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ORIGINAL ARTICLE

Plasma TGF-β1, MMP-1 and MMP-3 Levels in Chronic Pancreatitis

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Abstract Chronic pancreatitis (CP) presenting clinically with upper abdominal pain, as well as exocrine and endocrine insufficiencies, is characterized by irreversible morphological and functional alterations in the pancreas. The objective of the present study is to investigate the plasma levels of transforming growth factor-β 1 (TGF-β1), matrix metalloproteinases MMP-1 (collagenase) and MMP-3 (stromelysin) in CP. A total of 71 CP patients and 100 control subjects were considered for the study. Plasma levels of TGF-β1, MMP-1 and MMP-3 were determined by enzyme-linked immunosorbent assay in patients and control subjects. The plasma levels of TGF-β1 and MMP-1 were significantly elevated in patients compared to control group ($^{*}P = 0.0301$, $^{**}P < 0.0001$). However, there was no significant difference in the plasma levels of MMP-3 between patients and controls ($P = 0.3756$). The elevated levels of TGF-β1 and MMP-1 may influence the inflammatory reactions by enhancing the pancreatic stellate cell activation and deposition of extracellular matrix resulting in pancreatic fibrosis. Thus, the present study highlights the role of fibrogenic cytokine marker TGF-β1 and matrix metalloproteinases in the pathogenesis of CP.

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Introduction

Chronic pancreatitis (CP) is a progressive inflammatory process affecting the pancreas [1]. Clinically it is characterized by recurrent attacks of abdominal or back pain (in about 80% of patients), pancreatic stone deposition (in about 30% of patients), exocrine and endocrine pancreatic insufficiency in advanced stages (steatorrhea and diabetes mellitus, respectively). Males are about three to four times more likely to be affected than females with the disease exhibiting overt symptoms by middle age [2].

In human tissues, normal homeostasis requires intricately balanced interactions between cells and the network of secreted proteins known as the extracellular matrix (ECM). These cooperative interactions involve numerous cytokines acting through specific cell-surface receptors. The balance between the cells and the ECM when perturbed, results in a disease condition. The regenerative response of the damaged pancreas is assumed to be determined by a balance between newly synthesized and deposited ECM and degradation of ECM. Collagen is known to be responsible for the tensile strength of the healing wound during tissue repair, while fibronectin forms a scaffold to which cells migrating into the wounded area can attach [3].

Transforming growth factor β1 (TGF-β1) participates in the healing process, directing the migration of monocytes and fibroblasts and increasing synthesis and secretion of ECM [4, 5]. Matrix metalloproteinases (MMPs) are involved in degrading ECM. Pancreatic stellate cells (PSC) play a central role in the pathogenesis of pancreatic fibrosis and

their activation leads to release of MMPs. MMP-1 (collagenase) is the most ubiquitously expressed interstitial collagenase, a subfamily of MMPs that cleaves stromal collagens, and has a prominent role in collagen degradation. MMP-1 is a major proteinase of the MMP family that specifically degrades type I collagen, which is a major component of the ECM, as well as other fibrillar collagens of types II, III, V, IX, and X. As the collagen types are the most abundant proteins in the body, MMP-1 is critical for modeling and remodeling the ECM [6]. MMP-3 (stromelysin-1, EC#3.4.24.17) is a potent proteoglycan-degrading enzyme; its expression is induced in response to local conditions such as mechanical loading, inflammation, etc. MMP-3 is a potentially key enzyme which directly degrades components of the ECM including proteoglycans, laminin, fibronectin, gelatins, and collagens [7, 8].

To our knowledge, fibrosis markers predicting pancreatic fibrosis have not been assessed before in CP of Indian cohort. Therefore, the aim of the present study was to understand the role of TGF-β1, MMP-1 & MMP-3 as markers of pancreatic fibrosis in CP.

Materials and Methods

Study Subjects

A total of 71 CP patients referred to the Gastroenterology unit of Gandhi Hospital during the last two years were included in the present study. Confirmed cases of CP based on the clinical diagnosis, biochemical findings and imaging analysis were included in the study. A total of 100 asymptomatic control subjects visiting the hospital for general health checkup were also included in the study for comparative purposes. Written informed consent was obtained from all the subjects and the study was approved by Institutional Ethics Committee. The demographic characteristics such as sex, age, duration, familial incidence, addictions like smoking and alcohol consumption were noted in all the subjects based on a standard proforma.

Sample Collection

5 ml of blood was obtained from all the subjects in vacutainers with and without anticoagulant for the separation of plasma and serum respectively. These samples were stored at −70°C until further use.

Enzyme-Linked Immunosorbent Assay (ELISA)

The fibrosis markers were estimated in the plasma samples of all the subjects. Concentrations of TGF-β1 (Cat. No. KAC1688, Invitrogen®, California, USA), MMP-1 (Cat. No. QIA55, Calbiochem®, Merck KGaA, Darmstadt, Germany) and MMP-3 (Cat. No. QIA73, Calbiochem®, Merck KGaA, Darmstadt, Germany) were measured in duplicate using a commercial ELISA kit according to the manufacturer's protocols. The concentrations for each sample were calculated from the standard curve generated. The results were cross checked by analyzing the samples at random. The findings were similar on replicative study with the results being 100% concordant.

Statistical Analysis

Mean and standard deviation were calculated for all the groups. Student's *t* test was carried out to test the significance of the data at 5% and 1% levels.

Table 1 Demographic features of CP patients and control subjects

Variables	CP		Control		Odds ratio	*P* value
	n	%	*n*	%		
Total	71		100			
Gender						
Males	69	97	94	94		
Females	2	3	6	6	2.202(0.49–9.8)	0.472
Age						
<35years	25	35	70	70		
≥35years	46	65	30	30	0.233(0.122–0.444)	0.001**
Addictions						
Smokers	44	61	57	57		
Non-smokers	27	39	43	43	1.229(0.662–2.282)	0.532
Alcoholics	57	80	44	44		
Non-alcoholics	14	20	56	56	5.182(2.574–10.417)	0.001**

**$P < 0.001$

Table 2 Mean levels of TGF-β1, MMP-1 & MMP-3 in CP patients and control subjects

	CP	Controls	*t* value	*P* value
TGF-β1	215.35 ± 178.09	168.55 ± 100.22	2.1870	0.0301*
MMP-1	2.713 ± 1.8	1.62 ± 1.39	4.4778	0.0001**
MMP-3	66.92 ± 38.70	61.85 ± 35.35	0.0888	0.3756

*$P < 0.05$, **$P < 0.001$

Table 3 Mean levels of TGF-β1 in CP and control subjects with respect to epidemiological variables

	CP	Controls	*t* value	*P* value
Total	215.35 ± 178.09	168.55 ± 100.22	2.1870	0.0301*
Sex				
Males	208.41 ± 172.60	166.63 ± 109.70	1.9234	0.0561
Females	455.0 ± 275.77	177.5 ± 103.54	1.4933	0.1860
Age				
<35years	258.2 ± 139.59	165.23 ± 105.8	3.4556	0.0008**
35years	192.07 ± 130.95	161.05 ± 108.65	1.0773	0.2848
Alcoholics	197.64 ± 170.95	215.0 ± 65.0	0.7738	0.4405
Non-Alcoholics	287.5 ± 194.7	154.62 ± 107.33	2.9837	0.0046*
Smokers	185.80 ± 119.62	110.05 ± 64.88	4.0713	0.0001**
Non-smokers	263.52 ± 240.5	112.93 ± 76.35	3.8244	0.0003**

*$P < 0.05$, **$P < 0.001$

Results

The demographic characteristics of the CP patients and control subjects are presented in Table 1. A total of 71 CP patients and 100 control subjects were included in the present study. Among the patients, gender distribution of patients showed 97% of males and 3% of females respectively. Classification of the patients based on age below and above 35 years showed 35% and 65% respectively. 61% of the patients were smokers and 80% were alcoholics.

The mean TGF-β1 levels in CP patients and control subjects were 215.35 ± 178.09 and 168.55 ± 100.22 ng/ml, respectively (Table 2). The levels were found to be significantly elevated in the disease group compared to the control subjects ($t = 2.1870$, $*P = 0.0301$). When the comparison was made with respect to the demographic factors, a significant increase of TGF-β1 levels was observed in the patients of age group below 35 years, non-alcoholics, smokers and non-smokers when compared to the control group (Table 3). However, there was no significant difference in TGF-β1 levels within the various attributes of the disease group.

Table 4 gives the mean levels of plasma MMP-1 in CP patients and controls. The plasma MMP-1 levels were found to be significantly elevated in CP patients compared

Table 4 Mean levels of MMP-1 in CP and control subjects with respect to epidemiological variables

	CP	Controls	*t* value	*P* value
Total	2.713 ± 1.8	1.62 ± 1.39	4.4778	0.0001**
Males	2.712 ± 1.82	1.53 ± 1.34	4.77	0.0001**
Females	2.71 ± 1.67	2.65 ± 1.71	0.0431	0.96707
<35years	2.43 ± 1.57	1.53 ± 1.38	2.698	0.0083**
≥35years	2.85 ± 1.91	1.83 ± 1.43	2.5012	0.0146*
Alcoholics	2.64 ± 1.89	1.30 ± 1.21	4.7928	0.0001*
Non-Alcoholics	2.99 ± 1.38	1.81 ± 1.47	2.5502	0.0143*
Smokers	2.37 ± 1.79	1.37 ± 1.30	3.2523	0.0016*
Non-smokers	3.37 ± 1.66	1.66 ± 1.42	0.1848	0.8540

*$P < 0.05$, **$P < 0.001$

Table 5 Mean levels of MMP-3 in CP and control subjects with respect to epidemiological variables

	CP	Controls	*t* value	*P* value
Total	66.92 ± 38.70	61.85 ± 35.35	0.0888	0.3756
Males	66.52 ± 38.70	59.7 ± 35.51	1.1662	0.2453
Females	81.96 ± 36.21	70.83 ± 36.41	0.3747	0.7207
<35years	72.10 ± 38.84	56.06 ± 35.54	1.8902	0.0618
≥35years	64.44 ± 37.55	76.01 ± 32.47	1.3831	0.1708
Alcoholics	63.80 ± 39.27	64.8 ± 28.71	0.6542	0.472
Non-Alcoholics	75.45 ± 38.27	61.28 ± 36.95	1.022	0.0974
Smokers	64.44 ± 37.22	64.5 ± 27.60	0.0093	0.9926
Non-smokers	72.03 ± 40.40	60.69 ± 36.34	11.34	0.2278

*$P < 0.05$, **$P < 0.001$

to the control subjects ($t = 4.4778$; **$P = 0.0001$). Similar observation was found with respect to all the epidemiological variables.

Table 5 gives the mean levels of plasma MMP-3 in CP patients and controls, wherein no significant difference between patients and control group was observed.

Discussion

CP is characterized by profound alterations of ECM formation and composition. The detection of matrix-degrading activity in pancreatic tissue put forth to the concept of fibrosis as a result of tilt in the balance between matrix production and degradation. The proportion of alcoholics was significantly higher in CP patients than in healthy controls indicating addictions and male preponderance as significant variables in the etiopathogenesis of the disease.

In the present study, we determined the plasma levels of fibrogenic factor TGF-β1 along with MMP1 and MMP3. TGF-β1 has been shown to have a profibrotic role in a variety of organs including liver, skin, and kidneys [9–12]. Studies have shown that TGF-β1 promotes fibrogenesis not only by increasing collagen production but also by inhibiting MMPs in the pathway of collagen degradation. It has been demonstrated by Shek et al. [12] that activated PSCs also express activated TGF-β1, which up-regulates collagen-1 while down regulating the expression of MMP-3 and MMP-9 only and not on TIMP-1 expression. The pattern of expression of the mediators studied is similar to that described in parallel cell types of fibrosis of the liver and kidney and reinforces the hypothesis that there may be generic aspects to wound healing in organs [13].

The importance of TGF-β1 in pancreatic fibrogenesis has also been shown in transgenic mice overexpressing TGF-β1 in the pancreas. This necro-inflammatory pathway supports the hypothesis of Klöppel et al. [14] that fibrosis of the pancreas is induced by fibrogenic mediators stimulating PSC after initial tissue damage. Thus, increased levels of TGF-β1 observed in CP compared to control subjects may enhance the PSC activation and increase the deposition of ECM in fibrotic tissue [14].

With regard to the progression of fibrosis, MMPs qualify as ideal candidate proteins since their function is closely linked to the accumulation and degradation of ECM. Among several MMPs that are expressed, MMP-1 (collagenase) and MMP-3 (stromelysin) are crucial because of their capacity to degrade a broad spectrum of ECM molecules and activate other MMPs. Thus, the combination of low expression of collagenases and stromelysins results in the prevention of degradation of the fibrillar collagens deposited due to PSC activation. It is further strengthened by TGF-β1 influencing in decreasing the induction of MMP3 expression [12].

MMP-1 produced by cytokine-activated interstitial cells is one of the most important enzymes in degrading ECM. Interestingly, in the present study higher levels of MMP-1 observed in CP patients indicates a shift in the balance between matrix production and degradation more towards degradation, especially as MMP-1 is up-regulated, which acts as a key to activate the cascade of other MMPs and cytokines. Yu et al. found that MMP-1 combined with MMP-9/CD44 receptors of the cell membrane form MMP-1/19-CD44 complex, allowing the activated TGF-β1 to become active through hydrolysis to carry out its biological functions [15]. It is believed that MMPs not only appear in the downstream of inflammatory responses but also exert a positive feedback effect on cytokines. Therefore, they can be considered as important "regulators" of inflammatory responses [16].

The central role of TGF-β1 in the induction of fibrotic events provides a basis for TGF-β1 as a therapeutic target. The complex regulation of TGF-β1 production and activation offers a number of targets for TGF-β1 suppression. While cytokines influences MMPs expression, MMPs

themselves are able to up regulate cytokines to cause further damage to the pancreatic tissue. Thus the present study highlights the use of TGF-β1 as a prognostic marker as they are sensitive to CP. Further studies are warranted to confirm and address the cellular basis of the transiently expressed MMPs along with functional studies of the pro- and antifibrogenic potential.

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