic end-stage pulmonal diseases. As it is a difficult, time consuming and extremely expensive treatment, rodent model of lung transplantation seems to be convenient, cheap and reliable survey to observe physiological or pathological changes or to introduce a new treatment to the transplanted lungs.

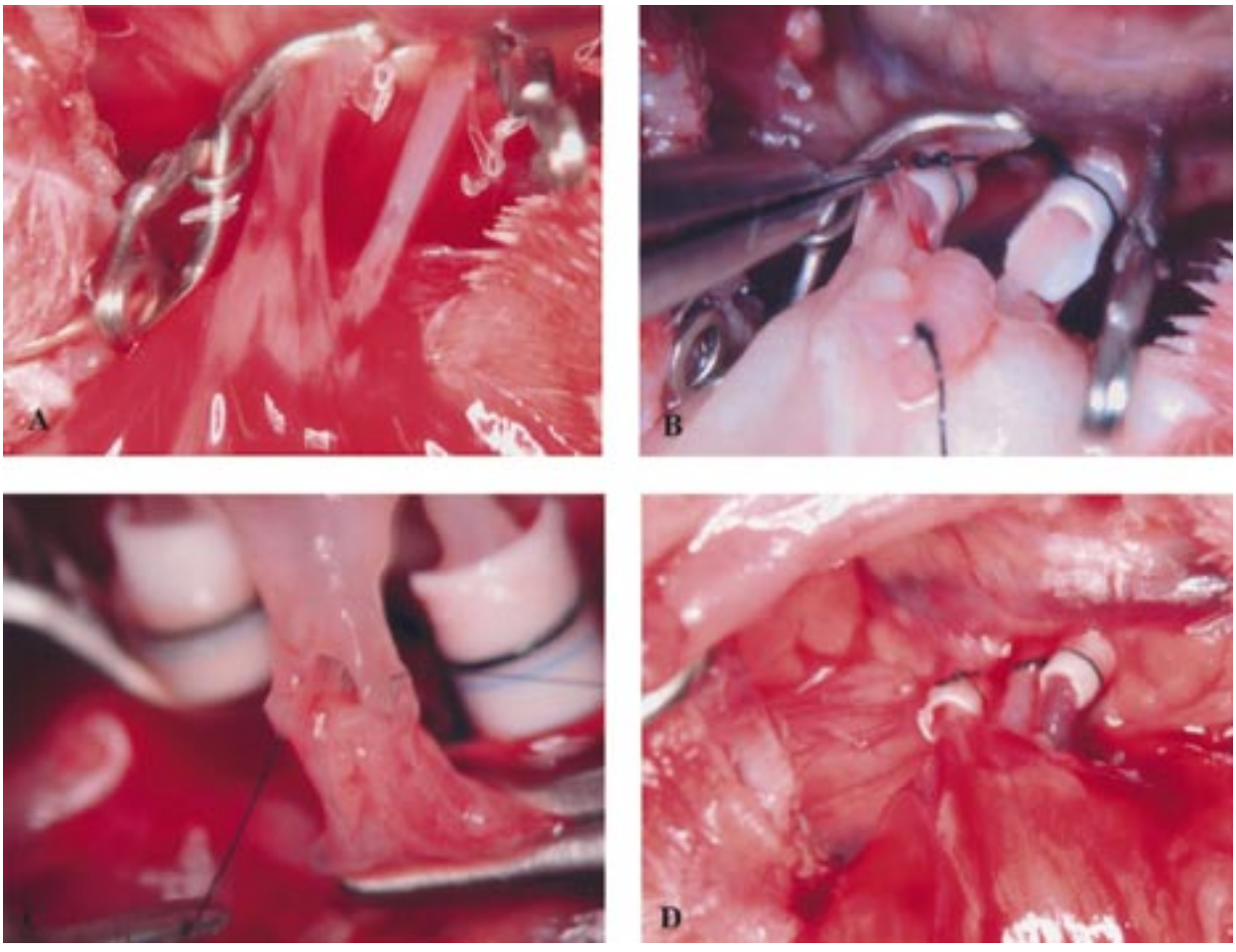
Thanks to the two years of author's scientific fellowship in the General Thoracic Surgery Ward in University – and Kanton--Hospital Insel in Bern, Switzerland, it is possible to describe here the process of mastery the rat lung transplantation.

Material and methods

Between June 2000 and December 2001 the author has performed the total of 197 orthotropic single left rat lung transplantations. The majority of 147 were allotransplantations done from Brown-Norway to Fischer F344 rats. 50 isotransplantations were done among the same bred of Fisher F344 rats. To observe the progress of learning the procedure, recipients were divided into four groups. Group I: 50 recipients grafted between 15.06.2000 and 13.09.2000, Group II: 50 recipients grafted between 14.09.2000 and 7.02.2001, Group III: 50 recipients grafted between 8.02.2001 and 8.09.2001, Group IV: 47 recipients grafted between 9.09.2001 and 17.12.2001.

Operative Procedure: All the microsurgical procedures were done under the magnification between 6 to 20x of the Olympus® SZX12 stereoscopic microscope, Japan. The microsurgical tools of Fehling, Miltex, Pilling – Germany

Figure 1A-D. Model of single left rat lung transplantation.



and Dumont Surgical, Switzerland were used. The donor and recipient procedure took approximately 120 minutes. The method of cuff technique for vessel anastomosis was used [1].

Donor: The animal was preanaestetized in the glass chamber inhaling 4% Halothane (SIGMA®, Buchs, Switzerland). Thiopental (Penthotal, Abbott AG) 50 mg/kg was injected intraperitoneally. Heparin (Liquemin, Roche Pharma, Switzerland) was administered by a peripheral vein (500 IU/kg). A tracheostomy was performed and the animal was ventilated through the tube (14 GA iv. Catheter, Insyte®, Spain) with FiO_2=1.0, f=100/min, TV=10 ml/kg by Harvard Rodent Ventilator model 683 (Harvard Apparatus, South Natick, Massachusetts, USA). After cutting the inferior vena cava and left appendix of the heart, a small silicon hose was inserted into the main pulmonary artery via the incision in the right ventricle. Both lungs were flushed with 20 ml of LPD solution (Perfadex®, Medisan Pharmaceutics, Uppsala, Sweden) at a pressure of 20 cm H_2O. The trachea was then tied in end-inspiratory position. The heart-lung block was removed and the left lung was separated ex-vivo from the heart and right lung. 24 gauge cuffs were placed around the pulmonary artery and vein and the vessels were everted and tied onto the cuff [1] and fastened with 8-0 monofilament thread (Surgipro, USSC, USA). The lung was stored in LPD solution at 10^0C until implantation.

Recipient: The recipient was anaesthetised by breathing Halothane (SIGMA®, Buchs, Switzerland) in a glass chamber, intubated, and anesthesia was maintained with 2% Halothane. A left thoracotomy under the 4th rib was performed. The neurovascular clips were put onto the left pulmonal artery (PA) and left pulmonal vein (PV). The left main native bronchus was ligated with 6-0 polifilament thread (Sofsilk, USSC, USA) and cut off. The incision was made in PV and PA) (*Fig.1A*). These vessels were flushed with 0.9% NaCl. The cuffs of the graft were inserted into the recipient's vessels respectively (*Fig.1B*). The 6-0 polifilament ligatures (Sofsilk, USSC, USA) were put around the cuffs. The native PA and PV were cut off beyond the anastomosis and native lung was taken away. The 9-0 Monosof (Tyco Healthcare, Wollerau, Switzerland) running over-and-over continuous suture was performed for bronchial anastomosis (*Fig.1C*). The ventilation and then the perfusion of the graft were restored by removing clips from left bronchus, PV and PA respectively (*Fig.1D*). The chest drain (24Gx3/4" infusion set, Terumo®, Belgium) was inserted to the left haemithorax and the thoracotomy was closed with four layers of continuous suture (4/0 Prolene, Johnson and Johnson®). The thoracic drain was removed after the animal restored spontaneous breathing and the animal was extubated.

Graft Function: On day 5 postransplant the animal was preanaestetized in the glass chamber inhaling 4% Halothane

Tabele 1.

197 transplantations: June 2000 – December 2001	GROUP I 1-50	GROUP II 51-100	GROUP III 101-150	GROUP IV 151-197
Time frame (months)	3	5	7	3
Complications (n)	20	5	4	1
Warm ischaemia time (minutes: average ± standard deviation)	35.6 ± 5.4	26.7 ± 4.4	24.8 ± 2.3	22.0 ± 3.1

(SIGMA®, Buchs, Switzerland). Thiopental (Pentothal, Abbott AG) 50mg/kg was injected intraperitoneally. Animal was ventilated via tracheostomy – Harvard Rodent Ventilator model 683 (Harvard Apparatus, South Natick, Massachusetts) with FiO_2=1.0, f=100/min, TV=10ml/kg. A thoraco-laparotomy in the anterior midline was performed. Neurovascular clips were put on the right main bronchus and right pulmonary artery in order to ventilate and perfuse only the left lung. After five minutes 300μl of blood was aspirated from the aortic arch to the syringe (Pico™ 50 Radiometer-Copenhagen, Denmark) to assess blood gas (Radiometer ABL 700 Serie, Copenhagen, Denmark). After cutting the inferior vena cava and left appendix of the heart, a small silicon hose was inserted into the main pulmonary artery via the incision in the right ventricle. The lungs were then flushed with 20ml of 0.9% NaCl under the pressure of 20cm H_2O. The tracheostomy tube was removed and the trachea was ligated in end-inspiratory position. The heart-and-lung block was cut out, the samples of lungs were put in 10% formaline solution (SIGMA®, Buchs, Switzerland) for histological analysis.

However, the blood gas analysis and formaline fixation are of no interest in this paper.

Results

There is a big difference in both complication rate and warm ischaemia time between the first group I and the other groups II to IV, which show consecutively only slight improvement. The results are shown in the *Tab. 1*.

The perioperative complications include: death, bronchial or vessel stenosis, bronchial fistula, haemorrhage from the anostomosis, anaesthesia overdose, vena cava rupture during chest drain removal, pleural empyema.

The complications due to not technical reasons, like lethal virus instillation, peritonitis due to intraperitoneal injection, expected and physiological rejection of the allograft were not classified in these results.

The warm ischaemia time is defined here as the time between taking the graft from $+10^0$C storage solution and putting it on the recipient till its complete reperfusion and reventilation.

Discussion

The rat lung transplantation technique has many variations; some authors prefer cuffless way to anastomose vessels and bronchi [2,3], though from the author's experience cuffs properly applied for the vessels allow for relatively easy and fast graft circulation restoration. Some others recommend no suture for these anastomoses [4] and that is probably good advice, as the continuous suture for the bronchus anastomosis make great difficulty for the surgeon, especially on the beginning and is the main cause of whole transplantation failure. In our experience we did not meet any serious complications due to reperfusion injury as the others describe [5], as the graft was excised from the living donor and properly stored. As the time for the $+10^0$C storage in this procedure is rather short (approximately 40 minutes), we presume that the disturbances caused by fatty acids metabolism [6] did not have big impact for our outcomes. The temperature indicated is presumed to be optimal for the living lung tissue storage [7]. The reperfusion injury is a cause of the tissue damage and it is proportional to the time the graft storage. Decrease of reperfusion pressure is thought to be of benefit to diminish this complication [8-10]. In our experience the faster this procedure is performed, the lesser influence of the reperfusion injury on the outcomes is.

There are many opinions about how fast one can master given surgical procedure. Basing on this material, completing of 50 transplantations allows the surgeon to feel confident about it, though further practise also improves the outcomes in lesser extent.

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Impact of interferon-alpha therapy on the serum level of alpha-fetoprotein in patients with chronic viral hepatitis

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Abstract

Purpose: Assessment of the long-term effect of interferon-α (IFN-α) treatment on the serum level of hepatocarcinogenesis marker – α-fetoprotein (AFP), in patients (pts) with chronic viral hepatitis (c.v.h.) type B and C.

Material and methods: Thirty seven pts (21 with HCV and 16 with HBV infection; 20 women, 17 men, aged 24--62) were included in the study. The pts were administered IFN-α in the dose of 9-15 MU per week, thrice weekly, for 16 weeks (HBV group) and 24-52 weeks (HCV group). The effectiveness of IFN-α treatment was evaluated on the basis of the HBV DNA and HCV RNA level in the blood. The serum AFP values were determined before and 4-7 years after IFN-α treatment.

Results: The baseline serum AFP level was increased in 26 out of 37 pts (70%) (14/21 from HCV group; 12/16 from HBV group). After the 4-7 years' follow-up it remained elevated only in 2 out of 37 pts (5%). AFP values significantly decreased after IFN-α treatment (17.58±19.09 IU/ml vs 7.95±21.78 IU/ml; $p < 0.05$; normal range 0-5 IU/ml) in both HBV and HCV, responder and non-responder groups.

Conclusions: IFN-α therapy significantly decreases the serum AFP level in patients with chronic viral hepatitis B and C. Its beneficial clinical effects have been observed both in responders and in non-responders. It could diminish the risk of liver carcinogenesis, however further studies are required to elucidate this issue.

Key words: alpha-fetoprotein, chronic viral hepatitis, interferon-alpha, carcinogenesis.

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Introduction

Hepatocellular carcinoma (HCC) is one of the 10 most common tumors in the world [1]. Hepatitis viruses, particulary the hepatitis B virus (HBV) and the hepatitis C virus (HCV), are major environmental causes of human hepatocellular carcinoma (HCC) world-wide, as they induce chronic liver disease and cirrhotic transformation of the liver [2,3]. These two major aetiological factors have been definitively incriminated in the pathogenesis of HCC. Approximately 300 million people are chronic carriers of HBV and about one percent of the world's population is infected with HCV. HCC for most patients is a terminal complication of chronic inflammatory and fibrotic liver diseases. With regrettably few exceptions, treatment of this neoplasm is largely palliative, and long-term survival is rare. Over one million patients die from HCC annually [4].

The molecular mechanism of HBV and HCV-related liver carcinogenesis is constantly beeing debated. There is evidence for biological effects of proteins of both viruses, which can directly modulate major transduction cellular signals controlling cell proliferation, differentiation and viability, but still the pathogenesis of HCC is poorly understood [5]. Current discussion focuses on the question of whether interferon-α, administered to patients with chronic liver disease (with or without biochemical or virologic response), delays or prevents cancer of the liver. The literature data suggests that interferon--α therapy may prevent hepatocellular carcinoma in patients with cirrhosis, particularly in those with HCV [6-10].

Serological tests (for alpha-fetoprotein) and ultrasonography are used in HCC screening [1]. AFP is α_1-globulin secreted by fetal hepatocytes and in a small amount by other cells of the fetal gastrointestinal tract. Physiologically, in human adults an increased AFP level is present in the serum of pregnant women. In pathology, measurements of alpha-fetoprotein (AFP) are an important tool in the care and management of patients with benign and malignant hepatic disorders. The elevation of serum AFP in benign hepatic diseases (acute and chronic viral hepatitis, toxic liver injury) is associated only with small, transient increases in serum [11-14]. Therefore, a quan-

Table 1. Characteristic of patients with CH B and CH C before IFN-α theraphy.

	CH B (n=16)	CH C (n=21)
Age* (years)	43±14	42±12
Range of age (years)	18-66	18-62
Sex:		
– female	7	13
– male	9	8
Source of infection:		
– medical procedures	13	20
– family contact	1	0
– professional exposure	1	0
– sexual contact	1	0
– unknown	0	1
Acute hepatitis in anamnesis	10	4
Duration of infection		
– (years)*	4±4	6±6
– (range)	(1-18)	(1-16)

* all data are expressed as mean ±SD; CH B – chronic hepatitis type B; CH C – chronic hepatitis type C; IFN-α (interferon-α).

tification of serum AFP has been widely used as a diagnostic marker for HCC. The concentration of AFP correlates with the tumor's size and after an effective surgical procedure it should decrease or even normalize. Measurements of serum AFP levels have also been used in screening the populations at high risk of human HCC such as those with cirrhosis or carriers of HBV and HCV viruses [12,14].

The aim of the study was to evaluate the influence of IFN-α treatment on the serum levels of the hepatocarcinogenesis marker (AFP) in patients with HBV and HCV-related chronic liver disease (CLD). For this purpose we have estimated and compared the serum AFP levels before, after therapy and during a 4-7 year follow-up.

Material and methods

Characteristics of the patients selected for the study. Thirty seven patients (pts), with chronic hepatitis B and C were selected for IFN-α treatment on the basis of the following criteria:

- age – over 18 years;
- duration of the disease before treatment – minimum 6 months;
- markers of active virus replication (in chronic hepatitis B: HBeAg, HBV DNA > 10 pg/ml; in chronic hepatitis C: HCV RNA in the serum or hepatic tissue);
- elevated activity of aminotransferases determined 3 or more times at monthly intervals of time before treatment;
- features of chronic hepatitis in morphologic biopunctate of the liver;
- no features of decompensation of the liver function: prothrombin time – normal or slightly prolonged, albumin levels > 3 g/dl, no signs of ascites or encephalopathy.

The patients were divided into the following groups:

Group I – 16 patients with chronic hepatitis B (CH B), included 7 women and 9 men, age range – 18-66 years.

Table 2. Diseases coexisting with CH in patients treated with IFN-α.

Disease coexisting with CH	CH B (n=16)	CH C (n=21)
Ulcer disease	3	5
Liver haemangioma	1	1
Cholelithiasis	1	2
Diabetes mellitus	0	3
Simple goitre	2	1
Angina pectoris	4	3
Arterial hypertension	2	4
Psoriasis	1	0

* CH B – chronic hepatitis type B; CH C – chronic hepatitis type C; IFN-α – interferon-α.

Table 3. Past diseases in patients treated with IFN-α.

Type of disease	CH B (n=16)	CH C (n=21)
Past surgery :		
Cholecystectomy	2	6
Appendectomy	4	5
Partial gastrectomy	0	1
Mammectomy	1	0
Strumectomy	1	1
Hysterectomy	4	4
Facial skeleton	0	1
Crural varices	0	1
Hodgkin's disease	2	0
Acute lymphocytic leucaemia	1	0

Group II – 21 patients with chronic hepatitis C (CH C), included 13 women and 8 men, age range – 18-62 years.

The characteristics of the patients of both groups are presented in *Tab. 1*.

The diseases coexisting with CH in both groups were determined on the basis of clinical evaluation and anamnesis and are compiled in *Tab. 2*.

At gastroscopy no active ulcer niche was found in any of the patients with coexisting ulcer diseases before IFN-α treatment. The course of the remaining diseases was stable. Two patients with CH B reported past Hodgkin's disease and one – acute granulocytic leukaemia – in all 3 patients a complete remission was observed before IFN-α treatment.

In the CH B group, one patient had earlier undergone mammectomy, another had been underwent to goitre surgery, 4 – operative procedures of the reproductive organs, 2 – cholecystectomy and 4 – appendectomy. In the CH C group, one patient had undergone partial gastrectomy, 6 – cholecystectomy, 4 – operations of the reproductive organs, 1 – goitre surgery, 5 – appendectomy, 1 – facial skeleton surgery and 1 – crural varices operation. The past diseases in both groups are listed in *Tab. 3*.

The patients treated with IFN-α in the HBV and HCV groups did not significantly differ with regard to changes in serum levels of aminotransferases, alkaline phosphatase, γ-glutamyltranspeptidase, bilirubin, albumin, γ-globulin, platelets and prothrombin time. Laboratory findings are shown in *Tab. 4*.

Table 4. Laboratory findings in patients with CH B and CH C before IFN-α theraphy.

	CH B (n=16)		CH C (n=21)	
	Mean	Range	Mean	Range
ALT (U/l)	294±206	57-798	160±109	61-578
AST (U/l)	212±200	27-990	111±63	48-320
AP (U/l)	114±51	48-321	90±28	45-169
GTP (U/l)	124±153	14-820	57±44	10-185
Bilirubin (μmol/l)	15±12	5-72	15±12	7-60
Albumins (g/dl)	4.26±0.48	3.46-5.97	4.40±0.40	3.50-5.56
Gammaglobulins (g/dl)	1.60±0.59	0.90-4.16	1.40±0.50	0.14-2.57
Platelets (G/l)	153±37	82-224	187±49	101-333
Prothrombin time (s)	16±2	12-20	15±2	12-20

Normal range: alanine aminotransferase (ALT): 5-40 U/l, aspartate aminotransferase (AST): 5-40 U/l, alkaline phosphatase (ALP): 35-125 U/l, gamma-glutamyltranspeptidase (GTP): 10-75 U/l, bilirubin: 5-17 μmol/l, albumins: 3.5-5.5 g/dl, gamma-globulins: 0.91-1.48 g/dl, platelets: 100-350 G/l, prothrombin time: 12-16 s.
* CH B – chronic hepatitis type B; CH C – chronic hepatitis type C; all data are expressed as mean ±SD.

Interferon α–2b (IFN-α) (Intron A, Schering-Plough) was administered subcutaneously, 3 x per week in a dose of 9 or 15 MU/week. Since some patients developed marrow suppression, the treatment was periodically discontinued or the dose reduced. The treatment duration was: 16 weeks in CH B patients and 24-52 weeks in CH C patients. After the treatment ended the patients were followed up once a month, the final efficacy of therapy was evaluated after one year. The serum AFP levels were checked every 6 months for 4-7 years following the treatment.

Laboratory tests. At the beginning of the treatment morphology with absolute granulocyte and thrombocyte count was performed after each IFN-α injection. Then these tests as well as alanine and aspartate aminotransferases (ALT, AST), alkaline phosphatase, gamma-glutamyltranspeptidase, bilirubin determinations were done once a week.

The levels of total protein, protein electrophoresis, coagulation parameters (prothrombin time), and renal efficiency (creatinine, urea) were evaluated every month. HBV DNA (using Digene Hybrid Capture System – Murex Diagnostica GmbH, Burgwedel) and HCV RNA (RT PCR) were determined before, in the middle and directly after the treatment ended, then a half and one year later. The serum AFP levels were measured by a RIA – AFP – PROP/J^{125}, MJ^{137}/ test.

Statistics Results are expressed as means±SD. Statistical analysis was performed using the unpaired Student's t-test, Mann-Whitney's U-test, the χ^2 test with Fisher's correction. The significance level was set at $p<0.05$. The statistical analysis was performed using SPSS PC+ software.

Results

The effectiveness of IFN-α treatment was evaluated in both groups directly after therapy and one year later. The lack of serum HBV DNA or HCV RNA one year after the treatment ended was assumed to be a complete and sustained response. Twenty out of 37 patients (54%) achieved a complete and sustained response to treatment (11/16 from the HBV group and 9/21 from the HCV group) and 17 showed no such a response.

The serum AFP levels were monitored every six months for 4–7 years following the treatment. The baseline serum AFP level was increased in 26 out of 37 patients (70%) (14/21 from the HCV group; 12/16 from HBV group).

After the 4–7 year follow–up it remained increased only in 2 out of 37 patients (5%) (1 from the HBV and 1 from the HCV group). The AFP values significantly decreased after IFN-α treatment (17.58±19.09 IU/ml vs 7.95±21.78 IU/ml; $p<0.05$; normal range 0-5 IU/ml) in both HBV and HCV, responder and non-responder groups. These results are presented in *Tab. 5*.

Table 5. Serum AFP levels in patients with CH B and CH C*.

Group of patients	Serum AFP levels (IU/ml)		p
	Before IFN-α	After IFN-α	
All (n=37)	17.58±19.09	7.95±21.78	p<0.05
Responders (n=20)	13.14±11.66	4.35±3.36	p=0.001
Non-responders (n=17)	26.09±25.96	13.92±35.31	p<0.05

* all data are expressed as mean ±SD; AFP: α-fetoprotein; CH B – chronic hepatitis type B;
* CH C: chronic hepatitis type C; IFN-α (interferon-α).

HCC developed after 5 year in one patient, who was a non-responder female with chronic HBV infection, with a baseline AFP level – 24.4 IU/ml and the follow-up AFP level (5 years after the treatment) – 29.42 IU/ml.

Discussion

The treatment of chronic hepatitis B and C remains difficult despite recent progress in this field. Due to prophylactic vaccination, HBV infections can be prevented and the whole situation seems to be under control. On the other hand, the problem of HCV infections, whose diagnosis by specific tests became possible only in 1989, continues to increase. There are about 100 million HCV carriers world-wide [1-3]. It should be stressed that these infections are statistically undetectable as the majority of them are asymptomatic. So the reported numbers show only "the tip of the iceberg".

With IFN-α in the treatment of hepatotropic virus infections there is some hope of fighting the disease. At present, there is no doubt that the drug administered in chronic hepatitis inhibits its progression in some patients [6-10,15]. However, the long-term effects remain highly unsatisfactory.

Strict selection criteria were used in the group of patients analysed by us. Thanks to that 91% of the patients completed their treatment. Similar results were obtained by other authors who used similar selection procedures [15]. All patients with CH B and CH C selected for interferon-α therapy had elevated

aminotransferases activity. As the literature data show in some CH B patients, HBsAg, HBeAg and HBV DNA are likely to be detected with normal or slightly increased levels of hepatic enzymes. Such patients respond poorly or do not respond to IFN-α treatment at all. They should not be selected for therapy but constantly followed up [15].

In our population, the mean ALT activity before treatment was lower in patients with CH C compared with the patients with CH B. Although, even normal activity of this enzyme does not objectively reflect the degree of liver damage in chronic HCV infections. Contrary to patients with CH B, the group with CH C and low aminotransferases activity better respond to IFN-α treatment [15].

The majority of patients treated in our Department received interferon-α in a dose of 5 MU 3 times a week. Some patients had their doses modified as the symptoms of marrow suppression developed, i.e. decreased absolute neutrophil and thrombocyte counts. The efficacy of IFN-α therapy in the examined population was not very satisfactory (54%), particularly in HCV patients (42%), in whom also the number of recurrences within one year following the treatment was higher (compared to the group with HBV infection).

At present, studies are being carried out to determine the role of IFN-α for HCC prevention in patients with HBV and HCV – related chronic liver disease. Our results show that IFN-α therapy could decrease the risk of liver carcinogenesis in these patients. It significantly decreases the serum AFP levels. Its beneficial effect was observed in responders as well as non--responder groups. During the follow-up, HCC was detected in one patient 5 years after completing IFN-α therapy. The patient was a non-responder, female with HBV infection and with increased baseline and follow-up AFP levels. On the basis of these results we recommend hepatocellular cancer screening at frequent intervals for every patient treated with IFN-α after therapy. The fact that there was no HCC development in patients with a complete and sustained response to IFN-α treatment provides hope for this therapy in chronic viral hepatitis. Further studies are required to clarify the role of IFN-α in preventing malignant hepatocyte transformation in patients with HBV and HCV-related chronic liver disease.

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IL-15 in the culture supernatants of PMN and PBMC and the serum of patients with Lyme disease

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Abtract

Purpose: The aim of this study was to estimate the production of interleukin-15 (IL-15) by neutrophils (PMNs) and peripheral blood mononuclear cells (PBMC) confronted with the serum levels of IL-15 in patients with Lyme disease.

Material and methods: PMN and PBMC were isolated from heparinized whole blood of patients. The cells were incubated for 18hs at 37°C in a humidified incubator with 5% CO_2. After 18hs incubation, supernatant was removed and assessed for IL-15 using ELISA kits.

Results: The results obtained showed significant increase in the ability of patient's PMNs and peripheral blood mononuclear cells (PBMC) to release IL-15. Although PBMC produced higher concentrations of IL-15 than PMN, the quantitative dominance of PMN in the peripheral blood suggest a significant role for these cells in the defense reactions controlled by this cytokine. Similar changes in the secretion of IL-15 by PMN and PBMC in patient group may be caused by the same regulatory mechanisms which influence the functional abnormalities of the cells examined.

Conclusions: A change in the ability of PMN and PBMC to release IL-15 may have various implications for the cellular and humoral response to the Borrelia burgdorferi (B.b.) infection in patients with Lyme disease.

Key words: interleukin-15, neutrophil, peripheral blood mononuclear cells.

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Introduction

Interleukin-15 (IL-15) is a pleiotropic cytokine which shares biological activities with IL-2. IL-15 uses both β- and γ-chains of the IL-2 receptor for binding and signalling. The IL-15 receptor (IL-15R) complex also includes a specific α subunit (IL-15Rα), distinct from the IL-2Rα chain. IL-15R is expressed on various cells of the immune response, including T and B cells, NK cells and more recently, peripheral blood neutrophils [1,2].

IL-15 plays an important role in both innate and adaptive immunity. It induces T cell proliferation and cytokine production, stimulates the locomotion and chemotaxis of T cells and delays its apoptosis. IL-15 has been shown to stimulate the growth and cytotoxicity of NK cells and to induce antibody-dependent cell-mediated cytotoxicity. It was also demonstrated that this cytokine induces macrophages function and B cell proliferation [1-3]. Recent investigation have shown that IL-15 potentiated several antimicrobial functions of normal neutrophils (PMNs) involved in the innate immune response against invading pathogens [4]. IL-15 was observed to enhance phagocytosis, NF-κB activation, IL-8 production and to delay apoptosis of these cells. In addition, IL-15 has been shown to prime the metabolic burst of PMN in response to N-formyl-methionyl-leucyl-phenylalanine (fMLP) [4-8].

Numerous in vitro and in vivo studies have suggested that neutrophils play a critical role in controlling spirochete Borrelia burgdorferi (B.b.) persistence that is responsible for Lyme disease. It has also been demonstrated that infectivity of the B.b. correlates with resistance to elimination by phagocytic cells [9,10].

The main source of IL-15 are macrophages/monocytes and lymphocytes as well as neutrophils [1,4,11,12]. There is no available data refering to PMN and peripheral blood mononuclear cells (PBMC) ability to release IL-15 in patients with Lyme disease. The changes in the secretion of IL-15 by these cells might modulate the PMN and PBMC activity in response to B.b. infection through autocrine or/and paracrine mechanisms.

Table 1. The mean concentrations of IL-15 in the culture supernatants of PMN and PBMC and serum of control and patients with Lyme disease.

	PMN unstimulated $\bar{X} \pm SD$	PMN LPS-stimulated $\bar{X} \pm SD$	PBMC unstimulated $\bar{X} \pm SD$	PBMC LPS-stimulated $\bar{X} \pm SD$	Serum (pg/ml) $\bar{X} \pm SD$
Control n=15	0.82±0.56	1.68[a]±0.69	1.94±0.79	3.21[a]±0.95	1.97±0.9
Patient group n=30	2.32*±1.62	2.76*±1.66	3.67*±1.85	3.86±1.61	5.46*±0.15

* – statistical differences with control ($p<0.05$)
[a] – statistical differences between unstimulated and LPS-stimulated cells ($p<0.05$)

The aim of this study was to estimate the production of IL-15 by neutrophils and peripheral blood mononuclear cells in comparison to the serum levels of IL-15 in patients with Lyme disease. Studies, involving estimation of IL-15 production by immunocompetent cells, such as PMN and PBMC, may be useful and would widen knowledge about IL-15 role in the course of B.b. infection.

Material and methods

Patients

We examined 45 patients (21 males and 14 females) aged between 44 and 72 years (mean ±58) with Lyme disease recognized according to EUCALB. The patients were treated in the Department of Infectious Diseases and Neuroinfections, Medical University of Bialystok. The diagnosis of Lyme borreliosis was based on the epidemiological and case history, and interpretation of serologic tests using ELISA and Western-blot methods. Of 30 patients in the study 13 were classified as early localized Lyme borreliosis in the form of erythema migrans, 13 as Lyme arthritis form. Blood samples were taken from each patient at presentation.

Control subjects (n=15) were healthy people aged from 32 to 53 years (mean 42.5 year).

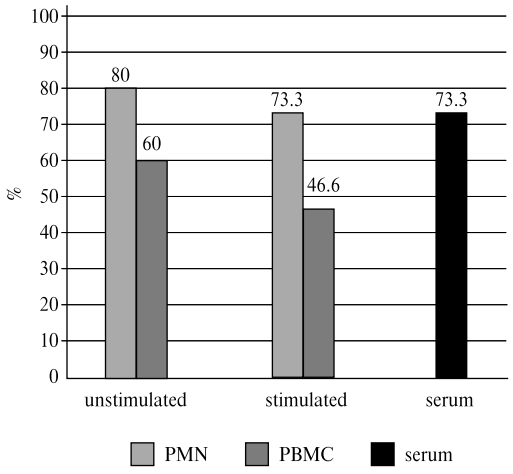
PMN and PBMC cultures

Cells were isolated from heparinized (10 U/ml) whole blood by Gradisol G gradient (1.115 g/ml). This method enables simultaneous separation of two highly purified leukocyte fractions: mononuclear cells (PBMC), containing 95% lymphocytes and polymorphonuclear cells (PMNs) containing 94% PMNs. The purity of isolated PMNs was determined by May-Grunewald-Giemsa-staining. The PMN were washed three times with RPMI-1640 medium (RPMI-1640 Medium, Gibco) and were suspended in the culture medium to provide $5x10^6$ cells/ml. Following culture, the viability of PMN was >93%, PBMC>94% as determined by trypan blue exclusion.

Culture medium consisted of RPMI-1640 medium supplemented with 10% autologous serum, 100 U/mL penicillin and 50 ng/mL streptomycin.

The cells were cultured in flat-bottomed 96-weel plates (Falcon) with LPS (100 ng/ml, Sigma-Chemical Co). The cells were incubated for 18 hs at 37^0C in a humidified incubator with 5% CO_2. After 18hs incubation, supernatant was removed and assessed for IL-15 using ELISA kits (Biosource).

Figure 1. The precentage of patients with the concentrations of parameters examined above the mean concentrations of control group.



Results

Unstimulated PMN derived from patients with Lyme disease secreted more IL-15 than those from normal subjects (*Tab. 1*). In 80% of patients, the concentration of IL-15 in the culture supernatants of PMN was significantly higher than in healthy persons (*Fig. 1*). In contrast to control group, LPS stimulation of patients PMN did not lead to a significant enhance in the production of IL-15 (*Tab. 1*). In 73.3% of patients, the concentration of IL-15 in the culture supernatants of LPS--stimulated PMN was higher than in control group (*Fig. 1*). There was no difference between patient groups with Lyme arthritis and erythema migrans.

To investigate the PMN contribution to the secretion of IL-15 among the leukocytes of peripheral blood, we also measured the IL-15 release by autologous PBMC under the

same culture conditions. The concentrations of IL-15 in the culture supernatants of PBMC from patients were higher than of those in control group (*Tab. 1*). In 60% of patients, unstimulated PBMC secreted more IL-15 than those from healthy subjects (*Fig. 1*). Similar to PMN, LPS stimulation did not result in higher IL-15 production by PBMC from patients (*Tab. 1*). In 46.6% of patients, the concentration of IL-15 in the culture supernatants of LPS-stimulated PBMC was higher than in control group (*Fig. 1*). The differences between IL-15 values in the culture of PBMC obtained from patients with different forms of Lyme disease were not significant.

There was no significant differences between the amounts of IL-15 secreted by unstimulated and LPS-stimulated PMN and PBMC (*Tab. 1*).

The mean concentrations of IL-15 in the serum of patients with Lyme disease were significantly higher than in control group (*Tab. 1*). The increased level of IL-15 has been found in 73.3% of patients (*Fig. 1*).

There was a correlation between the concentration of IL-15 in the culture supernatants of PBMC and the serum levels ($r=0.62$, $p<0.05$).

Discussion

PMNs are the primary effector cells in inflammation that extent beyond their capacities to function as phagocytes and cells releasing cytotoxic compounds. It was established that PMN have ability to synthesize and release the proinflammatory cytokines such as: IL-1, IL-6, OSM, IL-8, IL-12, IFN-α, TNF-α, GM-CSF, G-CSF, Gro-α, MIP-1α and MPI-1β and anti-inflammatory mediators such as TGF-β, IL-1Rα, sTNFRs [4,6,13,14]. The synthesis and secretion of these mediators by PMN can be changed at different pathological conditions [15,16].

The present study showed increase in the ability of PMN derived from patients with Lyme disease to release IL-15. Additionally, we have also found that autologous PBMC exhibited the same changes in the IL-15 secretion. Although PBMC produced higher concentrations of IL-15 than PMN, the quantitative dominance of PMN in the peripheral blood suggest a significant role for PMN in defense reactions controlled by IL-15 in patients with borreliosis. Similar changes in the release of IL-15 by PMN and PBMC may be caused by the same regulatory mechanisms which influence the functional abnormalities of the cells examined.

The high levels of IL-15 secreted by unstimulated PMN and PBMC from patient group, and simultaneous lack of response to additional LPS-stimulation in vitro, could be caused by the earlier activation of these cells in vivo due to direct or indirect effects of B.b. antigens. There are different data indicating that the outer surface lipoprotein of B.b., OspA and OspB are potent inducers of several inflammatory cytokines or chemokines [10,17-19]. It has been demonstrated that OspA can stimulate the production of IL-1, IL-6, IL-12, IFN-α, and TNF-α by mononuclear cells [17-19]. Furthermore, Oksi et al. showed that production of IL-4 by these cells was decreased but production of IFN-α was increased in patients with Lyme disease [20]. On the other hand, Diterich et al. demonstrated reduced release of TNF-α and IFN-α and enhanced secretion of IL-10 and GM-CSF by white blood cells [21]. Recent examination of Infante-Duarte et al. showed that B.b. and lipopeptides derived from B.b. outer surface lipoproteins induce IL-17 expression in Th cells that were different from "classical" Th1 and Th2 and were characterized by coexpression of the proinflammatory cytokines IL-17, TNF-α and GM-CSF [22].

A change in the ability of PMN and PBMC to release IL-15 may have various implications for cellular and humoral reactions in patients with Lyme disease. IL-15 secreted by PMN and PBMC may lead to the stimulation of each other's function in response to B.b. infection. The increase in IL-15 production by PMN as well as PBMC can also influence other cytokines secretion. IL-15 has been shown to stimulate mononuclear cells to release of IL-1, MCP-1, GM-CSF, IFN-α and TNF-α [6,23]. Moreover, Musso et al. showed that IL-15 induces secretion of the neutrophil chemotactic factor IL-8 that is effective in neutrophil activation suggesting an indirect role of IL-15 in the early steps of the inflammatory response to B.b. through increased amount of PMN infiltrating the tissues [8]. Additionally, our previous studies demonstrated that IL-15 induces in neutrophils not only IL-1β but also its regulatory protein – IL-1 receptor antagonist (IL-1Rα) [15]. Miller et al. reported that the balance between IL-1β and IL-1Rα influenced the pathogenesis of Lyme arthritis [24].

The changes in secretion of IL-15 by PMN and PBMC can lead to changes in their concentration in body fluid. The results of our study demonstrate markedly elevated serum levels of IL-15 in patients with Lyme disease. A direct correlation between the release of IL-15 by PBMC and the sera levels indicated significant influence of these cells on the level of IL-15 in blood. Athough there was no correlation between IL-15 release by PMN and the serum levels in patients, a coincidence between them was found.

In conclusion, the observations made in this study suggest an additional role of PMN and PBMC in the immune response of patients with Lyme disease. However, further examination, involving estimation the IL-15 secretion together with its regulatory protein, such as soluble IL-15Rα by these cells, in patients before and after treatment, might be helpful to confirm the clinical significance of the above observations.

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Concentration of interleukin-6 (Il-6), interleukin-8 (Il-8) and interleukin-10 (Il-10) in blood serum of breast cancer patients

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Abstract

Purpose: Interleukins may stimulate cancer cells growth and contribute to locoregional relapse as well as metastasis. Permanent synthesis and release of these cytokines leads to augmentation of their serum concentration that might be utilized as a marker of immunity status and immune system activation in prognosis and monitoring of the course of cancer.

Material and methods: Therefore, in the present study we assessed the concentration of IL-6, IL-8 and IL-10 in blood serum of breast cancer patients to determine whether it correlates with the disease progression.

Results: We showed statistically higher serum concentrations of IL-6, IL-8 and IL-10 in breast cancer patients in comparison with healthy women, which also correlated with clinical stage of breast cancer.

Conclusions: The present study indicates that elevated IL-6, IL-8, and IL-10 serum concentration, are strongly associated with breast cancer and correlate with clinical stage of disease. It was feasible that it can be used to diagnose women with breast cancer and to identify patients with a poor prognosis who may benefit from more aggressive management.

Key words: interleukin-6, interleukin-8, interleukin-10, breast cancer.

Introduction

Breast cancer is the most common cause of cancer-related deaths in women in industrialized nations and creates a significant public health problem. In Poland, over 11 000 new cases are diagnosed annually with 7 000 deaths attributed to this disease each year. The current trend in the incidence is expected to grow well into 21st century, despite strategies and campaigns aimed at risk factor reduction. Although significant improvements in therapy have occurred last years, most deaths from breast cancer are still caused by metastases that are resistant to conventional treatment. Therefore, novel approaches to the management of breast cancer must be developed.

The biology of breast cancer is complex, involving oncogenesis, evasion of host immune defense mechanisms, angiogenesis, invasion and metastasis. Recently, the contribution of interleukins, pleiotropic cytokines, to cancer progression has been demonstrated. IL-6, IL-8 and IL-10 exert a variety of effects on the immune system, acute-phase responses and hematopoiesis. Macrophages, monocytes and lymphocytes as well as cancer cells have been documented to produce and secrete IL-6, IL-8 and IL-10 [8,15,19]. These cytokines in an autocrine or paracrine manner induce in vitro growth of ovarian cancer, cervical cancer, prostate cancer, lung cancer, kidney cancer and melanoma cells [4,6,7,14]. Furthermore, their contributions to the tumor angiogenesis have been reported [3,9]. Moreover, it has been shown that treatment effectiveness and prognosis in course of malignancy are determined by the stage of the disease and activity of the immune system mechanisms modulated by different interleukins, e.g. IL-6, IL-8, IL-10 [10,16,20].

Therefore, in the present study we assessed the concentrations of IL-6, IL-8 and IL-10 in blood serum of breast cancer patients and healthy women and their correlation with clinical stage of breast cancer.

Material and methods

Analysis was performed in 45 breast cancer patients, diagnosed in Regional Cancer Center in Bialystok. Age of the patients ranged from 25 to 79 years. Clinical diagnosis was routinely confirmed by the histopathological examination of

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Table 1. IL-6, IL-8 and IL-10 serum concentration values in breast cancer patients and control group.

		Control group N=25	Breast cancer patients N=45
IL-6 concentration (pg/ml)	Median	3.3*	31.7*
	Range	1.56-8.6	6.25-100.0
	Percentage of elevated values	0	39 (86.7)
IL-8 concentration (pg/ml)	Median	5.2	40.1*
	Range	3.9-8.0	7.80-76.0
	Percentage of elevated values	0	36 (80.0)
IL-10 concentration (pg/ml)	Median	5.7	24.7*
	Range	3.9-8.8	5.6-37.0
	Percentage of elevated values	0	35 (77.8)

* – statistically significant differences ($p<0.05$)

Table 2. IL-6, IL-8 and IL-10 serum concentration values in breast cancer patients according to the clinical stage of the disease (TNM classification).

		Stage of the disease according to TNM classification			
		II A N=6	II B N=23	III A N=12	III B N=4
IL-6 concentration (pg/ml)	Median	18.7*	19.3*	40.9*	44.1*
	Range	6.25-30.6	7.8-36.4	7.8-96.0	8.4-100.0
	Number of patients with elevated values	5/6	20/23	11/12	3/4
IL-8 concentration (pg/ml)	Median	33.6	35.2	36.3	48.8
	Range	7.8-60.0	7.8-76.0	7.8-60.0	8.0-75.0
	Number of patients with elevated values	4/6	18/23	11/12	3/4
IL-10 concentration (pg/ml)	Median	18.9	19.6	29.9	26.0
	Range	5.6-29.0	6.4-32.0	6.2-35.0	6.9-37.0
	Number of patients with elevated values	5/6	18/23	9/12	3/4

* – statistically significant differences ($p<0.05$)

the tumor tissue samples. Of the 45 patients, 30 has suffered from ductal infiltrating carcinoma (carcinoma ductale infiltrans) and 15 from lobular infiltrating carcinoma (carcinoma lobulare infiltrans). Examined patients were in clinical stage IIA, IIB, IIIA and IIIB according to TNM classification. At the time of examination patients were not showing any signs of clinically overt active inflammatory process. Control group comprised 25 healthy women. Blood samples were collected before treatment initiation. Blood serum was stored in a freezer at -20°C.

Interleukin-6, -8 and -10 concentrations were determined in serum samples by enzyme-linked immunosorbent assays (ELISAs). ELISA reagents have been used that are commercially available in assay kits (R&D Systems). Median interleukins serum concentrations obtained from control group of healthy women were taken as reference values.

Statistical differences were analyzed by Student's t test and nonparametric Wilcoxon test. A value of $p<0.05$ was considered statistically significant.

Results

Tab. 1 presents serum concentrations of IL-6, IL-8 and IL-10 in breast cancer patients and healthy women (control group), respectively. With reference to the control group differences were considered statistically significant. Serum concentration of the IL-6 were increased in 39 (86.7%), IL-8 in 36 (80%) and IL-10 in 35 (77.8%) patients. The positive significant correlation between serum concentrations of the IL-6 and IL-8 ($p<0.05$) and negative significant correlation between serum concentrations of the IL-10 and interleukins IL-6 and IL-8 ($p<0.05$) was demonstrated. The distribution of the interleukins serum concentration according to the clinical stages of breast cancer is shown in *Tab. 2*. Higher serum concentrations of IL-6, IL-8 and IL-10 were seen in stage III of the disease compared to values obtained from stage II patients. Statistically significant differences were seen only with reference to the IL-6 serum concentration ($p<0.05$), whereas IL-8 and IL-10 serum concentrations were not significantly correlated with the stage of the disease.

Discussion

The main cause of death of breast cancer patients initially treated with radical surgery and/or radiotherapy is almost inevitable local or distant disease relapse. Recently a contribution of immune system disturbances to cancer cells growth and metastasis has been demonstrated. Presence of malignancy triggers interleukins cascade responsible for the modulation of activity of the whole immune system. IL-6, IL-8 and IL-10 profoundly affect immune response mechanisms [13,15]. These interleukins, produced by immunocompetent cells as well as cancer cells, exert their effects on various host cell types resulting in a variety of biologic responses, such as induction of proliferation and differentiation of immunocompetent cells or induction of acute-phase proteins [13]. Interleukins may also stimulate cancer cells growth and contribute to locoregional relapse as well as metastasis [4,5,7]. Permanent synthesis and release of these cytokines lead to augmentation of their serum level that might be utilized as a marker of immunity status and immune system activation in prognosis and monitoring of the course of cancer. Recently, a various aspects of clinical utilization of interleukins serum levels in ovarian cancer, cervical cancer, prostate cancer, kidney cancer and lung cancer have been evaluated [3,7,20]. It has been showed that breast cancer cells lines are able to produce and secrete IL-6, IL-8 and IL-10 [11]. However, results obtained by different authors clinically are somewhat inconsistent. Some authors [17,18,21] reported elevated IL-6 and IL-8 serum concentration before treatment initiation as indicators of poor prognosis in breast cancer patients. Others [10,16] did not reveal such dependence. Thus, clinical value of these interleukins serum concentration monitoring in breast cancer patients remains to be fully elucidated.

In the present study it has been demonstrated that breast cancer is associated with elevated serum concentrations of IL-6 and IL-8 that are known stimulators of angiogenesis as well as cancer cells proliferation and growth [1,9]. Moreover, significantly higher serum concentrations of IL-10 in breast cancer patients compared to control group subjects have been documented. In addition, high values of IL-10 serum concentration were associated with low serum concentrations of IL-6 and IL-8, whereas elevated values of IL-6 and IL-8 serum concentration were detected in breast cancer patients with confirmed low serum concentration of IL-10. Interleukin-10 is a multifunctional cytokine that may inhibit both immune cellular-type response and Th-1 (CD4 +) – mediated functions of immunocompetent cells that are able to produce IL-6 and IL-8 leading to the progression of malignancy [2,12,15]. Convincingly, host immune response as well as interleukins production by cancer cells resulting in increased values of IL-6, IL-8 and IL-10 serum concentration in advanced breast cancer patients.

In summary, the present study indicates that elevated IL-6, IL-8 and IL-10 serum concentration are strongly associated with breast cancer and it also correlate with clinical stage of disease. This can be possibly used to diagnose women with breast cancer and to identify patients with a poor prognosis who may benefit from more aggressive management.

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An evaluation of laryngeal cancer morbidity time trends in Lithuania

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Abstract

Purpose: To make assessments of the rates of cases of larynx cancer in Lithuania in the years 1978-2001 as well as possible trends of changes in the future.

Material and methods: The data contained in the Lithuanian Cancer Register for the period 1978-2001 about new cases as well as the data compiled by the Lithuanian Department of Statistics on the average number of population of Lithuania within the same period in the same age groups have been used in the course of the study. The data have been standardized by age using direct method, in accordance with the European standard; a regression analysis of larynx cancer case rates was made.

Results: After standardization of data for the period 1978-2001, tendencies of increase have been registered both among men and women: in 1978 the case rate per 100 000 population was 10.73 among men and 0.26 among women, in 2001 the corresponding data were 11.6 among men and 0.7 among women. Throughout the period the investigated case rate for men was higher than for women. An increasing average age of men and women suffering from this disease has been noticed: average age for men is annually increasing by 0.1566 years and for women – 0.0602 years. The forecast for men in the year 2006 is 13.88 cases per 100 000 population and 0.54 for women.

Conclusions: The increase of larynx cancer case rates is growing more rapidly among women than among men, and also average age of the patients is increasing. The forecast is that in 2006 the case rate will be growing up, and both men and women will get ill at older age.

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Key words: case rates, larynx cancer, prognosis, trends of changes.

Introduction

In Lithuania in 1995 larynx cancer accounted for 1,8 per cent of all malignant diseases, in 2000 – 1.5 percent. Within the period 1978-2001 average number of persons getting every year ill with larynx cancer was 179 men and 10 women. The index of cases for men per 100 000 population increased from 10.73 in 1978 to 11.6 in 2001, the corresponding figure for women has grown from 0.26 to 0.7.

The number of case rates in Lithuania, in comparison with case rates of such localization cancer in other European countries is not very high, however they are diagnosed late, stages III–IV predominating. According to data of Cancer Register – 2001 kept by the Institute of Oncology of the University of Vilnius (there under – IO VU), larynx cancer of stage III among men accounted for 38.5 per cent, stage IV – 21.3 per cent, among women – stage III – 50 per cent and stage IV – 18.8 per cent of cases [1-4].

Cancer of larynx is one of the most common malignancies in Europe, with about 52 000 new cases per year, of them 90% occurring in men. The yearly incidence rate for men in Southern and Northern Europe is between 18 per 100 000 and 6 per 100 000, respectively. For women incidence rate is not higher than 1.5 per 100 000 per year [5,6].

Increasing incidence has been reported from Canada, Italy, Denmark, the United States, Australia, especially among females. In Finland an overall decreasing incidence rate has taken place among males since early 1970s. This was caused exclusively by a decrease of supraglottic cancer cases. This is probably due to the strong decrease in the prevalence of smoking in Finland [7]. Among the European men population, the incidence of larynx cancer increases with age, with most carcinomas being diagnosed in individuals aged 65 or more (about 45% of all cases), and a peak incidence in the 6th and 7th decades with about 50 new cases per 100 000 per year [6].

Figure 1. Regression of larynx cancer case rates among men (comparison of standardized and non-standardized data).

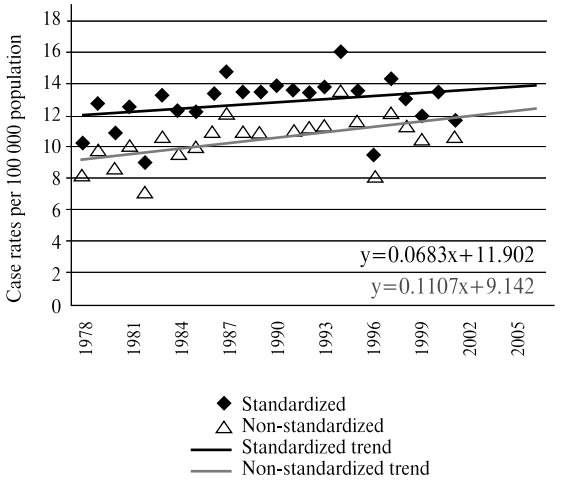
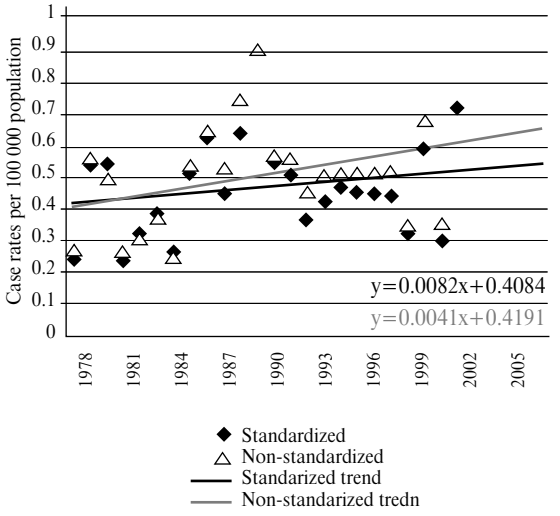


Figure 2. Regression of larynx cancer case rates among women (comparison of standardized and non-standardized data).



Examining the dynamics of the cancer of larynx the trends of growth both among women and men can be observed. An evident growth in the context of case rates is not recorded (although throughout the years some fluctuation is noticed) but calculating the case rates this index is increasing because of the reduction of population.

Material and methods

To investigate the number of case rates in Lithuania primary data contained in the Lithuanian Cancer Register within the recent 24 years (1978-2001) had been used. The data were grouped by sex (men, women), five-year age groups (totally 18 groups) in accordance with recommendations of WHO, namely, 0-4, 5-5, 10-14, 15-19, 20-24, 25-29, 30-34, 35-39… 70-74, 75-79, 80-84, 85+. The Department of Statistics has also collected data about the average number of population of Lithuania during the period under discussion in the age groups referred to above.

To assess the trends of development of case rates among men and women (the number of case rates per 100000 population) a direct regression analysis was employed.

Y = a + bX, where
Y – case rates of larynx cancer (theoretical value)
a – constant
b – slope of regression line or average absolute annual change (the number of cases per 100000 population)
X – time (year)

Statistical reliability of the slope rate of regression line has been checked using statistical hypotheses. To this end a calculation of probabilities (P_b) [8] was made.

$$P_b = 2P\left(\left|\frac{b-\beta}{S_b}\right| \geq Z_{P/2} | H_0 : \beta = 0\right) \quad b$$

Probability P_b expresses the lowest level of significance where zero hypothesis ($H_0\beta = 0$) can still be rejected.

Making regression analysis both standardized and non-standardized data have been used. Linear regression equations obtained have been used to assess the forecast case rates trends. Confidence intervals of the forecasts have also been assessed (with 95 per cent of confidence level) [9]. Besides absolute changes in the case rates corresponding to the slope of regression curve average annual growth of larynx cancer case rates have also been calculated disclosing, in per cent, average annual increase of the number of cases. Average annual growth rates in per cent have been calculated using geometrical average employing data obtained by using regression equations [9]. To make graphical comparisons a logarithmic scale was used because larynx cancer case rates among men are much higher than between women.

Examining the dynamics of larynx cancer case rates in the average age groups direct regression and weighted average methods were employed. To calculate the weighted average medians of the age groups were employed taking the number of cases as weights. After calculating average weighted age of the persons with larynx cancer, for every year of the period studied on the basis of the data obtained, regression analysis was made.

Results

Changes in the larynx cancer case rates among men

Within the period 1978-2001 in the average 179 men were having larynx cancer in Lithuania. The index of case rates per 100000 of population has increased from 8.16 person in 1978 to 10.7 person in 2001. The average case rate figure within the period under discussion was 10.5 persons per 100000 population (*Fig. 1*). After standardizing the data corresponding data were as follows: in 1978 10.73 cases per 100000 population, 11.6 persons in 2001, average case rate – 12.75. The fact that the average figure is higher than the data for both 1978 and 2001 evidences that, in comparison with actual data for the

Table 1. Parameters of regression analysis of larynx cancer case rates in Lithuania for the period 1978-2001 and the forecast for 2006.

Sex	Average (b)	P_b	Average annual change, per cent	Determination coefficient (R^2)	Forecast (95% intervals of confidence)
Men					
Rough	0.11	0.0068	1.06%	0.29	12.36 (10.98 – 13.73)
Standardized	0.07	0.1648	0.54%	0.09	13.88 (12.12 – 15.64)
Women					
Rough	0.008	0.0914	1.64%	0.12	0.65 (0.47 – 0.82)
Standardized	0.004	0.3428	0.88%	0.04	0.54 (0.38 – 0.70)

Figure 3. Comparison of larynx cancer case rates for men and women (standardized data).

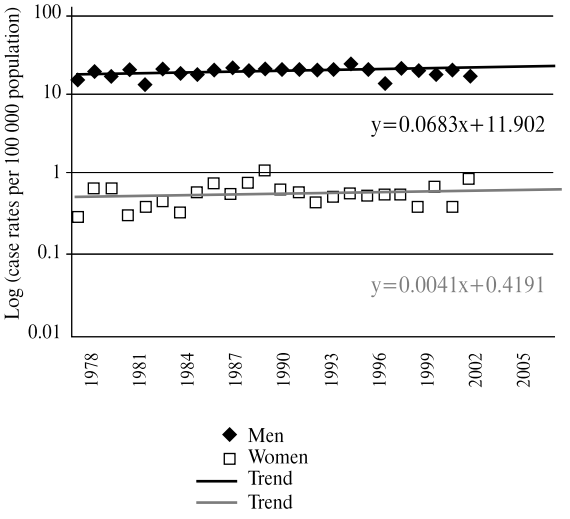
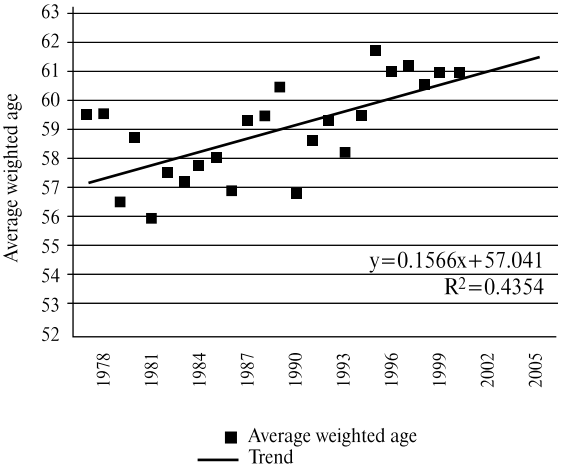


Figure 4. Age regression of men (weights – larynx cancer case rates).



period 1978-1994, beginning with 1995 a tendency of reduction of cases appeared (*Fig. 1*).

Changes in the larynx cancer case rates among women

Within the period under discussion (1978-2001) in the average almost 10 women, that is 18 times less than men, were suffering from the cancer of larynx every year. However, the case rate per 100 000 population increased from 0.28 persons in 1978 to 0.86 in 2001, that is, 3 times (actual non-standardized data) (*Fig. 2*). Average case rate for women within the period under discussion was 0.51 persons per 100 000 population. After standardizing the data the case rate per 100 000 population were as follows: 0.26 in 1978, 0.70 in 2001, average case rate – 0.47 (*Fig. 2*). Regression analysis of larynx cancer case rates for men and women is generalized in *Tab. 1*.

As can be judged by *Tab. 1*, after standardization of data average annual change, in per cent, in the case rates both for men and women has reduced by nearly 2 times. It shows that as people are getting older larynx cancer case rates are significantly influenced. Both for men and women quite low determination coefficients are observed showing that the models applied explain only a small part of the reasons for getting ill with the larynx cancer: in case of non-standardized data regression equation for men explains only 29 per cent of variation of larynx cancer case rates with time for men and only 12 per cent for women. After standardization of data preciseness and reliability of models reduces even more. Such conclusions are confirmed by the probabilities of the slope of regression line (P_b): after standardization of data the probabilities increase very much, that is, much doubts arise if the regression slope coefficient differs greatly from zero. Especially high probability (P_b) is observed investigating data for women because larynx cancer is not a very typical disease for women and therefore there are no such marked and reliable trends of case rates or morbidity trends. Judging by the data presented one can make a conclusion that in the future ceteris paribus larynx cancer case rates are expected to increase in 2006 with 95 reliability. Larynx cancer case rates per 100 000 men population are expected to be 10.98-13.73 and 0.47-0.82 for women (non-standardized data) (*Fig. 3*).

Analyzing larynx cancer case rates for men and women (*Fig. 3*) we have made a conclusion that larynx cancer case rates have a tendency to increase, yet the rates for men are faster. Average annual increase of absolute larynx cancer case rate for men is 0.0683 while for women it is only 0.0041. Comparison of annual average changes, in per cent, is somewhat complicated because the case rate for women is much less than for men,

Table 2. Parameters and forecasts of age of those having larynx cancer by 2006.

Sex	Average change (b)	P_b	Average annual change, per cent	Determination coefficient (R^2)	Forecast (95% intervals of confidence)
Men	0.16	0.00045	0.27%	0.44	61.58 (60.17 – 62.99)
Women	0.06	0.5925	0.10%	0.01	62.89 (58.78 – 67.00)

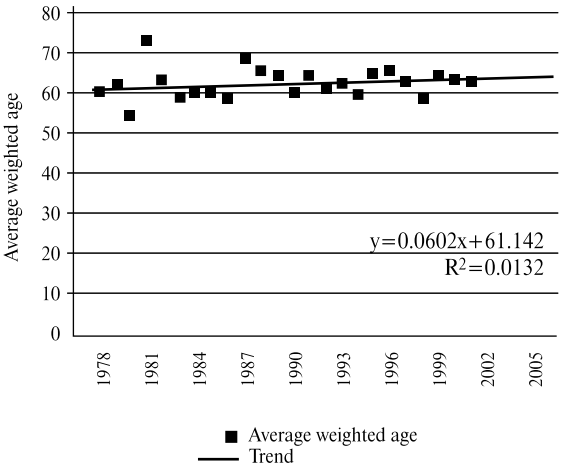
therefore the basis for making a comparison is less. Average annual larynx cancer case rate change for men is 0.54 per cent, while for women it is only 0.88 (standardized data). Thus, we can see that because of different basis for comparison (we have found that potential tempos of the case rate growth are bigger than absolute tempos of growth for men).

Regression analysis of larynx cancer case rates by age groups

Men At the beginning of the period investigated average age of men having larynx cancer was about 59.5 years while at the end of the period – already about 61 years (actual data). As can be judged by *Fig. 4*, men of older age groups are more often getting ill with this disease. If such tendencies remain in the future in 2006 one may expect that the average age of ill men will be about 61.58 years.

Women As can be judged by *Fig. 5*, average age of women having larynx cancer has within the period investigated did not undergo essential changes – in 1978 average age was 60 years and in 2001 – 62.63 years. It is probable that in 2006 the figure will reach 62.89 years (*Tab. 2*).

Figure 5. Age regression of women (weights – larynx cancer case rates).



Discussion

Analyzing larynx cancer case rates in Lithuania within the period 1978-2001 one may observe the tendencies of growth both among men and women (standardized actual data): in 1978 the case rate per 100000 population was 10.73 for men and 0.26 for women, while in 2001 corresponding data were 11.6 and 0.7. thus, throughout the period analyzed the larynx cancer case rate among men was 12.75 cases per 100000 population and 0.47 cases per 100000 women, that is, 27 times less than among men. It may first of all be explained by less widespread consumption of alcohol and smoking among women. Results of calculations show that both in 1978 and in 2001 the ratio of case rates among men and women remained similar although the gap is slightly decreasing – in 1978 the case rate of men established by a calculated trend equation was 28.5 times and in 2001 – 26 times higher than between women (standardized values).

The tendencies all over the world show that the above proportions are decreasing but the reduction is conditioned by the growth of the case rates among women. Judging by the data of the above Society the highest number of the case rates per 100000 population is in Spain (20 cases), slightly less in Poland, France and Italy. The data on the case rates in economically developed countries are generally 5-10 cases per 100000 population. At the same time in Sweden this figure is exceptionally low [2,5,6,11].

Investigating the age of those having the larynx cancer a tendency of growth within the average age both of men and women was registered. It may be accounted by the fact that longer and longer time is needed for cancerogenes to have an influence and, on the other hand, the better quality of medical treatment had positive effect. Then, average duration of life of men having the larynx cancer is increasing faster than that of women: the age of men is increasing annually by 0.1566 years while that of women – only by 0.0602. One may forecast that in 2005 the average life of men having the larynx cancer will reach 61.58 years and of women – 62.89 years.

Having made prognostic calculations of the larynx cancer case rates negative tendencies in the case rates both for men and women have been established. It is forecast that the case rate for men in 2006 will be 13.88 per 100000 population and for women – 0.54 cases (standardized data obtained using a calculated trend equation).

Conclusions

1. The average annual (at 1978-2001 period) increase in laryngeal cancer morbidity rates in Lithuania among males was 0.54%, among females – 0.88%. The age average of the sick persons had increased.

2. This analysis suggests that morbidity will rise until reaches 11.88 per 100 000 among males and 0.54 per 100 000

among females. The age average among the sick persons should increase if other conditions and previous trends continue.

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Cytokine and adhesion molecule concentrations in blood of patients with B-cell chronic lymphocytic leukaemia with regard to disease progression

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Abstract

Purpose: To examine concentrations of IL-8, E-selectin and VCAM-1 in blood plasma, culture supernatant and isolated broken lympocytes in patients with chronic lymphocytic leukaemia (CLL), at I and III stage of the disease according to Rai's classification and in healthy individuals constituting controls. Two types of lymphocyte cultures were carried out – nonstimulated and stimulated with mitogen.

Material and methods: A concentration assay of substances mentioned above was made using ready immunoenzymatic sets of the ELISA type.

Results: In all cases, plasma concentrations of IL-8, E-selectin and VCAM-1 were the highest in III stage of CLL and moderately increased in I stage comparing to control. The concentrations of IL-8 in culture supernatants and in broken lymphocytes and concentration of VCAM-1 in lymphocytes were increased in both stages of CLL. In contrast concentrations of E-selectin were decreased in lymphocytes in both types of culture. Significant decrease of E-selectin and VCAM-1 concentrations were observed in supernatants of nonstimmulated cultures.

Conclusion: Significant increase of E-selectin, IL-8 and VCAM-1 concentration in blood plasma may support higher activity and suggest the higher ability of leukaemic lymphocytes to migration. Increased IL-8 concentration in isolated, broken, stimulated lymphocytes may be a characteristic feature of the biochemical processes of leukaemic lymphocytes. The lowest values of E-selectin and VCAM-1 concentrations in culture supernatants and broken lymphocytes may suggest their degradation during in culture.

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Introduction

B-cell chronic lymphocytic leukaemia (CLL) belongs to the malignant lymphomas and originates from the immunological system. Its etiopathogenesis has not been fully explained yet. At present the scientists incline to the genetic theory. This states the point at which mutation is generated idiopathically or due to the activity of external agents, such as toxic chemical substances, organic substances, certain medications, and physical agents. The aberrations of chromosomes responsible for primary blastic lymphocyte transformation ultimately induce B-cell chronic lymphocytic leukaemia (B-CLL). These aberrations refer most frequently to a long arm of chromosome 13 (13q). They can also be produced by the surplus of long arm of the chromosome 14 (14q+). Neoplastic lymphocytes are blocked at the stage of G_0 division cycle, which protects them against maturation and death through apoptosis [1-3].

Interleukin-8 (IL-8) belongs to the chemokines, so-called substances having the characteristics of both cytokines and chemotactic agents. IL-8 is characterized by high biological activity and has IL-8R specific receptors belonging to a superfamily combining with G protein that binds the guanylic nucleotide. This plays a vital role in neoplastic invasiveness and metastasis [4-6]. E-selectin (endothelial leukocyte adhesion molecule 1; ELAM-1) takes part mainly in the first stage of migration i.e. leukocyte rolling. It is of interest that IL-8 is absent from nonstimulated endothelial cells, and the induction of its expression requires a new protein [7-9].

Vascular cell adhesion molecule 1 (VCAM-1) takes part in leukocyte adhesion to endothelial capillaries during inflammatory processes. An increase in its expression has been observed after IL-1β, IL-4 and IL-14 stimulation. Very-late-activated integrin 4 (VLAI-1) is a ligand for VCAM-1 [10,11].

IL-8 and VCAM-1 actively participate in lymphocyte migration, directly or indirectly, by means of appropriate

Table 1. Comparison of mean concentrations of IL-8 in the plasma, culture supernatants and in broken lymphocytes of patients at I and III stage of CLL and of healthy subjects.

Groups of study	Concentration of IL-8 (pg/ml)				
	Blood plasma	Cultures of lymphocytes			
		Nonstimulated		Stimulated with Neupogen	
		Supernatant (N0)	Broken lymphocytes (L0)	Supernatant (NS)	Broken lymphocytes (LS)
	X±SD p	X±SD p	X±SD p	X±SD p	X±SD p
Control group N=9 (C)	7.888±1.682	7.889±1.64	250.18±12.53	7.889±3.45	262.66±20.3
Patients in I stage of CLL n=9 (I)	12.76±1.684 I : C (p<0.02)	12.35±1.7 I : C (p<0.02)	512.86±95.54 I : C (p<0.02)	16.684±2.43 I : C (p<0.02)	558.74±92.7 I : C (p<0.02)
Patients in III stage of CLL n=9 (III)	15.851±1,691 III : C (p<0.02)	14.72±2.54 III : C (p<0.02)	873.82±253 III : C (p<0.0001)	17.03±2 III : C (p<0.02)	1318.7±231.7 III : C (p<0.0002)

ligands. Therefore the aim of this study was the determination of concentrations of IL-8 and VCAM-1 in the plasma, cell culture supernatants and broken lymphocytes.

Material and methods

The study material included: plasma, culture supernatant and isolated and broken lymphocytes obtained from patients with B-CLL cell (9 individuals in stage I and 9 in stage III according to Rai's classification) [12] and from 9 healthy volunteers of the same age (workers and students of the Medical University of Białystok).

The diagnosis of leukaemia 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 to peroxidases (POX), PAS and esterases. Leukaemic cell immunophenotyping was performed by means of a coulter flow cytometer EPICS XL using a group of antibodies to discriminate between B and T lymphocytes; CD19 and CD20 for B lymphocytes, and CD3, CD7 and CD8 for T lymphocytes, respectively.

In patients with CLL, examinations were carried out before treatment with cytostatics, corticosteroids, blood transfusions or possibly RTG therapy, 3-4 weeks after conventional treatment in patients at stage III of the disease. Patients with acute inflammation conditions caused by both bacterial and viral infections and allergies, were excluded from the studies.

The assay of E-selectin, IL-8 and VCAM-1 concentrations was performed on blood plasma, culture supernatant and broken lymphocytes with immunoenzymatic ELISA kits from the R&D Europe Systems (UK) and DPC Biermann GmbH (Germany). The assay procedure was not radically different from the instructions enclosed by the manufacturer. Lymphocytes were isolated using G Gradisol according to the method described by Zeman et al. [13]. Two types of cultures were always grown from the blood collected from each patient. These were nonstimulated (0) and Neupogen – stimulated in pairs. Roche Neupogen (granulocyte colony stimulating factor) in doses of 1 µl/4 ml was used to activate lymphocytes and to provoke them to blastic transformation. In each case the assay was performed in: a) the blood plasma, b) the culture supernatant without mitogens-(N0), c) a culture supernatant with Neupogen-(NS), d) broken mitogen-non-stimulated lymphocytes-(L0) and e) broken Neupogen stimulated – (LS) lymphocytes.

In order to eliminate erytrocyte impurities, hemolytic shock was induced using buffered, hypotonic liquid PBS without Ca^{++} and Mg^{++} ions. Intravital staining with trypan blue resulted in 92-98% viable cells for examination.

An isolated leukocyte culture was performed for 4 hours on the Parker medium according to a Kuhn and Gallin's method [14]. The isolated lymphocyte count with regard to the donor was between $4x10^6$ and $6x10^6$ of cells/1 ml of culture.

After incubation, the cells were washed and centrifuged, then broken by means of a Vibra Cell ultrasound homogenizer (Sonics Material, USA) at a power of 50 W with a frequency above 80 Mhz for a 20 seconds.

All data are displayed as mean ±SD. Comparisons between groups were performed using Student's t-test. A value of $p<0.05$ was deemed significant.

Results

The determination of IL-8 concentrations in the plasma of B-CLL patients showed their significant increase at stages I and III of the disease. The highest increase was in plasma at stage III of B-CLL ($p<0.02$) (*Tab. 1*).

In the culture supernatant, a significant increase was observed in IL-8 concentrations at the stages I and III of B-CLL patients in non-stimulated groups: (N0 I) ($p<0.02$), (N0 III) ($p<0.02$) and in stimulated (NS I) ($p<0.02$), (NS III) ($p<0.02$) in comparison to control groups. IL-8 concentrations

Table 2. Comparison of mean concentrations of E-selectin in the plasma, culture supernatants and in broken lymphocytes of patients at I and III stage of CLL and of healthy subjects.

Groups of study	Concentration of E-selectin (ng/ml)				
	Blood plasma	Cultures of lymphocytes			
		Nonstimulated		Stimulated with Neupogen	
		Supernatant (N0)	Broken lymphocytes (L0)	Supernatant (NS)	Broken lymphocytes (LS)
	X±SD p	X±SD p	X±SD p	X±SD p	X±SD p
Control group N=9 (C)	3.7548±0.9277	4.1702±0.308	0.832±0.136	0.498±0.123	0.409±0.094
Patients in I stage of CLL n=9 (I)	3.7284±0.6662 I : C N.S.	0.1564±0.015 I : C (p<0.002)	0.624±0.099 I : C N.S.	0.44±0.12 I : C N.S.	0.084±0.021 I : C (p<0.02)
Patients in III stage of CLL n=9 (III)	7.08±1.22 III : C (p<0.02)	0.3976±0.159 III : C (p<0.002)	0.63±0.099 III : C N.S.	0.44±0.12 III : C N.S.	0.084±0.021 III : C (p<0.02)

Table 3. Comparison of mean concentrations of VCAM-1 in the plasma, culture supernatants and in broken lymphocytes of patients at I and III stage of CLL and of healthy subjects.

Groups of study	Concentration of VCAM-1 (ng/ml)				
	Blood plasma	Cultures of lymphocytes			
		Nonstimulated		Stimulated with Neupogen	
		Supernatant (N0)	Broken lymphocytes (L0)	Supernatant (NS)	Broken lymphocytes (LS)
	X±SD p	X±SD p	X±SD p	X±SD p	X±SD p
Control group N=9 (C)	13.712±3.04	1.022±0.019	0.4718±0.051	0.1698±0.0315	0.359±0.067
Patients in I stage of CLL n=9 (I)	26.752±2.515 I : C (p<0.02)	0.247±0.033 I : C (p<0.002)	0.4478±0.029 I : C N.S.	0.2952±0.133 I : C N.S.	4.1±0.39 I : C (p<0.002)
Patients in III stage of CLL n=9 (III)	29.74±2.941 III : C (p<0.02)	0.247±0.103 III : C (p<0.002)	0.483±0.115 III : C N.S.	3.005±0.711 III : C (p<0.002)	4.24±0.13 III : C (p<0.002)

were found to be about twofold higher in stage III before stimulation and I and stage III of B-CLL after stimulation. The moderate increase was in B-CLL-patients at stage I in comparison to the control group.

IL-8 values in isolated, broken lymphocytes were generally higher in comparison to the IL-8 concentrations in the control groups; the highest level – sixfold higher – occuring at stage III of B-CLL after stimulation (LS III) ($p<0.0002$). A moderate increase – four-fold – was observed in lymphocytes at stage III non-stimulated ($p<0.0001$). Concentrations of IL-8 were about two-fold higher at stage I of B-CLL patients before ($p<0.02$), and after stimulation ($p<0.02$).

Concentrations of E-selectin were slightly higher in the plasma of B-CLL patients at stage I and approximately two--fold higher in stage III of B-CLL ($p<0.002$) in comparison to the control group (*Tab. 2*).

A significant decrease was observed in E-selectin concentrations in non-stimulated lymphocyte culture supernatant: (N0 I), (N0 III) ($p<0.002$) and similar in stimulated (NS I), (NS III) in comparison to the levels from the control groups. The lowest decrease in this selectin's concentrations was observed in the supernatant from non-stimulated culture of leukaemia cells.

E-selectin values in non-stimulated, isolated, broken lymphocytes of stage I and III B-CLL patients were lower in comparison to the control group. E-selectin concentrations were significantly lower in the stimulated group than in non--stimulated groups ($p<0.02$).

Concentrations of VCAM-1 were higher in the plasma of B-CLL patients at stages I and III of the disease ($p<0.02$) in comparison to concentrations in control group (*Tab. 3*).

A significant decrease was observed in VCAM-1 concentrations in (N0 I), (N0 III) ($p<0.02$) non-stimulated lymphocyte

culture supernatant in comparison to control groups. In contrast, a significant increase was noted at stage III of B-CLL after stimulation (NS III) ($p<0.002$).

VCAM-1 concentrations were seven-fold higher in isolated, broken lymphocytes after stimulation: (LS I), (LS III) ($p<0.002$) and only slightly higher in B-CLL at stage III before stimulation in comparison with the levels of stimulated lymphocytes in the controls.

Discussion

Chemokines and adhesion molecules actively participate in the lymphocyte migration from the blood to the surrounding tissue. They play especially important role in all stages of leukocyte migration, directly or indirectly by means of appropriate ligands. This process is observed wherever there are capillaries. However lymphocyte permeation takes place in the specialized capillary venules with high endothelium existing in lymphatic organs. This migration is possible due to chemokines, adhesion molecules and their receptors [1,6,15].

Chemotaxis and the second stage of migration depends on the lymphocytes being activated by chemokines. These are small proteins about 8-10 kDa in weight. Protein G is the basic link for this stage of migration [4]. IL-8 belongs to the most potent chemotactic factors of the immune system, having great importance in neoplastic invasions and formation of metastases. Protein G catalyzes changes of GTP to GDP (joined with protein G subunit α) after ligand conjunction with a specific receptor. Cytoplasmatic proteins after activation are transported through the cell to the nucleus. They play an important role in the activation of gene transcription [4,5,16]. The stimulus causes the activation of the genes; after that, it causes stimulation of the biosynthesis of biologically active proteins. This allowes leukocyte migration to the region of the homeostatic disturbance almost instantanously.

Therefore, we can see some tendency of the paradoxical activation of the leukocytes to synthesis and excretion substances like IL-8 in the course of CLL. Permanent activation of the lymphocytes by these interleukins creates a situation where leukocytes respond even to the smallest stimuli [17].

The results of our examinations show a significant increase of IL-8 concentrations in the blood plasma, culture supernatants and in isolated, broken lymphocytes. They may indicate intensification of IL-8 synthesis and excretion processes by the leukaemic lymphocytes. The conditions of the culture allow for the separation of lymphocytes from other types of cells. So most probably, the significant increase of IL-8 concentrations observed in our study corresponds to the excretory function of lymphocytes. It is interesting that processes of synthesis and excretion subside after a mitogene reaction. This may suggest the particular participation of external factors in the onset and progression of leukaemic processes.

On the surface of the endothelium vessel are adressins, which are ligands to lay receptors found on the surface of the lymphocyte. Coupling these ligands initiates different processes leading to migration through the vessel wall. Migration begins at the moment the cell approaches the vessel wall. Selectins L, E and P take part in stage I of adhesion – leukocyte rolling – along the internal wall of blood vessels and stage IV – diapedesis [2,6,17].

E-selectin manifests itself only on the surface of endothelium after stimulation, for example by IL-1, bacterial endotoxin (LPS), or tumor necrosis factor-α (TNF-α). In the advanced stages of CLL, its overproduction is most likely to occur. The soluble isoforms of this selectin can be expressed in the blood plasma at all times. The expression of sE-selectin is recognized as a final indicator of endothelium activation.

The increased E-selectin concentration found in the blood plasma is supported by some authors who discuss the activation of endothelium in the course of CLL [1,4,6,8]. A significant decrease in E-selectin concentration in I and III stages of CLL in culture supernatants and in broken lymphocytes has been found which may indicate degradation of E-selectin's soluble forms in the case of the culture. In the group after stimulation, a significantly decreased E-selectin concentration in comparison with nonstimulated groups seems to be of interest. The study of this dependence will allow us to find some way to understand the specific humoral activity of the endothelial cells.

Stage III – strong adhesion – takes part among other molecules from the immunoglobulin superfamily, which are co-receptors for integrin β_2 and are participants in the intercellular adhesion [18].

Soluble VCAM-1 is liberated from previously activated endothelial cells. It is known from the literature that concentrations of sVCAM-1 are higher in patients with inflammation, arteriosclerosis, coronary artery disease and leukaemia [19].

Our studies show a significant increase in sVCAM-1 concentrations in the blood plasma of CLL-patients which appears to confirm this assumption. We also observed a significant decrease of VCAM-1 concentrations in the culture supernatant (N0) in CLL-patients. It is proposed that this molecule was subjected to some biodegradation in the course of the culture. Also it may give evidence of the less than physiological resistance of this substance to exogenous agents. For example: temperature, partial pressure of CO_2 or a lack in the culture medium are factors which consolidate the structures of VCAM-1 to protect against degradation.

A significant increase of VCAM-1 concentrations in isolated, broken lymphocytes after Neupogen stimulation may indicate the tearing off and inactivation of the molecules bound with the membrane receptors of lymphocytes during the course of mitogenic action.

Changes in substances related to the cytokine network are characterized by proliferative disorders. Enhanced values of interleukins and adhesion molecules observed in our examination may give evidence of their increase during excretion. There are no dysfunctional regulation systems of lymphocyte function during early stage of CLL. Permanently ongoing processes act in order to prevent and control the growth of cells and their excessive migration to the surrounding tissues. In vivo cytokines may undergo decomposition, inactivation or destruction by substances located in the circulatory system [20-24]. Our studies appear to confirm this assumption. In blood plasma we observed the highest concentrations of cytokines in stage III in comparison with stage I of CLL. The repair processes take place only in the patient, where many cell lines react simultaneously. In isolated cultures, with specific conditions, we can

study the excretion function of only one type of cell. The significant increase of the investigated interleukin's concentrations in nonstimulated supernatant cultures in CLL may suggest the greatest ability of leukaemic lymphocytes to excrete them. The addition of Neupogens to the cultures, imitates the effect of mitogenes in a CLL-patient's body. A significant increase of IL-8 and VCAM-1 concentrations may support the essential role of mitogens in cell multiplication processes. The significant decrease of concentrations of E-selectin in nonstimulated groups is interesting. It is probably connected with the activation of degradation processes.

In conclusion, significant increase of E-selectin, IL-8 and VCAM-1 concentration in blood plasma may support higher activity and suggest the higher ability of leukaemic lymphocytes to migration. Increased IL-8 concentration in isolated, broken, stimulated lymphocytes may be a characteristic feature of the biochemical processes of leukaemic lymphocytes. The lowest values of E-selectin and VCAM-1 concentrations in culture supernatants and broken lymphocytes may suggest their degradation during of the culture.

These investigations may appear useful in the future as an additional diagnostic index of lymphocyte migration.

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Peripheral blood lymphocyte population in children infected with Helicobacter pylori

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Abstract

Purpose: Helicobacter pylori infection in children is associated with a chronic inflammatory process of gastric and duodenal mucosa, which may have a various clinical course ranging from asymptomatic and chronic inflammatory condition to gastric ulceration. The immune system may contribute especially to chronic gastric mucosa inflammation.

The aim of our study was to assess the levels of peripheral blood T ($CD3^+$, $CD4^+$, $CD8^+$) and B lymphocyte subpopulation ($CD19^+$) in children with Helicobacter pylori infection and to evaluate their relation to degree of antrum mucosa inflammation.

Meterial and methods: The study was performed in 32 children aged 7-18 years, hospitalized due to dyspeptic symptoms. The endoscopic examination of upper gastrointestinal tract was performed and gastric and duodenal mucosa was estimated in all patients.

The endoscopic and histological evaluation of gastric mucosa was performed according to the Sydney System [4]. The urease test (CLO-test – H. pylori) was made to estimate the severity of the infection.

Results: Moderate antrum mucosa inflammation was found in 41.2% of the examined. The highest percentage of children (58.8%) presented marked inflammation. No mild inflammation was found in children examined.

Conclusions: No correlation was found between lymphocyte levels and the degree of the inflammatory changes in antrum mucosa. The evaluation of peripheral blood lymphocytes performed in children with Helicobacter pylori infection suggests that T lymphocytes may play a predominant role in this infection.

Key words: Helicobacter pylori, peripheral blood lymphocytes, children.

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Introduction

Helicobacter pylori is the most important causative agent responsible for a chronic inflammatory process in duodenal and gastric mucosa. The structural components of bacterium dependent on its strain, induce the mobilization of the host's immune system to the immune response. However, its reaction varies with regard to the host's individual features and its age [1].

Most studies present the role of T lymphocytes and their assessment in blood and gastric mucosa. The data from the literature referring to adults indicate an increase in $CD4^+$ and $CD8^+$ lymphocytes in gastric mucosa of patients with chronic inflammation caused by Helicobacter pylori infection [2,3].

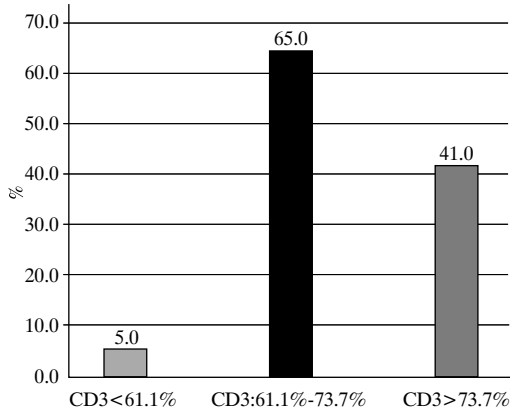
The purpose of the study was to assess the quantity of peripheral blood T ($CD3^+$, $CD4^+$, $CD8^+$) and B ($CD19^+$) lymphocyte subpopulation in children infected with Helicobacter pylori taking into consideration clinical symptoms or their absence. The results obtained were compared to the severity of gastric mucosa inflammation within antrum.

Material and methods

The study was performed in 32 children aged 7-18 years, hospitalized due to dyspeptic symptoms, in the III Department of Children's Diseases in the Medical University of Białystok. The endoscopic examination of upper gastrointestinal tract was performed and gastric and duodenal mucosa was estimated in all patients.

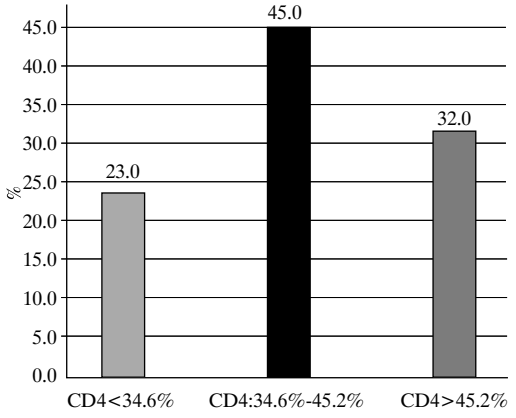
The endoscopic and histological evaluation of gastric mucosa was performed according to the Sydney System [4].

Figure 1. Evaluation of CD3 lymphocyte population in the course of Helicobacter pylori infection in children aged 7-18 years.



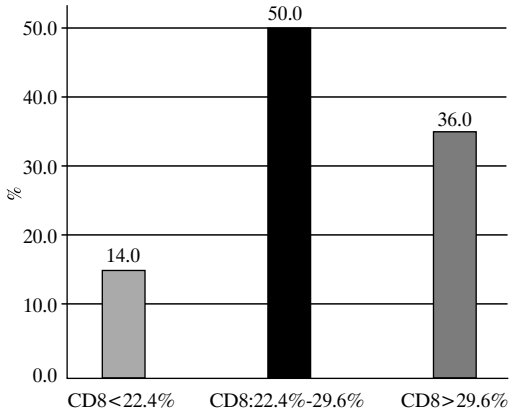
61.1%-73.3% – interval of CD3 referential values for children aged 7-18 years.

Figure 2. Evaluation of CD4 lymphocyte population in the course of Helicobacter pylori infection in children aged 7-18 years.



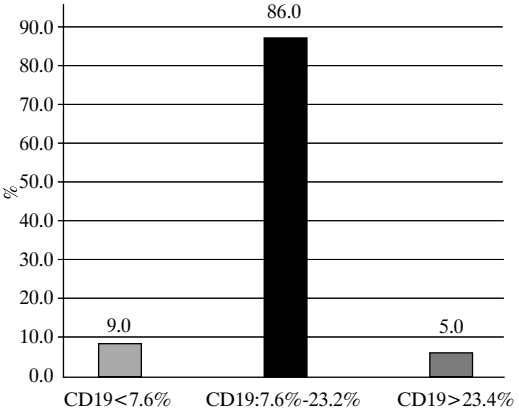
34.6%-45.2% – interval of CD4 referential values for children aged 7-18 years.

Figure 3. Evaluation of CD8 lymphocyte population in the course of Helicobacter pylori infection in children aged 7-18 years.



22.4%-29.6% – interval of CD8 referential values for children aged 7-18 years.

Figure 4. Evaluation of CD19 lymphocyte population in the course of Helicobacter pylori infection in children aged 7-18 years.



7.6%-23.2% – interval of CD19 referential values for children aged 7-18 years.

The urease test (CLO-test – H. pylori) was made to estimate the severity of the infection.

The histological assessment of antrum and corpus mocosa was carried out according to the Sydney System. It estimated the severity of Helicobacter pylori infection and the severity of antrum and corpus mucosa inflammation using hematoxylin and eosin (H-E) and modified Giemsa staining.

Peripheral blood lymphocyte subpopulations were examined with a flow cytometry instrument – EPICS XL of COULTER firm, using specific monoclonal antibodies against superficial antigen by means of triple fluorescence. The following types of peripheral blood cells were analyzed: $CD3^+$, $CD4^+$, $CD8^+$, $CD19^+$.

The values of lymphocyte populations examined in patients were compared to the referential values from the flow cytometry laboratory for the individual cells examined with regard to age intervals [5].

The study was performed in two groups of children:

1. group of 17 children (53.1%) aged 7-18 years with Helicobacter pylori – associated chronic gastric mucosa inflammation (8 girls, 9 boys; mean age – 12.9 years);

2. group of 15 children (46.9%) aged 7-15 years with dyspeptic symptoms but normal gastric mucosa and without Helicobacter pylori infection (7 girls, 8 boys; mean age – 12.0 years).

Both clinical and laboratory examinations were carried out with the consent of children's parents and approved by the Committee of Ethics at the Medical University of Białystok.

The results obtained were analyzed statistically. Statistical comparisons were done with Kołmogorov-Smirnov test. A p value <0.05 was considered to be significant. The results were presented in a graphic form.

Results

The evaluation of peripheral blood $CD3^+$ lymphocyte T population was performed using specific monoclonal antibod-

Figure 5. Evaluation of CD3 lymphocyte population in blood serum of children Helicobacter pylori infected and the severity of antrum mucosa inflammation.

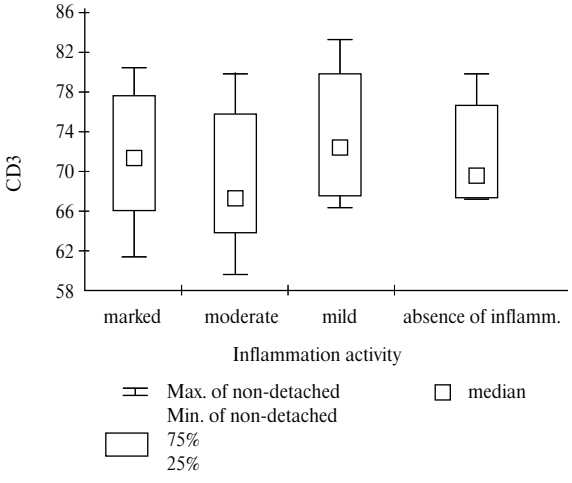


Figure 7. CD8 lymphocytes in blood serum of children infected with Helicobacter pylori and severity of antrum mucosa inflammation.

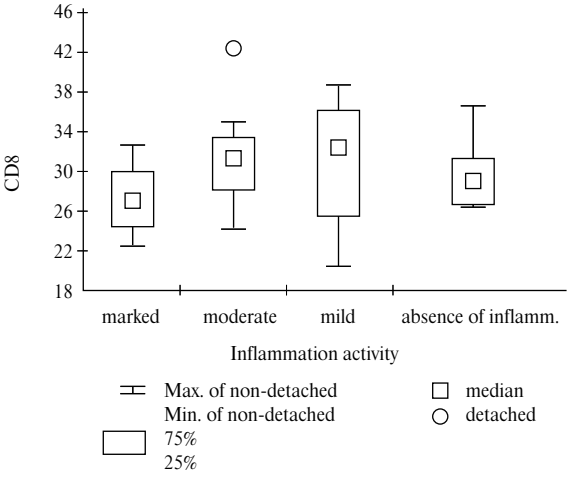


Figure 6. CD4 lymphocytes in blood serum of children infected with Helicobacter pylori and the severity of antrum mucosa inflammation.

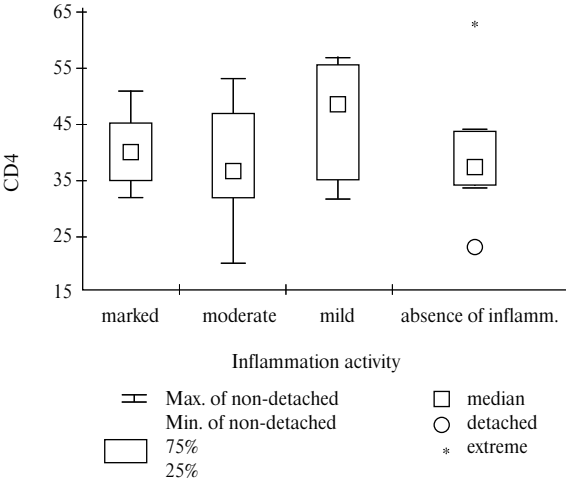
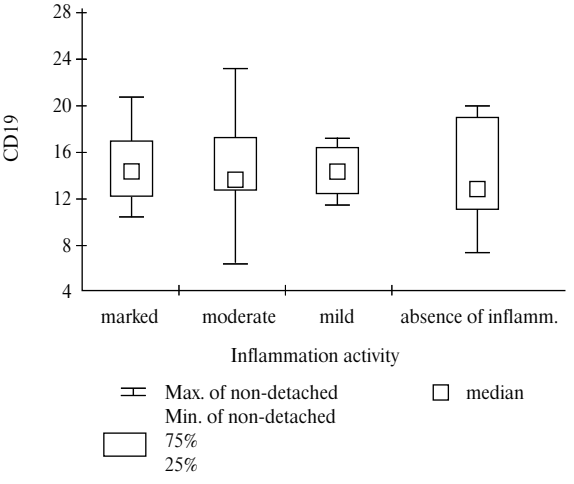


Figure 8. CD19 lymphocytes in blood serum of Helicobacter pylori infected children and severity of antrum mucosa inflammation.



ies against superficial antigen by means of triple fluorescence. It proved an increase in the percentage of these cells above the upper limit of referential value interval for the age in 41.0% of the examined. In 5% of children infected, the values of $CD3^+$ were below the lower limit of referential value (*Fig. 1*).

The evaluation of $CD4^+$ lymphocyte T population in the course of Helicobacter pylori indicated the values below the interval of referential values for the age of 7-18 years in 23% of children included in the study. The values of $CD4^+$ were above the upper referential value for this age group in 32% of the examined (*Fig. 2*).

In our study, the assessment of peripheral blood $CD8^+$ lymphocyte T population showed the values of these cells within the referential value interval in 59% of cases children infected with Helicobacter pylori and above the upper limit of norms accepted for this age in 36% of the examined (*Fig. 3*).

The evaluation of peripheral blood B lymphocytes ($CD19^+$) proved that their values were included in the referential value interval in 86% of children examined; they were below – in 9%, and above the age norms accepted – in 5,0% of the examined cases (*Fig. 4*).

The histological evaluation of gastric mucosa biopsies performed in children with infected Helicobacter pylori according to the Sydney System, made it possible to estimate the severity of mucosa inflammation. Moderate antrum mucosa inflammation was found in 41.2% of thesaces. The highest percentage of children (58.8%) presented marked inflammation. No mild inflammation was found in examined children. Moderate antrum mucosa inflammation measured by the number of infiltrating granulocytes was observed in 52.9% of cases, whereas marked inflammation was found in 47.1% of children Helicobacter pylori infected.

The analysis of percentage values of peripheral blood T and B lymphocyte subpopulations proved no statistically significant differences in comparison with the severity of gastric antrum mucosa inflammation in children Helicobacter pylori infected.

The following figures present the results obtained in our study: *Fig. 5, 6, 7, 8*.

Discussion

Antigen-specific and non-specific response of the immune system caused by Helicobacter pylori antigen stimulation leads to gastric and duodenal mucosa inflammation. This response may promote the infection elimination, but may also contribute to gastric mucosa damage.

In people with infected Helicobacter pylori, specific IgG and IgA antibodies in gastric secretion prove that gastric mucosa takes part in a immune response [6]. Helicobacter pylori colonization in the layer of mucus or under the epithelial cells of the stomach is accompanied by immunologically competent cells. Hence, it is not completely known how a cell response influences the bacteria colonization. Gunasekaran et al. [6] described the increase in the quantity of $CD4^+$ and $CD8^+$ within mucosa follicles and $CD8^+$ in gastric epithelium during Helicobacter pylori infection.

D'Elios et al. [7] proved that in patients with chronic non-ulceric gastric or duodenal inflammation, cytokines of Th1 profile were balanced by Th2 cytokin production, which might be crucial in inducing a protective response in such patients.

Bamford [8] showed that the profile of cytokines produced by $CD4^+$ phenotype lymphocytes isolated from gastric mucosa and exposed in vitro to urease during 48 hours was the same as in patients infected and not infected with Helicobacter pylori. Th1 type cytokines were found in both groups.

In our study, the assessment of $CD3^+$ lymphocytes indicated an increase in the percentage of these cells above the upper limit of the referential value interval for the age in 41% of children examined. The values of $CD3^+$ were below the lower limit of the referential value interval for the age only in 5% of children infected with Helicobacter pylori.

The assessment of $CD4^+$ lymphocytes in the course of Helicobacter pylori infection showed the values below the interval of referential values in 23% children aged 7-18 years.

According to Crabtree, $CD8^+$ and $CD22^+$ cells are the main lymphocyte subpopulation among the lymphocytes isolated from gastric mucosa in patients infected with Helicobacter pylori infection. Though, it is not known whether they have a cytotoxic or suppressive function [9].

In our examinations, the values of $CD8^+$ lymphocytes within the referential value interval were found in 50% of children Helicobacter pylori infected and above the upper limit of the age norms – in 36% of the cases.

The results of our study indicate that peripheral blood T lymphocytes as well as $CD4^+$ and $CD8^+$ cell subpopulation were enhanced in 1/3 of patients.

The cytometric examination carried out by Quiding-Jarbrink [10] showed that there was an increase in the production of $CD4^+$ and $CD8^+$ cell subpopulations in Helicobacter pylori infected patients with ulcerative duodenal inflammation and in asymptomatic ones. Patients with Helicobacter pylori infection manifested increased IFN gamma levels [7]. This and other cytokines produced in response to T lymphocytes may be responsible for the intensified secretion of hydrochloric acid in the stomach and lead to duodenal ulceration [10].

In other study, the humoral and cellular reactions were estimated in the group of adult patients with chronic gastric mucosa inflammation and Helicobacter pylori infection and the uninfected group but stimulated with the extract of Helicobacter pylori antigens. The similar reactions were observed in both groups, but a significantly higher level of IFN gamma was reported in patients stimulated with the extract of Helicobacter pylori antigens [11].

Additionally, Th1 cells were proved to recognize specifically Helicobacter pylori antigens. The predominance of Th1 type response is not profitable for the host and the infection with highly virulent Helicobacter pylori bacterium strains together with such factors as individual genetic characteristics may lead to chronic gastric mucosa inflammation and other diseases [11].

A proper immune response which would cause the shift of cytokine profile to Th2 response leading to the initiation of the protective response of the immune system against specific Helicobacter pylori antigens, would contribute to lessening the tissue damage of the host.

The values of B lymphocytes ($CD19^+$) were within the interval of referential values in 86% of the examined cases and their increase was found in 5% of our patients.

The assessment of peripheral blood lymphocytes in children with Helicobacter pylori infection seems to confirm a dominant role of T cell in this infection.

Helicobacter pylori infection – associated inflammatory changes in gastric mucosa may persist for a few to more than ten years manifesting themselves in neutrophil infiltration in the stage. The size of leukocyte infiltration correlates with the severity and activation of gastric mucosa inflammation within antrum and corpus [12].

The results of morphological examination of gastric mucosa proved that inflammatory changes in chronic gastric mucosa inflammation differed with regard to the severity of inflammation.

The analysis of the percentage values of peripheral blood lymphocyte subpopulations with regard to the severity of the inflammatory process in the stomach in the course of Helicobacter pylori proved no statistically significant differences for these lymphocytes. The results obtained were presented in figures.

Our earlier assessment of corpus and antrum mucus cells using monoclonal antibodies showed the increase in $CD8^+$ and $CD20^+$ lymphocytes in patients Helicobacter pylori infected [12].

Hatz et al. indicated a high percentage (95%) of T cells ($CD3^+$) in the mucosa of patients with Helicobacter pylori infection. $CD4^+$ cells predominated in the lamina propria and $CD8^+$ lymphocytes constituted 75-80% of intraepithelial lymphocytes (IEL). B cells ($CD22^+$) were rarely found and lymphoid follicles were absent, whereas in our study, lymphoid follicles were reported on average in more than half of patients infected with Helicobacter pylori. The results of our study are in agreement with other authors' findings [13-15].

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Comparative evaluation of gastric mucosa morphological changes in children and adults with positive IgG antibodies to Helicobacter pylori

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Abstract

Purpose: Helicobacter pylori colonization of gastric epithelium causes a local and systemic, cellular and humoral immune response. Despite this immune response involvement in the infection, its elimination from the organism does not take place and the process usually becomes chronic.

The purpose of the study was to establish the prevalence of gastric mucosa inflammation in children and adults with serum positive anti-Helicobacter pylori antibodies IgG.

Material and methods: The study included 171 patients comprising 109 (63.7%) children and 62 (36.3%) adults with IgG positive titre against Helicobacter pylori, who were qualified to the study basing on epidemiological examinations estimating the prevalence of Helicobacter pylori infection in the population of north-eastern Poland living in the country, town and city. All patients reported dyspeptic symptoms. The evaluation was performed basing on the morphological (endoscopy) and histopathological examinations estimating the changes in gastric mucosa of these patients.

Results: The evaluation of antrum and corpus gastric mucosa proved normal gastric mucosa in 34 children (31.1%) and 10 adults (16.1%) with positive IgG antibodies against Helicobacter pylori. The evaluation of the severity showed the predominance of moderate inflammation within corpus in children (37.6%) and marked inflammation in adults (45.1%).

Conclusions: The concentration of IgG antibodies against Helicobacter pylori in both groups was highest in patients with marked antrum gastric mucosa inflammation.

Key words: Helicobacter pylori, gastric mucosa inflammation, children, adults.

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Introduction

Helicobacter pylori infection causes gastric mucosa inflammation in both children and adults. Neutrophilic activation is an important feature of this inflammation [1,2]. Leukocytes are found in the pyloric part and the corpus of the stomach. They are located in the lamina propria of the mucosa, within the epithelium (especially at the neck of the nodules) and in the lumen of foveoli, where "foveolus abscesses" can be formed. The density of neutrophils within the epithelium is correlated with the extent of mucosa damage and the intensification of Helicobacter pylori infection [3]. Neutrophils are very sensitive indicator of the presence or absence of Helicobacter pylori and disappear in few days after its eradication. If the infiltrates are still found in the sample obtained after treatment and no bacteria are discovered, special staining should be applied to detect the bacteria [4,5].

However, lymphocyte aggregates are the most characteristic histological feature of gastric mucosa inflammation caused by this strain of bacterium. They are found in 100% of Helicobacter pylori infection cases. Their equivalent in a macroscopic picture is nodularity of the antral mucosa. Moreover, less or more diffused chronic infiltrate, which can differ (even by two grades) between the antrum and corpus, is observed in most of gastric mucosa samples in the histopathological examination [6].

The purpose of the study was to establish the prevalence of gastric mucosa inflammation in children and adults with serum positive IgG titre against Helicobacter pylori basing on the morphological (endoscopy) and histopathological examinations of changes in gastric mucosa of these patients [7].

Table 1. Macroscopic picture of changes in gastric mucosa, the results of urease test and the level of IgG antibodies against Helicobacter pylori in the patients examined.

Children							Adults					
	Present		IgG	Absent		IgG	Present		IgG	Absent		IgG
	N	%	U/ml	N	%	U/ml	N	%	U/ml	N	%	U/ml
Macroscopic picture	43	39.4	164.8	66	60.5	125.8	27	43.5	136.9	33	53.2	95.7
Urease test	65	59.6	157.4	44	40.3	117.3	39	62.9	132.0	23	37.0	85.7
Lymphatic follicles	65	59.6	186.3	44	40.3	132.9	19	30.6	157.5	40	64.4	110.0

Material and methods

The study was performed in 171 patients including 109 (63.7%) children and 62 (36.3%) adults with positive anti-Helicobacter pylori antibodies IgG chosen in epidemiological examinations estimating the prevalence of Helicobacter pylori in the population of north-eastern Poland in the country, town and city. After having established serum positive IgG, all patients underwent endoscopy. They also reported dyspeptic symptoms.

The evaluation of specific IgG antibody concentration was carried out by means of the screening quantitative test of Recom Well Helicobacter IgG of MIKROGEN GmbH firm to detect and identify directly serum IgG against Helicobacter pylori. The antigens used in this test are obtained by genetic engineering; they are optimal antigens without any impurities and proteins causing cross reactions. The use of CagA recombinant antigen enhances a diagnostic value of the test. The titre above 24 U/ml is regarded as positive.

The endoscopic and histological estimation of gastric mucosa was performed according to the Sydney Classification [8]. The urease test was made during endoscopy (CLO--test = H. pylori).

The rapid urease test for Helicobacter pylori detection in gastric bioptates was made using the kits produced and distributed by The Institute of Food and Nutrition in Warsaw.

The biopsy specimens of gastric mucosa obtained from the antepyloric area were placed on the blotting – paper dripped with distilled water from the firm kit. The change of color from yellow to amaranth was considered as the result indicating explicitly Helicobacter pylori infection.

The histological examination of antrum and corpus gastric mucosa was performed according to the Sydney System, evaluating the severity of Helicobacter pylori infection and for grading the gastritis (staining with hematoxylin and eosin (H-E) and modified Giemsa).

The endoscopic examinations of the upper part of the alimentary tract in children and adults, who due to clinical indications and with positive IgG against Helicobacter pylori agreed to gastroscopy, were carried out in the Endoscopic Laboratory of the III Department of Children's Diseases of the Medical University of Białystok.

Statistical analysis was performed using The Statistica 6 package. Student-t test for paris, Student-t for two mean values, median test were used. The tests were selected depending on conditions determined by the obtained values of the parameters.

Results

In our study, the analysis of the endoscopic picture of children with positive IgG showed macroscopic changes in 39.4% of children. A similar percentage was observed in adults (43.5%).

Positive urease test was found in 59.6% of children and 62.9% of adults. Lymphatic follicles were observed in 59.6% of children and 30.6 % of adults (*Tab. 1*).

Normal gastric mucosa of antrum and corpus was found in 34 children (31.1%) and 10 adults (16.1%) with positive IgG antibodies in serum.

The estimation of the severity of the inflammation (according to the Sydney System) in corpus showed the predominance of moderate degree – in children (37.6%) and marked degree – in adults (45.1%).

The estimation of the severity of the inflammation in antrum revealed the predominance of marked degree both in adults and children (*Tab. 2*).

The marked inflammation in antrum was found in both children (44.9%) and adults (36.7%). The moderate inflammation in corpus was higher in children than in adults, in whom the marked mucosa inflammation predominated – 45% of all examined (*Tab. 3*).

Discussion

Gastric mucosa inflammation (gastritis) is an acute condition of Helicobacter pylori infection both in children and adults [9]. In adults, the infection may be accompanied by primary peptic ulcer and very rarely by MALT lymphoma [10,11]. In children, there are no such disorders in the course of Helicobacter pylori infection, frequently, despite the infection, no clinical symptoms are reported.

Helicobacter pylori infection causes initially an acute inflammatory process in gastric mucosa, which after 3-4 weeks changes into a chronic process. In the acute stage of the infection, infiltrates of polymorphonuclear cells in the lamina propria, degenerative changes and intensified epithelium shedding can be observed. The spontaneous eradication of bacteria and the regression of inflammatory symptoms can take place exceptionally. However, in most cases, gastric mucosa inflammation continues and becomes chronic.

In the endoscopic examinations, that accompany Helicobacter pylori infection, are found normal gastric mucosa, or with eryhtema, erosions, ulceration, and antrum gastric mucosa

Table 2. The incidence of histopathological changes of corpus and antrum gastric mucosa in the patients examined.

CORPUS								
Children					Adults			
Hist-path changes	inflammation		activity		inflammation		activity	
	N	%	N	%	N	%	N	%
No changes	34	31.2	34	31.2	10	16.2	10	16.2
Mild	47	43.1	5	4.6	28	45.1	4	6.4
Moderate	26	23.8	41	37.6	19	30.0	20	32.3
Marked	2	1.8	29	26.6	5	8.0	28	45.1
ANTRUM								
Children					Adults			
Hist-path changes	inflammation		activity		inflammation		activity	
	N	%	N	%	N	%	N	%
No changes	34	31.2	34	31.2	9	14.5	9	14.5
Mild	6	5.5	4	3.7	6	9.6	4	6.4
Moderate	38	34.8	22	20.2	26	41.9	9	14.5
Marked	31	28.4	49	44.9	21	33.8	40	64.5

Table 3. Severity of corpus and antrum gastric mucosa inflammation and the level of IgG against Helicobacter pylori in the groups examined*.

Hist-path changes	CORPUS					
	Children			Adults		
	N	%	IgG U/ml	N	%	IgG U/ml
No Changes	34	31.2	110.5	10	16.1	52.4
Mild	5	4.6	139.3	4	6.4	37.2
Moderate	41	37.6	148.6	20	32.2	131.2
Marked	29	26.6	167.2	28	45.1	131.7
Hist-path changes	ANTRUM					
	Children			Adults		
	N	%	IgG U/ml	N	%	IgG U/ml
No changes	34	31.2	110.5	9	14.5	53.8
Mild	4	3.6	129.4	4	6.4	39.3
Moderate	22	20.1	141.6	9	14.5	118.3
Marked	49	44.9	163.4	40	36.7	132.0

* according to Sydney Classification

nodularity, especially in children [9,12,13]. If Helicobacter pylori infection is accompanied by duodenal ulcer in children, disseminated nodularity in antrum is observed. However, when Helicobacter pylori causes only primary gastric mucosa inflammation, nodularity is observed only in 50-60% of cases [14]. The authors quoted, observed no nodularity in the case of duodenal ulcer without co-existing Helicobacter pylori infection and in none of cases without ulceration and Helicobacter pylori infection. They based their observation on the material of 5000 children. In our earlier studies, nodularity of antrum occurred in 61.1% of children with Helicobacter pylori-connected gastric mucosa inflammation [7]. When nodularity is not visible during the endoscopic examination, blood exuding during biopsy seems to be helpful. It fuses with mucosa into 'a carpet of nodules'. This method is called hematochromendoscopy [14].

In the physiological conditions, the lymphoid tissue (lymphoid follicles) are not found in gastric mucosa [8].

The elements of the lymphoid tissue are produced by an inflammatory reaction associated with Helicobacter pylori infection. It was proved that in these follicles, Helicobacter pylori activated T lymphocytes, which stimulated B cell proliferation. Lymphoid follicles were no found in any other gastric mucosa inflammation [8].

Gastric mucosa stimulation by Helicobacter pylori antigens leads to its inflammation, which is characterized by the infiltration of neutrophils with lymphocytes and plasma cells comprising epithelium and mucosal membranes in the initial stage of the inflammation. Later lymphoidal cells form the follicle structures. The absence of M cells in gastric mucosa proves that there is no direct intake of antigens (including Helicobacter pylori antigens) in the stomach [15].

On the other hand, the epithelial cells in the inflamed mucosa express the antigens of MHC class II and the antigens of B7-1 and B7-2 taking part in the antigen presentation

[15,16]. Thus, local lymphoidal cells of mucosa take part in the effector activity of the immune response producing antibodies and cytokines. The presence of lymphoid follicles is a proof of this phenomenon and they are present in 60% of children and 30% of adults infected with Helicobacter pylori in our material. Lymphoid follicles were found in patients with moderate and marked antrum gastric mucosa inflammation.

Nodularity of antrum seen in the endoscopic examination can be observed for months and years after Helicobacter pylori eradication and ulcer healing. The absence of endoscopic changes in about 50% of children with Helicobacter pylori infection resulting from a focal mucosa inflammation indicates that the biopsies must be taken from the antrum, cardia and corpus as an integral part of an examination [17-19].

In our study, the analysis of the endoscopic picture of children and adults examined showed macroscopic changes in a similar percentage in both children and adults. A significant difference was found in the prevalence of antrum nodularity in children and adults (*Tab. 1*). Nodularity of antrum was accompanied by an increase in the serum concentration of IgG against Helicobacter pylori in children and adults examined. The highest mean serum concentration of specific IgG was observed in children (*Tab. 1*).

The density of inflammatory infiltrate was proved to correlate with the intensification and extent of bacterium colonization. Helicobacter pylori invades mainly the surface of gastric epithelium and is found in the highest quantity in the epithelium of the opening of gastric nodules. The generative cell exposure of the isthmus of gastric nodules to an inflammatory infiltrate may cause cell damage and result in intestinal metaplasia and /or nodule atrophy. The presence of neutrophils always indicates active chronic gastritis.

The localization and intensification of gastric mucosa inflammation in the course of Helicobacter pylori infection are changeable and always affects antrum, next cardia, and later corpus [17].

In our study, normal gastric mucosa of antrum and corpus was found in 34 children (31.1%) and 10 adults (16.1%) with positive IgG antibodies in serum.

The estimation of the severity of the inflammation (according to the Sydney System) in corpus showed the predominance of moderate degree – in children (37.6%) and marked degree – in adults (45.1%).

The estimation of the severity of the inflammation in antrum revealed the predominance of marked degree both in adults and children (*Tab. 2*).

Antigen-specific and nonspecific response of the immune system of gastric mucosa due to Helicobacter pylori infection determines the development of its inflammation. Both types of an immune response may take part in controlling the infection and at the same time they may contribute to gastric mucosa damage.

The serum concentration of specific IgG for Helicobacter pylori was highest in patients with marked antrum inflammation. The medium value of IgG against Helicobacter pylori in children (163 U/ml) was higher than its medium values in adults (132 U/ml) with marked antrum inflammation. The mean value of IgG in children with normal mucosa was 110.5 U/ml; in adults – it was lower and equaled 53.8 U/ml.

These mean values of IgG seem to indicate that generalized humoral response of the developmental age in Helicobacter pylori infection is more potent than in adults. In spite of the higher antibody production in Helicobacter pylori infections, it can often become chronic. The access of antibodies to infectious foci formed by Helicobacter pylori rods penetrating mucosal membrane may be hindered [20].

A generalized humoral response is a systemic response to superficial Helicobacter pylori antigens and cannot be a reliable indicator of active Helicobacter pylori infection. Tests for determining serum IgG concentration cannot be used to estimate the effectiveness of bacterium eradication, either, since the level of these specific antibodies is maintained for a long time after eradication.

The histological evaluation of mucosa inflammation activity of antrum and corpus in children and adults is presented in *Tab. 3*.

The marked inflammation in antrum was found in both children (44.9%) and adults (36.7%). The moderate inflammation in corpus was higher in children than in adults, in whom the marked mucosa inflammation predominated (45% of all examined).

A higher increase in both serum IgG level and in a quantity of follicles in percentages in children seems to indicate a stronger immune response to Helicobacter pylori infection in children than in adults.

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The incidence of some civilization diseases in families of children with food allergy/intolerance

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Abstract

Purpose: The aim of the study was to evaluate the incidence of chosen civilization diseases in families of children with food allergy/intolerance. We also wanted to indicate the need for developing and implementing activities preventing these diseases among children.

Material and methods: On the basis of information from questionnaires, two groups of children were distinguished: a group of 80 children suffering from food allergy/intolerance on elimination diet (GR1) and a group of 67 healthy children (GR2) on regular diet. In GR1, the elimination diet with soya bean preparations or casein hydrolysates was introduced before the age of 6 months and continued for at least 12 months. A high risk of hypercholesterolemia according to extended American Academy of Pediatrics criteria including hypertension, diabetes and obesity was determined for children in both groups.

Results: The research showed that 31.25% of children examined according to AAP criteria and 46.25% according to extended criteria had a positive family history of premature diseases of the circulatory system. The study proved that hypertension was the most frequent cause of morbidity in families of children from a high risk group and it was found in 67.7% of families with children on elimination diet and with a positive family history and in 78.7% of families with children from GR2 with a positive family history. Obesity, coronary heart disease, hypercholesterolemia, atherosclerosis and diabetes were listed consecutively.

Conclusions: Once a positive family history of cardiovascular diseases is discovered, systematic education promoting health in a family and complex evaluation of physical and psychomotor development of the children should follow. Arterial blood pressure and lipid profile in serum ought to be monitored to eliminate risk factors of these diseases for children.

Key words: elimination diet, civilization diseases, risk factors.

Introduction

Epidemiological studies have confirmed that infectious diseases, cardiovascular diseases and malignant tumors are the main causes of morbidity and mortality in the world. The published epidemiological reports also show a dramatic increase in the number of allergic diseases. Currently, this problem affects 10 to 35% of the population and there is a constant rising tendency [1].

The high incidence of allergic diseases in Europe, such as hay fever (10-20% of population), asthma (2.5-10%) [2], dermatitis atopica and other dermatoses (10-12%) [3], necessitates an introduction of an effective systematic preventive program to reduce their occurrence. In Poland, the number of people with allergic diseases doubled in the last seven years. Today, one-fifth of the overall Polish population suffers from some type of allergy. This number is even greater in large cities, where it affects one out of every four people.

Food allergy and food intolerance are most frequent at the developmental age. Food allergy is a hypersensitivity to certain foods and substances that have been ingested [4,5]. The term "hypersensitivity to foods" refers to a dietary intolerance conditioned by immunological mechanisms usually of an early hypersensitivity type (type I according to Gell and Coombs), late hypersensitivity caused by allergic lymphocytes (type IV) or immunological complexes (type III). The combination of these three types of allergic reaction is frequently reported. Food intolerance, unlike allergy, is conditioned by non-immu-

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Table 1. The number of children in particular groups, their age and sex.

		number of children		boys		girls		average age	
		n	%	n	%	n	%	x (month of life)	SD
GR1 (n=80)	family history (+)	37	46.2	25	67.5	12	32.4	46.3	15.1
	family history (-)	43	53.8	23	53.5	20	46.5	45.1	14.5
GR2 (n=67)	family history (+)	33	49.3	15	45.4	18	54.6	50.5	14.9
	family history (-)	34	50.7	15	44.1	19	55.9	48.9	14.3

Table 2. Body weight at birth and birth term.

		body weight at birth (g)								birth term					
		<2500		>2500 <3000		>3000 <3500		>3500		premature		at term		post-term	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%
GR1 (n=80)	family history (+) (n=37)	2	5.4	5	13.5	11	29.7	19	51.3	0	0.0	34	91.8	3	8.2
	family history (-) (n=43)	5	11.6	6	13.9	16	37.2	16	37.2	6	13.9	32	74.4	5	11.6
GR2 (n=67)	family history (+) (n=33)	0	0.0	5	15.1	15	45.4	13	39.3	1	3.0	28	84.8	4	12.1
	family history (-) (n=34)	1	2.9	4	11.7	15	44.1	13	38.2	3	8.8	28	82.3	2	5.8

nological mechanisms, e.g. food indigestion or intolerance to lactose [4,6].

Undesirable alimentary reactions are observed in about 5-10% of population in North America, affecting 13% of children. In Europe, this problem affects 0.3 to 7.5% of children and this estimate increases to 10-20% for the children with a positive atopy family history [1,3]. In 1997, Polish experts under the supervision of Professor Kaczmarski presented a report on food allergy and food intolerance. It indicated that food allergy affects 5-8% of children and is the main reason of their complaints [6]. Every food product can be an allergen, especially if it contains proteins. For children in their first three years of life, hypersensitivity is usually caused by cow milk proteins. Clinical symptoms appear in 2.2-2.3% of the whole developmental age population [4,6,7].

In spite of many clinical and laboratory studies, it is often still difficult to confirm food allergy/intolerance, as there is no an ideal conclusive diagnostic test. According to the recommendations introduced in 1993 by the ESPACI (European Academy of Allergology and Paediatric Immunology) Congress, the diagnosis of food allergy requires an affirmative, double blind placebo-controlled food challenge (DBPCFC) performed with a given food product. The oral stimulus test done according to the DBPCFC recommendation is conducted mainly in clinical centres that do research into food allergy/intolerance [6].

In everyday medical practice, the diagnosis of food allergy is based on a case and family history and a medical examination of a child. It is reported that 30-40% of cases with suspected food allergy are confirmed after performing DBPCFC [4,6]. The chosen treatment in such cases is an elimination diet which permanently or temporarily eliminates harmful food and simultaneously introduces other components of equivalent nutritional value.

The aim of the study was to evaluate the incidence of chosen civilization diseases in families of children with food allergy/intolerance and to point to the need for working out and introducing activities which would prevent these diseases among children.

Material and methods

The study involved 670 children attending 5 infant nurseries, 10 kindergartens and also the children under the care of the Psychosomatic Out-Patient Clinic of the Propaedeutics of Pediatrics Department, Białystok Medical University.

The questionnaire consisted of questions referring to the child's term of birth, its body weight at birth, breast-feeding period, the age at which elimination diet was introduced and the intake of chosen food groups. The detailed family history took into account the incidence and the age at which such diseases as hypertension, atherosclerosis, diabetes, obesity, high cholesterol level and smoking occurred in the family back to the second generation. Children from families where morbid problems mentioned above were reported before the age of 55, were included in a high risk group.

On the basis of the data from the questionnaires, two groups were distinguished: a group of 80 children with food

allergy/intolerance on elimination diet (GR1) and a group of 67 healthy children (GR2) on regular diet with a positive family history of circulatory system diseases, who constituted the control group.

In GR1, the elimination diet with soya bean preparations or casein hydrolysates was introduced before the age of 6 months and continued for at least 12 months. Children at high risk according to extended AAP criteria including hypertension, diabetes and obesity were separated in both groups.

Statistics. Statistical analysis was performed by means of descriptive statistics and an independence test chi^2 with Yates and Fisher's correction using STATISTICA computer program.

Results

The number of children in particular groups, their age and sex are presented in *Tab. 1*. The average age of the children was similar in both groups and equalled $3^{9}/_{12}$-$4^{2}/_{12}$ years (*Tab. 1*). The values of BMI were also comparable: GR1=15.48 (SD=1.65) and GR2=15.65 (SD=2.32). No differences regarding body weight at birth and birth term were noted in both groups (*Tab. 2*).

The study shows, that 38% of the children on the elimination diet were breast-fed only for 2-3 months. The period of exclusive breast-feeding for 47% of children in GR2 was longer and equalled 4-6 months ($p<0.005$). In both groups, total breast-feeding period (5.2 and 5.7 months) was far too short (*Tab. 3*).

Dairy products were introduced in the case of 80% of children in GR2 (on average in the 5.15th month of life, SD=2.73, at the earliest in the 1st month of life, in the 12th month at the latest). These products were present in the diet of only 25% of children in GR1. On average they were introduced later, in about 24th month of life (SD=9.31) – at 6 months of life at the earliest and, at the latest, at 42 months of life. The difference in the number of children who were never given dairy products is statistically significant with $p<0.001$ (*Tab. 3*).

No differences were found in fish, fried food or meat intake in the tested groups. Significant differences concerned butter and margarine intake ($p<0.05$). In the group of children on elimination diet, unlike in the control group, 'soft' margarine was used more frequently than butter (*Tab. 4*).

The study shows that in families of children from both groups (GR1 and GR2) the main morbid problems concern the cardiovascular system (*Tab. 5*). Particular diseases, their incidence back to the second generation and the age at which they started, are presented in *Tab. 6*. The average value, standard deviation and minimum and maximum values are shown in each case.

The research proved that hypertension was the most frequent morbid problem in families of children from a high risk group. It was found in 67.7% of families with children on the elimination diet and a positive family history and in 78.7% of families with children from GR2. Obesity, coronary heart disease, hypercholesterolemia, atherosclerosis and diabetes were listed consecutively. The average age for the occurrence of these diseases (except for diabetes) was before the age of 50, and obesity, hypercholesterolemia and hypertension even before the age of 45 (*Fig. 1*).

Additionally, the study made it possible to find the age when the first health problems started for each family member from the group with children on elimination diet (*Fig. 2*).

It is interesting that cardiovascular system diseases occurred earlier in case of parents than grandparents. On average, the disease symptoms manifested themselves at the age of 38 for the parents (in the case of mothers sometimes as early as 30 and for the fathers even at 21). In the second generation,

Table 3. Exclusive breast-feeding period, total breast-feeding time and age at which dairy products were introduced.

		exclusive breast-feeding period								total breast-feeding time								Age at which dairy products were introduced									
		<1 m		2- 3 m		4-6 m		>6 m		<3 m		3-6 m		7-12 m		>12 m		not introduced		<12 month of life		>12 month of life	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%	n	%
GR1 (n=80)	family history (+) (n=37)	8	21.6	14	37.8	15	40.5	0	0.0	11	29.7	16	43.2	8	21.6	2	5.4	27	72.9	0	0.0	10	27.1
	family history (-) (n=43)	8	18.6	16	37.2	19	44.1	0	0.0	13	30.2	22	51.1	5	11.6	3	6.9	33	76.7	1	2.3	9	20.9
GR2 (n=67)	family history (+) (n=33)	7	21.2	6	18.1	19	52.5	1	3.03	6	18.1	16	48.4	11	33.3	0	0.0	6	18.1	27	81.8	0	0.0
	family history (-) (n=34)	7	20.5	10	29.4	13	38.2	4	11.7	7	20.5	10	29.4	17	50	0	0.0	8	23.5	25	73.5	1	2.9

Figure 1. The mean age of the onset of chosen diseases in families in GR1 (box=mean and ±1 SD; bars=maximum and minimum).

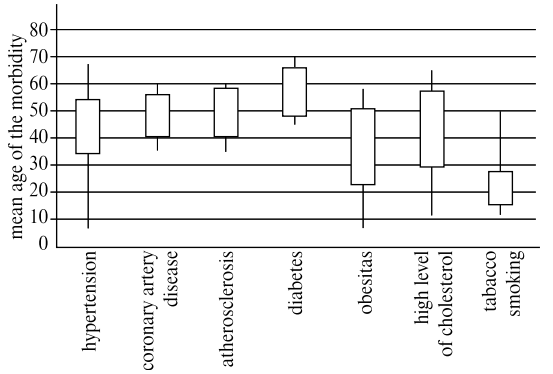
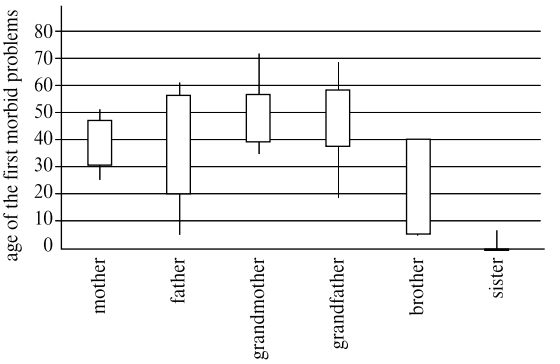


Figure 2. The age, at which the first morbid problems occurred for different family members in families of children on elimination diet (box=mean and ±1 SD; bars=maximum and minimum).



they occurred on average at the age of 46-47, at the earliest about 40. This points to a deterioration in the state of health of the middle-aged population.

The analysis of the results shows that tobacco smoking is particularly harmful. It was found that in 65% of families of children from GR1 with a positive family history, at least one person smoked, and the same was true for 100% of comparable families from GR2. There was a statistically significant difference in the occurrence of smoking addiction in the groups – it was much more frequent in the groups with the positive family history than in the groups with the negative one.

Table 4. Butter intake and margarine intake.

		butter intake							margarine intake							
		< 1 a week		1-2 a week		>2 a week		lack of data	< 1 a week		1-2 a week		>2 a week		lack of data	
		n	%	n	%	n	%	n	n	%	n	%	n	%	n	%
GR1 (n=80)	family history (+) (n=37)	22	59.4	1	2.7	14	37.8	0	9	24.3	2	5.4	26	70.2	0	0.0
	family history (-) (n=43)	14	32.5	11	25.5	17	39.5	1	19	44.1	5	11.6	18	41.8	1	2.3
GR2 (n=67)	family history (+) (n=33)	9	27.2	3	9.0	21	63.6	0	15	45.4	4	12.1	14	42.4	0	0.0
	family history (-) (n=34)	9	26.4	4	11.7	20	58.8	1	18	52.9	5	14.7	10	29.4	1	2.9

Discussion

The study indicates an urgent need for the preventive action among both groups of children: those with food allergy/ /intolerance as well as the healthy ones. The measures should be aimed at improving the state of health in the society. It was found that 31.25% of children examined according to AAP criteria and 46.25% according to the extended criteria including hypertension, obesity and diabetes had a positive family history of premature civilization diseases, mainly the diseases of the circulatory system. Therefore, a detailed interview with a patient should accompany a medical examination, to identify the children from the high risk group and to undertake early prevention activities. This opinion is supported by epidemiological analyses which predict that in 2020 about 1.5 million people out of 60 million children now living in the USA will suffer from cardiovascular diseases [8].

It is known that risk factors connected with the early occurrence of some diseases are mostly the same both for the adults and for the developmental age population. Obesity, high con-

Table 5. The main morbid problems in families of both tested groups.

Group	No	hypertension		coronary arterial disease		atherosclerosis		diabetes		obesity		high level of cholesterol (> 240 mg/dl)		tobacco smoking	
		n	%	n	%	n	%	n	%	n	%	n	%	n	%
Children on elimination diet (GR1)															
family history (+)	37	25	67.6	19	51.3	4	10.8	6	16.2	22	59.45	14	37.8	24	64.8
family history (-)	43	0	0.0	0	0.0	0	0.0	1	2.32	0	0.00	1	2.32	12	27.9
						n.s.									p=0.0416
Children on normal diet (GR2)															
family history (+)	33	26	78.7	19	57.5	16	48.4	13	39.3	18	54.5	17	51.5	33	100
family history (-)	34	0	0.0	2	5.8	0	0	3	8.8	0	0.0	2	5.8	12	35.2
						n.s.									p=0.0191

centration of total cholesterol and LDL-cholesterol are some of the factors that are often present already in childhood [9].

Clinical studies show that abdominal obesity is correlated with high values of arterial blood pressure, LDL-cholesterol and insulin. The area of atherosclerotic lesions in arteries depends on the scale of obesity, the arterial blood pressure and the HDL-cholesterol concentration [8].

In our study we found that arterial hypertension was the most frequent disease in the families of the examined children and that it started on average at the age of 44, at 7 at the earliest. This shows that arterial blood pressure should be monitored permanently in the children with a positive family history of circulatory system diseases. Obesity was found to be the second most frequently reported morbid problem. On average it manifested itself at the age of 37, but, like arterial hypertension, its first signs could be noticed as early as in the 7th year of life. This confirms the need for promoting a healthy diet, establishing proper eating habits and a healthy lifestyle in every period of life. It also points to the necessity of a systematic evaluation of nutrition in the child population.

The need for periodic lipid profile control in blood serum, especially for the population at high risk of circulatory diseases was proved by the results of research conducted within the Family Cardiology Prevention Program-Szczecin 2000. It involved children before the age of 12, whose mother or father had myocardial infarction or ischemic cerebral stroke before the age of 50. Higher concentrations of LDL-cholesterol and total cholesterol were found in this group of children when compared to those with a negative family history. Similarly, higher values of these parameters were observed in smokers' families independently of a positive family history of ischemia.

The implementation of dietary recommendations of the National Cholesterol Preventive Program in the group of children with high total cholesterol and LDL-cholesterol levels resulted in a decrease in these parameters after only 3 months [10]. The American Academy of Pediatrics in 1998 published instructions which recommended education about nutrition and other risk factors. This paper concentrate on one risk factor of atherosclerosis, i.e. hypercholesterolemia and recommend, monitoring cholesterol concentration in blood serum and introducing a diet in case of its increase [11].

According to AAP, the evaluation of cholesterol concentration in serum should be performed for children who have a parental or grandparental history (before 55 years of age) of invasive cardiovascular examinations or a documented myocardial infraction, angina pectoris, peripheral vascular disease, cerebrovascular disease or sudden cardiac death. Children of parents with increased cholesterol concentration in serum (240 mg/dl or more) and children with other risk factors and unknown family history were also included in this group [11].

In our study, cholesterol level exceeding recommended age norms was found at the age of 12 at the earliest. At present, a routine testing of cholesterol concentration in blood serum is not a sufficient or a necessary prognostic factor of coronary heart disease. However, the analysis reveals that on average an increased cholesterol level occurs at the age of 43, whereas the symptoms of coronary heart disease in the 47th year of life.

Cigarette smoking, increased blood pressure, low HDL-cholesterol, obesity, diabetes and lack of physical activity are some other risk factors connected with an early onset of coronary heart disease [8,9]. In the tested group atherosclerosis was a morbid problem occurring on average at the age of 42.9 (SD = 9.03) and diabetes at the age of 57 (SD = 8.8).

Numerous clinical studies confirm that atherosclerotic lesions may develop even in early childhood. Berenson [8] proved fatty streaks in the aorta in 50% of autopsies and fibrous plaques in coronary arteries in 8% of autopsies of children aged 2-15. The severity of lesions was directly pro-

Table 6. Chosen diseases in families, their incidence back to the second generation and the time of their onset.

	hypertension	coronary arterial disease	atherosclerosis	diabetes	obesity	high level of cholesterol (> 240 mg/dl)	tobacco smoking
Children on elimination diet (GR1)							
n	30	23	7	8	27	19	68
parents	4	3	2	1	8	6	36
grandparents	25	20	5	7	17	11	29
siblings	1	0	0	0	2	2	3
time (in years)							
x	44.06	47.47	49.28	56.62	36.85	42.94	21.54
SD	9.94	7.94	9.03	8.85	14.20	14.12	6.10
max	67	60	60	70	58	65	50
min	7	35	35	45	7	12	12
Children on normal diet (GR2)							
n	26	21	16	16	18	19	47
parents	1	0	0	0	3	1	25
grandparents	25	21	16	16	15	18	22
siblings	0	0	0	0	0	0	0
time (in years)							
x	48.92	47.19	50.75	52.56	41.06	48.79	20.36
SD	8.11	8.94	6.09	8.56	11.30	9.96	2.98
max	66	60	60	65	60	60	30
min	30	30	40	25	22	30	14

portional to the number of the discovered atherosclerotic risk factors. The elimination of risk factors leads to a decrease in morbidity and mortality caused by the diseases of the circulatory system. Our study shows that the main morbid problems in families of the examined children were connected with the diseases of the circulatory system. The incidence of arterial hypertension, obesity, increased cholesterol level, coronary heart disease, atherosclerosis and diabetes in the families of children with food allergy/intolerance is the same as in the families of healthy children.

The proper monitoring of progress in a dietary treatment of food allergy/intolerance demands a systematic periodic observation of a child as well as checking its development parameters. This creates many opportunities to promote health in families of these children. These prophylactic activities should be aimed at preventing not only allergic diseases but also other diseases of social concern, especially those of the circulatory system.

Primary and secondary prevention concerning diseases of social consequence should be undertaken at doctor's meetings

with an allergic child's family. It is vital to promote a healthy diet: breast-feeding, an appropriate diet for pregnant and breast-feeding women, hypo-allergic formulas and elimination diets in food allergy and intolerance.

Systematic, age-related physical activity, prevention of obesity, psychological support in a stressful situation and abstaining from smoking are some of the preventive activities. The earlier these measures are introduced, the more effective they are. They should ideally be adopted by the whole society, particularly people from a high risk group with a positive family history.

Disclosing a positive family history of cardiovascular diseases necessitates systematic education promoting health in a family, complex evaluation of physical and psychomotor development of the children, as well as monitoring arterial blood pressure and lipid profile in serum to eliminate the risk factors of these diseases in the child population.

Acknowledgements

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The usefulness of testicular atrophy index in the assessment of undescended testicle – preliminary report

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Abstract

Purpose: Cryptorchidism affects 2-8% of male newborns. There is a controversy regarding timing of surgery as well as indications for orchiopexy in boys with retractile testicle.

The aim of this study was to evaluate the clinical usefulness of testicular atrophy index (TAI) as a criterion of qualifying patients with undescended testes for surgery as well as of monitoring the results of treatment.

Material and methods: In 1999-2000, 105 cryptorchid boys, aged 1 to 15 years (mean 4.8) underwent unilateral orchiopexy. Dimensions and volume of testes were measured by means of scrotal US and TAI was calculated before and 1 year after surgery.

Results: Pre- and postoperative scrotal US measurements were analyzed in 35 boys divided into five age dependent groups. The preoperative TAI values ranged from 27.1% to 52.8%. The biggest loss in volume of affected testis was found in boys aged 4 to 10 years (35.4% to 52.8%). The TAI values measured one year after orchiopexy were lower than preoperative ones. Significant difference in TAI values, ranging from 18.16% to 36.43% were observed in boys between 2 and 10 years ($p<0.001$). In the youngest (0-2yrs) and the oldest boys (>10yrs) the difference was not statistically significant.

Conclusions: The testicular atrophy index (TAI) proved to be a valuable and objective tool for qualifying patients with undescended testes for surgery as well as for monitoring the results of treatment. Its value of 20% and more should be considered an indication for surgery in boys with retractile testes.

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Introduction

Cryptorchidism is a serious developmental disorder and still remains one of the most frequent anomalies of genitourinary tract in boys. It affects 2-8% of male newborns, but its incidence falls to 0.5-1% during the first year of age due to the spontaneous descent of the number of testicles. Cryptorchidism may lead to impaired fertility and germ-cells neoplasia, therefore early hormonal (after the first year of life) and surgical treatment (after the second year of life) is recommended [1,2]. Besides clinical examination, the ultrasound imaging (US) is the basis of the accurate diagnosis and the assessment of the treatment results [3-5]. Considerable advances that have occurred in this kind of imaging over recent years as improved transducer technology allow the objective and precise monitoring of testicular parameters [5-7].

The aim of this study was to determine clinical usefulness of the index of testicular volume decrease (testicular atrophy index – TAI) in the management of the patients with undescended testicle treated in authors' department [3,4].

Material and methods

In 1999-2000, 105 cryptorchid boys, aged 1 to 15 years (mean 4.8) underwent unilateral orchiopexy in the Department of Pediatric Surgery & Oncology in Lodz. To all these patients hormonal therapy prior to surgery (chorionic gonadotrophin, Biogonadyl) was administered without satisfactory results. The following parameters were analysed in all patients: age, side of anomaly, dimensions, volume and position of the testicle before and 1 years after surgery (orchiopexy).

Ultrasound studies were performed by experienced sonographers using Toshiba unit with linear array 7.5 and 10 MHz transducers. The largest measurements in each dimension were

Table 1. Results of scrotal US before orchiopexy (N=35).

Age range	No of patients	Mean volume of affected testis (ml)	Mean volume of contralateral testis (ml)	TAI (%)
0-2	5	0.44	0.63	27.10
2-4	10	0.56	1.09	35.40
4-6	8	0.57	1.20	44.20
6-10	6	0.66	1.17	52.80
>10	6	0.76	1.25	28.90

Table 2. Results of scrotal US after orchiopexy (N=35).

Age range	No of patients	Mean volume of affected testis (ml)	Mean volume of contralateral testis (ml)	TAI (%)
0-2	5	0.88	1.10	20.00
2-4	10	0.86	1.00	17.24
4-6	8	0.96	1.28	21.04
6-10	6	0.96	1.15	16.37
>10	6	0.81	0.85	22.40

recorded and used to calculate testicular volume (TV) using the empirical formula of Lambert: TV (ml) = 0.71 x (width x length x height)/1.000 [8]. The testicular atrophy index (TAI) of the affected testicle was calculated as: TAI = (contralateral testis volume – affected testis volume)/contralateral testis volume x100 and expressed as a percent [3].

Mean differences between pre- and postoperative TAI measurements were evaluated by paired Student's t-test.

Table 3. Comparison of testicular atrophy index (TAI) before and after orchiopexy (N=35).

Age range (yrs)	No of patients	TAI before surgery (%)	TAI after surgery (%)	Difference	Student t-test
0-2	5	27.10	20.00	7.10	NS
2-4	10	35.40	17.24	18.16	p<0.001
4-6	8	44.20	21.04	23.16	p<0.001
6-10	6	52.80	16.37	36.43	p<0.001
>10	6	28.90	22.40	6.50	NS

Results

Out of 105 patients, 58 (55.2%) presented with right and 47 (44.8%) with left undescended testicle. Pre- and postoperative scrotal US measurements of 35 boys were analyzed. Patients were divided into five age dependent groups as follows: 0-2, 2-4, 4-6, 6-10, over 10 years. Number of patients in each group, mean volume of affected and contralateral testis as well as TAI before operation are shown in *Tab. 1*. The TAI ranged from 27.1% to 52.8%. The biggest loss in volume of affected testis was found in boys aged 4 to 10 years.

Results of corresponding parameters measured one year after orchiopexy are presented in *Tab. 2*. TAI was significantly lower comparing with its preoperative values. Remarkable difference in TAI values, ranging from 18.16% to 36.43% were observed in boys between 2 and 10 years (three middle groups in each table) ($p<0.001$). In the youngest (0-2yrs) and the oldest boys (>10yrs) the difference was not statistically significant (*Tab. 3*).

Discussion

Accurate assessment of the position of undescended testicle and its volume compared with contralateral, healthy testicle gives the surgeon a basic knowledge in cryptorchid boys [1,2]. Testicular volume measurements are of particular importance in assessing the clinical significance of cryptorchidism [7,9,10]. The scrotal ultrasound offers the greatest accuracy of all clinical methods used to determine testicular volume [5,7,11]. Because fertility is undefined at this age impaired growth of affected in relation to healthy testicle seems to be the only objective indication for surgery. However, Lee et al. [12] found that in men with a history of unilateral cryptorchidism small testicular size at orchiopexy was not associated with decreased paternity (89.8%), abnormal hormone levels, a lower sperm count or decreased testicular volume in adulthood.

There is still controversy regarding indications for orchiopexy in the group of boys with retractile or so called "wandering testicle" [1,2]. Although the testicle found in the inguinal canal can be easily pulled manually to the scrotum, it is pushed back to the canal as soon as the grip is released. We think there is a place for watchful waiting in these patients. We recommend scrotal US to be repeated every 12 months since the initial presentation. If there is significant progressive decrease in affected testicle volume during the observation period, one should offer surgical treatment.

We believe, basing on the results of the earlier studies of one of us (J.N.), that 20% and higher testicular atrophy index (TAI) should be considered an indication for surgery both in patients with undescended testicle and varicocele [4]. Sayfan et al. [13] suggested that a 20% to 25% volume differential is clinically significant. In our patients TAI ranged from 27.1% to 52.8% at initial diagnosis.

Scrotal US, including colour Doppler, enables an exact morphological analysis of the late results after surgical correction of undescended testes. In Riebel et al. [10] material, 53% of previously maldescended testes showed abnormalities with regard to position, volume, structure and perfusion without any correlation with the patient's age at surgery or the time interval between surgery and US (2-11yrs).

Testicular atrophy is observed in approx. 5-10% of testes in long-term outcome after orchidopexy, but it is impossible to say, if it resulted from injury of blood vessel during operation or pre-existent primary damage (testicular dysplasia) [1,2].

Our data show that repair of undescended testicle results in the growth of affected testes in the treated group. The postoperative TAI values decreased from 18.16% to 36.43% depending on patient's age. The smallest difference in TAI measurments (no growth of affected testes) was observed in the youngest (0-2yrs) and the oldest boys (>10yrs). This find-

ing indicates that the best results of surgical treatment of undescended testes should be expected in boys aged 2 to 10 years.

Conclusions

Ultrasound assessment is important in detecting testicular volume (TV) differential in cryptorchid boys. It should be used routinely to determine growth impairment secondary to undescending of testes. The testicular atrophy index (TAI) proved to be a valuable and objective tool for qualifying patients with undescended testes for surgery as well as for monitoring the results of treatment. Its value of 20% and more should be considered an indication for surgery in boys with retractile testes.

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Oxyuriasis-induced intestinal obstruction in a child – case report

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Abstract

Purpose: The presentation of an unusual case of the tumor of ileum wall induced by pinworm infection in a 5-years-old child.

Material and methods: The record of a 4-years-old boy treated in the department of pediatric surgery was analyzed concerning the diagnostic difficulties. After 6 month from an episode of ileo-cecal intussusception successfully treated with a barium colon enema, the diagnosis of lymphoma was made and the resection of distant segment of small intestine was performed.

Results: No clinical and laboratory features of oxyuriasis could be stated before the onset of disease, during hospitalization and in the follow-up period. The hypertrophied and activated lymphatic tissue with a non-specific inflammatory reaction to the pinworms were seen in the wall of ileum, appendix and mesenteric lymph nodes. No neoplastic cells were found in the microscopic study of ileum, appendix, mesenteric lymph nodes and peritoneal lavage fluid.

Conclusions: The proper diagnosis of oxyuriasis may be difficult when the course is atypical. The enterobius vermicularis infestation as an etiologic factor should be taken into account in any case of abdominal pathology. However, the methods routinely used in "acute abdomen" including examinations of blood, urine and stool, repeated ultrasound and CT, are not reliable.

As the infestation may mimic neoplasm, the surgical treatment and microscopic examination can be necessary for the final diagnosis in some cases.

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Key words: children, enterobius vermicularis, ileus, oxyuriasis, pseudo-tumor, pseudo-neoplasm.

Introduction

The acute abdominal pain is frequently claimed by children younger than 6 years of age presented in surgical office. In these patients, the ileus, appendicitis, neoplasm and bleeding have to be excluded first.

The proper diagnosis of "acute abdomen" in young children is a challenging problem for general practitioners, pediatricians, and even for experienced pediatric surgeons. The meticulous anamnesis and clinical examination are effective in most of the cases. However, the clinical signs in the patients below 6 years of life are uncertain and difficult for interpretation. Even the sophisticated diagnostic methods including ultrasound imaging, computed tomography and MRI may be insufficient in some cases. The delayed and erroneous diagnosis may result in the worsening of prognosis and final outcome.

One of the possible causes of "acute abdomen" in children may be parasite infection. The Enterobius vermicularis is the most common parasite occurring in men affecting about 10% of population of the developed countries. The infection rate in children is even higher [1].

Enterobius vermicularis is regarded as an usually innocuous inhabitant of the intestinal tract. The classical symptoms of pinworm infestation are pruritus ani, enuresis and insomia.

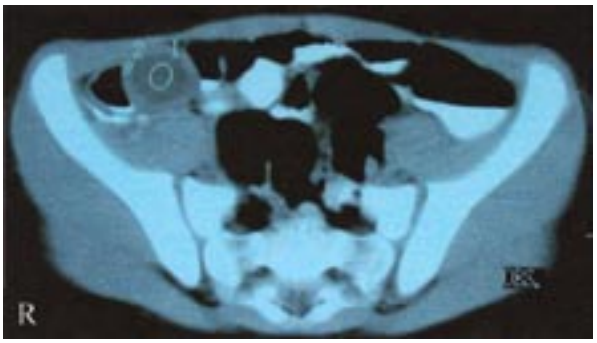
Material and methods

A 4-years-old male patient was presented to the Department of Pediatric Surgery because of acute abdominal pain, nausea and vomiting. The abdomen was soft but painful in the low right quadrant. Ultrasonographically, the ileo-cecal intussusception was diagnosed. The colon barium enema confirmed the diagnosis and was successfully used for a non-operative desinvagination. The ultrasound examination after two days

revealed thickened wall of distal ileum interpreted as a remnant swelling after the intususception. After the uneventful 3-day hospitalization, the child was discharged.

Six months later, an episode of not well localized abdominal pain, nausea, vomitus and moderate distension of abdomen was noted. The utrasound examination revealed a cystic tumor 3.5x4x3 cm in the region of cecum. No signs of generalized or local infection, disorders of coagulation system, blood cells and markers of neoplasm were revealed. No eosinophilia was observed. No ova were found in the feces. The spiral CT confirmed the ultrasound picture of an abnormal mass in the terminal ileum (*Fig.1*). A provisional diagnosis of lymphoma was made and the child was qualified to the open laparotomy for excisional biopsy.

Figure 1. Abnormal mass (28.6x25.3 mm), partially cystic, ventrally and laterally adjacent to the cecum with non-homogenous content surrounding by a thick capsule (4.2 mm). Spiral CT.



Results

During the operation, a solid, pale and macroscopically non-inflammatory tumor was found in the posterior-superior part of ileal wall ca 4 cm of the Bauhin valve. The mesenteric lymph nodes in the tumor region were slightly enlarged. No other pathological findings in the peritoneal cavity were seen. The tumor was resected with a margin of normal ileal wall, together with enlarged lymph nodes and with macroscopically not inflamed appendix (*Fig. 2* and *3*).

Histologically, the hypertrophied and activated lymphatic tissue and no neoplastic cells were found in the wall of ileum and appendix. The appendix and pseudo-tumor lumen contained the pinworms. In the enlarged mesenteric lymph nodes a non-specific inflammatory reaction was noted. No pathological findings were stated in the peritoneal fluid specimen.

The postoperative recovery was uneventful. The patient was discharged on the 7th postoperative day. The treatment with albendazole was performed and repeated after 24 days.

During the 10-months of follow-up no abdominal pain and any other pathological symptoms were observed. The psycho-physical development of the boy is correct as yet.

Figure 2. Tumor of the ileal wall 3x3x2.5 cm, partially obstructing the ileal lumen, proximally to the cecum. Intraoperative view.

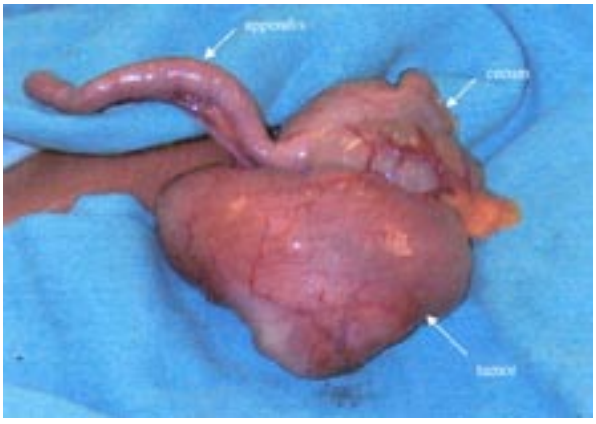
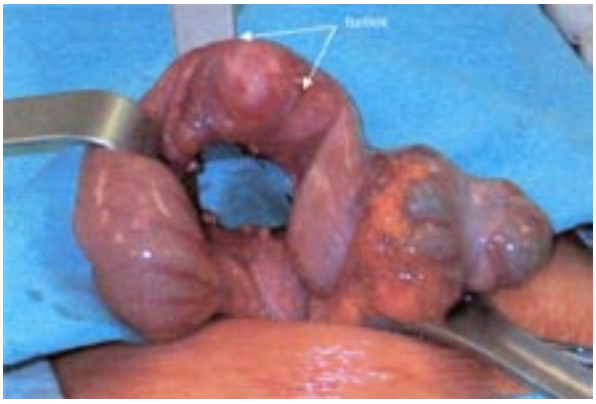


Figure 3. Partially prepared distal part of ileum containing pseudo-neoplastic tumor. Intraoperative view.



Discussion

The large bowel colonization by the Enterobius vermicularis is typical for massive infection [2,3]. The parasites are found in 0.5-40% of the removed appendices. As very susceptible to destruction, the pinworms are not always to be clearly demonstrated using a routine patomorphological examination of appendix [2]. The role of Enterobius vermicularis in pathogenesis of appendicitis is not fully cleared and the pinworms caused appendicitis is never diagnosed preoperatively.

Enterobius vermicularis infection, although rather sensitive to antiparasitic "two-doses, whole-family therapy", may result in the long term and even life-threatening complications, like granulomae of the liver, spleen, kidney, lung, fallopian tube, and uterus [3,6].

The most common sites for ectopic infestation of enterobius vermicularis are female genital tract and peritoneum. Interestingly, the pinworm infection outside the intestinal mucosa results rarely in evident inflammatory reaction. In most of the cases no physical signs are stated [7]. The absence of typical clinical signs of pinworm infection was observed in our patient.

It is stressed by some authors, that the pinworm infection may invade the intestinal wall, facilitate perforation and cause peritonitis [5,7]. However, the lesions of intestinal wall may

happen before the infection and serve as a mucosal break where the penetration of pinworms occurs [1].

Some authors [1,8] reported the relationship between parasitism and malignant diseases or morbid processes stimulating tumors.

A spontaneous development was shown experimentally of lymphoma in pinworm infected athymic mouse, which had been thought to be due to stimulation of proliferative reaction of lymphatic tissue [9]. A high number of parasite infection (up to 32%) has been reported in children with malignancies [10,11].

Colonization of ileal lumen with enterobius vermicularis is thought to be a frequent phenomenon. However, ileum is a rare location of pseudo-neoplastic tumors induced by enterobius vermicularis [1]. We were not able to find a description of such a tumor-like infiltration in the wall of small bowel in children.

The numbers of eosinophils in peripheral blood and peritoneal lavage fluid were not a good indicators of enterobius vermicularis infestation in our patient. This is confirmed by other authors [1].

The oxyuriasis should be always taken into account in children with a suspicion of tumor. Because of a high rate of pinworm infestation, which can mimic, obscure and overlap the abdominal pathology, all children with any signs suggesting pathology of intestinal tract should be routinely tested for the enterobius vermicularis.

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High cholesterol in patients with ECG signs of no-reflow after myocardial infarction

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Abstract

Purpose: Despite successful restoration of blood flow in epicardial artery after myocardial infarction (MI), some patients do not benefit sufficiently from modern revascularisation methods due to the impairment of microcirculation, also called no-reflow phenomenon. Hyperlipidaemia is well established risk factor of coronary heart disease and its detrimental actions on vessels are widely acknowledged. We attempted to investigate possible relations between hyperlipidaemia and electrocardiographic signs of no--reflow in myocardial infarction after successful primary angioplasty.

Materal and methods: A total of 150 consecutive patients with acute myocardial infarction (AMI) with ST elevation who underwent successful primary angioplasty were studied. ECG was obtained directly before and 30 minutes after successful reperfusion. ST segment deviation was measured. Lack of 50% reduction of ST-segment elevation in the lead with maximal initial elevation, 30 minutes after angioplasty was defined as ECG sign of no-reflow.

Results: ST-segment resolution occurred in 116 patients (77%), whereas 34 presented ECG signs of no-reflow (23%). Patients with persistent ST-segment elevation had higher blood LDL and total cholesterol (TC) levels than group with ST-segment restoration (146.5 vs. 128.7 $p<0.01$ and 219.5 vs. 200.9, $p<0.05$ respectively). Triglyceride, HDL, glucose on admission and fasting glucose levels did not differ significantly between groups. ECG signs of no-reflow were observed more often in patients with anterior AMI, history of prior myocardial infarction and longer pain-to--balloon time ($p<0.05$).

Conclusions: Positive relation between impaired tissue perfusion and high TC and LDL blood levels suggests that lipids may play a role in the pathogenesis of no-reflow phenomenon, possibly by impairment of endothelial function.

Key words: myocardial infarction, reperfusion injury, primary angioplasty, hypercholesterolemia, microvascular dysfunction.

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Introduction

Early restoration of the patency of infarct-related artery (IRA) has become nowadays the main goal of acute myocardial infarction (AMI) treatment [1]. Despite of successful primary angioplasty, a substantial number of patients do not benefit sufficiently because of impaired tissue-level perfusion [2]. Because of this fact it is essential to focus on microvascular perfusion assessment as a marker of successful interventions [3]. Impaired tissue-level flow at the level of microvasculatory bed is called no-reflow phenomenon [4,5]. No-reflow is associated with poorer recovery of left ventricular function and more frequent development of congestive heart failure and death [6]. Different methods of detecting impaired myocardial perfusion were described: scintygraphy, PET, contrast echocardiography and angiographic assessment. The most frequently used, because of its simplicity and universal availability, is electrocardiographic ST-segment elevation resolution [7-9]. Persistent ST-segment elevation (STe) among patients with patent IRA correlates well with impaired microvascular perfusion [8,9].

The pathogenesis of no-reflow has been extensively studied for the last few years. It is known that reoxygenation of previously ischemic myocardium, causes generation of reactive oxygen species, followed by complement activation, activation of platelets, increased expression of adhesion molecules, endothelial damage, neutrophil activation and subsequent inflammatory process in surrounding tissues [10,11]. Microembolisation of capillaries with parts of thrombus as well as clusters of plate-

lets and leukocytes also plays an important role in impairment of the microvascular flow. However, precise pathomechanism of no-reflow phenomenon remains largely unexplored.

The hyperlipidemia is well established risk factor of coronary heart disease and lipid disturbances play an important role in atherogenesis [12]. High blood concentration of cholesterol may impair endothelial function and haemostatic balance [13,14]. The role of lipids in ischemia and reperfusion injury, however, has not been established yet. Because endothelial function deficiency seems to play a role in no-reflow phenomenon, we attempted to investigate possible influence of high blood lipids level on its occurrence.

Material and methods

Study population

Between October 1999 and December 2001, 150 patients with acute myocardial infarction with ST elevation, successfully treated with primary angioplasty, were enrolled to this study. An informed consent was obtained before any procedure was performed. Successful PTCA was defined as a restoration of TIMI grade 3 flow and less than 50% residual stenosis. All patients were admitted within 12 hours of symptom onset, presented typical chest pain (lasting more than 20 minutes), more than 0.1 mV ST elevation in at least 2 contiguous leads. The group was composed of 116 (77%) men and 34 (23%) women, with mean age 59 + 12.62 (41%) patients had anterior AMI.

Patients were examined on admission: blood pressure, heart rate, Killip/Kimball class were noted. History included time of onset of symptoms, history of coronary heart disease, prior AMI, hypercholesterolemia, hypertension, diabetes mellitus and smoking. Presence of Q-waves in electrocardiography (ECG) and NYHA functional class were assessed on the day of discharge.

Everyone received aspirin and heparin prior to angioplasty. Other medications were administered according to clinical indications.

The whole group was divided into two subgroups due to electrocardiographic evidences of impaired tissue-level flow: Group A with significant at least 50% ST-segment resolution and Group B without it.

Electrocardiographic analysis

The analysis of ST segment resolution was perfomed as described by Wong et al. [15] with minor modifications. In brief: a 12-lead ECG recordings were obtained on admission, about 30 minutes after successful reperfusion and on discharge from hospital. Electrographic tracings were analysed by two independent investigators blinded to angiographic and clinical data. The ST segment deviation was measured 0.08 s after J point in the lead with maximal initial elevation and compared to isoelectric line between T and P waves. Lack of 50% reduction of initial STe 30 minutes after PTCA was defined as ECG sign of no-reflow phenomenon.

The 32-point Selvester QRS score (SQS) was measured at discharge to assess myocardial damage [16]. The Selvester score system is calculated from the amplitude and size of Q, R and S waves [16]. This parameter correlates well with the mass of infarction, thus providing an important clinical insight which may affect prognosis [17].

Lipids measurement

Total cholesterol (TC), HDL and triglycerides (TG) blood concentrations were measured at the time of admission with enzymatic kits produced by Biomerieux (France). LDL levels were calculated from Friedewald's formula: LDL=TC – HDL - 0.2 x TG (mg/dl).

Statistical analysis

Distribution of every variable was tested with Kolmogorow-Smirnov test. Subsequently the Student's t test or the Mann-Whitney U test was used for statistical analysis where applicable. In order to assess the influence of variables on resolution of ST elevation, we used logistic regression analysis, which yielded consistent results with abovementioned methods. Additional analysis of correlations between non-categorical variables and extent of the ST resolution in per cent was performed using Pearson or Spearman tests, where applicable. Data are expressed as mean ± standard deviation (SD). Relative frequencies are used to present categorical variables with actual number of patients being given in brackets. These variables were assessed with Chi^2 test. A p value of less then 0.05 was considered as statistically significant.

Results

Baseline characteristic

ST segment resolution 30 minutes after successful angioplasty of IRA was observed in 116 patients (77%), who were classified as group A, whereas 34 (23%) presented ECG signs of no-reflow (group B). Baseline characteristic of studied subgroups are presented in *Tab. 1*. Group B had more often anterior AMI (59% vs. 36%; $p<0.05$), higher heart rate on admission (83.2 ± 14.6 vs. 75.3 ± 17.9; $p<0.05$) and longer pain-to-balloon time (300.9 vs. 255.4; $p<0.05$). Age, gender, Killip class and blood pressure (BP) on admission were comparable in both groups. We did not find significant differences in both glucose levels on admission and fasting glucose between the both groups (*Tab. 1*).

Everyone received acetylsalicylic acid and heparin prior to the angioplasty. In both groups almost 60% of patients received stents. During hospital stay, overall 92% of patients were given β-blockers, 71% ACE inhibitors, 75% ticlopidine and 47% gp2b3a inhibitors. The frequencies of administrated drugs were not statistically different between Group A and B (*Tab. 2*).

ST recovery, medical history and risk factors

We observed that in group with ECG signs of no-reflow there were more patients with prior myocardial infarction (21% vs. 8%; $p<0.01$) and history of hypercholesterolemia (68% vs. 47%; $p<0.05$). Smoking, history of hypertension and diabetes mellitus were comparable in both subgroups (*Tab. 3*).

ST segment recovery and lipids levels

Patients with persistent STe had higher blood LDL and TC levels than group with ST segment restoration (146.5 + 36 vs.

Table 1. Baseline characteristics of patients.

	GROUP A	GROUP B	p value
Age (yrs)	59.8 (±11.1)	57.3 (±11.9)	ns
Gender (male)	77% (89)	79% (27)	ns
Systolic BP on admmision (mmHg)	143.5 (±25.7)	149.3 (±33.6)	ns
Diastolic BP on admission (mmHg)	90 (±15.8)	92.8 (±22.1)	ns
Heart rate on admission (beats/minute)	75.3 (±17.9)	83.2(±14.6)	<0.05
Killip/Kimball class	1.43 (±0.5)	1.59 (±0.7)	ns
Anterior AMI	36% (42)	59% (20)	<0.05
Pain-to-Balloon time (minutes)	255.4 (±117.8)	300.9(±132.8)	<0.05
Fasting glucose (mg/dl)	108.3 (±42.3)	108.2 (±25.8)	ns
Glucose on admission (mg/dl)	160.0 (±60.7)	155.1 (±59.1)	ns

Group A – Patients with >50% reduction of initial ST elevation 30 minutes after angioplasty
Group B – Patients without ST segment resolution 30 minutes after successful angioplasty

Table 2. Adjunctive therapy in patients enrolled to study.

	GROUP A	GROUP B	p value
Acetylsalicylic acid	100% (116)	100% (34)	ns
Heparin	100% (116)	100% (34)	ns
Gp2b3a inhibitors	46% (53)	53% (18)	ns
Ticlopidine	72% (84)	82% (28)	ns
ACE inhibitors	69% (80)	76% (26)	ns
β-blockers	93% (108)	88% (30)	ns
Stent	56% (65)	59% (20)	ns

Group A – Patients with >50% reduction of initial ST elevation 30 minutes after angioplasty
Group B – Patients without ST segment resolution 30 minutes after successful angioplasty

128.7±29.6; p<0.01 and 219.5 + 34.9 vs. 200.9±35.9; p<0.05, respectively, *Fig. 1*). We also found statistically significant correlation between total cholesterol, LDL and extent of STe in per cent (R=0.189 P<0.05 and R=0.242 p<0.05 respectively, data not shown), what strongly supports our findings. However, TG, HDL did not differ significantly between groups (*Fig. 1*).

ST resolution and clinical parameters

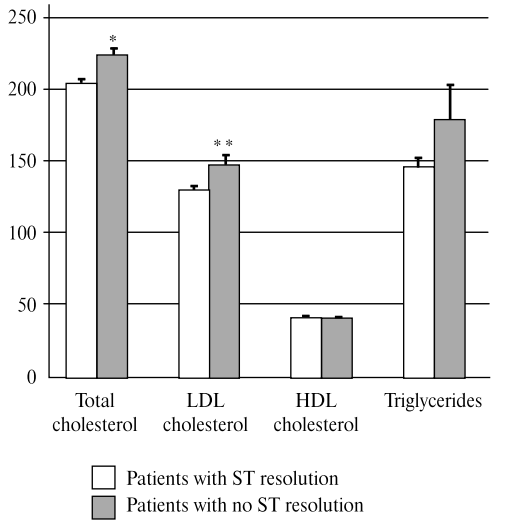
We did not find significant differences between both groups in respect to maximal CK and CK-MB activity, the length of stay in intensive cardiac care unit and presence of pathological

Table 3. Medical history and risk factors.

	GROUP A	GROUP B	p value
History of CHD	28% (32)	41% (14)	ns
Prior AMI	8% (9)	21% (7)	<0.01
History of hypercholesterolemia	47% (54)	68% (23)	<0.05
History of hypertension	42% (49)	47% (16)	ns
History of diabetes mellitus	14% (16)	18% (6)	ns
Smokers	62% (72)	50% (17)	ns

Group A – Patients with >50% reduction of initial ST elevation 30 minutes after angioplasty
Group B – Patients without ST segment resolution 30 minutes after successful angioplasty

Figure 1. Lipid levels in patients with (white boxes n=116) and without ST segment resolution (grey boxes n=34). Data are shown as mean ±SEM. * p<0.05 , ** p<0.01.



Q waves in ECG on discharge. The patients with ECG signs of no-reflow presented, however, significantly lower LV ejection fraction (44.5±9.3 vs. 40.9±9.6; p<0.05), higher NYHA functional class at the end of hospitalisation (2.2±0.5 vs. 2.1±0.3; p<0.03) and Selvester QRS scores (8±4.1 vs. 6.1±3.5; p<0.01) (*Tab. 4*). Both LV EF and Selvester QRS score significantly correlated with the extent of STe resolution in per cent (R=-0.199 p<0.05 and R=0.21 p<0.01 respectively, data not shown). This all may imply poorer prognosis of patients without ST resolution.

Discussion

Already several years ago, persistent ST elevation despite thrombolytic therapy has been used to stratify patients to the high-risk group, as these, in whom reperfusion was apparently

Table 4. Clinical parameters.

	GROUP A	GROUP B	p value
Peak of CK (IU/l)	2784.2 (±2626.1)	3097.9 (±2479.3)	ns
Peak of CKMB (IU/l)	283.5 (±217.5)	286.7 (±221.3)	ns
Ejection fraction (%)	44.5 (±9.3)	40.9 (±9.6)	<0.05
Selvester QRS score	6.1 (±3.5)	8 (±4.1)	<0.01
Q-wave AMI	89% (103)	94% (32)	ns
Days on Intensive Cardiac Care Unit	3.8 (±1.2)	3.7 (±1.3)	ns
NYHA class on discharge	2.1 (±0.3)	2.2 (±0.5)	<0.05

Group A – Patients with >50% reduction of initial ST elevation 30 minutes after angioplasty
Group B – Patients without ST segment resolution 30 minutes after successful angioplasty

unsuccessful [18]. More recently, it has been shown that also patients with normal flow restoration in the epicardial artery may have persistent ST elevation [8], which is strictly associated with impairment of tissue flow [8,9], and may have prognostic significance [15,19].

In this study we were able to show that patients with ECG signs of impaired microvascular flow following primary angioplasty in AMI, presented higher blood concentrations of total cholesterol and LDL-cholesterol. This finding was also supported by fact that these patients more often had hypercholesterolemia prior to infarction. Interestingly, patients without ST resolution more often had a history of prior MI or currently the anterior wall of LV was involved. Impaired tissue perfusion might be also linked to delayed reperfusion, as time pain-to-balloon was significantly longer in no-reflow group, but also to increased sympathetic activation, reflected by higher heart rate on admission. Both groups of patients have received comparable treatment. It is noteworthy that the frequency of gp2b3a antagonists and ticlopidine were very similar in both groups, despite reports that these drug might limit the occurrence of no-reflow.

Notwithstanding recent report by Iwakura et al. [20] on the influence of hyperglycemia during AMI on hampered microvascular flow, we did not notice similar phenomenon. In our study, the patients had, however, relatively lower glucose concentrations.

On the other hand, microvascular injury may have influence on the degree of myocardial damage. We did not observe significant differences in respect to maximal CK and CK-MB activity. Activity of these enzymes which was used, in contrast to concentration, assessed using immunoenzymatic methods, does not correlate best with the extent of the injury. Nevertheless, we found that patients without STe resolution had significantly lower ejection fraction, worse NYHA functional class and larger infarcts, as assessed with QRS Selvester score. On the other hand, we are not able to distinguish if these poor outcome predictors depend more on microvascular dysfunction or on other factors like: anterior localisation of MI, time from pain to reperfusion, history of previous MI, all of which were more frequent in group without STe resolution.

Our study suggests association between high blood lipids and no-reflow. The potential causative relation may be based on evidences of detrimental influence of lipids on endothelial function, oxidative stress and on pro-inflammatory properties of oxidized lipoproteins. The LDL-cholesterol and triglycerides impair endothelium dependent vasodilatation by lowering eNOS expression and NO bioactivity [13,21]. Lacking NO means not only impaired relaxation of vessel wall, but above all, lacking antiaggregatory and antithrombotic effects, as well as increased expression of adhesion molecules [11,22]. Moreover, the low density lipoproteins themselves stimulate leukocytes rolling and adhesion to the endothelial surface [23,24]. Lipoproteins are not only the target of oxidation, but they also promote generation of superoxide radicals [25,26]. In addition, cholesterol rich molecules influence growth and apoptosis of endothelial cells, as well as thrombogenesis and fibrinolysis [27-29]. Our investigation was not designed to find precise molecular explanation of observed correlations, but it reports for the first time new facts that may be in accord with the experimental data and may also help to elucidate possible mechanism of no-reflow phenomenon.

Although previous studies have established correlations between glycaemia and no-reflow phenomenon [20], our analysis did not find it. There are also differences between our results attaching hypercholesterolemia and total cholesterol, which did not correlate with impaired tissue perfusion in other studies [20]. Divergence can be due to different methods used to detecting microcirculatory flow.

Our study has several limitations. First of all we did not analyse follow-up of the patients, hence we may not comment on prognostic value of lacking STe resolution in our group of patients. Second, we based our study on measurement of ST segment changes, as a common and easily accessible marker of patent or impaired microvasculatory bed. This method, however, is secondary to other methods, like SPECT, PET or contrast echocardiography, in establishing no-reflow on the tissue-level. Our findings are still to be confirmed with mentioned techniques which still remain preferred methods of microcirculatory flow evaluation. Also ST segment changes may be assessed in different ways [7-9,15]. Until now there are no prospective comparative studies giving hard evidence supporting one of these methods.

Despite its limitations, our study raises important clinical issue of deleterious effects of hypercholesterolemia on microvascular perfusion after primary angioplasty in acute myocardial infarction. Hence, the relation between preexisting endothelial injury, due to high cholesterol levels, and reperfusion injury together with and molecular basis for the observed phenomenon in clinical setting, should be elucidated.

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Evaluation of skin barrier function in allergic contact dermatitis and atopic dermatitis using method of the continuous TEWL measurement

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Abstract

Purpose: The aim of study was to determine usefulness of the method of continuous TEWL measurement in the evaluation of skin barrier function in physiological conditions and in allergic contact dermatitis (ACD) and atopic dermatitis (AD).

Material and methods: Study was conducted on a group of 86 persons: 48 patients with allergic contact dermatitis, 18 with atopic dermatitis and 20 healthy individuals. Measurements of transepidermal water loss were made using custom-constructed device for continuous TEWL measurement. In each person the measurements of TEWL were made 4 times: measurement 0 (baseline) – before occlusion with 1% lauryl sulphate for 24 h, measurement 1-15 minutes after SLS patch removal, measurement 2-30 minutes after measurement 1 and measurement 3-30 minutes after measurement 2. Obtained data were statistically analyzed.

Results: TEWL ratio values obtained in measurement 0 were as follows in individual groups of patients: 13.20 ± 8.25 in the AD patients, 10.09 ± 8.29 in ACD patients and 9.02 ± 5.99 in control group. Analogous TEWL values in the subsequent measurements were: in measurement 1 – 16.08 ± 11.17; 11.63 ± 6.43; 17.39 ± 12.41, in measurement 2 – 23.72 ± 14.58; 14.71 ± 6.46; 17,55 ± 8.25, measurement 3 – 24.09 ± 14.93; 16.34 ± 6.32; 18.44 ± 8.26. TEWL ratio values were higher in both groups of patients as compared to control group but not statistically significant (p = 0.1778). After 24 h exposition to SLS, TEWL ratio values increased in all examined groups as compared to baseline (0) measurement. All measurements, except for measurement No 1 in AD group of patients, showed statistically significant differences. The highest increase of TEWL values were observed in group of AD patients.

Conclusions: Delay in skin reaction to SLS in patients with atopic dermatitis provides evidence for different properties of water barrier of the skin in this group as compared to healthy individuals. Increasing tendency in TEWL values 1 hour after SLS removal might reflect persistent damage to water barrier of the skin by detergent. Method of continuous assessment of water barrier of epidermis, through the possibility of multiple measurement by TEWL in examined periods of time, decreased the risk of mistake and increased accuracy of measurement. Measurement of TEWL values allows for assessment of otherwise unnoticed damage to water barrier of the skin.

Key words: skin barrier function, TEWL, continuous measurements, allergic contact dermatitis, atopic dermatitis.

Introduction

The outermost layer of the epidermis, the stratum corneum (SC), plays an essential role in protection the body from water loss. It is commonly known as a skin barrier. It has been shown that correct function of the water barrier depends on many factors, of which the most important are:

1. integrity of the stratum corneum [1,2]
2. proper degree of its hydration [3,4]
3. state of the intercellular lipid matrix [5]
4. size of the corneum cells [6].

A substantial number of non-invasive bioengineering techniques have recently been introduced with the aim to evaluate functional state of the skin barrier [7,8]. Skin surface hydration can be measured by electrical methods such as: measurement of resistance or conductance and capacitance [9-12]. The most direct method, expressing diffusional water loss through the skin, is transepidermal water loss measurement (TEWL). The

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principle of the method is based on measuring water vapour pressure gradient by the use of temperature and humidity sensors at two different levels above the skin surface. TEWL is then calculated and expressed in grams/m^2/h. Transepidermal water loss depends on a number of personal (age, examined region, skin temperature, sweat) and environmental (temperature and humidity, air circulation) factors [13-15]. The impairment of the water barrier and an increase in TEWL is found in pathological conditions with epidermis being damaged and in other diseases with dryness of the skin and matrix lipid disturbance [16-18].

The aim of study was to estimate usefulness of continuous TEWL measurement, using earlier constructed device [19], in evaluation of skin barrier function in physiological conditions, in allergic contact dermatitis (ACD) and atopic dermatitis (AD). We also aimed to determine the influence of SLS on transepidermal water loss in healthy persons and patients with ACD and AD.

Material and methods

Study was conducted on a group of 86 persons divided into 3 subgroups:

I. 48 – 31 female (F) and 17 male (M) – patients, at the age ranging from 17 to 62 (mean 41.5) years with allergic contact dermatitis;

II. 18 (8 F and 10 M) patients, age from 15 to 55 (mean 29.1) years with atopic dermatitis;

III. 20 (18 F and 2 M) healthy volunteers, age from 23 to 50 (mean 29.6) years as a control group.

ACD was confirmed by positive results of patch tests; Hanifin and Rajka criteria were used to recruit patients to AD group. Studies were conducted, after treatment cessation, in the state of remission of the skin lesions. Control group consisted of healthy individuals free of any allergic disease in familial and personal history.

Elicitation of irritant skin reaction by SLS

To determine the influence of SLS on skin barrier function 1% aqueous solution of SLS was used. A cotton patch 1 x 1 cm wet with 0.1 ml 1% SLS was applied for 24 hours on volar surface of forearm in occlusion. After removing the patch with SLS, visual evaluation of the skin was performed in four-step scale considering the presence of dryness, lichenisation and erythema: 0 – absence, 1 – weak, 2 – prominent, 3 – strong.

TEWL measurements

The measurements of transepidermal water loss were made by the use of custom-constructed device and earlier elaborated method was applied for continuous TEWL measurement (*Fig. 1*).

In all persons TEWL was measured 4 times on the volar surface of the forearm:

Measurement 0 – basal TEWL

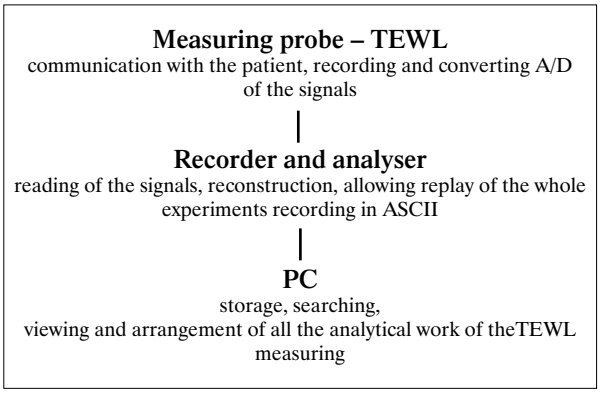
Measurement 1-15 minutes after SLS patch removal

Measurement 2-30 minutes after measurement 1

Measurement 3-30 minutes after measurement 2

The duration of each measurement was 2 minutes (*Fig. 2*).

Figure 1. Method for continuous measurement of TEWL.



In each person median TEWL ratio value and coefficient of variation were calculated for each of 4 measurements.

Statistical analysis

Difference in TEWL values and coefficient of variation between each group of examined persons were estimated using one-factor analysis of variance. Repeated measures analysis of variance was used to evaluate the influence of SLS on TEWL values and coefficient of variation in subsequent measurements. The differences between measurement 0 (baseline) and 1-3 were calculated using t-test for dependent variable.

Results

1. Assessment of the influence of disease and SLS on TEWL ratio values

TEWL ratio values in three examined groups are shown in *Tab. 1*.

Normal distribution of individual measurements has been rejected basing on Shapiro-Wilk test. Logarithmic transformation of data enabled parametric tests to be applied for the analysis [20]. One-factor variance analysis has been used in individual groups of examined persons in order to differentiate TEWL ratio values. Values 0 (baseline measurements) were analyzed for three groups. TEWL ratio values obtained in measurement 0 were higher in both groups of patients with relation to control group but not statistically significant.

Evaluation of the influence of SLS on skin was performed by the use of multidimensional variance analysis with repeated measurements. Increase in TEWL ratio values has been found in all examined groups of patients after 24 hours exposition to SLS as compared to baseline (0) measurement.

Measurements 1-3 have been analyzed in order to determine dynamics of changes in TEWL ratio values after removal of SLS from the surface of the skin. Distribution of mean TEWL ratio values (after logarithmic transformation) is shown in *Fig. 3*.

Student t test, used for dependent variables, was applied to compare analysis of measurement 0 with measurements 1-3 in individual groups (*Tab. 3*). No significant differences ($p=0.123$) were found between measurement 0 and 1 in atopic dermatitis

Figure 2. Continuous recording of TEWL during 2 min test.

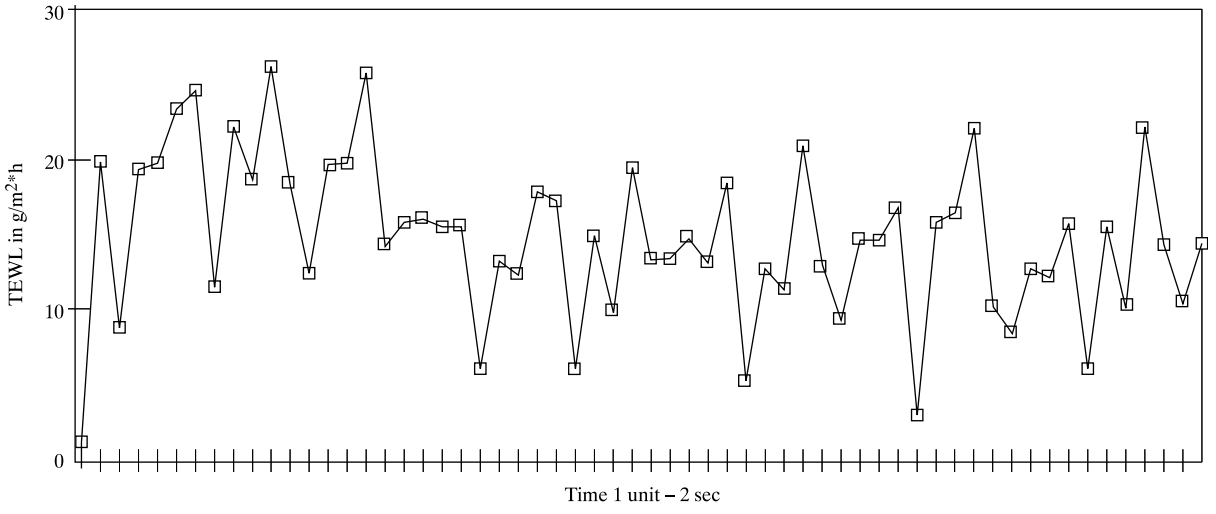


Table 1. Values of transepidermal water loss in patients with allergic contact dermatitis, atopic dermatitis and control group.

	Measurements			
	0 (baseline)	1	2	3
ACD - mean	10.09	11.63	14.71	16.34
Median	7.42	9.86	13.38	15.36
Standard deviation	8.29	6.43	6.46	6.32
AD - mean	13.202	16.08	23.72	24.09
Median	11.67	13.37	21.39	21.15
Standard deviation	8.25	11.17	14.58	14.93
Control group - mean	9.02	17.39	17.55	18.44
Median	7.41	13.54	16.35	16.91
Standard deviation	5.99	12.41	8.25	8.26

Figure 3. Distribution of mean values of logarithms of TEWL coefficients in patients with ACD, AD and control group.

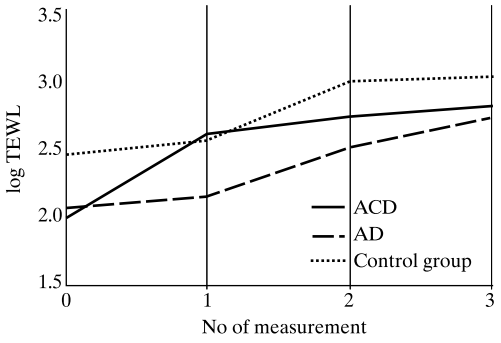
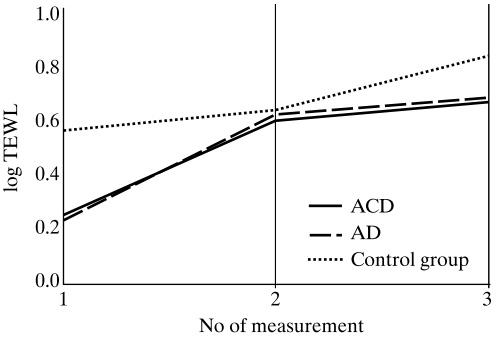


Table 2. Variation of TEWL values coeffictient.

	Measurements			
	0 (baseline)	1	2	3
ACD - mean	69.78	63.08	60.39	53.25
Median	64.24	63.3	61.61	53.48
Standard deviation	30.62	21.66	26.8	15.55
AD - mean	58.37	56.69	41.7	40.59
Median	55.69	54.81	37.09	30.65
Standard deviation	29.52	29.73	18.41	21.06
Control group - mean	67.34	58.8	47.3	44.9
Median	68.54	58.7	46.04	46.34
Standard deviation	16.04	26.19	16.18	16.73

Figure 4. Differences between measurement 0 and measurements 1-3.



group of patients. Remaining measurements showed statistically significant differences. *Fig. 4* shows graph of differences.

2. Evaluation of the influence of disease (ACD and AD) and SLS on variability of TEWL values

Variability of TEWL values is shown in *Tab. 2*.

Coefficient of variation values were analyzed. Basing on Shapiro-Wilk test normal distribution of results of individual measurements in all examined groups was rejected. Logarithmic transformation of data enabled the use of parametric tests for the analysis.

Analysis of TEWL ratio variability has been performed basing on measurement 0 in individual disease (atopic dermatitis vs. contact dermatitis) by the use of one-factor variance analysis. It did not show the influence of the disease on variability of the ratio ($p=0.151$)

Analysis of the influence of the disease and SLS on variability of TEWL ratio, calculated from measurements

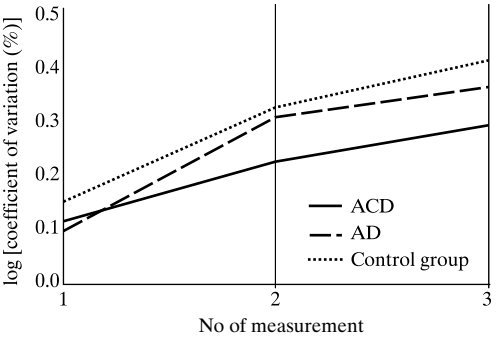
Table 3. Differential analysis of measurement 0 with measurements 1-3 in individual groups.

	P value		
	ACD	AD	Control group
Measurement 1	0.013	0.123#	0.013
Measurement 2	0.0001	0.0001	0.005
Measurement 3	0.0001	0.0001	0.0001

Table 4. Differential analysis of coefficient of variation of measurement 0 with measurements 1-3 in individual groups.

	P value		
	ACD	AD	Control group
Measurement 1	0.176#	0.57#	0.212#
Measurement 2	0.017	0.02	0.02
Measurement 3	0.0033	0.013	0.0053

Figure 5. Mean of values of differences in variation coefficient of baseline measurement and measurements 1-3.



0-3, was done by the use of multidimensional variance analysis with repeated measurements. It showed that variability of TEWL values does not significantly depend on examined group (p=0.108). TEWL ratio values were statistically significant in time-dependent manner (*Tab. 4*, *Fig. 5*).

Discussion

Baseline TEWL values did not differ among the groups of patients involved in the study. 24 hours of skin exposition to SLS caused increase in TEWL values in all examined groups of patients. The character of changes was similar, however, in group of patients with AD shortly after SLS patch removal (measurement 1), no significant changes in TEWL values were observed as compared to measurement 0. This "delay" of skin reaction to SLS in patients with AD, observed in case of coefficient of variation as well, might be reflective of changes in water barrier properties of skin in this group of patients. In case of coefficient of variation of TEWL values it concerned all examined groups of patients after SLS patch removal. It may indicate that skin irritation by SLS could be a second factor influencing TEWL values, independently on disease state.

With regards to basic TEWL values, no significant differences were found between skin of patients with allergic contact dermatitis during remission and healthy individuals, which is consistent with other studies [21,22]. It was shown, however, that skin sensitivity to SLS within unaffected part of the skin in patients with acute stage of contact dermatitis is increased. Agner et al. [23] was able to show significant differences in TEWL values in chronic phase of contact dermatitis which is in opposition to studies performed by Blichman and Serup who noted increase in TEWL values in acute as well as in chronic stage of contact dermatitis [24]. Increased TEWL values observed in examined patients, after skin exposition to SLS, became more explicit in the course of time. It may be indicative of a permanent damage to water barrier of the skin despite removal of the irritant factor. It is worth noting that other authors found higher TEWL values after few hours, or even few weeks, of exposure to 1% SLS. Multiple exposition to detergent caused damage to water barrier of the skin which persisted up to 9 weeks [25].

Despite a relatively high number of publications on different chemical compounds affecting TEWL values, most of them were uncontrolled, performed with temporary recording evaporimeter [26]. Results obtained by this method exhibited substantial inter-study variance. The main limitation of the above studies was, however, possibility of performing only single measurements during the course of clinical test. This could burden the final results with significant mistake.

Modified method, used in our study, decreased the risk of mistake and increased accuracy of measurement through the possibility of taking multiple measurements during 2 minutes of clinical test. We exploited the model used by others [27-29]. It was supplemented with additional triple TEWL measurements taken every 30 minutes which enable dynamic assessment of changes in skin permeability after SLS patch removal. Parallel determination of dispersion of results was additional criterion utilized in this test. Coefficient of variation, reflecting oscillation of measurements around the median value, has been found to be clinically significant.

It seems that coefficient of variation is owing to temporary changes in skin permeability to water, which is to a greater extent dependent on internal conditions than on external ones, such as air motion. In case of both atopic and contact dermatitis, coefficient of variation may reflect temporary changes in skin reactivity to different irritating compounds, such as SLS. To our knowledge there is no information in the literature on potential significance of such dynamic monitoring of water barrier of skin. In our studies we did not find changes in coefficients of variation in all examined groups but we did observe changes in TEWL values. TEWL ratio values were statistically significant in time-dependent manner. It is, however, difficult to judge which method of interpretation is more objective in its ability to assess changes in water barrier of the skin. It seems that evaluation of the status of skin barrier by other biophysical methods, like assessment of its electrical properties, could complement measurements based solely on TEWL method.

In conclusions: 1) delay in skin reaction to SLS in patients with atopic dermatitis provides evidence for different properties of water barrier of the skin in this group of patients as compared to healthy individuals, 2) increasing tendency in TEWL values, one hour after SLS removal, might reflect persistent damage to water barrier of the skin by the detergent, 3) our own method of continuous assessment of water barrier of epidermis, through the possibility of multiple measurement by TEWL in examined periods of time, decreased the risk of mistake and increased accuracy of measurement, 4) measurement of TEWL values allows for assessment of otherwise unnoticed damage to water barrier of the skin.

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Enhanced release of platelet factor 4 into the circulation in patients with atopic eczema/dermatitis syndrome

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Abstract

Purpose: The active participation of platelet in IgE-mediated inflammatory response is well documented. Platelet factor 4 (PF4), a platelet-specific protein may play an important role in the development of the atopic eczema/dermatitis syndrome (AEDS).

Objective: The aim of the study was to evaluate the activation state of circulating platelets in patients suffering from AEDS.

Material and methods: In vivo platelet activity was assessed by measuring plasma level of PF4 (enzyme-linked immunoassay method) in 9 males AEDS patients and 11 healthy, nonatopic subjects.

Results: Plasma PF4 was significantly increased ($p < 0.05$) in AEDS (31.88 ± 20.48 IU/ml) patients compared with control subjects (2.95 ± 0.6 IU/ml).

Conclusions: This result suggests that patients with AEDS may have increased in vivo platelet activation expressed by PF4 release. The aim of the study was to evaluate the activation state of circulating platelets in patients suffering from AEDS.

Key words: atopic dermatitis, platelet activation, platelet factor 4.

Introduction

Platelets play an important role in haemostasis and are actively involved in the inflammatory response. Moreover, altered platelet function has been reported in different forms of allergy [1]. There is evidence that platelet participates in the pathogenic mechanism of allergic diseases, including AEDS [2,3].

Platelet factor 4 (PF4) is regarded as a platelet-specific protein secreted when platelet is activated and belongs to the C-X-C chemokine family. Measurements of plasma levels of PF4 have been shown to be marker of platelet degranulation in vivo, can be used to detect activation of the circulating pool of platelets [4]. PF 4 is shown to be an inflammatory response mediator. In particular, by the fact that it can attract inflammatory cells to sites of inflammation [5,6]. Recently, by using an experimental model of AEDS, it has been demonstrated that PF4 may play an important role in the pathophysiology of this disease [7]. Taken together, we investigated whether patients with moderate AEDS exhibit increased platelet activity, assessed by circulating level of PF4. Moreover, AEDS belongs to the atopic triad and is characterised by high serum IgE levels. So we also analysed relationships between platelet activity and total IgE serum levels.

Material and methods

The investigation was carried out on non-smoking patients, treated at the outpatient department of Chair of Internal Disease, Allergology and Clinical Immunology, Zabrze.

The AEDS group consisted of 9 patients (9 males), the median age was 28 years (range: 25-30 years) and the median duration of recognized AEDS was 25 years (range: 18-26 years). The diagnosis of AEDS had been established by the criteria of Hanifin and Rajka [8]. All the patients had an elevated serum IgE levels (>500 kU/l) and positive skin prick tests to three or more different common airborne allergens. The disease activity was measured by assessing erythema, excoriation, cracking, dryness and lichenification. Only patients with moderate AEDS were included in the study. None of the patients suffered from concomitant inhalatory allergy, non had treatment with topical steroid or oral medications for 2-weeks before this study.

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Table 1. Plasma level of PF4 in patients with AEDS and healthy controls. Values presented as mean ± SD and median.

Analysed parameters	Healthy controls (n=11)	AEDS patients (n=9)	Statistical analysis
PF4 (IU/ml) mean ± SD median	2.95±0.6 2.5	31.88±20.48 32.5	p=0.00017

Control group consisted of 11 healthy non-smoking subjects with no signs of atopy who were matched for age and sex with the analysed group. They were instructed to take no medications (to avoid affecting platelet activation) for at least 10 days before the study.

An informed consent was obtained from the control subjects and patients. Permission of the University Ethics Committee was also obtained.

Blood sampling, measurement of PF4

The blood was obtained in the morning (7.00 to 8.00) after 25-minute rest with slight or no stasis from the antecubital vein into Diatube H tubes (Becton Dickinson) which were immediately placed in the ice/water bath for 20 minutes. Then the tube were centrifuged at 2500 g for 30 minutes at 4°C, and top third the volume of the resultant plasma supernatant were collected and frozen at -20°C for assay (not longer than 1 month).

PF4 was assayed by enzyme-linked immunosorbent assay (ELISA) kit (Diagnostica Stago, France) according to manufacturer's instructions. Samples were assayed in duplicates. PF4 levels were expressed in IU/ml. Precision of the assay was ±0.7 UI/ml in replicate determinations.

Details of the methods as well as references are given in the leaflets included in the sets.

Skin prick tests

Skin prick tests for common airborne allergens (Nexter-Allergopharma, Germany) were performed. A positive immediate skin response: wheal diameter of >3 mm and greater than or equal to that of the histamine control were selected for this study.

Other investigations

Serum total IgE were determined by ELISA kit according to the manufacturer's instructions (Nexter-Allergopharma, Berlin).

Total platelet counts were calculated by a direct method.

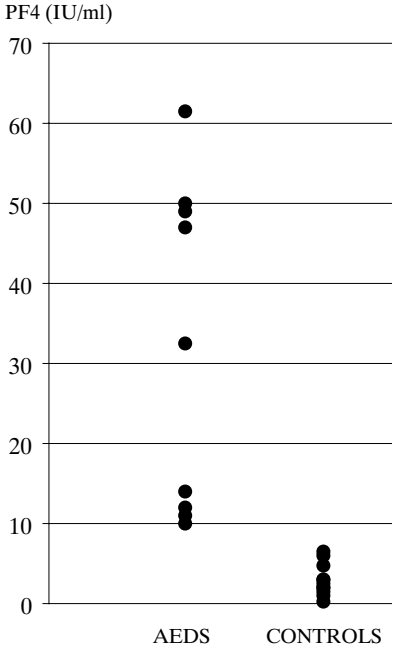
Statistical analysis

Data were compared between groups by Mann-Whitney's unpaired rank sum test. Correlation analyses were performed by Spearman's rank method. The results was presented as mean ±SD and median. P values less than 0.05 were considered significant.

Results

Plasma PF4 was significantly increased ($p < 0.05$) in the AEDS compared with the control group (*Tab. 1*). *Fig. 1* shows individual values of PF4 in patients with AEDS and healthy subjects.

Figure 1. Individual plasma levels of PF4 in patients with AEDS in comparison to the group of healthy individuals.



There were no significant differences in platelet number between the two examined groups (data not shown).

There was no correlation between plasma PF4 and IgE levels ($r = 0.083$, $n = 9$, $P > 0.05$).

As we failed to observe any changes in platelet number in persons who suffered from AEDS, we found it groundless to analyse relationship between the normal platelet counts and total IgE.

Discussion

Blood platelets can be activated by immune and non-immune stimuli. It has been suggested that in allergic disorders, IgE-dependent activation of platelet expressed by the release of mediators can be specifically triggered by the corresponding allergens [9]. The importance of circulating platelets and their mediators in atopic dermatitis is complex and still not completely understood [2,3]. In our previous study, platelet activation as measured by platelet aggregation in response to some aggregating agents in atopic dermatitis patients were not impaired [10]. As mentioned earlier, platelet activity can be

also determined by measurement of plasma levels of platelet-specific proteins, including PF4 [4]. Chemokines related to the beta subfamily, the so-called PF4 superfamily have been shown to modulate allergic inflammatory process and are considered to be important mediators of inflammation [5,6,11,12].

The results of our study revealed that PF4 plasma level was statistically significantly higher in patient with AD than in control group. These data may support the hypothesis of the occurrence of platelet activation in patients with AD. PF4 is a multifunctional protein specific to platelets, which may be involved in the recruitment and activation of inflammatory cells, including eosinophils and thereby contribute to the pathogenesis of skin inflammation associated with AD. It has been previously shown in a mouse model of AEDS that increased expression of PF4 may play an important role in the development of this disease [7]. Bruijnzeel et al. reported that eosinophils from the circulation of patients with atopic dermatitis exhibit potentiated migratory responses toward PF4 compared with eosinophils from normal donors [6]. Thus, the release of PF4 might lead to amplify inflammatory processes associated with atopic dermatitis and contribute to tissue injury.

Also, in other disorders platelets are sometimes present in an activated state in the circulation. There was evidence that platelet activation expressed by in vivo release of PF4 occurred during IgE-mediated airway reactions after allergen challenge in patients with allergic asthma [13]. Enhanced plasma level of PF4 in mast cell-dependent disorders, such as idiopathic cold urticaria has been described [14]. On the other hand, increased in vivo platelet activation has not been found in seasonal allergic rhinitis during natural pollen exposure [15]. The clinical significance of abnormal platelet activity in IgE-mediated human disease is not definitely established.

Conclusions

It seems that individuals with atopic dermatitis may have increased in vivo platelet activity, assessed by measuring plasma PF4. Platelet activity should be investigated further in larger groups of AEDS patients. Moreover, further studies are needed to assess whether this enhanced platelet activity correlates with severity of AEDS.

Acknowledgements

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Incidence of elevated LH/FSH ratio in polycystic ovary syndrome women with normo- and hyperinsulinemia

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Abstract

Purpose: The aim of this study was to determine the incidence of abnormal LH/FSH ratio in women with polycystic ovary with normo- and hyperinsulinemia and to assess the influence of elevated LH/FSH ratio on selected endocrine and biochemical parameters.

Material and methods: One hundred nineteen polycystic ovary syndrome women in reproductive age hospitalized between 1996 and 2000 in Division of Infertility and Reproductive Endocrinology at Poznan University of Medical Sciences were selected for the study. In all selected women LH and FSH serum levels were determined and LH/FHS ratio was calculated. These groups became the subject of a detailed clinical, hormonal and metabolic analysis, which was performed between 6th and 10th day of a natural or induced menstrual period.

Results: LH/FSH ratio greater than 2 was accepted as abnormal, and it was found in 54 women (45.4%; I group). Normal gonadotropin ratio was detected in 65 women (55%; group II). Statistically significant differences were noted between groups with normal and elevated LH/FSH ratio in the following parameters: BMI (body mass index), serum insulin, and LH levels. Further analysis revealed that the majority of women with elevated insulin concentrations belong to the group with normal LH/FSH ratio.

Conclusions: LH/FSH ratio is not a characteristic attribute of all PCOS women: in the present study this abnormality was detected in a subpopulation smaller than 50%. Most of the PCOS women with normal gonadotropin ratio belong to a group of patients suffering from hyperinsulinemia and obesity. Patients with hyperinsulinemia and excess of LH constitute a selected and distinct subgroup with increased adrenal androgenic activity.

Key words: polycystic ovary syndrome, hyperinsulinemia, LH, LH/FSH ratio.

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Introduction

Polycystic ovary syndrome [PCOS] affects approximately 5-10% of women in the reproductive age. It is characterized by hyperandrogenemia and clinical signs of androgenisation: hirsutism, acne with seborrhea, menstrual disorders with anovulation, and infertility. Additionally, the affected women may suffer from overweight or obesity of androgenic type (abdominal) with a typical waist to hip ratio (WHR) over 0.85 [11,12,18]. In polycystic ovary syndrome both the initial stadium of follicle growth, that is recruitment, and the growth with subsequent selection and domination proceeds irregularly. It results in the accumulation of a large number of small ovarian follicles producing predominantly androgens in thecal cells, with impaired aromatization to estrogens [1,8]. The exact pathogenic mechanism of PCOS is unknown. There are many hypothesis concerning causes of PCOS development and the concurrent coexistence of many interdependent disorders is also possible. Most attention is paid to the hypersecretion of LH and insulin resistance as well hyperinsulinemia. The oldest theory emphasized the relation between thecal cells stimulation with LH and the consequent androgen overproduction [3,8,20]. Lutropin is the best known androgen synthesis stimulator. However last decade brought new hypothesis emphasizing the role of insulin and insulin-like growth factor I (IGF-I) system [15,17].

Insulin resistance and hyperinsulinaemia were usually linked with diabetes mellitus type II (NIDDM) and obesity. Insulin resistance and secondary hyperinsulinemia are perceived as the main cause for the development of PCOS. Most probably insulin resistance in polycystic ovary syndrome is caused by post-receptor defects and it affects most frequently

patients with obesity. Ovarian morphologic abnormalities include theca and stroma hypertrophy and atresia of granulosa cells.

To date the pathogenic relationship between hypothalamic-pituitary system and insulin resistance in polycystic ovary syndrome have remained unexplained. Meanwhile there is still controversy over essential diagnostic investigations in PCOS. LH/FSH ratio greater than 2 has been considered as "gold standard" in PCOS diagnosis for a long time. Taking this into account it is an intriguing problem to recognize the role of LH and the possible associations with hyperinsulinemia and furthermore to evaluate the usefulness of gonadotropin ratio in PCOS diagnosis.

The aim of this study was:

1. To determine the incidence of abnormal LH/FSH ratio in women with polycystic ovary syndrome and with normoinsulinemia or hyperinsulinemia

2. To assess effects of increased LH/FSH ratio on remaining endocrine and biochemical parameters.

Study subjects

One hundred nineteen polycystic ovary syndrome women in reproductive age hospitalized between 1996 and 2000 in Division of Infertility and Reproductive Endocrinology at Poznan University of Medical Sciences were recruited to the study. PCOS was diagnosed on the grounds of the following clinical findings: testosterone >0.8 ng/mL, menstrual disorders – oligomenorrhoea or amenorrhoea, acne or hirsutism (>8 points according to Thomas and Ferriman scale).

In all selected women LH and FSH serum levels were determined and LH/FHS ratio was calculated. LH/FSH ratio >2 was accepted as abnormal. In both groups with normal and increased LH/FSH ratio insulin serum levels were determined in fasting state. These groups became the subject of a detailed clinical, hormonal and metabolic analysis, which was performed between 6th and 10th day of a natural or induced menstrual period.

Assays

Testosterone, LH, FSH, and prolactin were measured by specific chemiluminescence assays (Chiron Diagnostics GmbH, Fernwald, Germany). Serum levels of insulin were determined with ELISA assay – Enzymun Test Insulin (Boehringer Mannheim, Germany). Sex hormone binding globulin (SHBG) was measured by specific radioimmunoassay (Orion Diagnostica, Espoo, Finland).

Statistical analysis

Results are presented as arithmetical mean with standard deviation. Statistical analysis was done using Student's t-test after confirmation of the normal distribution. Differences at $p<0.05$ were considered statistically significant.

Table 1. Comparision of insulin concentration, LH, FSH, LH/FSH ratio and BMI in women with elevated and normal gonadotropin ratio.

Parameter	PCOS with LH/FSH>2 (n=54)	PCOS with LH/FSH <2 (n=65)	p <
BMI (kg/m^2)	24.4±5.3	27.1 ±7.1	0.05
Insulin (μU/mL)	11.2±7.5	16.9±10.5	0.001
LH (mU/mL)	14.7±6.8	6.1±3.0	0.0001
LH/FSH ratio	2.8±1.1	1.1±0.4	0.0001

Figure 1. Incidence of hyperinsulinemia in women with elevated gonadotropin ratio.

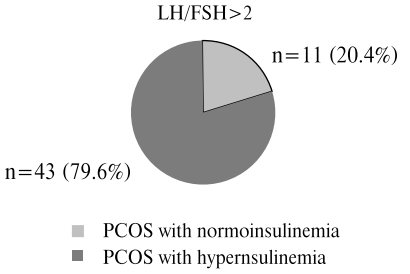
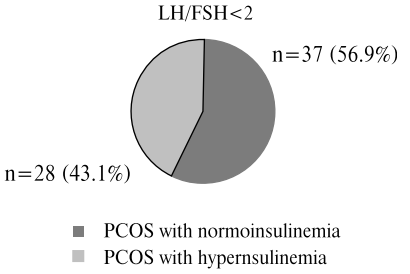


Figure 2. Incidence of hyperinsulinemia in women with normal gonadotropin ratio.



Results

Elevated LH/FSH ratio (LH/FSH>2) was found only in 45.4% of the studied PCOS women (54 out of 119).

Statistically significant differences were noted between groups with normal and elevated LH/FSH ratio in the following parameters: BMI, serum insulin, and LH levels (*Tab. 1*). However, there was no difference between groups with abnormal and normal gonadotropin ratio in total testosterone concentrations (1.01±0.3 ng/mL versus 1.02±0.4 ng/mL) and SHBG levels (48.89±28.4 versus 44.2±36.9 nmol/L). Also the average menstrual cycle length was similar in both groups: 54.2±28.7 days in a group with LH/FSH ratio >2 and 64.4±38.0 days in a group with normal gonadotropin ratio.

Having divided each group into patients with normo- and hyperinsulinemia it turned out, that the majority of women with elevated insulin concentrations belong to the group with normal LH/FSH ratio (*Fig. 1, 2*). Identification of subgroups with normo- and hyperinsulinemia among groups with normal and elevated LH/FSH ratio allowed for detection of significant differences in many parameters as demonstrated in *Tab. 2*.

Table 2. Clinical and endocrine parameters in normo- and hyperinsulinemic PCOS women according to gonadotropin ratio.

Parameter	PCOS with LH/FSH >2 (n=54)			PCOS with LH/FSH < 2 (n=65)		
	Normo-insulinemia (n=43)	Hyper-insulinemia (n=11)	p<	Normo-insulinemia (n=37)	Hyper-insulinemia (n=28)	p<
BMI (kg/m^2)	22.8±3.4	32.0±5.6	0.0001	23.3±4.0	33.0±7.0	0.0001
Hirsutism (points)	9.3± 3.9	10.0±2.5*	NS	8.8±4.6	7.3±4.4	NS
Testosterone (ng/mL)	1.0±0.3	1.0± 0.2	NS	1.1±0.4	1.0±0.3	NS
SHBG (nmol/L)	52.7±29.2	30.1 ±13.5*	0.05	57.7±41.2	20.9±8.0	0.001
FTI	9.0±5.9	15.4±10.8	0.001	10.1±9.6	20.1±12.0	0.001
Insulin (μU/mL)	8.4±3.5	25.7±6.3	0.001	9.4±4.5	27.1±7.5	0.001
LH (mU/mL)	13.9±5.7	17.8±9.5	NS	6.0±3.1	6.2±2.9	NS
FSH (mU/mL)	5.5±1.8	6.0±1.4	NS	5.8±2.0	5.9±1.5	NS
LH/FSH	2.7±1.1	3.0±1.3	NS	1.0±0.5	1.0±0.6	NS
DHEAS (μmol/L)	363.5±162	510±218*	0.05	419±178	367.2±139	NS

* $p<0.05$ in comparison to a group with hyperinsulinemia and LH/FSH <2

Discussion

Polycystic ovary syndrome is a subject of continuous studies concerning both pathogenesis, diagnostics methods, and therapeutics procedures. Nowadays, most attention is focused on the role of insulin resistance and hyperinsulinemia in development of the syndrome [10]. However, one problem remains important and controversial: how many PCOS women are affected by hyperinsulinemia. In the presented study of 119 women with ovarian hyperandrogenism, 33% of the subjects had elevated insulin serum levels. Literature data reports incidence of hyperinsulinemia and insulin resistance at 40% to 60% [9,13]. Insulin resistance is strongly associated with androgenic type of obesity (abdominal). In our study majority of patients with hyperinsulinemia presented with BMI>25, and the mean value in this group was over 30 kg/m^2.

At the end of 1980s LH/FSH ratio was still perceived as a "gold standard" for diagnosis of PCOS, and the coexistence of insulin resistance and hyperinsulinemia was only emerging as a potential pathogenic factor. The overproduction of LH and consequently the incorrect LH/FSH ratio is nowadays considered not to be a characteristic attribute of all PCOS patients. In this study elevated gonadotropin ratio was found only in 45.4 % of patients. Some studies assess the incidence of elevated LH/FSH ratio at even 94%. In 1975, Berger was the first to emphasize that one can differentiate a separate type of PCOS with normal gonadotropin level. At that time it was not associated with insulin resistance. Nowadays it is believed that elevated LH level occurs more rarely in a group of patients with insulin resistance and hyperinsulinemia, than in group without hyperinsulinemia. This observation was confirmed in a presented group of women, in which normal gonadotropin ratio 1: 1 was observed in up to 72% of patients with hyperinsulinemia. One may speculate that additionally to, that is considered to be a strongest androgen production stimulator, in women with normal LH level additional stimulators of steroidogenesis exist. Most probably it is insulin and IGF-I. Thus it could have been expected that the most severe clinical symptoms and greater androgen concentration would appear in women with hyperinsulinemia and overproduction of LH. However, the mean testosterone levels in the studied women, were independent of insulin and LH concentrations. Hirsutism of greater severity was observed in a group of women with hyperinsulinemia and LH/FSH ratio >2 when compared with women with hyperinsulinemia and normal gonadotropin ratio.

Interestingly, PCOS women with hyperinsulinemia and overproduction of LH, had significantly higher serum levels of dehydroepiandrosterone sulphate. In the remaining groups DHEAS concentration was normal. It is still not fully understood how insulin influences the adrenal androgen secretion. The negative correlation between insulin levels and dehydroepiandrosterone sulphate production have been found. On the other hand there are also studies that do not confirm correlation between insulin activity and adrenal androgen production [2].

Another interesting observation concerned sex hormone binding globulin. Remarkably lower SHBG concentration was noted in patients with hypernsulinemia, in comparison to the group without elevated insulin. Majority of available studies confirm low sex hormone binding globulin concentrations in women with insulin resistance and hyperinsulinemia. The negative influence of insulin on SHBG production in liver is well known [5,14,16]. It was unanticipated, though, to find significant differences in SHBG concentration in hyperinsulinemic groups with normal and high LH levels. In a group with elevated LH/FSH ratio SHBG globulin was higher than in a group with normal LH concentrations. It could be hypothesized that the excess of LH may reduce the influence of insulin on SHBG production. It is also interesting, that in the presented study, despite the higher index of free testosterone in women with hyperinsulinemia, clinical symptoms of hyperandrogenism were approximately similar in both of the groups. It is difficult to fully explain these results, we may only speculate that androgen peripheral activity was weakened by aromatization of androgens to estrogens, mainly to estron in excessively developed adipose tissue of those women.

PCOS is a very heterogeneous disorder of different phenotypes. In summary we would like to point out that: 1) LH/FSH ratio is not a characteristic attribute of PCOS women, 2) most of PCOS women with normal gonadotropin ratio belong to a subgroup of patients with hyperinsulinemia and obesity,

3) patients with hyperinsulinemia and excess of LH constitute probably a separate subpopulation with increased adrenal androgenic activity. Possibly, this is the group, in which two different pathogenic mechanisms interweave and present a characteristic clinical pictures of these women.

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Uterine contractility signals – an introduction to wavelet analysis

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Abstract

Purpose: **Analysis of the uterine contractility in the nonpregnant states has provided information about physiological changes during menstrual cycle. There is need to develop methods of recording uterine activity as well as mathematical interpretation of recorded time series. Wavelets are a new powerful tool for signal and image processing. The aim of this study is an introductory view of Fourier (one of the fundamental methods of investigating of biomedical signals) and wavelet transforms applications in the analysis of uterine contractions.**

Material and methods: **Spontaneous uterine activity of healthy patient and patient with dysmenorrhea was recorded by micro-tip two sensors catheter (Millar Instruments, Inc. USA). After amplification analogue signals were converted to digital. Signals were analysed using Fourier and wavelet transforms.**

Results: **Contrary to the Fourier decomposition, which is global and provides the information integrated over the whole signal, the continuous and discrete wavelet transforms allow to extract local and global variations of the recorded contractions. From the analysis of the coefficients of the wavelet transform we can assess various pattern of propagation: normal propagation, simultaneous propagation and inverted propagation.**

Conclusions: **This study is the introduction to the wavelet analysis of the uterine contraction signals. Wavelet transform provides insight into the structure of the time series at various scales. It allows to localise changes of the signal in time, providing additional information in comparison with the Fourier transform.**

Key words: uterine contractions, Fourier transform, wavelet transform.

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Introduction

Analysis of the uterine contractility in the nonpregnant states has provided information about physiological changes during the menstrual cycle [1]. There is need to develop methods of recording uterine activity as well as mathematical interpretation of recorded time series [2]. One of the fundamental methods of investigating various kinds of biomedical signals is the Fourier transform [3], which analyses signal in terms of periodic basis function (sine and cosine). Wavelets are a new powerful tool for signal and image processing. In the wavelet transform signal is decomposed into elementary components well localised in the time domain and in the frequency domain. This method has been successfully used in a number of fields. The wavelet transform have been used to analyse heart rate variability [4], EEG signals [5], electromyographic signals [6] and other kinds of biomedical signals [7].

The aim of this study is an introductory view of Fourier and wavelet transforms applications in the analysis of uterine contractions.

Data acquisition

Spontaneous uterine activity was recorded by a micro-tip catheter (Millar Instruments, Inc, USA). The study was approved by the regional ethics committee. The device consisted of two miniature pressure sensors (the distal sensor and the proximal sensor). The distance between sensors was 30 mm. The sensors produced electrical signals which varied in direct proportion to the magnitude of sensed pressures. After amplification, analogue signals were passed to IBM computer for conversion to digital form by means of an analogue-digital

Figure 1. Fourier analysis of normal uterine contractions.

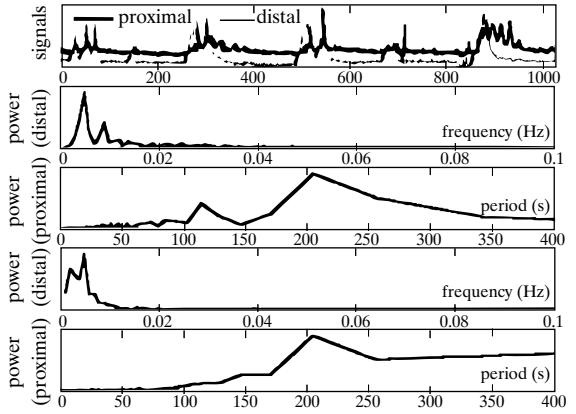
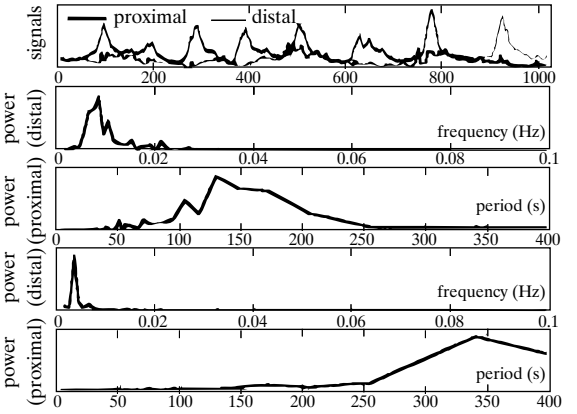


Figure 2. Fourier analysis of the contractions of the patient with dysmenorrhea.



(A/D) converter. Converted signals were recorded with a frequency 2 Hz on a computer hard disk. The sampling frequency may be changed in acquisition procedures. For analysis of the recorded signals programs written in MATLAB (MathWorks, Inc, USA), a high-performance language for technical computing, were used. Signal Processing Toolbox and Wavelet Toolbox appeared very helpful.

Fourier analysis

Intrauterine pressure signals may be analysed in the time domain or in the frequency domain. Parameters in the time domain such as area under curve recording (AUC), maximal amplitude of contractions and various statistical quantities (mean, standard deviation, median, skewness and so on) are easily computed even for short time window [7]. In frequency domain signal is decomposed by means of spectral analysis into its sinusoidal components. The Fourier transform (FT) of a signal x(t) is defined as [2]

$$X(f)=\int_{-\infty}^{+\infty} x(t)\exp(-2\pi ift)dt, \qquad (1)$$

where f is the frequency in cycles per unit time, usually in cycles per second (Hz).

The power spectrum or power spectral density function is given by

$$P(f)=|X(f)|^2. \qquad (2)$$

We can plot the power as a function of frequency. This information (frequency-based distribution of power) is very important from the medical diagnostics point of view. Fourier transform has some drawback. Frequencies that occur in one part of signal may be quite different from frequencies in the other part of it. There is no information about the localisation of the individual frequency components. Power spectrum gives only the frequency composition integrated over the whole signal or the whole analysing segment of the signal.

Basis function

$$\exp(-2\pi ift)=\cos(2\pi ift)-i\sin(2\pi ift), \qquad (3)$$

are perfectly localised in frequency domain, but they are not localised in time domain, they are infinite in time. *Fig. 1* shows an example of Fourier spectral analysis of uterine activity signals of normal patient performed by means of fast Fourier transform (FFT). For convenience, there are also the power versus period plot. Power spectra for distal and proximal signals are very similar. In *Fig. 2* there are the results of Fourier decompositions of the distal and the proximal signals of patient with dysmenorrhea. The power spectra for distal and proximal uterine activities are quite different (*Fig. 1*).

The Fourier transform is a global one. We have no information about localisation of the frequencies in time. Such information gives us the windowed Fourier transform (WFT), also known as short time Fourier transform (STFT) [9].

$$X(f,\tau)=\int_{-\infty}^{+\infty} x(t)w(t-\tau)\exp(-2\pi ift)dt, \qquad (4)$$

$X(f,\tau)$ is a two dimensional function of time and frequency, is the window outside of which the signal is suppressed (*Fig. 2*).

The spectrogram SPEC is defined as the square modulus of STFT

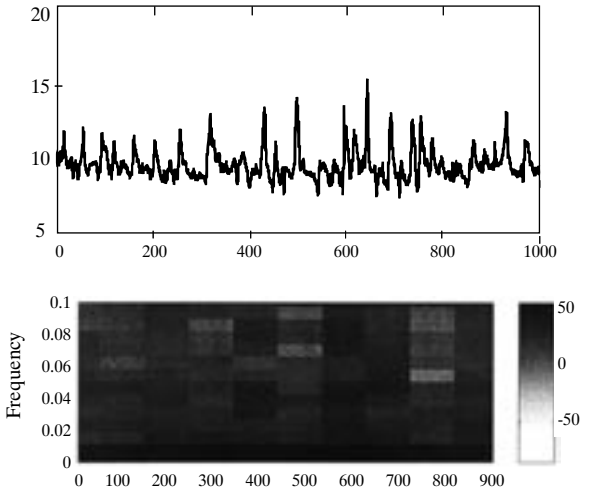
$$SPEC(f,\tau)=|X(f,\tau)|^2. \qquad (5)$$

$SPEC(f,\tau)$ is the energetic version of short time Fourier transform, it provides the energy distribution on a time-frequency plane. Often spectrogram is defined as the magnitude of STFT. Time-frequency localisation obtained by means of STFT is not precise. The product of the frequency and the time resolutions is constant

$$\Delta f\cdot\Delta t=\text{const}, \qquad (6)$$

so the increase of resolution in one domain causes the decrease of resolution in the other one. In uterine contraction activity signals low frequencies play the dominant role, thus the resolution in time domain is poor. Biomedical signals are in general very complicated. Sharp spikes, noise, non-stationarity result in Fourier decomposition of such time series into harmonic function being not satisfactory. *Fig. 3* shows that it is very difficult

Figure 3. The uterine contraction signal and its spectrogram.



to extract diagnostic information from spectrogram of uterine contraction activity signal (*Fig. 3*).

Wavelet transform

The wavelet transform is a new mathematical tool for the analysis of signals and images. The definition of the wavelet transform is similar to that of the Fourier transform. Instead of the periodic functions (sine and cosine) we use wavelets. The continuous wavelet transform (CWT) of a signal x(t) is given as [10]

$$CWT[x(t)]=W(a,b)=\int_{-\infty}^{+\infty}x(t)\psi_{a,b}(t)dt=\int_{-\infty}^{+\infty}x(t)\frac{1}{\sqrt{a}}\psi(\frac{x-b}{a})dt \quad (7)$$

where: $\psi_{a,b}(t)=\frac{1}{\sqrt{a}}\psi(\frac{x-b}{a})$ is the analysing wavelet (mother wavelet),

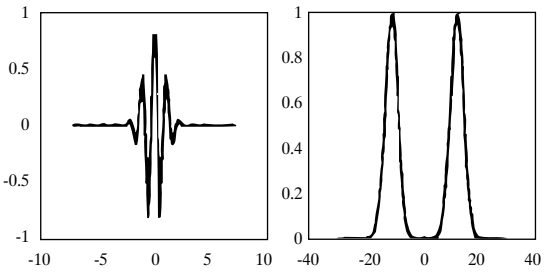
a – determines dilation, b – specifies translation and $(-\infty<a, b<\infty, a\neq 0)$.

In other words 'a' means scale and 'b' means position. Thus the equation (1) may be rewritten in a simpler form

$$W(a,b)=W(\text{scale, position})=\int_{-\infty}^{+\infty}x(t)\psi(\text{scale, position})dt \quad (8)$$

The term scale, used in wavelet analysis, is similar to the scale used in geographical maps. We can obtain small scales (high frequencies) or large scales (low frequencies) components of the analysed time series. In the wavelet transform we have an infinitive set of possible basis functions. Among them are Daubechies wavelets, Mexican hat wavelet, Meyer wavelet, Haar wavelet, Morlet wavelet, Coiflet wavelet, Spline wavelet and others. All of these functions are well localised in time and well localised in frequency. *Fig. 4* shows the Morlet wavelet and the magnitude of the Fourier transform of that wavelet $|\Psi(\omega)|$. We can see a good localisation of these functions in the time domain and in the frequency domain (*Fig. 4*).

Figure 4. The Morlet wavelet $\psi(t)$ and the magnitude of the Fourier transform $\psi(t)$ of that wavelet.



For admissible mother wavelet (the Fourier transform of this function has neither a zero frequency component nor infinite frequency components) the equation (1) there is a convolution integral. Thus, wavelet analysis can be viewed as a filtering process. Coefficients W(a, b) are obtained by band-pass filtering signal x(t) by wavelet ψ. If wavelet coefficients are large the resemblance between the signal and the wavelet is strong, otherwise it is slight.

It is possible to analyse a signal in time-scale plane with relative accuracy. Time-frequency plane is equivalent to time-scale plane. The wavelet spectrogram which is also named wavelet scalogram or shorter scalogram is defined as [10,11].

$$WSCAL(a,b)=|W(a,b)|^2. \quad (9)$$

Scalogram shows the distribution of energy in the time-scale plane. Scalogram can also be defined as a the magnitude of wavelet coefficients. Large scale corresponds to a stretched wavelet, small scale corresponds to a compressed wavelet (*Fig. 5*).

Fig. 5 shows us four scalograms of uterine contractions time series. We can see that for the normal contractions patterns the distal and the proximal signals are very similar. In the case of dysmenorrhea contractions, patterns are quite different. The local maxima of the absolute values of the wavelet coefficients reveal the occurrence of sharp time series variations. Local minima are connected with the occurrence of slow signal changes. The sharp temporal variations are zooming in over all scales.

Discrete wavelet transform

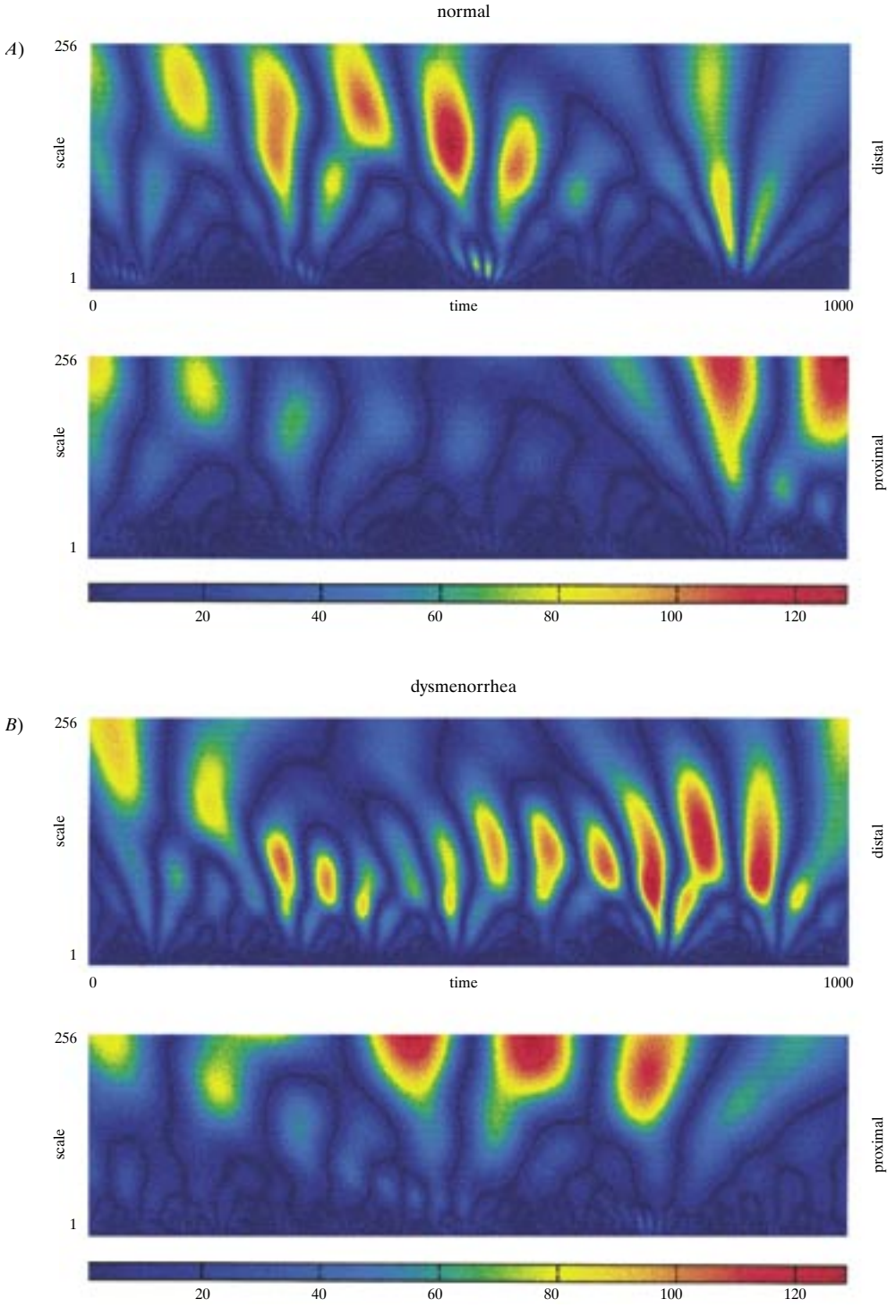
The disadvantages of the continuous wavelet transform are computational complexity and redundancy. They can be reduced by discretising scale 'a' and position 'b'. In dyadic representation, scale and position are based on power of two, so the wavelet decomposition works like a cascaded octave band-pass filter. Octave is the interval where the frequency at the end is twice the frequency at the beginning. For a forward discrete wavelet transform

$$DWT[x(t)]=W(j,k)=\sum_{n\in Z}x(t)\psi_{j,k}(t), \quad (10)$$

the scale and position are based on power of two ($a=2^j$, $b=k2^j$ – dyadic representation), j, k are the level and position parameters and $\psi_{j,k}(t)=2^{-j/2}\psi(2^{-j}t-k)$.

Wavelet coefficients W (j,k) = W (level, position) at the

Figure 5. A) the scalograms of normal uterine contractions, *B*) the scalograms of contractions of patient with dysmenorrhea.



lower parameter j represent the characteristic of the signal at higher frequency and vice versa. In discrete wavelet decomposition, wavelet coefficients are computed for $j = 1, 2, 3, \ldots$. At each level j the number of W(j,k) is reduced by 2^j. In practise, the maximum of 11 steps of wavelet decomposition is sufficient.

An efficient way to compute the discrete wavelet transform is the multiresolution signal decomposition algorithm (MRSDA), known also as the Mallat algorithm [12]. In the multiresolution algorithm time series is decomposed into a collection of orthonormal function, which are obtained by transactions and dilations of a scaling function $\varphi(t)$ and the wavelet $\psi(t)$. In practise, here the signal is convolved with a pair of quadrature mirror decomposition filters (QMF's) – lowpass filter L and highpass filter H and afterwards results are downsampled by a factor of two. This operation splits the signal bandwidth in half. As the results approximation coef-

Figure 6. The discrete wavelet decomposition of a signal.

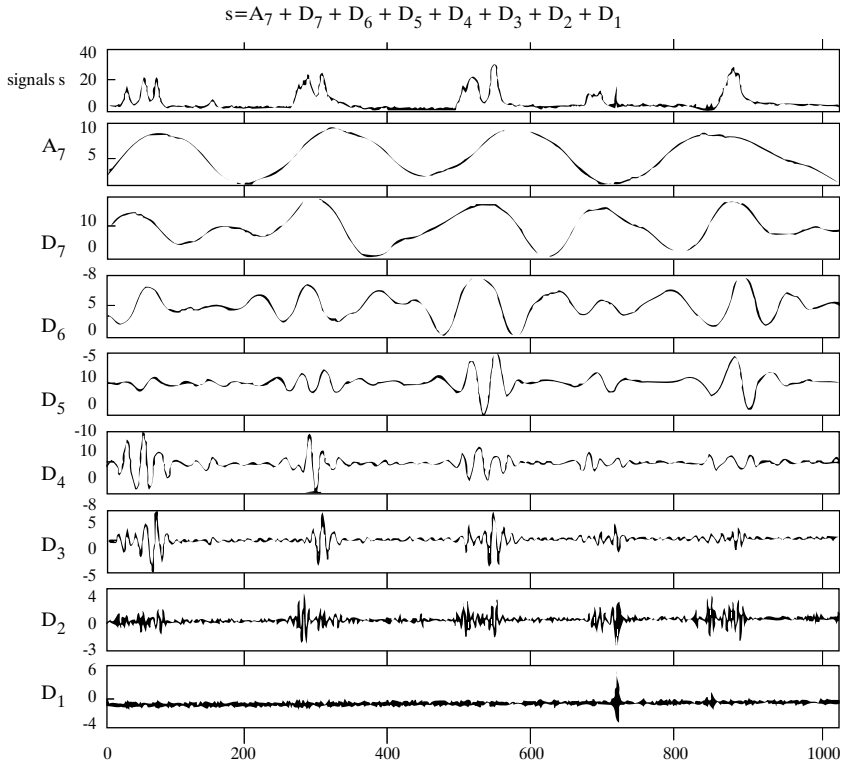
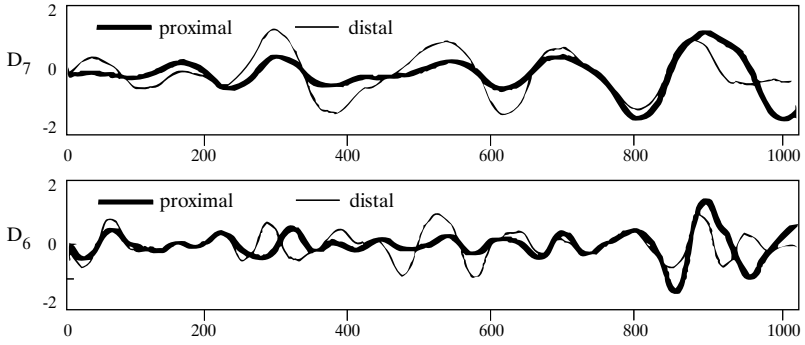


Figure 7. Assessing the propagation of the signal from details D_6 and D_7.



ficients cA_1 and detailed coefficients cD_1 are obtained. The process is iterated but only the approximation coefficients are further processed. From wavelet coefficient obtained by means of Mallat algorithm we can reconstruct approximations A_j and details D_j, so for a given reference level J, signal x(t) may be expressed as:

$$x(t)=A_J(t)=\sum_{j\le J} D_j(t), \qquad (11)$$

Approximations are the high scale low frequency components of the signal. Details are low scale high frequency component of the signal.

Fig. 6 shows how the time series is built from its approximation and details components. The level of performed discrete wavelet transform was 7. We reconstructed approximation A_7 and details $D_j(1\le j\le 7)$. This seven wavelet decomposition represents the following frequency bands (from D_1 to D_7): 0.25-0.5 Hz; 0.125-0.25 Hz; 0.0625-0.125 Hz; 0.03125-0.0625 Hz; 0.015625-0.031125 Hz; 0.0078125-0.015625 Hz; 0.00390625--0.0078125 Hz. These are equivalent to the following period bands: 2-4 s; 4-8 s; 8-16 s; 16-32 s; 32-64 s; 64-128 s; 128-256 s. Approximation is the component between 0Hz and the lowest frequency at the frequency band at level 7 (*Fig. 6*).

Propagation of uterine contractions signals

By means of the wavelet transform we can determine the propagation of the uterine contraction signals [13]. From the details D_j we can assess the time lag between the distal and proximal signals. There are various patterns of propagation of contractions:

– normal propagation – the uterine fundus contracts before the uterine os, positive lag,

– inverted propagation – the uterine fundus contracts after the uterine os, negative lag,

– simultaneous propagation – the uterine fundus and the uterine os contract simultaneously, lag equals 0.

Fig. 7 shows that from the details D_6 and D_7 we can assess the direction of propagation of the signal. It can be seen that in both cases the uterine fundus contracts before the uterine os. The proximal signals are delayed in relation to distal signals (*Fig. 7*).

Concluding remarks

This study is the introduction to the wavelet analysis of the uterine contraction signals. We discuss both the Fourier transform and the wavelet transform. Wavelet transform provides insight into the structure of the time series at various scales. It allows to localise changes of the signal in time, providing additional information in comparison with the Fourier transform. Wavelet analysis may be used in the research on dysmenorrhea and endometriosis as an additional tool.

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The evaluation of dentition status in HIV-infected patients

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Abstract

Purpose: The aim of the study was the evaluation of dentition status in patients infected with HIV.

Material and methods: We examined 30 HIV+ patients, aged 20-46 and 30 non-infected subjects as the control group. Oral hygiene and dentition status were estimated. Oral hygiene status using simplified OHI – plague index according to Green and Vermilion. Dentition status was analysed using decay intensity index (DMF) as well as teeth loss index according to Rogowiec. The results were analysed in dependence on HIV infection with regard to infection time.

Results: The results point to a high intensity of decay in HIV+ patients (23.66). There was a positive correlation between infection time and decay intensity and teeth loss evaluated using Rogowiec index. Unsatisfactory oral hygiene status (OHI≥1) was observed in 53.33% of infected patients. There is a relation between infection time and oral hygiene status. OHI – plague index increased in patients with infection time longer than 5 years up to 2.99 (patients with shorter than 5 years infection time – 1.17 and the control group – 0.57).

Conclusions:

1. There is a positive correlation between HIV infection and dentition status and oral hygiene.
2. Infection time influences index values: decay intensity, teeth missing, and oral hygiene.
3. HIV+ subjects are patients of high necessity of therapy and because of their basic disease they should come within broadened health education and prophylactic activities.

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Key words: HIV infection, dentition status, oral hygiene status.

Introduction

The report of joint program of the United Nations concerning HIV/AIDS and the World Health Organization, published in October 2000, has indicated rapid increase in the number of HIV infection in the Eastern Europe. Although Russia is the most affected by HIV infection, Poland also reveals increase in number of infected subjects from year to year [1]. According to the report of the National Department of Hygiene, only in December 2002, 54 Polish subjects were infected with HIV.

The infection spreads mainly by blood-derivative means, more rarely by sexual way and sporadically by other ways of transmission. Among all HIV-infected in Poland, 70% are drug-addicts who use intravenous narcotics [2,3].

Increasing number of AIDS patients and HIV-infected subjects increases the possibility of contact between infected people and medical care staff. A dentist is a first contact physician who can contribute to early diagnosis of the infection on the basis of case history and oral changes. Oral diseases in sero--positive patients belong to the group of changes characteristic for HIV infection diagnosis. Some of them, e.g. parodontal disease is connected with HIV and ulcerative gingivitis is strongly connected with HIV. On the basis of the observation, in 1992, the World Health Organization has divided disease processes of the oral cavity occurring in the course of HIV infection into 3 groups [3]: Group I – changes strongly connected with HIV, Group II – changes occurring more rarely but connected with HIV, Group III – changes probably occurring in HIV.

The aim of the study was the evaluation of dentition status in patients with HIV. Few studies concerning oral status of HIV-infected patients have been published, which is the reason for the present study. A positive aspect of our examination is the inspiration for discussion among dentists on their cooperation with sero-positive patients.

Table 1. Dental decay intensity expressed with the mean DMF in examined groups.

Examined group	Number of subjects	D	M	F	DMF
Infected (1)	30	12.93	9.10	1.63	23.66
Non-infected (2)	30	6.07	3.40	5.57	15.04
Statistical analysis – $p<0.05$ for groups		1 and 2	1 and 2	1 and 2	1 and 2

Table 2. The mean value of DMF and its components with regard to time of infection.

Examined group	Number of subjects	D	M	F	DMF
Infected <5 years (I)	16	15.06	5.69	1.69	22.44
Infected >5 years (II)	14	10.50	13.00	1.57	25.07
Non-infected (III)	30	6.07	3.40	5.57	15.04
Statistical analysis – $p<0.05$ for groups		1 and 3	1 and 2	1 and 3	2 and 3
			2 and 3	2 and 3	

Table 3. Missing teeth according to Rogowiec in the examined groups.

Examined group	Number of subjects	Molars/ /premolars	Canines	Incisors
Infected (1)	30	5.50	0.60	1.37
Non-infected (2)	30	2.83	0.20	0.43
Statistical analysis – $p<0.05$ for groups		–	–	–

Table 4. Missing teeth according to Rogowiec in examined groups depending on infection time.

Examined group	Number of subjects	Molars/ /premolars	Canines	Incisors
Infected <5 years (I)	16	3.94	0.44	0.56
Infected >5 years (II)	14	7.29	0.79	2.29
Non-infected (III)	30	2.83	0.20	0.43
Statistical analysis – $p<0.05$ for groups		1 and 2	1 and 3	1 and 2
		2 and 3		2 and 3

Material and methods

We carried out the examination in the group of 30 HIV+ patients, 11 women and 19 men, 20-46 years of age. The control group consisted of 30 subjects; respectively chosen as far as sex and age were concerned. Oral examination was conducted at artificial light using diagnostic dental equipment. Oral hygiene and dentitions were evaluated. Oral hygiene status was presented using a simplified Oral Hygiene Index (OHI – plague) according to Green and Vermilion [4-6]. Dentition status analysis was performed using intensity decay index (DMF – Decayed, Missing, Filled) together with the analysis of particular components of mean DMF [1,7] as well as tooth loss index according to Rogowiec [8].

The results were analyzed regarding HIV infection taking into account time of infection. Time between the moment of potential infection and the examination was considered as infection time. Thus, the patients were divided into 3 groups: group I – patients whose infection time did not exceed 5 years, group II – patients with more than 5 years of infection time, and group III – healthy subjects.

The results were analyzed statistically using Statistica.

Results

The results point to 100% of dental decay occurrence in both examined groups.

The intensity of dental caries, expressed with the mean DMF, is significantly higher in infected patients (DMF=23.66) than in the control group (DMF=15.04). Considering particular components of DMF number in the group of HIV-positive patients we should notice a high number of teeth with active decay/caries process (D=12.93), which exceeds remaining components, i.e. the number of extracted teeth (M=9.10) and the number of filled teeth (F=1.63). Adverse relations could be seen in the control group in which 5.57 of filled teeth, 6.07 teeth with decay/caries, and 3.40 extracted teeth were observed per a person. Statistically significant differences between the examined groups and the controls concern the mean number of DMF index and its components. The result of comparison of filled teeth mean number is particularly unfavorable as the component was several times lower than in the controls. *Tab. 1* presents the data described above.

Dentition status with regard to infection time is presented in *Tab. 2*. Comparing data we can notice that decay intensity, expressed with DMF index, is higher by 2.63 in group II than in group I and by 10.03 than in the controls (group III). We analyzed the structure of particular compounds of mean DMF and observed a correlation between the time of infection, the number of teeth with active decay process (D) and the number of teeth extracted (M). At an early period of infection, the values of D index are high (15.06), approximately 3 times higher than these of M index (5.69). Afterwards, there are changes in these relations, i.e. the number of teeth with active decay process decreases to 10.50 while the number of extracted teeth and the compound increase (M=13.00). The mean number of filled teeth (F) remains stable in both infected groups, group I – 1.69 and group II – 1.57.

Tab. 3 shows mean values of missing teeth index according to Rogowiec [8]. There are generally higher values in HIV-infected patients as compared to non-infected subjects; it concerns all tooth groups. The highest loss was observed in the lateral part (morals and premolars) in sero-positive patients who lost mean 5.50 of teeth, i.e. by 2.67 more teeth than in the controls. The anterior part also reveals higher tooth

Table 5. Oral hygiene status in examined population.

Examined group		Number of subjects	OHI pl X	OHI pl					
				<1			≥1		
				n	%	x	n	%	x
Infected	<5 years	14	1.17	8	57.14	0.46	6	42.86	1.69
	>5 years	16	2.99	6	37.50	0.50	10	62.50	2.23
	Total	30	2.14	14	46.67	0.43	16	53.33	2.03
Non-infected	Total	30	0.57	22	73.33	0.37	8	26.67	1.12

loss in infected patients and in the part of incisors it reaches the value of 1.37, canine teeth – 0.60 while in non-infected subjects the values were three times lower and were 0.43 and 0.20, respectively.

Tab. 4. presents the analysis of missing teeth depending on infection time. The highest number of missing teeth was observed in patients infected for more than 5 years. The mean number of extracted molar and premolar teeth reached 7.29 while missing teeth index in the lateral part in the rest of patients was approximately two times lower and in group I was 3.94 and in group III – 2.83. After a period of time, infected patients showed the increase in missing front teeth index. Subjects infected for less than 5 years (group I) missed mean 0.56 of incisors while group II had 2.29 incisor extracted. A similar relation could be seen as far as canines were concerned; group I showed 0.44 of missing teeth and 0.79 in group II. Statistical analysis revealed significantly higher tooth loss in subjects with a long duration of infection than in patients infected for less than 5 years and in non-infected. It concerned missing teeth both in the anterior and lateral parts.

Tab. 5. presents oral hygiene status in examined population. It shows that 53.33% of patients with HIV had unsatisfactory status of oral hygiene (the value of OHI index ≥1) while 26.67% of the control group revealed high values of this index. The observation was confirmed by mean OHI-plague index, which equaled 2.14 in infected patients and several times exceeded that of the controls (0.57).

Data presented in *Tab. 5*. point to the relationship between infection time and oral hygiene status. OHI-plague index in patients infected for less than 5 years was 1.17 and increased together with duration time of infection up to 2.99 in patients infected for more than 5 years. The percentage of patients with shorter infection time with poor oral hygiene was 42.86% and increased to 62.50% in patients with longer infection time.

Discussion

HIV-positive subjects made a small percentage of people inhabiting Podlasie province. However, as the number of subjects infected increases, their dentitions and dental treatment needs become a problem, which has to be solved. It should be noticed that 76.7% of examined HIV+ patients are addicted to narcotics. A high percentage of addicted in the examined group makes us compare our results and those of Szymaniak et al. [6], who evaluated drug-addicts dentition status. In their studies, DMF index was 18.8 while our studies revealed 23.66 in HIV+ patients and was higher by 4.86. A higher mean number of DMF in infected patients occurs as a result of more teeth with active decay process and extracted teeth with similar number of filled teeth. The analysis of our results shows a high intensity of decay in HIV+ patients, which is confirmed by some authors [9,10] and denied by others [11].

As far as drug-addicts are concerned, it seems that the degree of their dentition damage is proportional to addiction time [7]. This opinion is confirmed by our studies concerning HIV-positive patients. After a period of time, DMF index increases and its particular compounds are changed. A high number of teeth with active decay process in patients with a short infection time (P=15.06) decreases in patients with longer infection time (P=10.50). The number of extracted teeth increases from 5.69 in group I to 13.0 in group II. The number of filled teeth does not change. Such a structure of DMF compounds suggests that HIV+ patients, despite high decay intensity, in the first period of infection did not undergo conservative treatment and extractions are the only effect of visits at the dentist's.

Baqui et al. [9] evaluated relation between tooth decay advancement and the level of HIV+ patient viral load. Their results showed that from 42.9% to 69.2% of HIV--positive patients were characterized by a high intensity of decay (DMF>20) and a percentage of patients with high DMF index increased with the increase in viral load.

As far as missing teeth index is concerned, its higher values are generally observed in HIV+ patients. The analysis shows that despite young age of infected people, tooth loss concerned anatomical tooth groups. A high index of missed teeth in the anterior part in HIV+ patients, which in case of both incisor and canine teeth is three times higher than in the controls, attracts attention. The results are confirmed by Szymaniak et al. [6] studies concerning missing teeth in drug-addicts.

Our studies confirmed the opinion that the degree of dentition damage is proportional to HIV infection time. While analyzing the results we observed statistically significant smaller teeth loss in patients with shorter infection time with reference to all groups of teeth. However, we could not find any reports on the relation between particular teeth group loss and infection time.

Oral hygiene reflects dentition status to a certain extend. Our results show the relation between high intensity of decay and poor status of the oral cavity in HIV+ patients. We also observed a correlation between infection time and OHI-plague and DMF index values. Both worse oral hygiene and dentition status characterized HIV-positive patients with long period of infection as compared to those with shorter time of infection and non-infected subjects. However, there are no reports con-

cerning oral hygiene status in HIV-positive patients. Thus, the comparison is not possible.

As most HIV+ patients are those addicted to narcotics, it can be assumed that intensity of illness process in the oral cavity occurs due to social and psychic disintegration and desistance of oral hygiene [12,13]. During taking drugs, the hierarchy of values and needs changes. After discontinuance, there occurs fear of dental treatment and lack of acceptance by the dentist. It causes postponing visits which leads to marked destruction in dentition.

In conclusions: 1) there is a positive correlation between HIV infection and dentition status and oral hygiene, 2) infection time influences index values: decay intensity, teeth missing, and oral hygiene, 3) HIV+ subjects are patients of high necessity of therapy and because of their basic disease they should come within broadened health education and prophylactic activities.

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Release of hydroxyl ions from calcium hydroxide preparations used in endodontic treatment

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Abstract

Purpose: The purpose of our study was to compare the in vitro release of hydroxyl ions from several calcium hydroxide preparations used in endodontic treatment.

Material and methods: Equal quantities of the materials – nonsetting (pure calcium hydroxide, Biopulp, Calcicure), setting – canal sealers (Sealapex, Apexit) and points were placed in dialisis tubes which were then immersed in deionized water. The release of hydroxyl ions from the preparations was measured by the median pH of the deionized water used for dialisis, by means of a pH-meter. The results of our study were analized by means of Tukey's resoneable correlation. Significance difference (one-way variance analysis ANOVA) and Pearson's linear correlation coefficient (r^2).

Results: Nonsetting preparations of calcium hydroxide have a significantly higher capability of hydroxyl ions release in comparison with sealers and points, irrespective of time ($p<0.05$). Sealapex and "plus" points released hydroxyl ions to a much greater extent than both Apexit and "regular" points at most periods of the experiment ($p<0.05$). Apexit released significantly more of hydroxyl ions than "regular" points, and Sealapex more than "plus" points in the later periods of the experiment ($p<0.05$). The pH values of dialysis samples of all materials correlated positively with time and the pH. Almost all materials reached a maximum on the 8-th day of the experiment.

Conclusions: To achieve maximum concentration of hydroxyl ions in tissues: • for temporary root fillings nonsetting preparations of calcium hydroxide should be chosen rather than points and they should be placed for at least one week, • for permanent root fillings it is more recommended to use Sealapex than Apexit as a sealer.

Key words: calcium hydroxide, release of hydroxyl ions.

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Introduction

Calcium hydroxide is widely used in dentistry and especially in endodontics as a dressing in periapical tissue inflammation, in cases of tooth root fracture, mechanical or inflammatory perforations as well as in the process of apexification [1-4]. The therapeutic effectiveness of calcium hydroxide dressing materials is based on the release of hydroxyl ions causing an increase in pH [5]. This alkaline pH promotes the action of alkaline phosphatase, a vital enzyme in the calcification, which requires an optimal pH of 10.2 [5,6].

The calcium hydroxide preparations used as dressing materials for temporary or final root canal filling are available as either setting, or nonsetting compounds and points and may release hydroxyl ions in varying degrees thus exerting different therapeutic effects. From the clinical point of view, it is therefore reasonable to measure the release of hydroxyl ions from different types of calcium hydroxide preparations available. It is also important to know whether the pH of these preparations changes with time.

The aim of our study was firstly to compare the in vitro release of hydroxyl ions from the various calcium hydroxide preparations used in endodontic treatment and secondly to determine whether their pH altered with time.

Material and methods

The calcium hydroxide preparations used in our study are summarized in *Tab. 1*. The methodology provided by Economides et al. was used in the study [7].

Table 1. Calcium hydroxide preparations used in present study.

Group	Product	Code	Manufacturer	Batch number	Composition
1 - nonsetting preparations	"pure" calcium hydroxide	PCH	Dental Therapeutics AB/Gillevagen, Sweden	510200	The ready – made paste consisting of pure calcium hydroxide and destilled water q.s.
	Biopulp *	BP	Chema Elektromet/ /Rzeszów, Poland	010201	Calcium hydroxide, bibasic calcium phosphate, magnesium oxide, sodium chloride, potassium chloride, dehydrated sodium carbonate
	Calcicure #	CR	VOCO/Cuxhaven, Germany	10946/M/97	Radiopaque water – based calcium hydroxide paste
2 - setting preparations	Apexit	AT	Vivadent Ets./Schaan, Liechtenstein	533281AN	Calcium hydroxide, hydrogenized calaphony, silicon dioxide, paraffin oil, calcium oxide, zinc oxide, calcium phosphate, polidimethylosiloxane, alkyl ester of phosphorid acid, trimethylohexanediosalicylate
	Sealapex	SX	Kerr Italia S.p.A/ /Salerno, Italy	68432	Calcium oxide, barium sulphate, zinc oxide, sub-micron silica, titanium dioxide, zinc stearate
3 - calcium hydroxide points	"Regular"	RCHP	Roeko GmbH/ /Langenau, Germany	367097	Calcium hydroxide, guttapercha, colouring agent
	"Plus"	PCHP	Roeko GmbH/ /Langenau, Germany	366896	Calcium hydroxide, guttapercha, sodium chloride, surfactant, colouring agent

* Biopulp was mixed with destilled water (pH 7.5) (Polpharma SA/Starogard Gdański, Poland, 031199) in proportion 0.07 g/0.03 g, respectively

\# The manufacturer does not give details about the composition

The materials of groups 1 and 2 were prepared according to the manufacturers' instructions. Equal quantities (0,1g) of the substance were placed in dialysis tubes (Sigma Aldrich Chemie GmbH, Steinheim, Germany, batch number D9277) which were then placed in deionized water of 6.6 pH. Four samples of each of the test materials were prepared.

The release of hydroxyl ions from each preparation was determined by measuring the pH value of the deionized water used for dialysis by means of a pH-meter model 5170 (ELWRO, Częstochowa, Poland). The pH value was measured directly after placing the samples in the water and after 30, 60, 90, 120 minutes and on the 2-nd, 8-th and 15-th day. The materials were kept in an incubator model Cultura (Almedica AG, Galmiz, Swiss) at a temperature of 37°C throughout the period of observation.

The results of our studies were analyzed by means of Tukey's reasonable significant difference (one-way variance analysis ANOVA) and Pearson's linear correlation coefficient (r^2). Statistical analysis was performed on the basis of computer programm Statistica 5.0 PL.

Results

The results obtained in our study are presented in *Fig. 1* and *Tab. 2,* and *3*. The figure shows that the greatest increase in pH occurred in the first 30 minutes of experiment. The pH of the samples increased gradually reaching maximum values on day 8 of the experiment and then decreased over the next seven days. A further slight increase in pH was only observed in the calcium hydroxide "plus" points and in "pure" calcium hydroxide.

As it can be seen in *Tab. 2*, a positive correlation between pH and time was found for all materials in this experiment – the highest for "pure" calcium hydroxide and the "plus" points ($r^2=0.72$) and the lowest for "regular" points ($r^2=0.18$). A positive correlation was observed between the pH values of particular samples – the highest for Biopulp, a nonsetting paste, and "pure" calcium hydroxide ($r^2=0.99$) and the lowest for "plus" and "regular" points ($r^2=0.74$).

The correlation between the pH values of almost all the test materials and time (with the exception of "regular" points) and the correlation between pH values in particular samples was statistically significant ($p<0.05$).

Tab. 3 shows that nonsetting calcium hydroxide preparations (group 1) presented the highest initial alkaline pH, ranging from 9.93 ± 0.06 to 12.24 ± 0.01 for "pure" calcium hydroxide, from 10.10 ± 0.08 to 12.08 ± 0.05 for Biopulp and from 10.39 ± 0.20 to 12.09 ± 0.04 for Calcicure. These differences were statistically significant with regard to Calcicure and "pure" calcium hydroxide, Calcicure and Biopulp after 30, 60, 120 minutes and also on the second day of the experiment ($p<0.05$).

Canal sealers (group 2) and calcium hydroxide points (group 3) presented lower values of pH (*Tab. 3*).

In group 2, Sealapex pH – from 7.89 ± 0.03 to 11.77 ± 0.05

Figure 1. Correlation between pH and time with regard to particular materials.

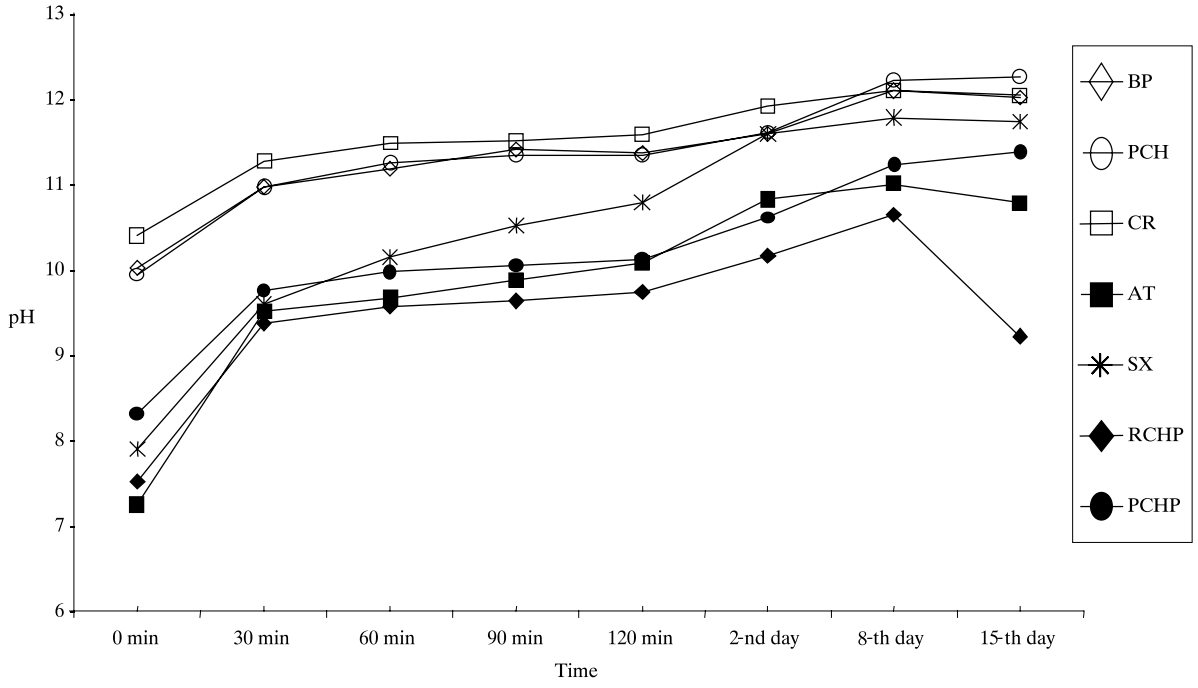

Table 2. Correlation between the pH values of particular materials and the time, and correlation between preparations in the whole period of the experiment.

	Time	BP	PCH	CR	AT	SX	RCHP	PCHP
Time	–	◊	■	◊	◊	◊	○	■
BP	◊	–	◄	◄	◄	◄	■	◄
PCH	■	◄	–	◄	◄	◄	■	◄
CR	◊	◄	◄	–	◄	◄	■	◄
AT	◊	◄	◄	◄	–	◄	■	◄
SX	◊	◄	◄	◄	◄	–	■	◄
RCHP	○	■	■	■	■	■	–	■
PCHP	■	◄	◄	◄	◄	◄	■	–

◄ – almost complete correlation $0.9 < r^2 < 1$; ■ – very high correlation $0.7 < r^2 < 0.9$; ◊ – high correlation $0.5 < r^2 < 0.7$; ○ – low correlation $0.1 < r^2 < 0.3$

– was higher than that for Apexit – from 7.24±0.12 to 10.99±0.21 – and these differences were statistically significant at almost all periods of the experiment (except at 30 minutes) ($p<0.05$).

In group 3, alkaline pH ranged from 7.51±0.43 to 10.66±0.07 for "regular" points and from 8.30±0.43 to 11.22±0.22 for "plus" points and these differences were statistically significant in all periods of the experiment ($p<0.05$).

Moreover, significantly higher values of pH were observed in group 1 in comparison with group 2 and 3 at almost all periods of the experiment (except the 2-nd and the 15-th day) ($p<0.05$).

Among the materials of group 2 and 3, the pH of Sealpex was higher than the pH of both types of points after 60, 90, 120 minutes, and also in the 2-nd and 8-th day of the experiment. The pH of Apexit was higher than the pH of "regular" points only in the last five periods of the experiment. The differences were statistically significant ($p<0.05$).

Discussion

The therapeutic effect of calcium hydroxide is due to its breakdown into calcium and hydroxyl ions. Hydroxyl ions show an affinity to various biologically active substances [8]. Their antimicrobal activity is important since microbes cause the majority of endodontic diseases [9].

The antibacterial activity of hydroxyl ions is related to the formation of a potent alkaline medium leading to the destruction of lipids, the main component of bacterial cell membrane, and their causing structural damage to bacterial proteins and nucleic acids [8]. Numerous publications support the theory regarding bactericidal activity of calcium hydroxide [10-14].

The results of our in vitro studies, indicating that nonsetting calcium hydroxide preparations (group 1) release hydroxyl ions most easily, are consistent with other authors' findings [1,3,7,9,15,16]. The pH values obtained in our study proved

Table 3. Mean pH values of calcium hydroxide preparations analyzed during the experiment.

Preparations	Time							
	0 min	30 min	60 min	90 min	120 min	2-nd day	8-th day	15-th day
BP (a)	10.01 ±0.08	10.96 ± 0.01	11.17 ± 0.04	11.40 ± 0.05	11.36 ±0.02	11.58 ± 0.00	12.08 ±0.05	12.00 ±0.09
PCH (b)	9.93 ±0.06	10.96 ±0.03	11.24 ± 0.05	11.33 ± 0.03	11.33 ± 0.09	11.60 ±0.01	12.21 ±0.04	12.24 ± 0.01
CR (c)	10.39 ±0.20	11.25 ±0.04	11.47 ± 0.02	11.50 ± 0.03	11.56 ± 0.02	11.90 ± 0.02	12.09 ± 0.04	12.03 ± 0.01
AT (d)	7.24 ±0.12	9.50 ±0.03	9.66 ±0.03	9.87 ± 0.03	10.06 ± 0.06	10.81 ± 0.07	10.99 ± 0.21	10.77 ± 0.31
SX (e)	7.89 ±0.03	9.58 ±0.06	10.14 ± 0.08	10.50 ± 0.04	10.77 ± 0.02	11.58 ± 0.09	11.77 ± 0.05	11.72 ±0.04
RCHP (f)	7.51 ± 0.43	9.36 ± 0.13	9.56 ±0.10	9.62 ± 0.12	9.73 ±0.10	10.15 ± 0.07	10.63 ± 0.07	9.21 ± 0.96
PCHP (g)	8.30 ± 0.43	9.74 ± 0.13	9.96 ±0.9	10.04 ± 0.10	10.11 ± 0.11	10.60 ± 0.11	11.22 ± 0.02	11.37 ± 0.13
The differences statistically significant for $p<0.05$ between particular preparations	a,b,c – d,e,f,g d – e,g f – g	a,b – c,d,e,f,g c – d,e,f,g d,f – g e – f	a,b – d,e,f,g c – d,e,f,g d – e,g e – f,g f – g	a – d,e,f,g b – c,d,e,f,g d – e,f,g e – f,g f – g	a,b – c,d,e,f,g c – d,e,f,g d – e,f e – f,g f – g	a,b – c,d,f,g c – d,e,f,g d – e,f,g e – f,g f – g	a,b,c – d,e,f,g d – e,f,g e – f,g f – g	a,b,c – d,f d – e,f e – f f – g

to be significantly higher for Calcicure than for the rest of the materials in group 1 after 30, 60 and 120 minutes as well as on the second day of the experiment ($p<0.05$). Beltes et al. [1] also mention a high alkaline potential of Calcicure. Canal sealers (group 2) and points (group 3) were characterized by much weaker capability of releasing hydroxyl ions. As for sealers, a higher alkaline pH was found for Sealapex in comparison with Apexit and these differences were statistically significant at almost all periods of the experiment, except for 30 minutes ($p<0.05$). This was despite a higher percentage of calcium hydroxide in Apexit (31.9%) than in Sealapex (24%). Staehle et al. [16] presented similar findings when examining root dentine alkalization produced by applying various calcium hydroxide materials, particularly Sealapex, in root canals. However, they observed that application of Apexit did not cause an increase in the pH of hard tissue. Gordon and Alexander [17] also indicated a greater ability of Sealapex to release hydroxyl ions, in comparison with other calcium hydroxide sealers.

Among calcium hydroxide points, the pH of "regular" points rose from an initial 7.51 ± 0.43 at the beginning of the experiment to 10.63 ± 0.07 after 8 days. In the second week, the pH of the samples decreased rapidly to the level of 9.21 ± 0.96. Our results differ from the results obtained by Economides et al. [7], who observed that the maximum pH of "regular" points was 9,5. The above divergence could be caused by the different times of the pH measurements.

"Plus" points were characterized by a significantly higher release of hydroxyl ions in comparison with "regular " points. Their pH increased from the first to the last day of the experiment and ranged from 8.30 ± 0.43 to 11.37 ± 0.13. We could not find any data on calcium hydroxide "plus" points found in the literature, which could be compared to the results of our studies. These materials are characterized by a lower percentage quantity of calcium hydroxide (52% of $Ca(OH)_2$) in comparison with "regular " points (58% of $Ca(OH)_2$), but additionally, they contain sodium chloride and surfactant a surface active agent which improves the solubility of points and facilitates the release of ions.

This proves that ion accessibility is connected with the addition of substances that inhibit or initiate the release of ions, not with the calcium hydroxide content in preparations. This conclusion is also shared by other authors [9,16-18].

Time seems to be a vital factor in relation to the effective therapeutic action of calcium hydroxide preparations. In the present studies, the pH of all the dialysis samples increased gradually with time reaching the highest values on the 8-th day of the experiment. An exception was the "plus" points in which the pH reached the highest value after 2 weeks. A positive correlation between the pH values of the dialysis samples and time was found for all materials. This corresponds with other authors findings [5,17].

The low solubility and poor ability to diffuse make it difficult for compounds based on calcium hydroxide [8] to reach maximum pH levels in a short period of time. The results of our in vitro studies show that they should be applied to canals for a minimum of 7 days to achieve maximum therapeutic effectiveness.

In conclusion, present in vitro experiment suggest that, to achive maximum concentration of hydroxyl ions in tissues two issues should be contemplated: 1) for temporary root fillings nonsetting preparations of calcium hydroxide should be chosen rather than points and they should be placed in situ for at last one week, 2) for permanent root fillings it is more recommended to use Sealapex than Apexit as a sealer.

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Effects of ebelactone B on cathepsin A activity in intact platelets and on platelet activation

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Abstract

Purpose: Previous in vitro studies have demonstrated that a potent antihypertensive agent ebelactone B inhibits cathepsin A/deamidase activity. The aim of our studies was to assess the effects of this inhibitor on cathepsin A activity in intact platelets and on platelet activation events.

Material and methods: PRP or washed human platelets from healthy volunteers were pre-incubated with different concentrations of ebelactone B (1-10 μM) for 10-60 min. Cathepsin A activity in platelets was assayed colorimetrically using Cbz-Phe-Ala at pH 5.5. Expression of platelet activation markers GpIIb/IIIa and P-selectin on non-activated or agonist-activated platelets (ADP, TRAP) was measured by flow cytometry.

Results: Pre-treatment of platelets for up to 60 minutes with 10 μmol/l ebelactone B, that effectively inhibits cathepsin A activity in platelet lysate, did not affect this activity in intact platelets. Exposure of PRP to 10 μmol/l ebelactone B alone, or before platelet activation with ADP or TRAP caused only a small but non-significant increase in P-selectin and GpIIb/IIIa expression on the platelet surface, as demonstrated by flow cytometry analysis.

Conclusions: The lack of cathepsin A inhibition by ebelactone B in intact platelets indicates that this inhibitor does not enter cells. Therefore, a potential antihypertensive significance of this compound may be through the inhibition of cathepsin A/deamidase released from activated or damaged cells. In vitro ebelactone B seems to exert no effect on platelet activation. Further studies are underway to determine whether ebelactone B administration affects platelet activation events in experimental model of hypertension in rats.

Key words: ebelactone B, cathepsin A, human platelets, platelet activation markers.

Introduction

Cathepsin A (EC 3.4.16.1) is a lysosomal serine peptidase present in all examined mammalian cells and tissues [1], including human blood platelets [2,3]. In vitro it hydrolyzes various synthetic and natural biologically active peptides both at acidic pH (carboxypeptidase activity) and at neutral pH (deamidase and esterase activities) [4]. For example, human platelet cathepsin A deamidates substance P, endothelin 1 and enkephalinamides, converts angiotensin I to angiotensin II, and also cleaves bradykinin [2]. Therefore, it is postulated that after secretion from activated platelets or other cells such as macrophages, cytotoxic lymphocytes NK and LAK cathepsin A/deamidase may play a role in the local metabolism of the biologically active peptides [2,5,6]. Moreover, earlier in vitro studies have demonstrated that deamidase activity of cathepsin A is inhibited by an effective antihypertensive agent ebelactone B [5], which is a potent inhibitor of urinary carboxypeptidase Y-like kininase [7,8]. However, the role of cathepsin A/deamidase and the significance its inhibition in cell physiology is still unknown.

In the present study we investigated whether ebelactone B can inhibit cathepsin A activity in intact blood platelets. We also investigated the effect of ebelactone B on platelet activation, because some of the antihypertensive drugs are known to reduce platelet hyperactivity in hypertension [9-13]. Cathepsin A activity was assayed at pH 5.5 using a highly specific synthetic substrate Cbz-Phe-Ala, which is frequently employed for determination of this activity in crude extracts. The level of platelet activation induced by ADP or TRAP was assessed by determining P-selectin and GpIIb/IIIa expression on platelet surface using the flow cytometric method.

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Material and methods

Cbz-Phe-Ala, ebelactone B, and ninhydrin reagent solution were purchased from Sigma Chemical Co, St. Louis, MO USA. ADIAflow Gp (Platelet) kit containing mouse IgG-Fluorescein isothiocyanate (FITC) and antibodies against GpIIb/IIIa complex (CD41a) and P-selectin (CD62P), TRAP (thrombin receptor agonist peptide), calibration beads solution, fixative reagent was purchased from American Diagnostica; ADP was from Sigma (St. Louis, MO, USA).

PRP or washed human platelets (5×10^8 cells/ml) in HEPES--Tyrode's buffer, pH 7.4 (136 mmol/l NaCl, 2.68 mmol/l KCl, 1 mmol/l $MgCl_2$, 0.36 mmol/l NaH_2PO_4, 11.9 mmol/l $NaHCO_3$, 3.4 mmol/l HEPES, 0.1% glucose, 0.35% BSA) were incubated without or with 1.5 or 10 μmol/l ebelactone B at 37°C for 15-60 min. After centrifugation, platelet pellets were washed 3-times to remove the inhibitor, and finally resuspended in „assay buffer" (50 mmol/l sodium acetate buffer containing 100 mmol/l NaCl, 500 mmol/l sucrose and 1 mmol/l EDTA at pH 5.5) containing 0.5% Triton X-100 for determination of cathepsin A activity.

Cathepsin A activity was assayed using Cbz-Phe-Ala, as described earlier [3]. Briefly, the reaction mixture (500 μl) contained the substrate (5 mmol/l), cell lysate (50-100 μg of total protein) and "assay buffer". After 60 minutes of incubation at 37°C, the reaction was stopped by the addition of 500 μl of 10% TCA. The aliquots were taken to determine the amount of the released C-terminal alanine by the colorimetric ninhydrin method [14]. Enzyme activity was expressed as μmoles of the alanine released from the substrate per min per mg of protein.

For flow cytometry analysis, we used platelet rich plasma (PRP) obtained by centrifugation (150 μg, 15 min, 23°C) of fresh human blood from healthy volunteers as described in ref. 15. Samples were preincubated with ebelactone B (1-10 μmol/l), or vehlicle (DMSO 0.01%) for 10 or 60 minutes. Then the surface expression of GpIIb/IIIa or P-selectin on non-activated platelets or on platelets activated with ADP (4 μg/ml) for 2 minutes or with TRAP was determined according to the manufacture procedures using flow cytometer (Becton-Dickinson).

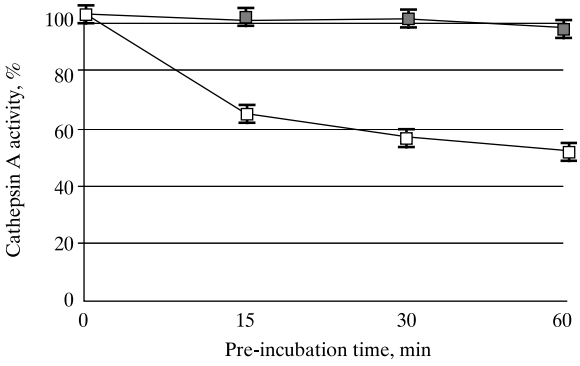
Protein concentration in platelet lysates was determined by the method of Bradford [16] using Bio-Rad reagent with bovine albumin as a standard.

Statistical analysis of the data was performed by using Student's t-test. All results are expressed as mean ± standard deviations. $P < 0.05$ was considered statistically significant.

Results

The effect of ebelactone B on cathepsin A activity in platelet lysate or in intact platelets pretreated with this inhibitor for 15-60 minutes is presented in *Fig. 1*. Cathepsin A activity was effectively inhibited by 10 μmol/l ebelactone B in platelet lysate. The percentage of this inhibition was about 37% and 50% after 15 and 60 minutes of preincubation with this inhibitor, respectively. The lower concentrations of the ebelactone B (1 or 5 μmol/l) were less effective (not shown). In contrast, exposure of intact platelets to 10 μmol/l ebelactone B for up to 60 minutes, followed by washing of the platelet pellets to remove the inhibitor, before lysis, did not result in a decrease in cathepsin A activity. The same results were obtained during preincubation of PRP with ebelactone B, followed by washing of the platelet pellets to remove extracellular inhibitor before lysis (not shown).

Figure 1. Comparison of cathepsin A activity in platelet lysate and in platelets pretreated with ebelactone B. Means ± SD.



The intact platelets (-■-) or platelet lysate (-□-) were incubated with 10 μmol/l ebelactone B (remove) at 37°C for 15, 30 or 60 minutes. After centrifugation of intact platelets, the platelet pellets were washed 3-times to remove the inhibitors, and finally resuspended in "assay buffer" containing 0.5% Triton X-100. Cathepsin A activity was assayed at pH 5.5 using 5 mmol/l Cbz-Phe-Ala after 60 min incubation. The maximal cathepsin A activity in the absence of the inhibitor (4.8 U/mg) was designated as 100%.

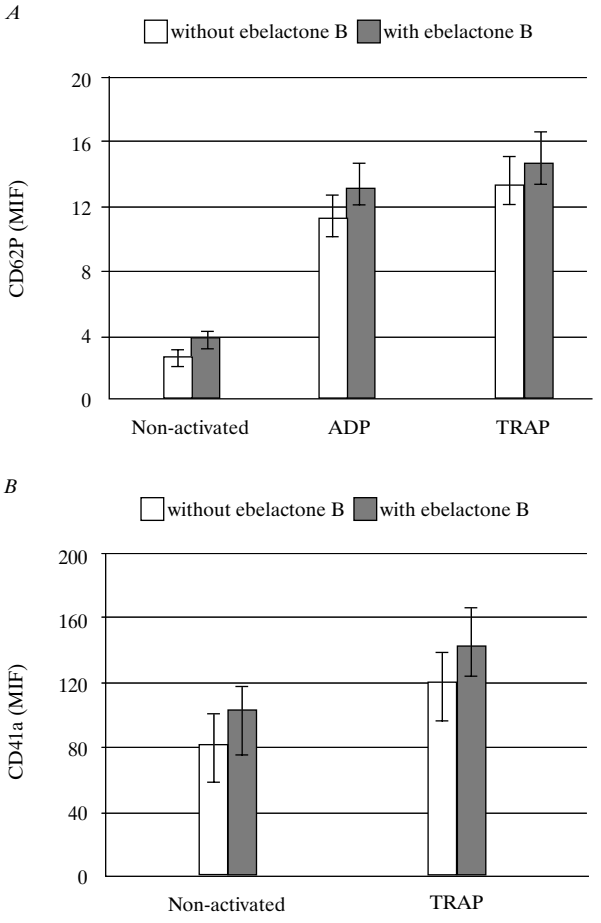
Next, we investigated whether ebelactone B affects platelet activation events using CD62P (P-selectin) and CD41a (GpIIb/IIIa) antibodies (*Fig. 2*). The expression of these markers on platelets pretreated with ebelactone B was measured before and after platelet activation with ADP or TRAP, and the mean fluorescence intensity values (MIF) were determined using flow cytometry. As seen in *Fig. 2*, in the control PRP, not treated with ebelactone B, activation of the platelets resulted in an increase in the platelet surface binding of monoclonal antibodies directed against P-selectin (CD62P) (*Fig. 2 A*) and GpIIb/IIIa (CD41a) (*Fig. 2 B*). After exposure of platelet rich plasma to 10 μmol/l ebelactone B, the mean fluorescence intensity values (MIF) of both P-selectin and GpIIb/IIIa were not statistically different between non-activated platelets and activated platelets.

Discussion

Ebelactone B is an effective antihypertensive agent that prevents the development of deoxycorticosterone acetate-salt hypertension in rats through the inhibition of urinary carboxypeptidase Y-like kininase [7,8]. In vitro it also inhibits cathepsin A/deamidase which shares many enzymatic properties with carboxypeptidase Y-like enzyme, including the ability to hydrolyze some bioactive peptides involved in the development of hypertension [2,4].

In the present study, we demonstrate that ebelactone B, at concentration of 10 μmol/l, which effectively inhibits cathepsin

Figure 2. Effects of ebelactone B on P-selectin (A) and GpIIb/ /IIIa (B) expression during platelet activation. Means ± SD.



PRP was preincubated for 60 minutes without or with 10 μmol/l ebelactone B, and then the platelets were activated by ADP (4 μg/ml) for 2 minutes or TRAP according to the manufacture procedure. Expression of CD41a and CD62P were measured by flow cytometry.

A activity in platelet lysate, does not inhibit this activity in intact platelets. It indicates that ebelactone B does not enter cells. Therefore, a potential antihypertensive significance of this compound may be through the inhibition of cathepsin A/deamidase released from activated or damaged cells, as was shown by others for urinary carboxypeptidase Y-like kininase, derived from the kidney [7,17]. The lack of cathepsin A inhibition by ebelactone B in intact platelets also indicates that this inhibitor cannot be used to study the role of cathepsin A in platelet physiology and pathology. The only known cell-permeable inhibitor, which almost completely inhibits cathepsin A activity in intact platelets and other cells, is a microbial product lactacystin/β-lactone [18,19]. However, it also inhibits a cytosolic multienzyme proteinase complex (proteasome), which is responsible for nonlysosomal proteolysis in all examined cells [20].

A number of reports have demonstrated that the antihypertensive drugs such as angiotensin II (AT) [9] receptor antagonist losartan or rilmenidine [21] are potent antiplatelet agents in hypertension [11-13]. In contrast, captopril and enalapril, the inhibitors of angiotensin-converting enzyme (ACE), have no effects on platelet activation events [22,23]. In aggreement with these studies, in our model system, cathepsin A/deamidase inhibitor ebelactone B seems to exert no effect on platelet activation. It causes only a small but non-significant increase in expression of P-selectin, a component of α-granule membrane that mediates the adhesion of activated platelets to monocytes and neutrophils. Ebelactone B has also no effect on expression of GpIIb/IIIa receptor that is critical for platelet aggregation. These markers are frequently used to test platelet activation by flow cytometric analysis under various pathological conditions, including hypertension [9,10,15]. Therefore, further studies are necessary to find out whether ebelactone B affects platelet activation events in experimental model of hypertension.

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The pattern – reversal visual evoked potentials in children with migraine with aura and without aura

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Abstract

Purpose: Studies of the visual evoked potentials (VEP) in migraine have yielded contradictory results. Several investigators suggested that VEP may be helpful test in diagnosis of a child with headache. The aim of our study was to compare interictal pattern-reversal visual evoked potentials (PR-VEP) in children and adolescents with migraine and tension-type headaches and to evaluate VEP parameters in migraine with and without aura.

Material and methods: The study was carried out in 93 children and adolescents aged 7-18 years with attack headaches.

Results: 51 children had diagnosed migraine. In this group 30 children (59%) had migraine without aura (MO), 12 children (23.5%) migraine with aura (MA) and 9 (17.5%) patients other variants of migraine (MV): hemiplegic, ophthalmoplegic, basilar. In control group 42 children were classified as tension-type headaches. All children had PR-VEP performed in headache – free period, without prophylactic treatment. The P100 mean latency was significantly longer in migraine than in tension-type headache. Amplitudes N1-P100 and P100-N2 were siginificantly larger in migraneurs compared with tension-type headache. The mean amplitudes of N1-P100 and P100-N2 were significantly lower in MA compared with group MO. There were no statistically significant differences of other PR-VEP parameters between MA, MO, MV.

If we compare individual results of each patient with migraine with mean value ± 2 standard deviations (SD) of tension-type headaches group, only 25% have VEP abnormalities of latency or amplitude above 2SD value in tension-type headache group.

Conclusions: The diagnosis of migraine in children actually remains predominantly based on medical history, due to low sensitivity and specifity of electrodiagnostic tests in headaches. However PR-VEP may support the diagnosis of migraine in some cases. VEP could be also helpful method in studying the pathogenesis of different forms of migraine. VEP abnormalities in migraine can be related to a cortical spreading depression and a central neurotransmitter alterations.

Key words: visual evoked potentials, migraine, tension-type headache.

Introduction

Headache is a common complaint in children. The diagnosis and the therapeutic approach is based solely on medical history and physical examination. Electrophysiological techniques of evoked potentials (EP) could be useful to support the diagnosis. The visual function in migraneurs differs from those of normal subjects and patients with other type of headaches. Visual aura is a well-known feature of migraine and the majority of migraine attacks with or without aura are acompanied by photophobia [28]. Migraine patients are more sensitive to enviromental light stimuli [11].

Studies of the cortical visual evoked potentials (VEP) have yielded contradictory results. Several investigators did not find differences between migraneurs and healthy control subjects [4,7,19,29,35,40]. However, others reported changes of VEP amplitudes, latencies and habituation response. Some authors demonstrated increased P100 latencies [13,17] and larger P100 amplitudes [3,6,17,20] in migraneurs. However, some authors showed shorter N75 and P100 latencies in migraneurs [3,20]. Increased amplitudes and P100 response assymetry in migraine with aura in comparison with cases without aura was reported [34]. In the last years disturbances of VEP habituations in migraine were reported [1,2,12,33].

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Table 1. Latencies and peak–to–peak amplitudes of pattern – reversal visual evoked potentials in children with migraine and tension–type headache (mean±standard deviation).

	Migraine (n=51)	Tension-type headache (n=42)
P 100 latency (ms)	109.99±12.63*	106.80±4.42
N1-P100 amplitude (μV)	16.03±5.53*	12.18±4.41
P100-N2 amplitude (μV)	14.65±5.58*	10.08±4.84
N1 latency (ms)	74.33±5.46	73.96±4.43
N2 latency (ms)	152.83±12.23	158.98±7.08

* p<0.05 vs mean value in tension-type headache group

Such studies have been performed mainly in adults. A very few clinical reports of VEPs in migraine in children and adolescents have been published. Lahat et al. [15,16] reported in children significantly larger amplitude between P100 and N2 with migraine compared with other types of headaches. They suggested VEP study may be helpful in diagnosis of a child with headache when symptopms may not be characteristic. However, in other studies there were no statistically significant differences of VEP parameters in children 3-14 years old with different types of headaches [26] and between groups of children with migraine and control subjects [18].

The aim of our study was to compare interictal pattern – reversal visual evoked potentials (PR-VEP) in children and adolescents with migraine and tension-type headache and to evaluate VEP parameters in migraine with and without aura.

Material and methods

The study was carried out on 93 children and adolescents aged 7-18 years with attack headaches, reffered for evaluation to Pediatric Neurology Department between April 2000 and September 2001. Migraine or tension-type headache was diagnosed according to the criteria of Headache Classification Comitee of International Headache Society from 1988. All patients were examined by a neurologist. EEG and CT scan were performed if necessary.

All children were headache – free for at least 2 days before electrophysiologic study. Visual acuity and fixatiom of all subjects were normal. None of patients received prophylactic anti-migraine therapy before electrophysiologic test.

MEDELEC Sapphire Premiere was used for VEP recording. Patients were seated 1 metre in front of the TV monitor. Checkboard pattern of black and white squares subtending 16' of arc at reversal frequency of 2 Hz was presented. Subjects with one eye patched were instructed to fixate on the middle of the screen. The responses were recorded from silver chloride electrodes on the scalp in occipital region in points Oi and Oz. The refferal electrode was in Cz. The bandwidth was 1 Hz to 100 Hz. During uninterrupted stimulation blocks of 128 responses were averaged. Analysis time was 300 ms. Peaks N1, P100 and N2 were analysed.

Table 2. Latencies and peak–to–peak amplitudes of pattern – reversal visual evoked potentials in children with migraine without aura (MO), migraine with aura (MA), variants of migraine (MV). (mean±standard deviation).

	Migraine without aura (n=30)	Migraine with aura (n=12)	Variants of migraine (n=9)
P 100 latency (ms)	108.74 ±14.50	114.68 * ±8.11	106.88 ±4.86
N1/P100 amplitude (μV)	17.38 ±5.19*	13.38 ±3.95 #	15.04 ±8.37*
P100/N2 amplitude (μV)	16.45 ±4.81*	10.09 ±4.07 #	14.07 ±6.46 *
N1 latency (ms)	74.48 ±5.05	75.82 ±4.21	71.81 ±7.41
N2 latency (ms)	154.08 ±12.84	151.79 ±11.33	150.03 ±11.43

* p<0.05 vs value in tension-type headache group
# p<0.05 vs value in migraine without aura group

The mean latencies N1, P100, N2 and amplitudes N1-P100, P100-N2 were compared by Student t test for unpaired samples. A „p" value below 0.05 was considered significant.

The study was approved by the Ethics Comittee of Medical University in Białystok (R-I-003/140/99).

Results

51 children aged 12.43±2.86 years: 31 boys and 20 girls had diagnosed migraine. In this group 30 children (59%) had exclusively migraine without aura (MO), 12 children (23.5%) classical migraine with aura (MA) and 9 (17.5%) patients other forms of migraine (MV) with focal neurologic signs: hemiplegic, ophthalmoplegic, basilar. In control group 42 children aged 13.02±2.89 years: 14 boys and 28 girls were classified as tension-type headaches. All patients in this group had episodic tension-type headaches: less than 15 days with headache per month.

The mean latencies and amplitudes of the PR-VEP in group of all cases of migraine (M) and tension-type headache (TH) are shown in *Tab. 1*. The P100 mean latency: (109.99±12.63 ms) was significantly longer in migraine than in tension-type headache (106.80±4.22 ms). Amplitides N1-P100 (16.03±5.53 μV) and P100-N2 (14.65±5.58 μV) were siginificantly larger in migraneurs compared with tension-type headaches, respectively: 12.18±4.41 μV and 10.08±4.84 μV. There were no differences of mean latencies N1 and N2 peaks compared M and TH groups (*Tab. 1*).

The comparison of the PR-VEPs results in different forms of migraine are summarized in *Tab. 2*. There were no statistically significant differences of all PR-VEP mean values of latencies: P100, N1, N2 between migraine with aura, without aura and other migraine variants. However, tendency to longer P100 latencies in migraine with aura was observed. The mean amplitudes of N1-P100 and P100-N2 were significantly lower in migraine with aura compared with group without aura (*Tab. 2*).

We compared also VEP parameters of each patient with diagnosed migraine with mean value +2 standard deviations (SD) in tension-type headache group (TH). 13 children (25%) have P100 latency above 2SD, 13 children (25%) have amplitude N1-P100 above and only 6 (11%) have P100-N2 amplitude value above mean +2SD in TH group.

Discussion

Our study showed that the mean latency of P100 and also the amplitudes N1-P100, P100-N2 are larger in migraine compared with tnsion-type headache. These results correlate cardinally with previous studies in children [15,16]. However, in study of 78 children aged 3-14 years there were no significant differences in the mean values of VEP between different types of headache: migrane with and without aura or tension-type headaches [26]. Earlier studies, which suggested that using VEP is an objective test in patients with migraine [10,13,21,22] were later criticized based on inappopriate choice of study groups [26] and erroneous interpretation of VEP results [36,37].

The large discrepances between various investigations are probably caused by several factors. Important causes are differences in selection of examined groups: all forms of migraine, migraine with aura or without aura only, migraine equivalents. They were compared to different controls: healthy asymptomatic subjects [18,29,40], non-specific headaches [15,16], tension-type headache [26]. In this study, we chose comparing with tension--type headache and demonstrate prolonged mean P100 latency in group of all children in migraine, but not in common migraine without aura. This result seems to depend mostly on migraneurs with visual aura, where mean P100 latency was significantly longer. However, other incestigators observed that increase P100 latency is not related to the presence or absence of an aura [14]. Second cause of disagreement among various studies is irreproducibility of results because of differences in methods: position of recording electrodes, parametres of stimulation: flash or checkboard pattern, frequency, size of pattern, stimulus intensity. It was confirmed that VEP habituation or potentiation was found for small check stimuli, but not for medium sized checks [29]. Differences in VEP were dependent also on spatial frequency. Only at high frequency 2 and 4 cycles per degree was the latency N2 prolonged in migraine with aura and did it tend to be delayed in migraine without aura [23]. Important factor seems to be duration of migraine. P100 amplitude is increased in cases of short duration, but in addition it showed a sharp decline with duration to below normal in cases with a long history of migraine with aura [14]. There were also found significant correlation between duration of illnes and percentage of VEP assymetries in migraine with aura [34]. However comparing 2 groups of patients with migraine for 2 years or less and other with disease for 10 years and more, Yucesan et al. [41] concluded that migraine duration has no influense on PR-VEP. VEPs are related also on time relationship to attack. Amplitude of PR-VEP in migraine with aura and migraine equivalents increases, when recorded soon after attacks, especially with 10 days [34]. During the attack VEP habitation was less pronounced than the day before and during the 2 days following the attack VEP habitation were replaced by potentiation [12].

The pathnomechanism of VEP alterations in migraine is not clear. They have been interpreted as markers of anatomical lesions of the optic pathway due to ischaemia during the attacks. The changes of P100 amplitude and latency in children with migraine with aura in our study support the hypothesis that visual dysfunction in aura might be secondary to a loss of inhibitory GABAergic interneurons in the visual cortex, due to repeated parenchymal insults during the aura [5]. Because in most studies none correlation of VEP with duration and severity of migraine was found [32,39], some investigators suggests that visual evoked potentials abnormalities are congenital features [1]. The mutation in the gene CACNL1A4 was identified for familiar hemiplegic migraine [24]. Abnormal genes in other types of migraine are possible.

It has been also postulated that electrophysiologic abnormalities can be related to a central neurotransmitter alterations involving the input modulation mechanism: lack of inhibition or increase in excitation [10,38]. Serotonin is thought to play a pivotal role in migraine pathogenesis [8]. So, low interictal activity in the raphe cortical serotonergic pathway could be responsible for the low preactivation level of sensory cortices [33]. The normalisation of VEP just before and during the migraine attack might reflect an increase in the cortical preactivation level due to enhanced activity in raphe – cortical serotonergic pathway [12].

Furthermore, an inverse correlation between increased P100 amplitude and lowered serum magnesium levels was found in children suffering from migraine with and without aura in a headache – free period [3]. Magnesium is known to play an important role in the release of neurotransmitters (serotonin, dopamine, GABA, catecholamines and excitatory amino acids) and in the regulation of calcium channel in neural cells. A reduction of magnesium levels in blood and saliva in juvenile and adult migraineurs has been reported [9,31]. This reduction could be associated with spreading cortical depression, central neurotransmitter release and platelet hyperaggregation [27]. These data seem to support the hypothesis that hypomagnesemia could facilitate transmission in the visual pathway by means of hyperexcitability of neural cells [3].

In studies of sequential blocks of PR-VEP there were found major differences between migraneurs and healthy controls. The potentiation of the response in migraine contrasting with habitation in control subjects is characteristic [1,2,33]. The strong negative correlation between the initial amplitude of VEP and their amplitude increase during subsequent averaging, confirms that the response potentiation in migraine is due to a reduced preactivation level of sensory cortices [2]. Habituation of PR-VEP in healthy humans is maximal after 12 minutes, at the time when there is a decrease of stimulation enhanced by lactate levels in the occipital cortex [30]. Interictal habituation deficit in cortical information processing in migraine may suggest lactate accumulation in sensory cortices during sustained activation [1].

Althought we observed statistically significant changes of mean values VEP parameters in migraine, if we compared inividual results of each patient with migraine with mean value +2 standard deviations (SD) of tension-type headaches group, only 25% have VEP abnormalities above 2SD in tension-type headache group. It suggests very low sensitivity of this test. In

group of children investigated by Lemka [18] 50% patients with migraine had P100 latency more than 2SD above mean value of control healthy subjects. In other study sensivity of VEP in migraine in children was 67%, and the specifity was 83% [15]. The relatively low sensivity may reflect the inability to distinguish, basing on clinical symptoms alone between migraine and other headaches in children.

In conclusion, the diagnosis of migraine in children to remains predominantly based on medical history, due to low sensitivity and specifity of VEP in headache. However PR-VEP may support the diagnosis in some cases. PR-VEP could be helpful method in studying the pathogenesis of different forms of migraine. VEP abnormalities can be related to a cortical spreading depression and a central neurotransmitter alterations.

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