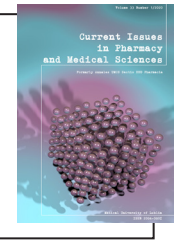
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Disturbances of extracellular protein metabolism in ceruleininduced pancreatitis

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ABSTRACT

Chronic pancreatitis (CP) is still a serious clinical problem due to the significant difficulties in its diagnosis, especially in the initial stages of development. Among the mechanisms that mediate the pathogenesis of CP and lead to pancreatitis-related disorders is unregulated activation of proteolytic enzymes, namely, matrix metalloproteinases (MMPs). The aim of our study was to determine the disturbances of protein metabolism under the conditions of CP alone or in combination with diabetes type 1 (CP+DT1). Herein, CP was induced in the nonlinear male rats by intraperitoneal injection of cerulein (5 μg·kg^{-1} of body weight; five times during fives day). DT1 was modeled in the rats with CP by a single intraperitoneal injection of streptozotocin (65 mg·kg^{-1} of the body weight). The levels of MMP-2 and -9 were determined by enzyme-linked immune sorbent assay, and the level of low and middle molecular weight (LMMW) substance was measured spectrophotometrically, while the peptide fractions were analyzed by size exclusion chromatography. The present study revealed a significant increase of MMP-2 and MMP-9 levels in the serum, liver and pancreas of the rats with CP and CP+DT1. Elevated levels of MMPS may act as a factor for the initiation of subsequent cascade of events resulting in the development of pancreatitis-associated complications. Pathogenesis of chronic pancreatitis alone or in combination with diabetes type 1 has been accompanied by the formation and accumulation of LMMW substance, changes in peptide composition and level of individual peptides in the tissues of the rats. Such alterations are among key triggers of amplification of metabolic disorders under chronic pancreatitis.

INTRODUCTION

According to world statistics, the overall incidence of chronic pancreatitis is about 50 per 100.000 people, but its prevalence tends to grow rapidly [1]. Making an accurate diagnosis of chronic pancreatitis is difficult due to vague and non-specific clinical symptoms, especially at the early stage of disease. Despite numerous studies, the underlying mechanisms that mediate the pathogenesis of chronic pancreatitis and lead to pancreatitis-related disorders have not clearly been elucidated. Therefore, detailed investigation of molecular pathways of pancreatitis development is important to improve diagnostic approaches and identify the potential markers of this disease. Pathogenesis of pancreatitis is associated with impairment of proteolysis [2]. Activation of proteolytic enzymes within the pancreas is considered as an early event in the progression of pancreatitis that could trigger the irreversible destruction of pancreatic parenchyma and ductal structures with further fibrous scar tissue formation. These alterations are considered as key triggers of some pancreatitis-associated complications, namely, fibrosis or/and malignant transformation. Both disorders are thought to relate to a change in the balance between synthesis and degradation of extracellular matrix proteins that are provided by matrix metalloproteinases. Among the severe consequences of chronic pancreatitis is diabetes type 1 [3]. Extensive damage of pancreas tissue results in permanent loss of exocrine and endocrine functions and impairment of insulin synthesis. According to the NHS, diabetes type 1 occurs in around 50% of all people with chronic pancreatitis (www.diabetes.co.uk). The current

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research has been conducted to estimate the levels of matrix metalloproteinases, total protein, peptides, and accumulation of low and middle molecular weight substances in the pancreas, liver, and blood serum of the rats with chronic pancreatitis and the rats with chronic pancreatitis under type 1 diabetes development.

MATERIALS AND METHODS

Fifty nonlinear white adult male rats, 135±10 g, were used in this study. All procedures were carried out in accordance with National Institute of Health Guidelines for the Care and Use of Laboratory Animals, as well as the European Community Council Directive of 24 November 1986 for Care and Use of Laboratory Animals, and were approved by the Ethical Committee of Taras Shevchenko National University of Kyiv.

In the study, chronic pancreatitis was induced by intraperitoneal (i.p.) injection of cerulein (Sigma, St. Louis, MO, USA) diluted in physiological saline (5 $\mu g \cdot kg^{-1}$ of the body weight), five times per day at one hour intervals. Control rats were received equal volumes of 0.9% NaCl injected i.p. The injections were carried out within five consistent days. After the last day of cerulein injection, the rats were kept at standard conditions for the following nine days. Pancreatitis development was confirmed by high serum amylase levels.

On the 14th day from the start of the experiment, half of the animals from the CP group were randomly selected and were used for inducing diabetes type 1. DT1 was induced in the 16 h-fasted rats by a single i.p. injection of streptozotocin (STZ; Sigma, USA), in a dose of 65 $mg \cdot kg^{-1}$ of the body weight dissolved in 0.5 mL of freshly prepared 0.01 M citrate buffer, pH 4.5. The other animals from the CP group and all control rats received equal volumes of vehicle alone. The diagnosis of diabetes type 1 was verified based on high concentration of blood glucose (higher than 15 $mmol \cdot L^{-1}$), as well as high glycosylated hemoglobin level. Thus, there were 3 experimental groups:

1. control (n=10);
2. CP (n=20); and
3. CP+DT1 (n=20).

At the 44th day of the experiment, the animals were fasted overnight, and then sacrificed. The serum was prepared by centrifugation at 1000 g for 30 min of blood samples previously incubated at 37°C at least 30 min. The liver and pancreas were immediately collected after the animals have being sacrificed. The tissue (1 g) were homogenized in 10 mL ice-cold 50 mM Tris-HCl buffer (pH 7.4) and further centrifuged at 12 000 g for 30 min at 4°C. The level of MMP-2 and -9 was measured by enzyme-linked immunosorbent assay, following the standard protocol [4].

Samples of the sera, homogenates of liver, and pancreas were diluted with 50 mM Tris-HCl buffer (pH 7.4) containing 137 mM to obtained concentrations of proteins at 10 $\mu g \cdot mL^{-1}$. The primary MMP-2 antibody (4D3:sc-53630, Santa Cruz Biotechnology, Inc., USA), MMP-9 antibody (7-11C:sc-13520, Santa Cruz Biotechnology, Inc., USA), and corresponding secondary antibody conjugated to horseradish peroxidase (Sigma-Aldrich, USA) were used. The protein concentration was determined according to the method described by [5], using crystalline bovine serum albumin as the standard protein, and the absorbance was measured at 595 nm. The low and middle molecular weight (LMMW) substance fractions were obtained according to [6].

The optical density of the samples was determined with a spectrophotometer Smart SpecTMPlus (BioRad, USA) at 254 nm in the case of LMMW substance determination, and at 210 nm in the case of peptide determination.

The level of peptides was calculated using a calibration curve prepared with CBZ-glycil-glycine dipeptide of 0.26 kDa as a standard. The peptide fraction was analyzed by size exclusion chromatography via a Sephadex G 15 column (Bio Rad, USA). The areas under the peaks of the chromatographic curves were calculated using the OriginLab (v 9.1). The molecular weight of peptides was also estimated using calibration curve. For this purpose, the column was previously calibrated with standard marker solution (lysozyme, 14.3 kDa; insulin, 5.7 kDa; vitamin B12, 1.35 kDa) [7].

The data of biochemical estimations were reported as mean±SEM for each group (n=10). Statistical analyses were performed using one-way analysis of variance (ANOVA). Differences were considered to be statistically significant when $p<0.05$.

RESULT AND DISCUSSION

Pancreatitis is a pathological state strongly associated with uncontrolled activation of proteolytic enzymes. Among the enzymes that potentially could be involved in the progression of pancreatitis and the development of pancreatitis-related complications are matrix metalloproteinases (MMPs) – a family of enzymes responsible for the breakdown of the extracellular matrix, as well as the basal membrane of the vessels [8]. The present findings showed a significant increase of the level of MMPs, such as MMP-2 and MMP-9 in the liver and pancreas of the rats with CP, as well as CP+DT1 (Table 1). The increased level of both MMPs in the serum of the rats indicates the disorders of tissue integrity and the release of MMPs from the damaged organs into the bloodstream. Given into account that MMP-9 is an inducible enzyme [9], the elevated level of this metalloproteinase is explained, first of all, by the accumulation of pro-inflammatory cytokines and their influence on the MMP-9 gene transcription. The increased level of trypsin at the early stages of pancreatitis development serves as an additional factor triggering the conversion of proMMP-9 into the enzymatically active form [10]. The over-production of MMP-2, -9 is considered a poor prognostic indicator under chronic pancreatitis.

These MMPs can affect cell functions both directly and/or indirectly. For example, MMP-mediated degradation of the collagen of basement membrane is one of the mechanisms of pathological activation of pancreatic stellate cells, which, in turn, produce excessive amounts of extracellular matrix components, as well as express cytokines that leads to the development of pancreatic fibrosis and enhancement of inflammation within the organ. These changes may interact with one another and amplify themselves, resulting in the disorders of cellular processes. We noted that the level of some MMPs is elevated in the metastatic tissue,

suggesting these molecules are essential component of the tumorigenesis. Considering the fact that MMP-2 is expressed at very low level under normal physiological conditions, but its expression is increased dramatically in tumor and stromal components of many malignancies [11], the accumulation of MMP-2 in the pancreas of the rats with CP and CP+DT1 could indicate the tendency of pancreatic cells to undergo cancer transformation.

The proteolytic imbalance, alongside the presence of inflammation is accompanied by an accumulation of low and middle molecular weight metabolites, with molecular weight up to 5,000 Da. These at high concentrations act as endogenous toxins [12]. Herein, some of LMMW substances are neurotoxic, while other molecules can inhibit biosynthesis and affect the oxidative phosphorylation. Generally, their negative influence is complex, is realized at different cellular levels and leads to the damage of macromolecules, changes of cell membranes composition, as well as disturbances of the intracellular signaling pathways. The long-term increase of the concentration of these compounds is considered as confirmation of the development of a non-specific state of endogenous intoxication [13].

Taking into account all these facts, we analyzed the level of LMMW substances under chronic pancreatitis conditions. The increased level of LMMW substances in the tissue and especially in the serum of the rats with CP and CP+DT1 (Table 1) at the 44th day of the experiment indicated the systemic nature of the endogenous intoxication and serves as an adverse prognostic marker. The accumulation of LMMW substances in the serum could be due to the impairment elimination of these compounds from the bloodstream. This assumption has been confirmed by the result regarding the total protein concentration in the serum of the rats with CP and CP+DT1. The detoxification potential of plasma is partly determined by albumin, which is involved in the elimination of endogenous toxins by transporting them to the liver and kidneys [14]. Therefore, the decrease of total protein concentration (Table 1) including albumin might lead to amplification of endogenous intoxication under pancreatitis conditions.

Table 1. The level of MMPs, LMMW substances, total peptide and protein content in the serum, liver and pancreas of the rats CP and CP+DT1

		MMP-2	MMP-9	LMMW substances rel.un·mL^{-1}	Total peptides μg·mL^{-1}	Total proteins μg·mL^{-1}
		%				
Serum	Control	100±5	100±5	0.07 ±0.03	0.71 ±0.01	26.32 ±1.45
	CP	175±7*	325±16*	0.23 ±0.05*	0.82 ±0.19	19.52 ±1.12*
	CP+DT1	275±14*#	325±17*	0.35 ±0.07*	0.92 ±0.17*	21.00 ±1.10*
				rel.un·g tissue^{-1}	μg · g tissue^{-1}	μg· g tissue^{-1}
Liver	Control	100±5	100±5	9.85 ±0.21	19.96 ±0.65	42.75 ±0.25
	CP	200±10*	260±12*	32.64 ±0.74*	47.48 ±0.65*	40.32 ±0.11
	CP+DT1	242±11*#	300±15*#	16.89 ±0.25*#	24.44 ±0.52#	26.24 ±0.65*#
Pancreas	Control	100±5	100±5	30.17 ±0.21	113.39 ±1.62	45.47 ±0.15
	CP	240±10*	433±22*	49.47 ±0.68*	61.20 ±0.66*	49.90 ±0.12
	CP+DT1	200±11*#	235±11*#	29.88 ±0.15#	33.31 ±0.73*#	27.59 ±0.34*#

Values are expressed as mean±SEM (n=10); *p<0.05 – significantly different from the corresponding control; #p<0.05 – significantly different from the corresponding CP group

Since diabetes mellitus has been recognized as an inflammatory state, this may be one of the reasons that mediated more significant decrease of total protein level in the group of rats with diabetes type 1. It is well-known that some pro-inflammatory cytokines can directly inhibit the protein synthesis. As the peptides are an integrative part of the fraction of LMMW substances, we determined the total level of peptides in the tissue of the rats with CP and CP+DT1. According to the result (Table 1), the total concentration of peptides in the serum and their content in the liver of the rats were increased. The significant decrease of total peptide levels in the pancreas of the rats in both experimental groups may result from cell damage and the release of their content, including peptides, into the bloodstream.

There are a number of studies indicating that acinar cell necrosis, and in particular, recurrent necrosis, is one of the most serious complications of pancreatitis. Total content of peptides in the biological fluids and tissues represents the peptide pool, which is important for maintenance of homeostasis. The peptide pool of each organ is quite specific in composition and in individual peptide content. This is associated with the set of proteolytic enzymes, their regulators and composition of substrate proteins, which are slightly varied for each different organ. Considering that the repertoire of peptides changes dynamically according to the physiological or pathological states, the investigation of peptide pool could be useful in providing the prognostic criterions for monitoring of disease progression. The results of chromatographic analysis of the peptide fractions derived from the tissue of the rats with chronic pancreatitis are presented in the Table 2. As can be seen from the table the pathogenesis of CP alone and in combination with CP DT1 was characterized by changes of peptide composition, as well as individual peptide level.

Table 2. Molecular weight (Da) and level of individual peptides (%) in the peptide pool derived from the serum, liver and pancreas of rats with CP and CP+DT1

		Number of peaks					
		1	2	3	4	5	6
Serum	Control	1017 Da (50%)	689 Da (20%)	545 Da (18%)	444 Da (12%)	-	-
	CP	1112 Da (14%)	934 Da (37%)	711 Da (28%)	658 Da (4%)	543 Da (8%)	465 Da (9%)
	CP+DT1	1342 Da (10%)	1077 Da (24%)	843 Da (6%)	787 Da (25%)	586 Da (17%)	415 Da (8%)
Liver	Control	1104 Da (39%)	746 Da (61%)	-	-	-	-
	CP	1345 Da (4%)	1074 Da (54%)	740 Da (38%)	416 Da (4%)	-	-
	CP+DT1	1086 Da (27%)	839 Da (5%)	734 Da (18%)	701 Da (27%)	630 Da (21%)	415 Da (2%)
Pancreas	Control	1110 Da (10%)	952 Da (43%)	736 Da (30%)	670 Da (10%)	399 Da (7%)	-
	CP	1282 Da (15%)	1053 Da (36%)	802 Da (27%)	711 Da (16%)	412 Da (6%)	-
	CP+DT1	1198 Da (8%)	976 Da (38%)	692 Da (30%)	589 Da (19%)	401 Da (5%)	-

The most significant alteration was detected in the liver of the rats with CP+DT1. The appearance of peptides that could not be found in the control indicates the intensification of catabolic processes. Since peptides actively take part in the regulation of long-term metabolic processes, the change of peptide composition could be an important factor affecting homeostasis under chronic pancreatitis. Due to structural similarity to biologically active endogenous peptides,

some components of the peptide pool may shift cellular metabolism by binding with active or/and regulatory centers of enzymes and affect in this way the activity of key enzymes. Additionally, they could block cell receptors and thus disturb the cellular signaling pathways.

CONCLUSION

In conclusion, the pathology of chronic pancreatitis is characterized by the prevalence of catabolic reactions and significant accumulation of low and middle molecular weight substances, as well as changes in content of individual peptides simultaneously with decrease of total protein content. The more than twofold increase of the level of matrix metalloproteinase that we saw indicates disorders in the metabolism of extracellular proteins.

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