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• BRIEF REPORTS •

Effect of interleukin-10 and platelet-derived growth factor on expressions of matrix metalloproteinases-2 and tissue inhibitor of metalloproteinases-1 in rat fibrotic liver and cultured hepatic stellate cells

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Abstract

AIM: To examine the expressions of matrix metalloproteinases-2 (MMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in rat fibrotic liver and in normal rat hepatic stellate cells, and to investigate the changes in their expressions in response to treatment with interleukin-10 (IL-10) and platelet-derived growth factor (PDGF).

METHODS: Rat models of CCl_4-induced hepatic fibrosis were established and the liver tissues were sampled from the rats with or without IL-10 treatment, and also from the control rats. The expressions of MMP-2 and TIMP-1 in liver tissues were detected by S-P immunohistochemistry, and their expression intensities were evaluated in different groups. Hepatic stellate cells (HSCs) were isolated from normal rat and cultured *in vitro* prior to exposure to PDGF treatment or co-treatment with IL-10 and PDGF. MMP-2 and TIMP-1 levels were measured by semi-quantitative reverse transcriptional polymerase chain reaction (RT-PCR).

RESULTS: CCl_4- induced rat hepatic fibrosis models were successfully established. The positive expressions of MMP-2 and TIMP-1 increased obviously with the development of hepatic fibrosis, especially in untreated model group (84.0% and 92.0%, $P<0.01$). The positive signals decreased significantly following IL-10 treatment (39.3% and 71.4%, $P<0.01$ and $P<0.05$) in a time-dependent manner. TIMP-1 mRNA in PDGF-treated group was significantly increased time-dependently in comparison with that of the control group, but PDGF did not obviously affect MMP-2 expression. No difference was noted in TIMP-1 and MMP-2 expressions in HSCs after IL-10 and PDGF treatment ($P>0.05$).

CONCLUSION: MMP-2 and TIMP-1 expressions increase in liver tissues with the development of fibrosis, which can be inhibited by exogenous IL-10 inhibitor. PDGF induces the up-regulation of TIMP-1 but not MMP-2 in the HSCs. IL-10 inhibits TIMP-1 and MMP-2 expressions in HSCs induced by PDGF.

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INTRODUCTION

Liver fibrosis and its end-stage sequelae cirrhosis represent a major worldwide health problem. By definition progressive fibrosis occurs when the rate of matrix synthesis exceeds matrix degradation[1]. Considerable evidence suggests that the hepatic stellate cells (HSCs) are central to the fibrotic process. HSCs are normally located in the perisinusoidal space as quiescent vitamin A-storing cells secreting low levels of extracellular matrix (ECM). Following liver injury, increased synthesis of extracellular matrix constituents occurs in combination with other phenotypic changes (also called activation) of HSCs into myofibroblast-like cells. It has been demonstrated by the analysis of freshly isolated HSCs that a number of these phenotypic changes take place, including increased expression of extracellular matrix constituents and the expression of α-SMA[2,3]. This activated phenotype of HSCs subsequently becomes the major source of the interstitial collagens[4-6]. It has been suggested that HSCs are also a source of matrix-degrading metalloproteinases (MMPs), indicating their participation in matrix remodeling[7-9]. As a family of neutral proteinases, MMPs act on a variety of substrates[10]. Different expression profiles of MMPs influence the outcome of ECM components, resulting in preferential accumulation of interstitial collagens, type I in particular, in the fibrotic liver. MMPs are tightly regulated at the levels of transcription, secretion, and proteolytic activation, and their activities are governed by tissue-derived inhibitors[11]. The expression of tissue inhibitors of MMPs (TIMP) has also been demonstrated in human fibrotic liver disease and animal models of liver fibrosis[12]. At present, 4 TIMPs have been characterized[13], all being low-molecular-weight proteins sharing structural similarities. Individual members of the TIMP family display selective affinities for different members of the MMP family[14]. TIMP-1 controls mostly the activity of MMP, particularly MMP-1, whereas TIMP-2 is the major inhibitor of MMP-2[15]. MMP/TIMP balance is thought to play a pivotal role in the development of liver fibrosis, but their direct interaction *in vivo* has not yet been clarified. In the present study, the expressions of MMP-2 and TIMP-1 in rat fibrotic liver and in HSCs were examined and their changes were investigated in the presence of interleukin (IL)-10 and PDGF.

MATERIALS AND METHODS

Materials

One hundred clean male Sprague-Dawley rats weighing 140-180 g

(provided by Shanghai Experimental Animal Center) were divided randomly into control group ($n = 24$), model group ($n = 40$) and IL-10 treatment group ($n = 36$). All the rats were bred under routine condition (room temperature of 22±2 °C, humidity of 55±5%, with light/dark alternating every 12 h and free access to water and food. The feed was provided by BK Company in Shanghai, China).

Preparation of rats

Rats in control group were given intraperitoneal injection with saline at 2 mL/kg twice a week, and those in model and IL-10 treatment groups received intraperitoneal injection with 500 mL/L CCl_4 (dissolved in castor oil) at 2 mL/kg twice a week. From the third week, rats in treatment group were given intraperitoneal injection with IL-10 at 4 μg/kg (dissolved in saline) 20 min prior to CCl_4 injection. All injections were performed on Mondays and Thursdays after measurement of the rats' body weight. In the 5th wk, 3 rats in model group and 2 in treatment group died; in the 7th wk, 8 and 4 rats in these two groups died, respectively, and in the 9th week, another 10 and 6 died. At this time point, 3 rats in control group also died. In the 5th, 7th and 9th wk, 7 to 10 rats in each group were sacrificed to collect their liver samples, which were fixed in 40 g/L formaldehyde and embedded with paraffin.

Immunohistochemistry

Rat liver tissues were sectioned at the thickness of 4 μm. After deparaffinization with xylene and dehydration with graded ethanol, the sections were incubated in PBS containing 30 mL/L H_2O_2 to remove endogenous peroxidases and then in PBS containing 0.1 mol/L citrate to saturate the nonspecific binding sites. After incubation with goat anti-rat MMP-2 and TIMP-1 monoclonal antibodies, the sections were treated with instant S-P immunohistochemical reagents (American Zymed Company) and then incubated in a buffer solution containing 3,3-diaminobenzidine tetrahydrochloride (DAB) and H_2O_2 for visualization, followed by dehydration and mounting procedures. Microscopic examination of the sections was then performed.

Result assessment

Reactions were graded and scored according to their intensities and percentage of the positive cells respectively as follows: Zero score for negative reaction, 1 score for pale yellowish staining, and 2 scores for dense yellow or brown staining; 0 score for a percentage of positive cells below 5%, 1 score for one between 6% and 25%, 2 scores for one between 26% and 50%, and 3 for one over 50%. The eventual score of the section was derived from the product of the 2 scores for staining intensity and positive cell percentage, and graded as negative (-) result for a score lower than 1, positive result (+) for one between 2 and 3, and strong positive result (++) for one over 4. Ridit analysis was utilized to assess the difference between the groups.

Hepatic stellate cells isolation and culture

Male Sprague-Dawley rats weighing 450-500 g were used for isolation of HSCs. Rat liver nonparenchymal cells were isolated by means of sequential perfusion with collagenase and pronase E as described previously[16]. Buoyant HSCs were separated from the resulted cell suspension by elutriation over a Nycodenz gradient centrifugation. Desmin immunocytochemistry demonstrated a purity of isolated HSCs over 95%. The HSCs were then seeded into plastic tissue culture flask at the density of 1×10^6/mL in DMEM containing 100 mL/L fetal calf serum (FCS), and incubated at 37 °C with 50 mL/L CO_2. The culture medium was replaced 24 h after plating and every 48-72 h thereafter. The subsequent passages of HSCs were diluted to 5×10^4/mL before seeded into 50 mL culture flask containing DMEM medium supplemented with FCS.

PDGF treatment of HSCs

The cultured HSCs were divided into 6 groups: the first and sixth groups serving as control were cultured in 3 mL DMEM medium for 2 and 24 h, respectively, with the second, third, fourth and fifth groups cultured in 3 mL DMEM medium in the presence of 20 ng/mL PDGF for 2, 4, 8 and 24 h, respectively. The cells were then harvested for reverse transcriptional-PCR (RT-PCR).

Co-treatment of HSCs with IL-10 and PDGF

Cultured HSCs were divided into 8 groups: the first and second groups (blank control) were cultured in 3 mL DMEM medium for 2 and 24 h, respectively, and the third, fifth and seventh groups (negative control) cultured in 3 mL DMEM medium containing 20 ng/mL PDGF for 2, 12 and 24 h, respectively, while the fourth, sixth and eighth groups were cultured in 3 mL DMEM medium containing both 20 ng/mL IL-10 and 20 ng/mL PDGF for 2, 12 h and 24 h, respectively. The cells were then harvested for RT-PCR.

RT-PCR for MMP-2 and TIMP-1

Total RNA was isolated from HSCs using Gentra reagent (USA) according to the protocol provided by the manufacturer. The A_{260}/A_{280} of total RNA ranged between 1.8 and 2.0. After treatment with Dnase-I (1-2 μg), total RNA was reversely transcribed into complementary DNA (cDNA) with oligo (dT) using cDNA synthesis kit, and 2 μL cDNA product was then used as the template to amplify specific fragments in a 25 μL reaction system. PCRs using *Taq* polymerase reaction were carried out with an initial denaturation at 95 °C for 5 min, followed by 25 cycles at 94 °C for 45 s, annealing at 60 °C for 30 s, at 72 °C for 60 s, with a final extension at 72 °C for 7 min. The primer sequences used for MMP-2 were 5'-GTGCTGAAGGACACCCTCAAGAAGA-3' (sense) and 5'-TTGCCGTCCTTCTCAAAGTTGTACG-3' (antisense), and those for TIMP-1 were 5'-GCCATGGAGAGCCTCTGTGG-3' (sense) and 5'-GCAGGCAGGCAAAGTGATCG-3' (antisense); the primers for β-actin as the internal control were 5'-GAGCTATGAGCTGCCTGACG-3' (sense) and 5'-AGCACTTGCGGTCCACGATG-3' (antisense).

Electrophoresis and semi-quantitative analysis

PCR products underwent 2% agarose gel electrophoresis and were visualized with ethidium bromide. The expected products' sizes were 604 bp for MMP-2, 310 bp for TIMP-1 and 410 bp for β-actin. Bio Imagine System was applied to detect the density of the bands of PCR products. The expression levels of MMP-2 and TIMP-1 were calculated by the ratio of their band densities of PCR products to that of β-actin. Semi-quantitative detection was repeated for 5 times. SPSS 10.0 software was used to analyze the difference between the groups.

RESULTS

MMP-2 and TIMP-1 expressions in liver tissues

The positive rate of MMP-2 in control group, model group and IL-10 treatment group was 9.5%, 84.0% and 39.3%, respectively, and that of TIMP-1 was 23.8%, 92.0% and 71.4%, respectively. The granular positive products were localized in the cytoplasm of hepatocytes and biliary epithelial cells. In control group, the positive expressions of MMP-2 and TIMP-1 were weak and found mainly in endothelial cells and hepatic cells. In model group, positive expressions increased obviously with the development of hepatic fibrosis, distributing in biliary epithelial cells, fibroblasts and muscular cells. In treatment group the

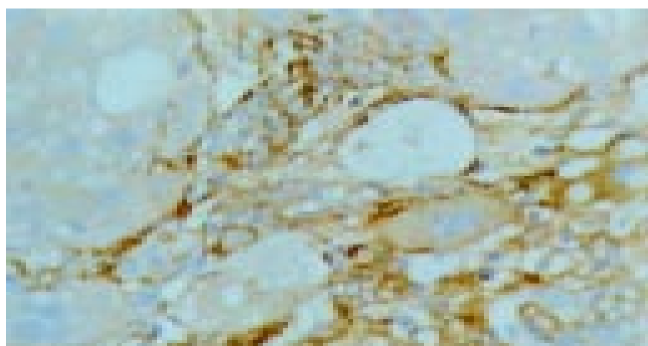

Figure 1 MMP-2-positive cells in the model group (S-P method, ×200).

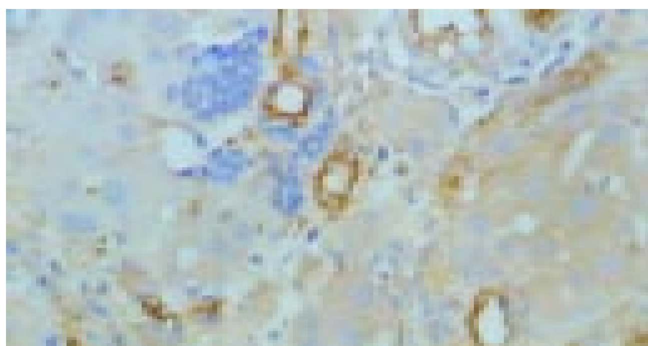

Figure 2 MMP-2-positive cells in IL-10 treatment group (S-P method, ×200).

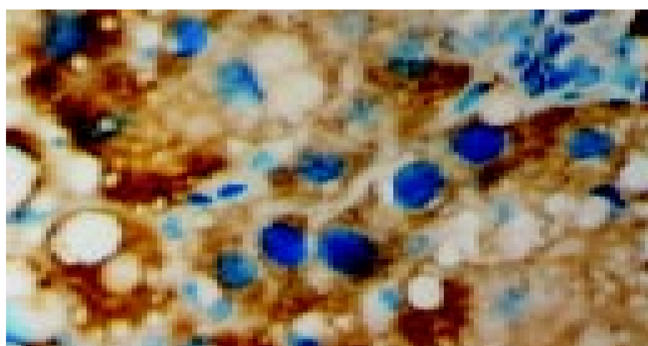

Figure 3 TIMP-1-positive cells in the model group (S-P method,

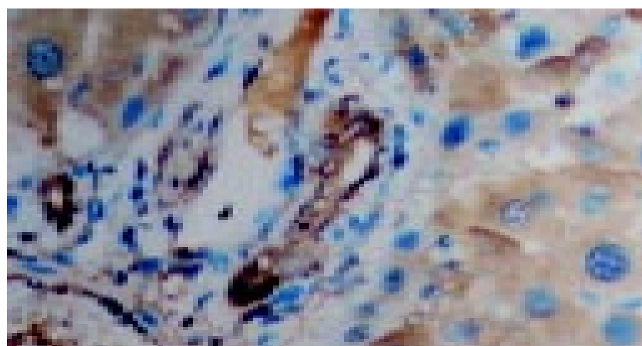

Figure 4 TIMP-1-positive cells in IL-10 treatment group (S-P method, ×400).

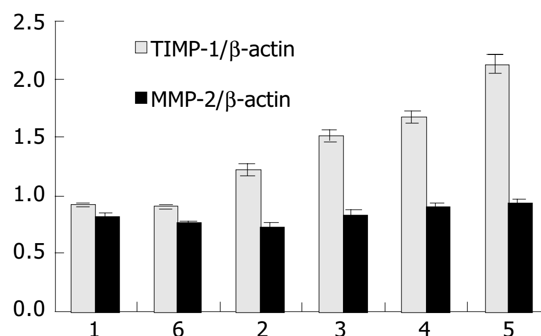

Figure 5 Effects of PDGF on TIMP-1 and MMP-2 expressions in HSCs. 1: Control group (2 h); 2: PDGF-treated group (2 h); 3: PDGF-treated group (4 h); 4: PDGF-treated group (8 h); 5: PDGF-treated group (24 h); 6: Control group (24 h).

Intensities of MMP-2 and TIMP-1 immunoreactivities

Comparison of MMP-2 and TIMP-1 positive expression levels between the 3 groups is shown in Table 1. Ridit analysis showed significant difference between the 3 groups (P<0.01). Higher expression levels of MMP-2 and TIMP-1 in model group were detected than in control group (P<0.01). In treatment group, IL-10 treatment resulted in decreased immunoreactivities for MMP-2 and TIMP-1 (P<0.01 and P<0.05 respectively). The expression levels of MMP-2 and TIMP-1 in different phases of hepatic fibrosis are listed in Table 2. With the development of hepatic fibrosis, the intensities of MMP-2 and TIMP-1 immunoreactivities increased gradually, and the difference was

Table 1 Comparison of MMP-2 and TIMP-1 immunoreactivities between control, model and treatment groups

Group	MMP-2					TIMP-1			
	n	-	+	++	Ridit value	-	+	++	Ridit value
Control	21	19	2	0	0.312	16	5	0	0.277^b
Model	25	4	13	8	0.712^d	2	14	9	0.684^d
Treatment	28	17	10	1	0.451^e	8	18	2	0.503^g

^{b}P<0.01 *vs* treatment group; ^{c}P<0.05; ^{d}P<0.01 *vs* control group; ^{e}P>0.05 *vs* control group; ^{g}P<0.05 *vs* model group.

Table 2 Comparison of MMP-2 and TIMP-1 immunoreactivities measured at different time points in model group

Wk	*n*	MMP-2				TIMP-1			
		-	+	++	Ridit value	-	+	++	Ridit value
5	8	1	7	0	0.378	1	6	1	0.36^{ab}
7	8	2	5	1	0.388	1	5	2	0.418^e
9	9	1	1	7	0.709	0	2	7	0.698^c

^{b}P<0.01 *vs* wk 7 and 9; ^{a}P<0.05 *vs* wk 9; ^{c}P<0.05 *vs* wk 7; ^{e}P>0.05 *vs* wk 5.

significant (P<0.05).

Relative quantities of MMP-2 and TIMP-1 mRNA in HSCs

Expression of TIMP-1 in PDGF-treated HSCs was significantly increased time-dependently as compared with that in control cells (P<0.01). There was no difference in MMP-2 expression between PDGF-treated HSCs and control cells (Figure 5). After treatment with IL-10 and PDGF, expressions of TIMP-1 and MMP-2 in HSCs were similar to those in negative control groups (P>0.05), without changes over time (Figure 6).

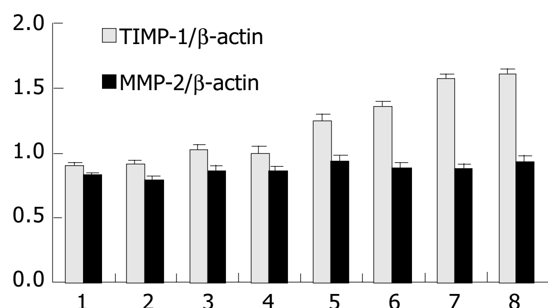

Figure 6 Effects of IL-10 and PDGF on TIMP-1 and MMP-2 expressions in HSCs. 1: Blank control group (2 h); 2: Blank control group (24 h); 3: Negative control group (2 h); 4: Treatment group (2 h); 5: Negative control group (12 h); 6: Treatment group (12 h); 7: Negative control group (24 h); 8: Treatment group (24 h).

DISCUSSION

Liver fibrosis is thought to be a progressive pathological process that leads ultimately to deposition of excess matrix proteins in extracellular space[17], and destroys normal liver architecture to finally result in cirrhosis. In extracellular space, matrix degradation occurs predominantly consequent to the action of a family of enzymes known as matrix metalloproteinases[18]. These enzymes are secreted by cells into extracellular space as proenzymes, which are then activated by a number of specific mechanisms. MMP-2 (gelatinase A) produced by activated HSCs, as demonstrated by immunohistochemistry and *in situ* hybridization[19,20], plays an important role in remodeling the basement membranes as it degrades several of the collagen components including collagen IV, laminin and fibronectin[21]. In other tissues, such as lung, kidney and heart, MMP-2 expression is also increased during fibrogenesis[22-25]. Recent studies showed that inhibition of MMP-2 activity[26] or blockade of MMP-2 synthesis[21] might effectively prevent mesangial cell proliferation and collagen I synthesis *in vitro*, indicating the possible role of MMP-2 as a growth factor and activator for mesangial cells, performed probably through an autocrine pathway[27]. In human liver fibrosis or in rat models of CCl_4-induced liver fibrosis, the expression of MMP-2 mRNA was increased by several fold, and HSCs expressed MMP-2 when activated by *in vitro* culture[27,28], as is consistent with our findings in this study.

TIMPs are the most important family of molecules involved in regulation of extracellular MMP activity[29-32]. TIMPs are produced by a wide variety of cells and often cosecreted with MMPs, providing local autoregulation of MMP activity. Recognized now as a multifunctional protein, TIMP-1 has been reported to stimulate steroidogenesis, inhibit angiogenesis, and induce changes in cell morphology[11,33]. It has been shown that TIMP-1, but not TIMP-2, can enter the nuclei of several types of cells, which further suggests that TIMP-1 may also act as a transcription factor[34,35]. We found that during the development of liver fibrosis, TIMP-1 expression in the liver was markedly up-regulated. Increased serum TIMP-1 levels have been documented in patients with chronic active liver disease in correlation with histological degree of human liver fibrosis[36]. Liver TIMP-1 protein levels are also closely correlated with the histological degree of liver fibrosis. The above findings suggest that TIMP-1 plays an important role in the development of liver fibrosis[12,30,37]. Recent findings indicate that HOE077, a prolyl-4-hydroxylase inhibitor, may prevent fibrosis by inhibiting the expression of liver type I procollagen and TIMP-1 mRNAs as well as proline hydroxylation and stellate cell activation, resulting in reduced expression of procollagen and TIMP-1 mRNAs[38]. Experiments with liver-targeted TIMP-1 transgenic (TIMP-Tg) mouse have shown significantly attenuated ability of reversing spontaneous liver fibrosis in TIMP-Tg mice as compared with the control mice[39]. But the exact role of TIMP-1 in liver fibrogenesis has not yet been clarified. In another experiment with TIMP-Tg mice, the direct effect of TIMP-1 overexpression on CCl_4-induced liver fibrosis was examined, it was found that TIMP-1 did not induce liver fibrosis by itself, but strongly promoted liver fibrosis development, in other words, TIMP-1 behaved not as the initiation factor, but as a strong promoter of liver fibrosis[40].

PDGF is a major mitogen for connective tissue cells and some other cell types. This dimeric molecule consists of disulfide-bonded, structurally similar A- and B-polypeptide chains that combine to form homo- and hetero-dimers, and is involved in autocrine and paracrine stimulation of cell growth in several different pathological conditions. Evidence showed that recombinant PDGF stimulated proliferation of nonconfluent myofibroblasts and collagen production in confluent cultures of myofibroblasts without increasing cell number, demonstrating the importance of PDGF in pathogenesis of liver fibrosis[41-45]. It has been established that human and rat HSCs are able to migrate according to the concentration gradients of chemotactic factors[46-48]. The best characterized chemotactic factor for HSCs identified so far is PDGF-BB[46,47,49], known also as the most potent mitogen for HSCs and overexpression during active hepatic fibrogenesis[50]. Our findings in this study showed that after PDGF treatment, the expression of TIMP-1 increased significantly in HSCs possibly because PDGF-promoted HSC proliferation and activation, indicating the importance of PDGF in pathogenesis of liver fibrosis. We also found that the expression of MMP-2 did not increase in HSCs after PDGF treatment, which is suggestive of the irrelevance of MMP-2 in fibrogenesis induced by PDGF.

IL-10, originally isolated from mouse helper T cells, is a cytokine that regulates a number of interleukins. It inhibits synthesis of several cytokines by T lymphocytes and activates monocytes, and was therefore originally named cytokine synthesis inhibitory factor[51]. Recent studies showed that IL-10 also acted on connective tissue cells such as fibroblasts, inducing, for instance, transcriptional inhibition of the expression of type I collagen, which is the major component of extracellular matrix[52]. IL-10 also possesses antifibrogenic properties by down-regulating profibrogenic cytokines like TGF-β_1 and TNF-α[53,54]. Our previous studies indicated that IL-10 could produce antifibrogenesis effect on CCl_4-induced rat hepatic fibrosis. In this study, we found that IL-10 down-regulated expression of MMP-2 and TIMP-1 in rat fibrotic liver, possibly another way that IL-10 exerts its effect of antifibrogenesis. In our previous study, PDGF was found to markedly promote the contraction and proliferation of HSCs and expressions of collagen type I and III as well as TGF-β_1 in HSCs, which was significantly inhibited by IL-10[55]. But results of the present experiment showed that at the dose of 20 ng/mL, IL-10 did not inhibit TIMP-1 and MMP-2 expressions in PDGF-treated HSCs, indicating that the inhibitory effects of IL-10 on HSCs may not involve TIMP-1 and MMP-2.

In summary, the present study demonstrates that positive

expressions of MMP-2 and TIMP-1 in rat liver tissue increase with the development of hepatic fibrosis, and MMP-2 and TIMP-1 play an important role during the development of liver fibrosis. Exogenous IL-10 decreases the expression of MMP-2 and TIMP-1 in liver tissues. PDGF increases the expression of TIMP-1 in HSCs, possibly through promoting HSC proliferation and activation, and this effect is not inhibited by IL-10.

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