Met-enkephalin in the liver as a marker of hepatocellular damage in chronic viral hepatitis type B and C

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Abstract

Purpose: The aim of the study was to assess the liver Met-enkephalin concentration in chronic viral hepatitis type B and C as well as in liver cirrhosis in order to estimate the role of opioid system in pathogenesis of liver disease.

Material and methods: The concentration of Met-enkephalin was examined in liver tissue of 103 consecutive patients with chronic hepatitis type B and C. Control group consisted of uninfected patients. Met-enkephalin concentration was analyzed in relation to the degree of hepatic necroinflammatory activity and the extent of fibrosis estimated by histopathological examination of liver bioptates and compared to such parameters as age, sex and concomitant diseases.

Results: Significant differences in Met-enkephalin concentration were found between cases with advanced fibrosis (stage 3 and 4 acc. to Batts and Ludwig classification) and cases with fibrosis classified as stage 2 ($p<0.05$). Met-enkephalin concentration was higher in HCV infected patients in comparison to HBV infected patients ($p<0.05$) and uninfected controls ($0.05<p<0.1$). There wasn't found any correlation between Met-enkephalin level and necroinflammatory activity in the liver, age, sex and concomitant diseases.

Conclusions: Met-enkephalin concentration measurement in the liver tissue seams to be a useful method for differentiation of stage 2 from stages 3 and 4 of severe liver fibrosis. There is increased concentration of Met-enkephalin in liver tissue in HCV infected patients in comparison to HBV infected or uninfected individuals. The degree of necroinflammatory activity in the liver as well as sex and age of patients with chronic hepatitis do not correlate with changes in opioid system.

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Introduction

Many of endogenic opioid substances derived from precursor proteins as proopiomelanocortine, proenkephaline and prodynorphine have been described. Most of these substances are composed of five amino acid peptides Leu- or Met-enkephalin. There are several types of receptors (with possible subclasses) which can bind them. Various specificity of peptide-receptor binding, different agonist and antagonistic effects in different tissues with possible cross reactions make the system, participating in multiple human systems regulation, extremely complicated.

The alterations of the opioid system in patients with severe liver diseases have been described. Some reports [1,2] showed elevated Met-enkephalin level in serum in patients with acute liver damage. The level was several times higher then in control group and decreased subsequently to normalization of aminotransferases. It was shown that in patients with decompensed liver cirrhosis and ascites [3,4] Met-enkephalin level was several times higher then in patients with cirrhosis but without ascites. It is possible that small opioid peptides play an important role in pathogenesis of ascites [3]. It was reflected by their blood vessels dilatation ability [5-7] and the presence of Met-enkephalin receptors in visceral blood vessels [8]. Met-enkephalin concentration is the highest in patients with liver cirrhosis and ascites as well as cholestatic liver diseases [2]. Enkephalins elevated in serum may cross blood-brain barrier [9,10] and cause cerebral dependence – naloxon reversible analgesia [11]. It may be responsible for increased patients' susceptibility to morphine [12]. The administration of opioid antagonists in cirrhotic patients can produce an effect which is similar to the withdrawal syndrome observed in opiate drug-addicts [13,14].

Itching of the skin in cholestatic syndromes become less intense after nalmefan or nalokson, this suggests the role of

Table 1. **Characteristic of patients**

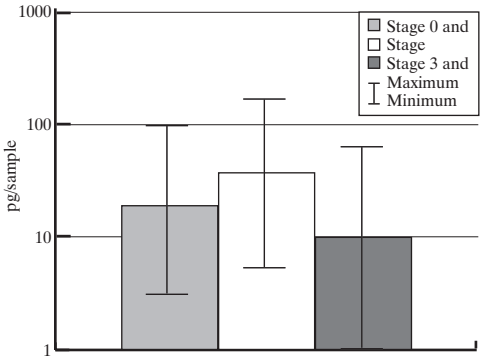
Women	Men	Mean age	HCV infection	HBV infection	Uninfected	Additional diseases
28 (27%)	75 (74%)	47	26 (25%)	54 (53%)	23 (22%)	28 (28%)

opioid system in its pathogenesis. This hypothesis is supported by observation that itching may be induced by morphine and other opioids [15].

Opioid system is thought to act as a link between neuroendocrine and immune systems [16]. Immunocompetent cells are both the source [17] and target for opioids through δ, μ, κ receptors [18]. Opioids released from immunocytes in inflammatory infiltrates can produce analgesia [19]. Moreover, it was shown in experiments *in vitro* that opioid peptides activate or inhibit lymphocytes and macrophages [20-23].

Currently available data on the effect of opioids on the immune system, encouraged us to make the hypothesis that Met-enkephalin might be the important cytokine regulating inflammatory process. The second hypothesis we have tried to verify are postulated relations between hepatic opioid system and process of hepatic fibrosis as well as chronic viral hepatitis type B and C.

Figure 1. **Met-enkephalin concentration in groups of patients according to degree of fibrosis. The results are presented in log scale**



Material and methods

Thick-needle biopsy samples of liver tissue were obtained from 103 consecutive patients with chronic HBV and HCV infected (*Tab. 1*) and from uninfected controls (biopsies were performed because of some other diagnostic indications).

Liver biopsies were performed with local anesthesia, according to Menghini's method with 1.6 mm needles. Informed written consents were obtained from all patients and the study protocol was approved by the local ethical committee. A small part from 2 mm long piece of a liver tissue was used in this study and remaining portion was examined histologically.

The liver tissue was taken to 500 μl of 0.5 N HCl and kept at -70°C until further processing. Tissues were homogenized and centrifuged for 15 min, 1500 rpm at 4°C. Supernatant (200 μl) was neutralized with 2 ml of 0.06 M phosphate buffer (pH 10.2). Native enkephalin was purified on Porapak columns with 250 mg of Porapak (Waters, 100-120 mesh) in 3 ml of absolute ethanol. Porapak slurry was prepared by degassing overnight 25 g of this material in 350 ml of absolute ethanol. Shortly before applying the samples, columns were equilibrated with 6 ml of redistilled water. Loaded column were washed with 6 ml of redistilled water and enkephalin was eluted with 3 ml of absolute ethanol, and then lyophilized by 16 h (Heto Holten). Lyophilized samples were reconstituted with 100 μl of 0.06 M phosphate buffer (pH 6.5) containing 0.2% bovine serum albumin. 50 μl of specific antiserum 18R2 diluted 1:10000 and 50 μl of 125-I-Met-enkephalin (~1500 cpm) were added and samples were incubated for 24 h at 4°C. After incubation 50 μl of 1% rabbit gamma globulin as second antibody was added and further incubated for 30 minutes at 4°C. Separation of bound from free complex was performed by adding 250 μl of 25% PEG. After 30 minutes of incubation, samples were centrifuged at 2000 rpm at 4°C for 30 minutes, supernatant was discarded, and pellets were counted in gamma-counter (LKB). The results were presented as pg per sample and pg per 1 g of total supernatant protein, according to Exton method.

The liver tissue was examined histologically with hematoxylin and eosin, Masson trichrome for collagen and Gomori for argentophilic fibers stainings. Activity of necroinflammatory process and degree of fibrosis were evaluated according to semi-quantitative five-degree Batt-Ludwig's scale [24].

Statistical analysis

Randomized group of 103 patients was statistically analyzed according to severity of fibrosis, HBV or HCV infections, activity of inflammation, serum ALT level, sex, age and concomitant diseases.

The data were not normally distributed and therefore non-parametric statistical analysis was applied using the Mann-Whitney U-test. Wilcoxon's test based on differences in Met-enkephalin levels between different determinations of the peptide in the same bioptate was done as the evaluation of the method involved, p value less then 0.05 was considered significant.

Results

Statistically significant differences were found between cases of moderate fibrosis assessed as stage 2 and advanced stages classified as close to cirrhosis or cirrhosis (*Fig. 1*). Significant differences were found between HCV and HBV infected patients ($p<0.05$) as well as between HCV infected and uninfected patients ($0.05<p<0.1$) (*Fig. 2*).

Figure 2. **Met-enkephalin concentration in groups of patients infected with HBV, HCV and uninfected controls. The results are presented in log scale**

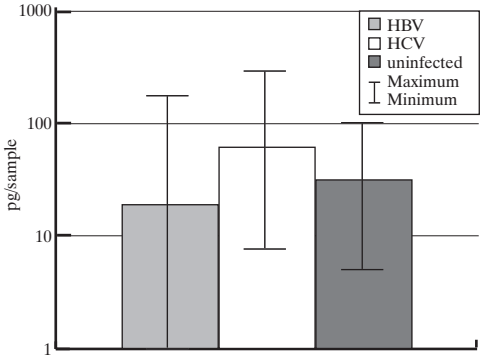
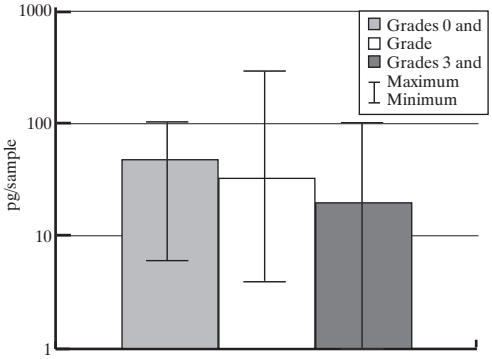


Figure 3. **Met-enkephalin concentration in groups of patients according to degree of necroinflammatory process (grades 0 to 4 acc. to Batts-Ludwig scale). The results are presented in log scale**



No correlation was found between Met-enkephalin level and necroinflammatory activity (*Fig. 3*), intralobular or portal inflammatory activity and serum ALT level.

Statistical analysis revealed no correlation between opioid level in liver tissue and such attributes as age, sex and concomitant diseases.

Met-enkephalin concentration of randomly selected samples was re-assessed in order to evaluate accuracy and repeatability of diagnostic method. Using non-parametric Wilcoxon's test, no statistically significant differences were found between obtained results. Thus, repeatability of involved method seems to be satisfactory.

Spearman's rank correlation coefficient for Met-enkephalin in bioptate and per 1 g of sample was 0.7344, which was statistically significant ($p<0.01$). It means that Met-enkephalin assessment in biopsy specimen could be used to estimate Met-enkephalin concentration in 1 g of bioptate proteins.

Discussion

Liver tissue in patients with chronic hepatitis consists of hepatocytes which are infiltrated by immunocompetent cells. These cells are responsible for necroinflammatory process which reflects activity of immune reactions. Well documented relations between opioides and immunological system [16,17] suggests possibility of correlation between altered Met-enkephalin concentration in liver tissue and severity and origin of hepatitis. This study showed a decrease of Met-enkephalin concentration in patients with the highest necroinflammatory activity in the liver. Increased Met-enkephalin concentration in liver was found in patients with chronic viral hepatitis type C in comparison to patients with HBV infection and control group. Liver damage due to HCV and HBV is characterized by immunologic reactions and differences in direct pathogenic effects of viruses can be observed. HCV, in contrast to HBV, stimulates autoimmune processes [25,26] and differ in mechanism of humoral and cellular immunity. The core proteins of HCV participate in hepatocyte damage but HBV is not directly cytopathogenic and reactions in liver tissue are caused by immunologic phenomena [27]. It seems to be possible that HCV directly affect the opioid concentration in cells, as we can see in HIV infected patients. In HIV infected the increase of β-endorphine was reported as a consequence of infection of susceptible cell [28]. However, it is impossible to determine which mechanism is responsible for observed alterations in liver enkephalin system.

The observed changes in Met-enkephalin concentration in patients with advanced liver fibrosis can be helpful in differentiation of patients with moderate fibrosis and classified as close to cirrhosis or cirrhosis. Liver cirrhosis leads to profound changes in opioid system reflected by increase in serum opioids, syndrome of chronic central opioid receptor stimulation and hypothetical participation in pathogenesis of ascites [3]. In patients suffering from primary biliary cirrhosis (PBC), serum Met-enkephalin is negative prognostic factor, its increased levels correlate with decreased survival rate [29]. Some authors [30] described significantly higher levels of serum Met-enkephalin in stages 3 and 4 in comparison to stages 1 and 2 of PBC. Decreased Met-enkephalin level in the liver with advanced fibrosis observed in our study can be caused by the same mechanism as in PBC. Low hepatic Met-enkephalin concentration in the most severe cases of fibrosis corresponds to other reports of normal enzymatic enkephalin degradation systems in liver tissue [31]. It is well known that the liver uptake of small biologically active proteins such as opioids plays an important role in clearing them from circulation [32]. Defect of non-endocytal energy dependent liver uptake may be a reason of Met-enkephalin reduction in the liver with the most advanced fibrosis, and serum Met-enkephalin elevation, which was widely reported in literature [3,4,30]. Proenkephalin together with its deriving products contribute to cellular proliferation and differentiation [33]. Liver cirrhosis is defined as progressive fibrosis with hepatocyte degeneration and parallel process of tissue regeneration. Liver Met-enkephalin may play auto- or paracrinic regulatory role in cell proliferation and differentiation. Decreased liver Met-enkephalin concentration may reflect progressive deterioration of the process of liver tissue regeneration.

In conclusion, Met-enkephalin concentrations measurement is not effective in distinguishing between a degree of necroinflammatory activity evaluated by liver histology and by serum

ALT. Decreased Met-enkephalin concentration in the liver with advanced fibrosis as well as increased level in patients with HCV infection suggest relationship between opioid peptides and both process of fibrosis and origin of liver damage.

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