2013 © Curr. Issues Pharm. Med. Sci. Vol. 26, No. 1, Pages 57-60

Current Issues in Pharmacy and Medical Sciences

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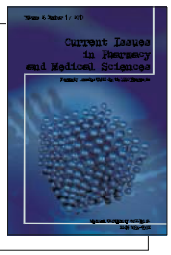

Gelatinase activities and TIMP-2 serum level in alcohol cirrhosis and chronic pancreatitis

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ABSTRACT

There are some divergent data concerning the role of matrix metalloproteinase (MMP)-2, MMP-9 and tissue inhibitor of MMP (TIMP)-2 in the pathogenesis of alcoholic cirrhosis (AC) and chronic pancreatitis (CP). Our objective was to evaluate the activity of MMP-2, MMP-9 and TIMP-2 serum levels in patients with AC and CP. Twenty-one patients with diagnosis of AC and twenty-two with CP admitted to the outpatient clinic for a control visit were enrolled. All results were compared with age and sex-matched control group (n=19). The sera obtained from venous blood were stored at -70°C for further analysis. Activity of MMP-2 and MMP-9 were evaluated with gelatin zymography, TIMP-2 serum level was analyzed with the usage of ELISA method. A significant decrease of serum MMP-2 activity was noted in sera of AC and CP patients in comparison with control. Activity of MMP-9 was elevated only in CP patients and TIMP-2 serum level was elevated only in AC patients. Decreased activity of MMP-2 in AC patients can contribute to cirrhosis development. The high level of MMP-9 in serum related to CP patients theoretically can exacerbate the inflammatory process within the pancreas.

Keywords: alcoholic cirrhosis, chronic pancreatitis, MMP-2, MMP-9, TIMP-2, zymography

INTRODUCTION

Matrix metalloproteinases (MMPs) are family of zinc-dependent endopeptidases that are responsible for the degradation of extracellular matrix (ECM) proteins under pathological and physiological conditions [15]. Moreover, the involvement of selected MMPs (eg. gelatinases: MMP-2 and MMP-9) in the regulation of activity of different biological compounds (as. chemokine CXCL-8, tran- sforming growth factor-β, interleukin 1β – IL1β) makes the examination of these enzymes in numerous diseases interesting [10,23]. The fluctuations in MMPs activities and their natural inhibitors (tissue inhibitors of matrix metalloproteinases, TIMPs) were observed in various liver [9,19,28] and pancreas diseases [13,17,25].

Cirrhosis is the final stage of numerous liver disorders. Its major cause in Western world is alcohol abuse. The ECM remodeling that occurs in the progress of cirrhosis leads to abnormal collagen deposition and fibrosis thereafter. Liver fibrosis is a complex dynamic process that reflects the balance between ECM synthesis and degradation. Previous studies indicated that selected MMPs could be considered as predictor of liver fibrosis [5]. MMP-2 that is highly expressed in myofibroblasts may have a profibrogenic role [20]. The hepatic expression of MMP-2 is elevated during disease progression [18]. Our previous work revealed the decrease of serum MMP-2 activity in the serious cirrhosis stage [22]. We suggested that the low MMP-2 activity could contribute to intense fibrosis due to the decrease of the collagenolytic enzyme activity. In addition, other authors indicated low MMP-9 values in chronic active hepatitis C and cirrhotic patients than in healthy controls [2].

Chronic pancreatitis is relatively common pancreas disorder related to alcohol abuse. Instead of a clear relationship with alcohol consumption, the pathogenesis of CP leaves many questions. Primary injury of acinar cell initiates chronic inflammatory process in pancreas followed by fibrosis and calculi formation [30]. The role of MMPs in the pathogenesis of CP is the target of the se-

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DOI: 10.12923/j.2084-980X/26.1/ a.12

lected studies. The disturbances of MMPs activity can affect the ECM remodeling and exacerbate the inflammatory process within pancreas [25].

The aim of the current study was to evaluate the gelatinase activities and TIMP-2 level in sera obtained from patients with alcoholic cirrhosis and chronic pancreatitis.

MATERIAL AND METHODS

Patients. Twenty-one patients with AC and twenty-two with CP were randomly enrolled during the control visit in Gastroenterology Outpatient Clinic. The local ethics committee of the Medical University of Lublin, Poland, in accordance with current legislation on this field, approved the protocol as well as the details of the informed consent. None of the study patients presented clinical or biochemical symptoms of the inflammation (normal level of c-reactive protein and white blood cells count).

The diagnosis of cirrhosis was based on clinical features, laboratory tests, imagine diagnostics (ultrasonography) and history of heavy alcohol consumption (<80g/day for 5 or more years until the enrollment) [8,24]. The stage of cirrhosis was estimated according to Child-Tourcott-Pugh criteria (CP score) [4,29]. Five patients presented stage A, nine patients presented stage B and seven- presented stage C of CP score.

The diagnosis of CP was established on clinical features (repeated attacks of acute pancreatitis, pain, malabsorption and diabetes) and imaging studies: ultrasonography (USG) or computed tomography (CT) of abdomen. USG demonstrated ductal dilation, parenchymal changes like enlargement or atrophy, presence of pseudocyst and calcification. CT revealed pancreatic calcifications, focal or diffuse enlargement of the pancreas, ductal dilation, and/or vascular complications.

The venous blood samples were obtained and after the centrifuge, the serum was collected for further analysis.

Biochemical assays. MMP-2 and MMP-9 activities were determined by previously published method [16,22]. Briefly, the samples, in a volume of 12 μl, consisted of supernatant and sample buffer with 10% sodium dodecyl sulfate (SDS) in a ratio of 4:1. The separation was done on a 10% polyacrylamide gel with 0.05% gelatin type A from porcine skin (Sigma-Aldrich, Poole, Dorset, UK). After electrophoresis, two 30-min washes were done with buffer containing: 50 mM Tris-HCl, pH 7.2 10 mM $CaCl_2$, 0.02% NaN_3 and 2.5% Triton X-100. The incubation was performed for 18 h at 37°C in the above buffer containing 1% Triton X-100. Gels were stained with 0.1% Coomassie Blue R-250 in 30% ethanol and 10% acetic acid and destained in 30% ethanol and 10% acetic acid. MMP-2 and MMP-9 activity was detected as clear bands on the blue background. Enzymes were identified by comparing their migration pattern with a molecular mass standard (Fermentas, Burlington, Canada), and MMP-2 and MMP-9 standards (R&D Systems, Minneapolis, USA). Quantification of zymograms was performed using a computer scanner (1200 dpi) and ImageJ software 1.42q (National Institutes of Health, Bethesda, MD, USA). The activity of gelatinases was expressed as the optical density (OD) of the substrate lysis zone.

Commercially available enzyme-linked sandwich immunoassay (ELISA) was applied to the estimation of TIMP-2 serum level (R&D System, MN, USA). All procedures were performed according to the manufacturer instruction. Optical density was read at wave length 450 nm (correction 540 nm).

Gelatinase activities and TIMP-2 level were compared with age and sex-matched healthy group (n=19). The study protocol was approved by the Ethical Committee of the Medical University of Lublin (Poland).

Statistical analysis. ANOVA with Tukey-Kramer tests, as well as Kruskal-Wallis with Dunn's multiple comparison post-hoc tests, were used as parametric and nonparametric tests respectively. Statistically significant values were considered when $p < 0.05$. Statistical analysis was performed with the use of the computer-assisted software GraphPad InStat v. 3.06. (San Diego, USA).

RESULTS

Gelatinolytic activity was detected at 72 kDa molecular weight (corresponding to pro-MMP-2), 92 kDa (corresponding to pro-MMP-9) and 130 kDa (corresponding to MMP-9/NGAL, a heterodimer with neutrophil gelatinase B–associated lipocalin) in all analyzed samples. The direct precursors of active forms of gelatinases (92 and 72 kDa forms) were assigned to the analysis (Fig. 1).

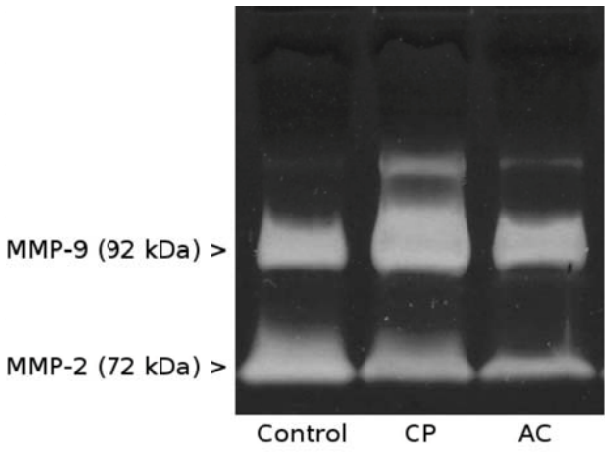

Fig. 1. Representative zymogram of serum gelatinolytic activity of patients' with chronic pancreatitis (CP) and alcoholic cirrhosis (AC). 92 kDa corresponds to pro-MMP-9, 72 kDa corresponds to pro-MMP-2. Significant decrease of 72 kDa band was observed in AC and CP patients. Elevation of 92 kDa band was noted in CP patients in comparison to control.

A significant decrease of serum MMP-2 activity was noted in sera of AC and CP patients in comparison with control. In contrary to MMP-2 the activity of MMP-9 was elevated in CP patients in comparison with control and AC patients. The higher concentration of TIMP-2 in serum was observed in AC patients that was statistically increased in comparison with CP and control patients. All results and patients characteristic are presented in Table 1.

Table 1. Characteristics of patients. AC – alcoholic cirrhosis, CP – chronic pancreatitis. OD – optical density. Results of MMP-2 and MMP-9 serum activity was expressed as mean and standard deviation (round bracket). TIMP-2 serum level was expressed as median, 1st and 3rd quartiles [square brackets].

	Control	AC	CP	Statistics
Age, range	41.3, 25-60	42.8, 29-61	45.7, 30-68	* p = ns
Sex, female/male)	11 / 8	7 / 14	8 / 14	** p = ns
MMP-2 (OD)	6848 (1770)	5159 (1527)	4059 (1394)	* p = 0.0002
MMP-9 (OD)	7649 (2011)	7831 (3951)	11822 (4214)	* p = 0.0025
TIMP-2 (ng/ml)	109 [98-125]	173 [133-211]	97 [88-116]	*** p = 0.0003

* ANOVA, ** Chi-square, *** Kruskal-Wallis tests.

DISCUSSION

MMP-2 and MMP-9 present different mechanism of expression. MMP-9 belongs to inducible metalloproteinase with the expression activated by numerous proinflammatory cytokines as tumor necrosis factor α (TNF-α) or IL-1β [27]. Typically, the activity of MMP-9 increases during acute phase of diseases, especially diseases with inflammatory pathogenesis [11,12]. Moreover, MMP-9 proves positive feedback to amplify the inflammatory process due to the ability to activate its inducers; TNF-α and IL-1β, as well as another proinflammatory chemokine CXCL-8 [27]. Contrary to MMP-9, the second gelatinase, MMP-2, is constitutively expressed [31]. This enzyme plays a significant role in the chronic phases of the disease [14]. Previously, it was noticed that the elevated activity of MMP-2 in an ischemic stroke is the positive prognostic factor for patients' outcome due to the involvement of this enzyme in regeneration processes [21].

Our current study concerns two diseases of digestive tract; alcoholic cirrhosis and chronic pancreatitis. Despite the chronic character of both examined stages, the serum activity of gelatinases differ in AC and CP patients. Previously, we noticed the relationship between the gradual decrease of serum MMP-2 activity and the stage of liver cirrhosis [22]. Currently, we confirm the lower activity of MMP-2 in serum of cirrhotic patients. In addition, we found higher TIMP-2 serum level in AC in comparison with control. TIMP-2 is the strong natural inhibitor of MMP-2 [6]; therefore, both above mentioned observations, decrease of MMP-2 and increase of TIMP-2 level, suggest that the reduction of MMP-2 activity is related to cirrhosis development. Other experiments revealed the contrary results; the hepatic expression of MMP-2 was steadily increased with disease progression, and the expression of MMP-9, was transiently elevated in hepatitis C virus-induced cirrhosis [18]. However, the experimental study of Onozuka et al. showed that MMP-2 deficient mice with two kinds of fibrosis, cholestatic and toxin-induced, exhibited extensive liver fibrosis as compared with wildtype mice [26]. Also, the study of Bruno et al. is in agreement with our current results. The authors revealed the MMP2/TIMP1 and MMP9/TIMP1 ratios (that can be considered as the *in vivo* activity indicators of MMP-2 and MMP-9 respectively) were lower in cirrhotic patients than in healthy controls [2]. In our study MMP-9 activity of AC patients did not differ from control, however, we observed the elevation of MMP-9 activity in sera of CP patients. The data concerning the role of gelatinases in CP are rather scant. According to the literature, MMP-9 serum level can be considered as a biochemical marker for the severe acute pancreatitis [3]. The MMP-9 serum level in mild acute pancreatitis did not differ in comparison with healthy control subjects. Other study noticed higher MMP-9 serum level in the patients with chronic recurrent pancreatitis that is not at variance with our current observation [1]. The high level of MMP-9 in serum of CP patients can theoretically exacerbate the inflammatory process within the pancreas due to activation of biological compounds such: TNF-α, IL-1β and CXCL-8. Moreover, MMP-9 is involved in insulin degradation that leads to diabetes development in the course of CP [7].

On the basis of our work we concluded that the reduction of MMP-2 serum activity is related to alcohol cirrhosis, as well as the raised MMP-9 serum activity is related to chronic pancreatitis. Further studies should estimate the clinical usefulness of both gelatinases as the biomarkers of AC and CP.

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