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• VIRAL LIVER DISEASES •

Methodologic research on TIMP-1, TIMP-2 detection as a new diagnostic index for hepatic fibrosis and its significance

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Abstract

AIM: **To set up a new method to detect tissue inhibitors of metalloproteinase-1 and -2(TIMP-1 and TIMP-2) in sera of patients with hepatic cirrhosis, and to investigate the expression and location of TIMP-1 and TIMP-2 in liver tissue of patients with hepatic cirrhosis, and the correlation between TIMPs in liver and those in sera so as to discuss whether TIMPs can be used as a diagnosis index of hepatic fibrosis.**

METHODS: **The monoclonal antibodies (McAbs) of TIMP-1 and TIMP-2 were used to sensitize erythrocytes, and solid-phase absorption to sensitized erythrocytes (SPASE) was used to detect TIMP-1 and TIMP-2 in the sera of patients with hepatic cirrhosis. Meanwhile, with the method of *in situ* hybridization and immunohistochemistry, we studied the mRNA expression and antigen location of TIMP-1 and TIMP-2 in the livers of 40 hepatic cirrhosis patients with pathologic diagnosis.**

RESULTS: **With SPASE, they were 16.4% higher in the acute hepatitis group, 33.3% higher in the chronic hepatitis group, and the positive rates were 73.6% and 61.2% respectively in sera of hepatic cirrhosis patients, which were remarkably higher than those in chronic hepatitis and acute hepatitis group ($P<0.001$). In 40 samples of hepatic cirrhosis tissues, all of them showed positive expression of TIMP-1 and TIMP-2 mRNA detected with immunohistochemistry or *in situ* hybridization (positive rate was 100%). Expression of TIMPs in different degrees could be found in liver tissue with cirrhosis. TIMPs were located in cytoplasm of liver cells of patients with hepatic cirrhosis. There was a significant correlation between serum TIMPs level and liver TIMPs level.**

CONCLUSION: **SPASE is a useful method to detect the TIMP-1 and TIMP-2 in sera of patients with hepatic cirrhosis, and TIMP-1 and TIMP-2 can be considered as a useful diagnostic index of hepatic fibrosis, especially TIMP-1.**

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INTRODUCTION

SPASE (solid-phase absorption to sensitized erythrocytes) is an immunological detecting method possessing the similar principle with ELISA(enzyme-linked immunosorbent assay) and SPRIA(solid-phase radioimmunoassay)[1]. Erythrocytes sensitized by antibodies are used as the indicator, taking the place of enzyme or isotope labeled antibody. The result is judged by the hemagglutination phenomena. SPASE has the same sensitivity and specificity as ELISA and SPRIA, and is so simple and rapid as RPHA (reverse passive hemagglutination)[2,3]. The monoclonal antibodies (McAbs) of TIMP-1 and TIMP-2 were used to sensitize erythrocytes. SPASE was used to detect TIMP-1 and TIMP-2 in the sera of patients with hepatic cirrhosis, and proved pretty well. At the same time, with the method of *in situ* hybridization and immunohistochemistry, the mRNA expression and antigen location of TIMP-1 and TIMP-2 in the livers of 40 hepatic cirrhosis patients diagnosed by pathology were studied.

MATERIALS AND METHODS

Materials

The U shape 96 well plexiglass microhemagglutination plate was used as the solid phase support. The solid-phase antibody (McAbs of TIMP-1 and TIMP-2) was purchased from Maxim Biologic Technology Corp, America, (No: MAB-0282, MAB-0283). Formaldehyde-chicken or sheep erythrocytes were sensitized by TIMP-1 and TIMP-2 McAb, according to the method introduced by Han[4] to prepare for sensitized erythrocytes. The concentration of McAbs was 50-100mg·L^{-1}. The positive samples that simulated positive sample were obtained from the sera of hepatic cirrhotic patients pathologiclly diagnosed, and normal human sera were used as the negative control, and PBS as the blank control. To test the thermal stability, McAb was bathed at 60℃ water with different time, and its activity was detected by RIA.

We collected 408 serum samples from Tangdu Hospital and Xijing Hospital affiliated to the Fourth Military Medical University, and Southwest Hospital affiliated to the Third Military Medical University, The First and Fourth Hospitals of Chinese PLA. The diagnosis of viral hepatitis accorded with the diagnosis standard revised by the Fifth National Academic Conference[5] for Infectious and Parasitic Diseases. According to the standard, there were 128 serum samples of acute hepatitis, 174 of chronic hepatitis, and 106 of hepatic cirrhosis. 40 liver samples were collected from the pathologic departments of Tangdu and Xijing Hospitals affiliated to the Fourth Military Medical University. All the 40 samples were proved pathologiclly with nodular hepatic cirrhosis. Male,40; female,8;the mean age, 50 years.

Methods

Procedure of SPASE 50mg·L^{-1} McAb were added into U shape 96 well plexiglass micro hemagglutination plate (100 μL·well), to make one layer and the unnecessary solution should be removed, the sparing solution could be reused, and then dried under room

temperature, fixed at 56℃, washed with PBS 3-4 times, to get rid of the unfixed McAb molecule. 100μL per well of the prepared serum sample (diluted at the ratio volume of 1:10) was added, reacted at 37℃ for 1h, washed with PBS 3-4 times, and then 50μL per well of McAb sensitized erythrocytes were added. After shaking mixed, they were standing for 1h at 37℃ or 2h at room temperature. The results were determined according to the absorbed condition of erythrocytes (like RPHA). The erythrocytes depositing on the bottom of the well was considered as negative result, showing a spot or a little circle in the center of the well without scattered erythrocytes around. The positive result was determined when the erythrocytes were absorbed and a layer spread on the bottom of the well.

Immunohistochemical staining The laboratory procedure referred to references 6-12.SP immunostaining was performed as described by streptomycin avidin-peroxidase immunochemistry kit (purchased from Maxim Biological Technology Company). Briefly, the liver samples were embedded with paraffin, and serial sections at 4μm thickness were prepared. Paraffin was removed from the sections with xylene and rehydrated with graded ethanol. After the antigens were repaired, unspecific immunoglobulin-binding sites were blocked by a 20min preincubation with 100mL·L^{-1} normal human sera. The sections were then incubated with monoclonal antibody against TIMP-1 or TIMP-2 at 4℃ overnight, and then secondary antibody was added at 37℃ for 30-40min, avidin-peroxidase at 37℃ for 20min, and finally DAB was added to be stained. After the sections were washed several times, they were counterstained with hematoxylin, dehydrated with ethanol, rinsed in xylene, and the sections were mounted with gum for microscopic examination and photography. To make sure of the reliability and specificity of the result of immunohistochemical staining, rabbit sera and PBS were used to replace the first antibody in our control test. 10 normal liver tissues were also used as the normal control samples.

Liver tissue *in situ* hybridization The investigation procedure referred to related references[13-17]. The *in situ* hybridization kit was purchased from Boshide Biological Technology Limited Company (Wuhan, China, No. MK1549). *In situ* hybidization was performed according to the manufacturer's direction. Briefly, the paraffin embedded serial sections(thickness 4μm), were dried at 80℃, and their paraffin was removed by xylene and rehydrated with graded ethanol. The sections were acidified in HCl for 30min, and blocked in 3mL 300mL·L^{-1}H_2O_2 for 10min before digestion in proteinase K for 30min, and then dehydrated with graded ethanol. After prehybridization at 37-40℃ for 2h, the labeled cDNA probes of TIMP-1 and TIMP-2 were denatured in hybidization buffer at 95℃ for 10min,then -20℃ for 10min, added on tissues which had been prehybridized at 37℃ overnight. Sections were washed in turn with 2×SSC, 1×SSC, 0.2×SSC, and Buffer I, blocking water was added at room temperature for 20min, and then rabbit anti-digoxin serum at 37℃ for 60min, biotinylated goat anti-rabbit serum at 37℃ for 30min, SABC at 37℃ for 30min, finally DAB was added to be stained. After several times of washing, the sections were counterstained with hematoxylin, dehydrated with ethanol, rinsed in xylene, and mounted with gum for microscopic examination and photography. (1) Blank control: prehybridization solution was replaced by the cDNA probes of TIMP-1 and TIMP-2 to be hybridized with the positive liver sections; (2) Negative control: the *in situ* hybridization was performed with 10 normal liver sections.

Semi-quantitative index was used to determine the results of immunohistochemistry and *in situ* hybridization: no positive cells (-); positive cells occupied hepatocytes of hepatic lobulfe less than than1/3(+); 1/3~2/3(++); more than 2/3(+++).

RESULTS

Methodologic Optimization

The best laboratory condition and influence factors To study the related factor of sensitivity and specificity of this technique, the simulated positive samples were used, and we concluded that there were two main factors might directly influence the experiment condition. One was the concentration of coated antibodies and the other the density of sensitized erythrocytes. Much higher or less concentration of coated antibodies would decrease the sensitivity, while 50-100mg·L^{-1} might be the best (Table 1). There was a close relation between the density of sensitized erythrocytes, which is the indicator, and at 2.5·L^{-1} the sensitivity appeared to be the best (Table 2).

Degree of accuracy and repetitive test The coefficient of variation (CV) in one lot of samples in the same plate was 6.06%. The coefficient of variation (CV) in different lot of samples in different plate was 7.65%. Both of them were less than 10% (Table 3), which showed that this technique was provided with good degree of accuracy and repetition.

Table 1 Relationship between coated McAb and the sensitivity of SPASE

Coated McAb (mg·L^{-1})	Serum titer							Negative control
	1:1	1:10	1:20	1:50	1:100	1:200	1:500	
12.5	+++	++	+	-	-	-	-	-
25	++++	+++	++	+	-	-	-	-
50	++++	++++	++++	++++	++	+	-	-
100	++++	++++	++++	+++	++	+	-	-
200	++++	+++	+++	++	+	-	-	-
400	+++	++	++	++	+	-	-	-

Table 2 Relation between the density of sensitized erythrocytes and the sensitivity of SPASE

Density of sensitized erythrocytes	Serum titer							Negative control
	1:1	1:10	1:20	1:50	1:100	1:200	1:500	
1.5·L^{-1}	+++	++	+	-	-	-	-	-
2.5·L^{-1}	++++	++++	++++	+++	++	+	-	-
3.0·L^{-1}	++++	++++	+++	++	+	+	-	-
6.0·L^{-1}	+++	+++	++	+	-	-	-	-
10.0·L^{-1}	++	+++	++	-	-	-	-	-

Table 3 Repetitive experiment of samples

	Well numbers	($\bar{x}$±s)	CV(%)
In the same lot	15	0.642±0.0389	6.06
In different lot	5	0.575±0.044	7.65

Table 4 Results of detecting TIMP-1 and TIMP-2 in different sera

Clinical type	*n*	TIMP-1		TIMP-2	
		Positive number	%	Positive number	%
Acute hepatitis	128	21	16.4	16	12.6
Chronic hepatitis	174	59	33.9	48	27.6
Hepatic cirrhosis	106	78	73.6	65	61.3

Clinical Application

Serum samples The 408 serum samples of patients with liver disease were used to detect TIMP-1 and TIMP-2 with SPASE. The

positive rates were 73.6% and 61.2% respectively, which were remarkablly higher than those in chronic hepatitis and acute hepatitis group. There was a significant statistical difference (P<0.001, Table 4).

Immunohistochemistry By immunohistochemistry detection, the positive signal as brown particles were scattered or diffused only in cytoplasm other than nuclei in liver cells. Table 5 represented the result of detecting TIMP-1 and TIMP-2 of 40 liver samples of hepatic cirrhosis, in which, for TIMP-1, 28 samples were (+++), 70.0% of cytoplasma; 4 were (++), 10.0%; 8 were (+), accounted for 20.0%. For TIMP-2, 22 samples were (+++), 55.0% of cytoplasma; 10 were (++), 25.0%; 8 were (+), 20.0%. 10 normal liver tissues were negative. There was no positive signal after abridging the first antibody or the second one, and there was no positive signal when the first antibody replaced by rabbit serum or PBS, which proved that the results of immunohistochemistry detecting were specific.

***In situ* hybridization** The positive signal of *in situ* hybridization showed brown particles, and distributed in the cytoplasm, scattered or diffused. There was no positive signal in nuclei. All the 40 samples of hepatic cirrhosis tissues showed positive expression of TIMP-1 and TIMP-2 mRNA, and the positive rate was 100%. Table 6 showed the intensity of TIMP-1 and TIMP-2 mRNA expression in the liver samples. For TIMP-1 mRNA, 32 samples were (+++), 80.0% of cytoplasma; 6 were (++), 15.0%; 2 were (+), 5.0%. Of TIMP-2 mRNA, 22 samples were (+++), in which 55.0% of cytoplasma; 16 were (++), accounted for 40.0%; 2 were (+), 5.0%. The expression intensity of TIMP-1 mRNA was stronger than that of TIMP-2 mRNA. There was no positive signal when TIMP-1 cDNA probes or TIMP-2 cDNA probes were replaced by the prehybridization solution, and when the 10 normal liver tissues were hybridized with TIMP-1 cDNA probes or TIMP-2 cDNA probes, all were negative. These proved that the results of*in situ*hybridization were specific.

Table 5 Expressing of TIMP-1 and TIMP-2 in liver

Group	*n*	TIMP-1				TIMP-2			
		-	+	++	+++	-	+	++	+++
Normal group	10	10	0	0	0	10	0	0	0
Hepatic cirrhosis	40	0	8	4	28	0	8	10	22

Table 6 Expressing of TIMP-1 mRNA and TIMP-2 mRNA in liver of hepatic cirrhosis

Group	*n*	TIMP-1				TIMP-2			
		+++	++	+	-	+++	++	+	-
Hepatic cirrhosis	40	32	6	2	0	22	16	2	0
Normal liver	10	0	0	0	10	0	0	0	10

DISCUSSION

SPASE is a new method used to detect the TIMP-1 and TIMP-2 in sera of patient with hepatic cirrhosis. During the setting up of this technique, we preliminarily tried to find out how to treat the influence factors and choose the condition. We consider that this method can not only possess both the advantages of SPA and IHT (indirect hemagglutination test), but also avoid the radiation pollution of RIA or cancer-causing danger of ELISA. Meanwhile, this method possesses many other advantages, such as wider range of usage, less influence factors, saving McAb, being easy to operate and judge detecting results. In a word, this is a specific, sensitive, rapid and economic method. There are two main factors that may influence the laboratory condition. Firstly, lower concentration of coated antibodies may result in fewer antibodies combined on the surface of support, which won't be able to catch antigens, while higher density may result in overlap of antibodies on the surface of support, which will have effect on the space position of conjunction of antigens and antibodies, and then antigens can't be effectively caught. Secondly, acting as the indicator system, a reverse correlation exists between the density of sensitized erythrocyte and sensitivity of detection. When the density of sensitized erythrocyte is higher than $3\cdot L^{-1}$, the detection sensitivity will be significantly decreased, while clarity of the result would be impaired if the density is lower than $1\cdot L^{-1}$. Because the erythrocytes can only react with one layer of the agent on the surface of solid support, excessive erythrocytes didn't combine with agents and would deposit at the bottom of wells, then the judgment of the results would be affected. For this reason, a layer of the sensitized erythrocytes with adequate density is better. The density we used in practice is about $2.5\cdot L^{-1}$, which is lower than that used in RPHA. In present study, the way of coating the antibodies was improved, and proved that the method of heating-fixed antibodies was feasible. Sheep erythrocytes and chicken erythrocytes sensitized by formaldehyde were detected with sensitization test, and no specific difference was observed except a little faster sedimentation rate of the former. This insured the source of erythrocytes.

The occurrence and progress of hepatic cirrhosis were the result of the interaction between hepatocytes and extracellular matrixes (ECM)[18-25]. The increase of ECM synthesis and decrease of ECM degradation will result in excess deposition of ECM in liver. More important reason of the excess deposition of ECM is the decrease of ECM degradation in the late stage. The metrical metalloproteinases played a leading role during the degradation of ECM[26-35]. MMPs were a group of zinc-ion dependent enzymes, which created conditions for further degradation of other proteinases though reducing the stability of helical structure of collagen and changing the secondary structure of substrates. TIMPs were a group of polypeptides with the ability of inhibiting the function of MMPs. TIMPs would inhibit the degradation of ECM through two main ways, which is non-covalent modification or conjugated with proenzyme. Research work showed that TIMP could be divided into four classes: TIMP-1, TIMP-2, TIMP-3, and TIMP-4. However, only TIMP-1 and TIMP-2 could be detected in liver[37-44]. All the 40 samples of hepatic cirrhosis tissues, showed positive expression of TIMP-1 and TIMP-2, and the positive rate was 100%. TIMP-1 and TIMP-2 were expressed at the same time, and were located in the liver cytoplasm, not in the nuclei. The expression of TIMP-1 was more obvious than that of TIMP-2. No expression of related antigens of TIMP-1 and TIMP-2 were detected. All these indicated that TIMP-1 did play an important role in the development of liver fibrosis and liver cirrhosis. The inhibitory effect of MMPs was enhanced with the high level expressing of TIMP-1 and TIMP-2 which resulted in the decrease of degradation of ECM, the deposit of ECM and the development of liver fibrosis and liver cirrhosis. The cause of the higher expression of TIMP-1 probably lay in two main reasons. First, the different classes of MMP resulted in the different inhibition activity of TIMP to MMP. TIMP-1 to procollagenase (MMP1) and TIMP-2 to gelatinase -A (MMP2),and gelatinase -B (MMP9) had assumed stronger inhibition activity. During the degradation of ECM, the main one was MMP1, and collagens Ⅰ,Ⅲ were the main object of MMP1. Second, the promotion of TIMP-1 was 10 times more than TIMP-2 to the proliferation of cells (including fibroblast, epithelial cells, endothelial cells, and smooth muscle cells). Furthermore, this action had no relation with the inhibition action of TIMP to MMP[45-59].

In situ hybridization was mainly used to observe the characteristics and accurate location of gene expression. The high sensitivity and strong specificity of this technique will be preferable to

study the pathogenesis of hepatic fibrosis and to demonstrate the diagnosis. Recently, digoxin has become a widely used non-isotope labeled compound characterized by its perfect specific and stability. Its sensitivity is almost the same as isotope, but without pollution. So, it is easily accepted and used to label TIMP-1 and TIMP-2 cDNA probe to detect the paraffin sections of liver. The results showed that this technique was high sensitive and specific. The rates of positive expressions of TIMP-1 and TIMP-2 were 80.0% and 55.0% respectively. The location of TIMPs expression was in cytoplasm of hepatocyte, except nuclei, and the mRNA expression of TIMP-1 was stronger than that of TIMP-2, which was in accordance with the results of immunohistochemistry, and further proved that the TIMPs played a key role in the development of hepatic fibrosis and hepatic cirrhosis.

It is now known that there is a noticeable increase of TIMP-1 in the injured liver, which takes place earlier and increases faster, therefore, more and more researchers has regarded it as the diagnostic index of hepatic fibrosis. Detecting TIMP-1 and TIMP-2 with SPASE was used as a quick labortory diagnosis of hepatic fibrohosis. The sera from hepatic cirrhosis patients pathologically confirmed and normal people served as positive and negative control respectively. The results of detecting TIMP-1 and TIMP-2 in the sera of 408 patients with hepatic disease showed that positive rate of TIMPs was higher in sera of hepatic cirrhosis patients than that of acute or chronic hepatitis(P<0.001). TIMPs in sera of chronic hepatitis patients were apparently higher than those of acute hepatitis patients. This conclusion enumerated above was Supported by the detecting results of TIMP-1 and TIMP-2 by using immunohistochemistry and in situ hybridization.

Recently, Murawaki *et al*[60-62] has detected TIMP-1 and TIMP-2 in sera of patients with chronic liver disease by means of ELISA, and found a good relation between TIMP-1 and TIMP-2 in sera and in liver. The sensitivity and specificity of TIMP-1 were higher than those of TIMP-2. In injured liver, especially in fibrotic liver, TIMP-1 predominated, and the degree of TIMP-1 was remarkably related to the severity of hepatic fibrosis. Compared with TIMP-1, the specificity and sensitivity of TIMP-2 were inadvisable for diagnosis of hepatic fibrosis. So, TIMP-1 was more important than TIMP-2 in the determination of histological change of hepatic fibrosis[63-70]. This was proved by our study of the expression of related antigens and the location of mRNA of TIMP-1 and TIMP-2. The expression of related antigens in liver could be reflected through detecting TIMPs in sera. So, TIMP-1 and TIMP-2 could be considered as useful diagnostic index of hepatic fibrosis, especially TIMP-1. Because viral hepatitis is common in China[71-99], liver fibrosis is the focus of research[18-24,100,101]. So serum TIMP-1 and TIMP-2 will find wide use in practice in the future.

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