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• BASIC RESEARCH •

Expression of TIMP-1 and TIMP-2 in rats with hepatic fibrosis

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Abstract

AIM: To investigate the location and expression of TIMP-1 and TIMP-2 in the liver of normal and experimental hepatic fibrosis in rats.

METHODS: The rat models of experimental immunity hepatic fibrosis (n=20) were prepared by the means of immunologic attacking with human serum albumin (HSA), and normal rats (n=10) served as control group. Both immunohistochemistry and *in situ* hybridization methods were respectively used to detect the TIMP-1 and TIMP-2 mRNA and related antigens in liver. The liver tissue was detected to find out the gene expression of TIMP-1 and TIMP-2 with RT-PCR.

RESULTS: The TIMP-1 and TIMP-2 related antigens in livers of experimental group were expressed in myofibroblasts and fibroblasts (TIMP-1: 482±65 *vs* 60±20; TIMP-2: 336±48 *vs* 50±19, P<0.001). This was the most obvious in portal area and fibrous septum. The positive signals were located in cytoplasm, not in nucleus. Such distribution and location were confirmed *by situ* hybridization (TIMP-1/*b-actin*: 1.86±0.47 *vs* 0.36±0.08; TIMP-2/*b-actin*: 1.06±0.22 *vs* 0.36±0.08, P<0.001). The expression of TIMP-1 and TIMP-2 was seen in the liver of normal rats, but the expression level was very low. However, the expression of TIMP-1 and TIMP-2 in the liver of experimental group was obviously high.

CONCLUSION: In the process of hepatic fibrosis, fibroblasts and myofibroblasts are the major cells that express TIMPs. The more serious the hepatic fibrosis is in the injured liver, the higher the level of TIMP-1 and TIMP-2 gene expression.

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INTRODUCTION

Chronic viral hepatitis, alcoholism and schistosomiasis are the most common diseases in China[1-9]. At present, two main strategies for the treatment of chronic liver diseases are anti-viral therapies and anti-fibrotic therapies[10-14]. Hepatic fibrosis is a main pathologic basis of chronic liver diseases, particularly caused by viral hepatitis, and cirrhosis as a severe outcome is the end stage of various chronic liver diseases with increased synthesis and/or inhibition of matrix degradation. Although important progress in hepatic fibrosis has been achieved in the last decades, its mechanism is still debated at present. The formation of hepatic fibrosis is a response to inflammation, but it is interesting that hepatic fibrosis is not found in acute liver injury. It has been proved by the experiment *in vitro* that apoptosis could not be found in inactive hepatic stellate cells (HSC). This may imply that the mechanism of hepatic fibrosis is complicated, and many questions are being explored[15-23].

Hepatic fibrosis is a pathological process with the overlapped extracellular matrix (ECM) protein. The latest evidence suggests that the change of ECM mainly is regulated by metalloproteinases (MMPs). Hepatic fibrosis is formed because the specific tissue inhibitors of metalloproteinases (TIMPs) inhibit ECM degradation[24-28]. Which cells can express and produce TIMPs? Until now, there still are different views on the involvement of TIMPs in normal liver tissue and experimental hepatic fibrosis rats. The expression and location of TIMPs antigens and TIMP mRNA are measured in rat livers with mAb and cDNA probes of TIMP-1 and TIMP-2 by immunohistochemical staining, and gene expression of TIMP-1 and TIMP-2 is observed by PCR technique.

MATERIALS AND METHODS

Animal experiments

Forty adult female Wistar rats, weighing 120-150 g (provided by Experimental Animal Centre of Fourth Military Medical University) were employed in the study. The rats were randomly divided into 2 groups. A rat model of hepatic fibrosis was produced by immunological attacking with human serum albumin (HSA), using the method introduced by Wang *et al*[29]. Anti-mouse monoclonal antibody IgG was purchased from Coulter Co.(France). Twenty healthy female Wistar rats were regarded as control group. Animals survived from the experimental attack were randomly allocated as follows. All rats were injected with 0.5 mL HSA diluted with normal saline (0.5 mL equals to 4 mg HSA) and same quantity of an incomplete Freund' s adjuvant (Sigma), once every 14 days for the first two times, then once every 10 days, 2 times. Ten days after the last injection, serum antibody was measured. Positive rats were chosen for experiment through coccygeal vein injection of HSA, twice a week, 2.5 mg for each at the first week, and then gradually 0.5 mg- increase once for each to 4.5 mg, and this dose was maintained for 2 months. All animals were sacrificed under narcosis, and their livers were immediately excised. Part of liver specimen was frozen in liquid nitrogen, part fixed in 40 g/L formaldehyde, the rest was fixed with glutaraldehyde, and investigated with electron microscope.

Immunohistochemical staining of TIMP-1 and TIMP-2

According to the methods previously described[30-33], the serial paraffin sections of liver samples at 4 μm thickness were performed for SP immune staining described by streptomycin

Table 1 Sequences of TIMP-1, TIMP-2 and β-actin primers

	Primer	Nucleic acid sections	Position (bp)
TIMP-1	Positive strand	5'-TTCGTGGGGACACCAGAAGTC-3'	
	Antisence strand	5'-TATCTGGGACCGCAGGGACTG-3'	482
β-actin	Positive strand	5'-GGAGAAGATGACCCAGATCA-3'	
	Antisence strand	5'-GATCTTCATGAGGTAGTCAG-3'	234
TIMP-2	Positive strand	5'-GTTTTGCAATGCAGATGTAG-3'	
	Antisence strand	5'-ATGTCGAGAAACTCCTGCTT-3'	540
β-actin	Positive strand	5'-ACCCCCACTGAAAAA-3'	
	Antisence strand	5'-ATCTTCAAACCTCCATGATG-3'	120

avidin-peroxidase immunohistochemistcal kit (Maxim Biological Technology Company, USA). Anti-mouse monoclonal antibodies of TIMP-1 and TIMP-2 were also obtained from Maxim.The sections were deparaffinizd and rehydrated. After retrieval of the antigens, nonspecific binding sites were blocked with 100 mL/L normal serum for 20 min.The sections were incubated with monoclonal antibody against TIMP-1 or TIMP-2 at 4 ℃ overnight, and then with secondary antibody at 37 ℃ for 30-40 min, avidin-peroxidase at 37 ℃ for 20 min, and finally with DAB to be colorated for 10 min, and counterstained with hematoxylin, dehydrated with ethanol, rinsed in xylene, and mounted with gum for microscopic examination and photography. To ensure the reliability of the experiment, rabbit serum and phosphate buffer were used instead of monoclonal antibody and secondary antibody, respectively. In addition, 10 healthy liver tissues were selected for control group. The background density of positive cells from 5 microscopic fields at random was measured, and its mean value was used for statistical analysis with *SPSS* 10.0

In situ hybridization

The experiment was performed as previously described[33,34] using *in situ* hybridization kit (Boshide Biological Technology Limited Company, Wuhan, China, *No* MK1549). Briefly, the serial paraffin sections (thickness 4 μm) were dried at 80 ℃, then deparaffinizd by xylene and rehydrated in graded ethanol, acidified in 1 mol/L HCl for 30 min, and blocked in 30 ml/L H_2O_2 3 mL for 10 min before digestion with proteinase K for 30 min, and then dehydrated with graded ethanol. After prehybridization at 37-40 ℃ for 2 h, the labeled cDNA probes were denatured at 95 ℃ for 10 min, then at -20℃ for 10 min, added onto liver tissue sections which had been prehybridized at 37 ℃ overnight. The sections were washed with 2×SSC, 1×SSC, and 0.2×SSC respectively. Buffer I was added, and then blocking solution was added at room temperature for 20 min, and then rabbit anti-digoxin at 37 ℃ for 60 min, biotinylated goat anti-rabbit at 37 ℃ for 30 min, SABC at 37 ℃ for 30 min, finally DAB was added to develop color. After several washings, the sections were counter-stained with hematoxylin, dehydrated with ethanol, rinsed in xylene, and mounted with gum for microscopic examination and photography. Blank control: TIMP-1 and TIMP-2 cDNA probes for positive hepatic tissues were replaced by pre-hybridization solution. Negative control: *in situ* hybridization was performed in 10 normal liver tissues. Semi-quantitative results were determined by *in situ* hybridization. (-) as no positive cells, (+) as positive cells <1/3 of all hepatic cells, (++) as positive cells between 1/3-2/3 of all hepatic cells, (+++) as positive cells >2/3 of all hepatic cells in a lobule.

PCR amplification

PCR primers of TIMP-1 were designed according to the whole TIMP-1 cDNA sequence of rats[35]. The PCR primers of TIMP-1 and TIMP-2 are listed in Table 1.

Total RNA of the liver was extracted with an isolation system (Promega). PCR was performed in 20 μL reaction volume containing 2 μL cDNA, 2 μL 10×buffer, 2 μL (2 mmol/L) 4×dNTP, 10 mmol/L primer (2 μL TIMP-1, 2 μL β-*actin* and 2 μL TIMP-1, 2 μL β-*actin*, respectively) and 1U *Taq* DNA polymerase. The samples were subjected to 30 thermal cycles, each consisting of 2 min at 97 ℃ for predenaturation, 30 s at 94 ℃ for denaturing, 30 s at 56 ℃ for annealing, 50 s at 72 ℃ for extension and 7 min at 72 ℃ for final extension after the last cycle. Ten μL sample of PCR product was subjected to electrophoresis in 20 g/L agarose gel with TAE buffer at 50 V for 1 h. After stained with ethidium bromide, quantitative analysis was performed. The ratios of TIMP-1/*b-actin* and TIMP-2/*b-actin* were regarded as the expression levels of TIMP-1 and TIMP-2.

Pathologic observation

Some liver sections were stained with hematoxylin and eosin, while other sections for Von Gieson and Masson special staining. The liver samples were also fixed with glutaraldehyde, and examined with electron microscope.

Statistical analysis

Was performed with *SPSS* 10.0.

RESULTS

Pathologic findings

In liver tissues from rats with hepatic fibrosis, hyperplasia of the lattice fibers and collagenous fibers was observed in portal area and extended outwards. Hyperplasia surrounding the central vein observed was distributed along hepatic sinus and connected each other. The hepatic lobules were encysted and separated by collagen bundles. The normal structure of lobules was destroyed, and pseudolobules formed. Infiltration of inflammatory cells was found around the portal area and central vein. The structure of liver tissues was normal in control.

Under electron microscopy, proliferation of activated hepatic stellate cells (HSC) surrounded by collagen fibers was found in early stage, in which abundant rough endoplamic reticulum and lipids were present. Eventually, with the deposition of collagen bundles, myofibroblasts formed in portal area, and the deposition of collagens produced a wide compartment. Lots of collagen fibrils resided within the space of Disse. A vast amount of swelling mitochondria and some lipids were detectable in degenerative hepatocytes.

TIMP-1 and TIMP-2 expression and localization

TIMP-1 and TIMP-2 antigens in the liver from experimental rats were detected in myofibroblasts, fibroblasts and vascular endothelial cells predominantly in the portal area and fibrous septum. Expression of TIMP-1 and TIMP-2 exhibited as brown particles in cytoplasm. No positive expression was found in nucleus. There was only a mild positive expression in vascular

endothelial cells of the normal rat liver. Image pattern analysis showed that the expression in the experimental group was much stronger than that in the control group (Table 2, Figure 1), so did the *in situ* hybridization (Figure 2). In order to confirm the specificity of immunohistochemical experiment, the first antibody and second antibody were replaced by at serum and buffer, respectively. Negative results were found in controls, with specific confirming experiment *for in situ* hybridization, and no positive result was found.

Table 2 Expression of TIMP-1 and TIMP-2 related antigens in rat liver

Group	*n*	TIMP-1	TIMP-2
Experimental group	20	482.50±65.00	336.50±48.32
Normal group	10	59.8±20.31	49.86±18.54

$P<0.001$.

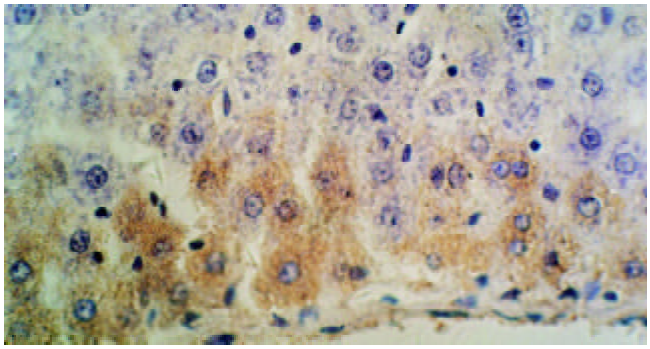

Figure 1 Expression of TIMP-1-related antigens in liver tissue from rats with experimental fibrosis (Immunohistochemical staining ×400).

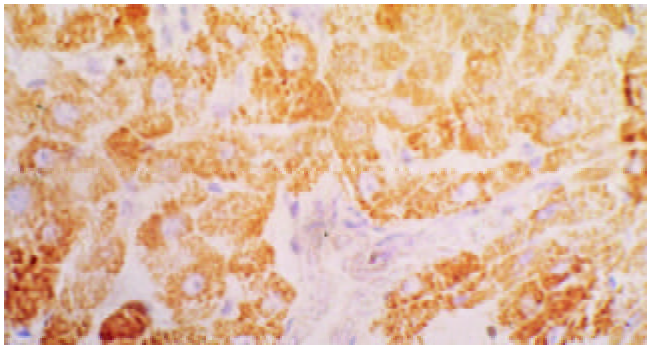

Figure 2 Expression of TIMP-1 mRNA in liver tissue from rats with experimental fibrosis (*in situ* hybridization ×400).

TIMP-1 and TIMP-2 gene expression

The expression of TIMP-1 in the liver of normal rats was strikingly low, while its expression in experimental group was significantly higher than that in the normal group (Table 3).

Table 3 Gene expression of TIMP-1 and TIMP-2 in rat liver

Group	*n*	TIMP-1 / β-*actin*	TIMP-2 / β-*actin*
Experimental group	20	1.86±0.47	1.06±0.22
Normal group	10	0.36±0.08	0.36±0.08

$P<0.001$.

DISCUSSION

It is necessary to establish and develop an ideal animal model of hepatic fibrosis, so as to investigate the etiology or pathogensis, to explore the effective diagnosis or treatment and to select or evaluate anti-fibrotic medicines. An ideal animal model should be very similar to the characteristics of human disease, with distinct stages of disease and a low mortality. The model should be economically and easily established without difficulty to get the animals.

Researchers began to establish the hepatic fibrosis model of rats with carbon tetrachloride (CCL_4) in 1936, which was an early study on animal model of hepatic fibrosis and cirrhosis. Since it was repeatedly improved, the experimental model has been widely employed. People gradually established various models, such as rat model by bile duct ligation (Zimmermann, 1992), rabbit model with schistosomiasis (Dunn, 1994), rat model by thioacetamide (Okanoue, 1954), *etc*. However, these models with various characteristics are not very ideal to the animal experiment because of their common shortcomings that the hepatic fibrosis lasted shortly and was absorbed spontaneously soon.

In 1966, Paronetto and Popper[36] have proved that an immune response could induce liver injury. The animals injected repeatedly with the globulin or swine albumin or the serum from swine, bovine or horse developed hepatic fibrosis after 5 weeks, and cirrhosis after 10 weeks. However, the model had a high mortality because of allergic reaction. We studied various models and improved the model developed by Wang *et al*[29]. The survival rate of rats obviously increased and the fibrosis lasted for more than 363 days when the rats were injected with a small quantity of dexamethasone through its caudal vein soon after the first or secondary attack. The fibrosis was proved pathologically and the formation of fibrosis belonged to the type III allergic reaction induced by the immunocomplex of albumin. Under electron microscopy it was proved that the pathological process stimulated the proliferation of hepatic stellate cells (HSC), which would transform to the myofibroblasts and secrete a large amount of collagens.

At present, it was found that TIMP-1 in the injured liver increased early and obviously, and many researchers thought that TIMP-1 was a very important promoting factor in the process of hepatic fibrosis[27,32,37], and that it could inhibit MMPs (such as MMP-1) to deposit ECM. Up to now, only TIMP-1 and TIMP-2 were found in liver and TIMP-1 increased more obviously than TIMP-2, and strong expression of TIMP-1 reflected the severity of hepatic fibrosis. With the method of immunohistochemistry, we found that TIMP-1 and TIMP-2 were expressed obviously only in myofibroblasts or fibroblasts of livers from experimental group, mostly in portal area and fibrous septum, while very mildly in vascular endothelial cells of livers from control group. Such a distribution was also shown in the results of *in situ* hybridization.

The results of *in situ* hybridization demonstrated that TIMP-1 mRNA was expressed in hepatocytes and in almost all mesenchymal cells, especially strong in lipocytes of inflammation area of the liver induced by CCL_4 or bile duct ligation model[38]. Our results indicated that it was strongly expressed only in myofibroblasts, fibroblasts and vascular endothelial cells, mainly in portal area and fibrous septum. The results of immunohistochemistry were similar. Whether the expression difference is due to various models or other causes is still unknown.

From the gene expression levels of TIMP-1 and TIMP-2 we suggested that the more severe the hepatic fibrosis was, the higher the gene expression levels of TIMP-1 and TIMP-2 were, and that the strong expression of TIMP-1 inhibited the degeneration of collagen by MMP-1 and pro MMP-9, and that the expression of TIMP-2 inhibited MMP-2, MMP-9, *etc*. to promote the deposition of ECM. The continuous deposition of collagen fibers in the liver finally resulted in hepatic fibrosis.

The concentrations of TIMP-1 and TIMP-2 in peripheral blood indicated their gene expression levels. Iredale *et al*[39] isolated hepatocytes, HSC and Kupffer cells from the liver of experimental hepatic fibrosis model, but failed to find TIMP-1 mRNA in hepatocytes with Northern hybridization in 1997. We detected the expression of TIMP-1 in hepatocytes, HSC and Kupffer cells with PCR technique, and the expression of TIMP-1 was mild in normal rat liver, but strong in fibrotic rat liver. Therefore, further experiments might prove no expression of TIMP-1 in hepatocytes. The strong expression of TIMP-1 in fibroblasts and myofibroblasts of fibrotic liver probably resulted from the activation of HSC. In fact, hepatic fibrosis was essentially a "repair" reaction to chronic liver damage because of the cell-cell interaction mediated by cytokines and the activation of HSC induced by the interaction of mesenchymal cells. The persistence of HSC activation was induced by repeated liver damage. Thus, the activation of HSC was mainly in the pathologic process of hepatic fibrosis[19,21].

Although TIMP-1 and TIMP-2 were closely related to hepatic fibrosis, study on TIMPs has just begun. Thus far, the mechanism of strong expression of TIMP-1 and TIMP-2 in fibrotic liver is still unknown, and other characteristics of TIMPs are being studied[40-43]. To enhance the study on TIMPs has become very important for the diagnosis, treatment and pathogensis of hepatic fibrosis[44-47].

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