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Changes in TIMP-1 and -2 expression in the early stage of porcine serum-induced liver fibrosis in rats

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ABSTRACT

It is widely recognized that tissue inhibitors of metalloproteinases (TIMPs), especially TIMP-1 and -2, play a key role in the progression of hepatic fibrosis. In the present study, we examined the changes in TIMP-1 and -2 expressions in the early stage of porcine serum (PS)-induced liver fibrosis in Brown Norway (BN) and Wistar rats. The rats were injected intraperitoneally with 0.5 ml/head of PS twice a week for up to 8 weeks and examined at 2, 4 and 8 weeks. Hepatic fibrosis and inflammatory cell infiltration developed at 4 and 8 weeks in BN and Wistar rats, respectively, and formation of pseudolobules was detected at 8 weeks in rats of both strains. The expression of liver TIMP-1 and -2 mRNAs significantly increased at 8 weeks in rats of both strains. At the same time, TIMP-1 and -2 activities were also detected in the liver of both strains. On the other hand, the expression of serum TIMP-1 and -2 proteins increased earlier (at 4 weeks for TIMP-1 and at 2 or 4 weeks for TIMP-2) than that of liver TIMP-1 and -2 mRNAs did. Although there are some reports suggestive of why the elevation of serum TIMP-1 and -2 proteins preceded that of liver TIMP-1 and -2 mRNAs, the exact reason is still obscure. In conclusion, the present study showed for the first time the mode of TIMP-1 and -2 expression and activity in the early stage of PS-induced rat liver fibrosis model.

Introduction

Hepatic fibrosis is a pathological process with deposition of extracellular matrix (ECM) proteins, especially type I and III collagens (Xu et al., 2004). The change of ECM is mainly regulated by matrix metalloproteinases (MMPs) (Abraham et al., 2005), and the activity of MMPs is tightly regulated by the amount of active protein and the concentration of tissue inhibitors of metalloproteinases (TIMPs) (Parsons et al., 2004). Although it has been said that the formation and prognosis of hepatic fibrosis are well related with MMPs/TIMPs (Ninomiya et al., 2001; Vaillant et al., 2001), it is now widely recognized that the TIMPs (TIMP-1 and -2) play a key role in the progression of hepatic fibrosis (Arthur, 1995; Boeker et al., 2002; Chen et al., 2002; Mitsuda et al., 2000; Nie et al., 2006; Tsushima et al., 1999; Xu et al., 2004; Zhang et al., 2003). TIMP-1 controls most MMP, in particular, MMP-1, whereas TIMP-2 is the major inhibitor of MMP-2 (Fridman et al., 1992; Howard et al., 1991).

Injured liver tissues show more expression of TIMP-1, and in turn, this would help interstitial fibrils to accumulate (Garcia et al., 2002; Yoshiji et al., 2002). An increase in serum TIMP-1 has been identified in human patients with chronic hepatitis, alcoholic cirrhosis, and primary biliary cirrhosis (Boeker et al., 2002; Li et al., 1994; Murawaki et al., 1993). Moreover, it has been reported that serum level of TIMP-1 reflects the change of liver TIMP-1 in patients (Leroy et al., 2004; Murawaki et al., 2001; Xu et al., 2004; Zhang et al., 2003).

Hepatic fibrosis and its end-stage sequelae cirrhosis represent a major worldwide health problem. Nowadays several kinds of animal model of liver fibrosis have been developed for the study on the mechanisms of liver fibrosis as follows: models induced by carbon tetrachloride (CCl_4) (Doi et al., 1991; Nie et al., 2006; Siller-Lopez et al., 2004), radiation (Geraci et al., 1992), D-galactosamine (Uetsuka et al., 1997), thioacetamide (Tani et al., 1998), alcohol (Xu et al., 2004), and bile duct ligation (Knittel et al., 2000; Kossakowska et al., 1998; Siller-Lopez et al., 2004). These liver fibrosis models are so-called post-necrotic liver fibrosis. On the other hand, porcine serum (PS)-induced rat hepatic fibrosis (Baba et al., 2004; Bhunchet et al., 1996; Hironaka et al., 2000; Shiga et al., 1997) is a model that shows humoral immune response to PS probably regulated by MHC class II molecules and inflammatory cells (Baba et al., 2005) and accompanies little hepatocytes injury (Baba et al., 2004; Bhunchet et al., 1996; Shiga et al., 1997). In addition, Baba and Doi (2004) reported that the strain difference between Brown Norway (BN)

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and Wistar rats in the development of PS-induced liver fibrosis might be closely related to the difference in the mode of MHC class II-related genes expression.

It is suggested that there are differences in participation of TIMPs in the progression of liver fibrosis among models (Knittel et al., 2000; Kossakowska et al., 1998; Nie et al., 2006; Xu et al., 2004). For example, Nie et al. (2006) reported that the correlations between TIMP-1 expression and liver fibrosis are different between immune-induced and CCl_4-induced rat liver fibrosis models. As to PS-induced rat liver fibrosis model, there is only one report referring to TIMP-1 expression. Namely, Hironaka et al. (2000) described that in rat liver fibrosis model induced by PS-treatment for 8 weeks, gadolinium chloride-treatment activates Kupffer cells to produce MMP-13 and subsequently reduces ECM but it does not change TIMP-1 expression. However, there are no reports of sequential changes in TIMPs expression in PS-induced rat liver fibrosis model. This study was therefore carried out to examine the sequential changes in TIMP-1 and -2 expressions in the early stage of PS-induced hepatic fibrosis model in BN and Wistar rats. BN rats are well known to show high immunoreactivities (Haczku et al., 1995; Uyama et al., 1995) and Wistar rats are frequently used in toxicological studies.

Materials and methods

Animals

Thirty 5-week-old male rats each of the BN/Crj (BN) (body weight: 90 (mean) ± 10 g (SD)) and Crj:Wistar (Wistar) strains (140 ± 10 g) were purchased from Charles River Japan Co., Kanagawa. The animals were housed 3 per cage using an isolator caging system (Niki Shoji Co., Tokyo) in an air-conditioned animal room (temperature: 23 ± 2 °C; relative humidity: 55 ± 5%; lighting:12 h-light and 12 h-dark cycle) and fed commercial pellets (MF, Oriental Yeast Co., Tokyo) and tap water *ad libitum*. The animals were subjected to the experiment after acclimation for one week.

Treatments

The animals of each strain were divided into two groups; PS-treated and control groups. The animals of the PS-treated group were intraperitoneally (i.p.) injected with 0.5 ml/head of PS (COSMO BIO Co., Ltd., Tokyo) twice a week (Monday and Thursday) for up to 8 weeks. The animals of the control group were given physiological saline in the same way. At 2, 4 and 8 weeks of experiment, 5 animals of each group of each strain were killed by blood sampling from abdominal aorta under ether anesthesia at 24 h after the last injection on Thursday, respectively.

The protocol of this study has been approved by the Animal Care and Use Committee of Graduate School of Agricultural and Life Sciences, the University of Tokyo.

Histopathology

The liver excluding the left lateral lobe was fixed in 10% neutral buffered formalin. 4-μm paraffin sections were stained with hematoxylin and eosin (HE) or Masson's trichrome (MT), and subjected to histopathological examination. The left lateral lobe of the liver obtained from each animal was frozen and stored at −80 °C until used for the further-mentioned examinations by semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) and gel reverse zymography.

Enzyme-linked immunosorbent assay for serum TIMP-1 and -2

The levels of serum TIMP-1 and -2 were measured by the indirect ELISA method with solid-phase antigen. In brief, microplate wells were coated with serum samples at 4 °C overnight and then incubated at room temperature for 1 h in 1% bovine serum albumin–phosphate buffered saline (BSA–PBS) containing 0.01% thimerosal. After washing with PBS containing 0.05% Tween 20 (PBST), serially diluted rabbit anti-human TIMP-1 or -2 antibody (Sigma, Missouri, USA) was introduced to the microplate wells and incubated at room temperature for 1 h. After another washing, the wells were incubated with biotinylated anti-rabbit IgG antibody (Pharmingen, San Diego, CA, USA) at room temperature for 1 h. After final washing, the wells were incubated with horseradish-peroxidase-conjugated streptavidin (DAKO, Glostrup, Denmark) at room temperature for 1 h. The wells were then washed and incubated with *o*-phenylenediamine (Wako, Osaka Japan) and H_2O_2 in dark at room temperature for 30 min. The enzyme reaction was stopped with 4N H_2SO_4. Absorbance at 492 nm was read using Model 550 Microplate Reader (Bio-Rad Laboratories, CA, USA). The results were expressed in titers, calculated based on the titers of the standard plasma obtained from MMP/TIMP Positive Control (Calbiochem, San Diego, USA). Data were represented as the mean ± standard deviation (SD) of 5 animals, and statistical analysis was done between the PS-treated and control groups using Student's *t*-test.

Semiquantitative RT-PCR for liver TIMP-1 and -2

RT-PCR was performed on TIMP-1 and -2 mRNAs in the liver. Total RNA was extracted from a half of the frozen left lateral lobe using ISOGEN (Nippon Gene Co., Ltd., Japan). The first strand cDNA was synthesized using oligo $(dT)_{12-18}$ primer and Superscript II RNase H-Reverse transcriptase (Invitrogen, CA, USA). PCR was performed using oligonucleotide primers sets corresponding to the cDNA sequences: TIMP-1, TIMP-2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 1). In brief, 100 μl reaction mixture containing 76.5 μl RNase-free water, 10 μl 10 × PCR buffer (100 mM Tris–HCl buffer, 500 mM KCl, and 15 mM $MgCl_2$; Takara Shuzo Co., Ltd., Japan), 10 μl dNTP (Takara), 1 μl cDNA sample, and 0.5 μl 250 units recombinant *Taq* DNA polymerase (Takara) was prepared. After preheating at 95 °C for 5 min, PCR reaction (cycle number: Table 1) was performed using Takara PCR Thermal cycler SP (Takara) as follows: denaturation at 95 °C for 1 min, annealing at 58 °C for 1 min, and extension at 72 °C for 1 min. The PCR products were electrophoresed in 2% agarose S (Nippon Gene) or 1 × TBE buffer (89 mM Tris-aminomethane, 89 mM Boric acid, 10 mM EDTA). The gels were stained with ethidium bromide (Sigma-Aldrich Co., USA). Fluorescent bands were visualized using a UV-CCD video

Table 1
Primer sequences and cycle numbers.

Gene	Sense primer (5′–3′)	Antisense primer (5′–3′)	Cycle number
TIMP-1	GCTAAATTCATGGGTTCCCCAG	TTGCTGAGCAGGGCTCAGATTA	34
TIMP-2	GCAATGCAGACGTAGTGATCAG	CCTGTGGTTTAGGCTCTTCTTC	32
GAPDH	GCTTCACCACCTTCTTGATGTC	GAGTATGTCGTGGAGTCTACTG	21