

# Expression of tissue inhibitor of matrix metalloproteinases-1 during aging in rat liver

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## Abstract

**AIM:** To investigate the expression and role of tissue inhibitor of matrix metalloproteinases-1 (TIMP-1) during natural aging in rat liver and to detect the expression of matrix metalloproteinase-2 (MMP-2) and MMP-9.

**METHODS:** The rats were divided into 3-mo-old group ( $n = 5$ ), 10-mo-old group ( $n = 5$ ) and 24-mo-old group ( $n = 5$ ). Histopathologic changes of liver were observed with HE and Masson stain. The location and protein expressions of TIMP-1 were determined by immunohistochemistry and Western blot; message RNA (mRNA) levels were measured in livers from rats of various ages by semi-quantitative reverse transcriptional polymerase chain reaction (RT-PCR). In addition, the expression of MMP-2 and MMP-9 was assessed by RT-PCR and Western blot.

**RESULTS:** Histologic examination showed that the aging liver had excessive fatty degeneration and collagen deposition. Immunohistochemical staining showed that TIMP-1 related antigen in livers was located in cytoplasm. The protein expression of TIMP-1 was significantly higher in the oldest animals and the mRNA expression was increased significantly in the 24-mo-old rats ( $t = 4.61, P = 0.002 < 0.05, 24\text{-vs-}10\text{-mo-old rats}$ ;  $t = 4.31, P = 0.003 < 0.05, 24\text{-vs-}3\text{-mo-old rats}$ ). The expression of MMP-2 and MMP-9 had no change during aging; the ratios TIMP-1/MMP-2 and TIMP-1/MMP-9 in aging liver were significantly higher than those in maturation and young livers.

**CONCLUSION:** TIMP-1 may play an important role in the process of liver aging.

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**Key words:** TIMP-1; Aging; Rat liver

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## INTRODUCTION

Aging affects organs, tissues, and cell types of the same organism in different ways. Aging is the most complex phenotype and can affect physiology, impairs function and increases susceptibility to all major chronic diseases<sup>[1]</sup>. In liver, age-related morphologic changes are the variable degrees of fibrosis and deposition of extracellular matrix (ECM). There is evidence that the change of ECM is mainly regulated by MMPs. Hepatic fibrosis is formed because specific TIMPs inhibit ECM degradation<sup>[2-4]</sup>. TIMP-1 is a very important promoting factor in the process of hepatic fibrosis<sup>[5]</sup>. The expression of TIMP-1 is also related to the aging of many organs, but few data are available on the changes of TIMP-1 during liver aging. We conducted this study to determine whether TIMP-1 was related to liver aging.

## MATERIALS AND METHODS

### Animals

Wistar rats (both sexes) provided by the Experimental Animal Center of General Hospital of PLA were studied at the age of 3, 10 and 24-mo (five animals per age group).

All studies were performed with the approval of Experimental Animal Committee in our hospital. The animals were fed under standard conditions and fasted for 4 h, then killed using pentobarbital sodium anesthesia. The liver was removed, immediately frozen in liquid nitrogen and stored at -80 °C, except for a portion cut out for histology.

### Histopathological examination

Liver samples from each rat were fixed in 40 g/L formaldehyde, embedded in paraffin, stained with hematoxylin-eosin (HE) and Masson trichrome collagen stain and then examined under an optical microscope.

### TIMP-1 immunohistochemical staining

The location and expression of TIMP-1 were detected by immunocytochemical staining with the ABC kit. Serial paraffin sections of liver samples at 4 μm thickness were deparaffinized in xylene and gradually rehydrated in alcohol. After retrieval of the antigens, nonspecific binding sites were blocked with normal serum for 20 min. The sections were incubated with polyclonal antibody against TIMP-1 (Santa Cruz) at 4 °C overnight, then with secondary antibody at 37 °C for 30 min, avidin-peroxidase at 37 °C for 20 min. The positive stains

**Table 1** Primer sequences and sizes of TIMP-1 and MMP-2, MMP-9 in expected PCR products for RT-PCR

Genes	Primer sequences	Product size (bp)
TIMP-1	Sense: 5'-GCCCAACCCACCCACAGA-3' Antisense: 5'-TTTGAAGGGATGGCTGAACAG-3'	405
MMP-2	Sense: 5'-GAGAAAAGCGCAGCGAGTGACG-3' Antisense: 5'-TTCCCCGCAAGCCCAAGTG-3'	145
MMP-9	Sense: 5'-CCACCGAGCTATCCACTCAT-3' Antisense: 5'-GTCCGGTTCAGCATGTTTT-3'	159
$\beta$ -actin	Sense: 5'-GGCATCTGACCCTGAAGTA-3' Antisense: 5'-GCCGATAGTGATGACCTGACC-3'	565

were shown as brown color by peroxidase substrate solution DAB. The sections were finally counterstained with hematoxylin, dehydrated, and mounted. As a negative control, PBS was used instead of the primary antibody.

### RT-PCR of TIMP-1 and MMP-2, MMP-9

We examined the expression of TIMP-1 and MMP-2, MMP-9 in liver from rats of various ages. Total RNA was extracted from livers using TRIzol reagent according to the manufacturer's instructions, then reversed by transcribing it into cDNA. Total RNA (5  $\mu$ g) and random primer (2  $\mu$ g) in DEPC water were denatured at 80 °C for 5 min, then 5  $\mu$ L 5 $\times$  reverse transcriptase buffer, 2  $\mu$ L 20 mmol/L dNTPs, 1  $\mu$ L M-MLV reverse transcriptase (200 U) and DEPC water were added to the total volume of 15  $\mu$ L. The reaction was performed at 37 °C for 1.5 h and at 100 °C for 5 min to inactivate the reverse transcriptase. PCR was performed in a 25  $\mu$ L reaction mixture containing 1  $\mu$ L reactant, 2.5 U *Taq* DNA polymerase and 20 pmol primers, and heated for 5 min at 95 °C for pre-denaturation, and then subjected to 35 PCR cycles, each cycle consisting of denaturation at 95 °C for 45 s, annealing at 60 °C for 45 s and extension at 72 °C for 45 s. TIMP-1 and MMP-2, MMP-9 genes were amplified with specific primers, the gene for  $\beta$ -actin was used as an internal control (Table 1). The amplified products were electrophoresed on 12 g/L agarose gel containing 0.5 g/L ethidium bromide and visualized under UV light. The density and area of each band were analyzed using the Gelworks system (UVP, USA). The ratios of TIMP-1/ $\beta$ -actin, MMP-2/ $\beta$ -actin and MMP-9/ $\beta$ -actin were regarded as the expression levels of TIMP-1 and MMP-2, MMP-9.

### Western blot for TIMP-1 and MMP-2, MMP-9

TIMP-1 and MMP-2, MMP-9 protein extracts from rat livers were subjected to Western blot analysis. Liver samples were homogenized in ice-cold extraction buffer RIPA and freshly added protease inhibitors (aprotinin 1  $\mu$ g/mL, leupeptin 1  $\mu$ g/mL, PMSF 10 mmol/L). The homogenates were centrifuged (4 °C, 10 min, 1 500 r/min), the supernatant was decanted and saved on ice. Protein concentrations were measured using the Bio-Rad method. The proteins were separated on SDS-polyacrylamide gel and transferred electrophoretically onto nitrocellulose membrane using a mini electrophoretic transfer apparatus (Bio-Rad). The nitrocellulose membrane was blocked with 100 g/L defatted milk in TBST (20 mmol/L Tris-HCl buffer, 9 g/L NaCl, 1 mL/L Tween-20) for 1 h at room temperature, and then incubated overnight at 4 °C in primary antibody to TIMP-1 or MMP-2, MMP-9 (Santa

Cruz). After washing with TBST, the membrane was incubated for 1 h at room temperature with horseradish-peroxidase secondary antibody (Santa Cruz). Immunoreactive bands were visualized with enhanced chemiluminescence (ECL, Amersham).  $\beta$ -actin was used as the internal control.

### Statistical analysis

Statistical analysis was performed using the SPSS statistical software program. Values were expressed as mean $\pm$ SD. The significance of the difference was calculated by two-tailed Student's *t* test.  $P < 0.05$  was considered statistically significant.

## RESULTS

### Histopathologic changes of liver

The mature and young livers showed normal lobular architecture with central veins and radiating hepatic cords and a normal distribution of collagen (Figure 1). The aging liver also showed a normal lobular architecture with central veins and radiating hepatic cords, but had excessive fatty degeneration and collagen deposition extending from central veins or portal tracts (Figure 2).

### TIMP-1 expression and localization

Immunohistochemical staining showed that the TIMP-1 antigen in livers was located in cytoplasm but not in nuclei. Its expression in aging liver was much stronger than that in mature and young livers (Figure 3).

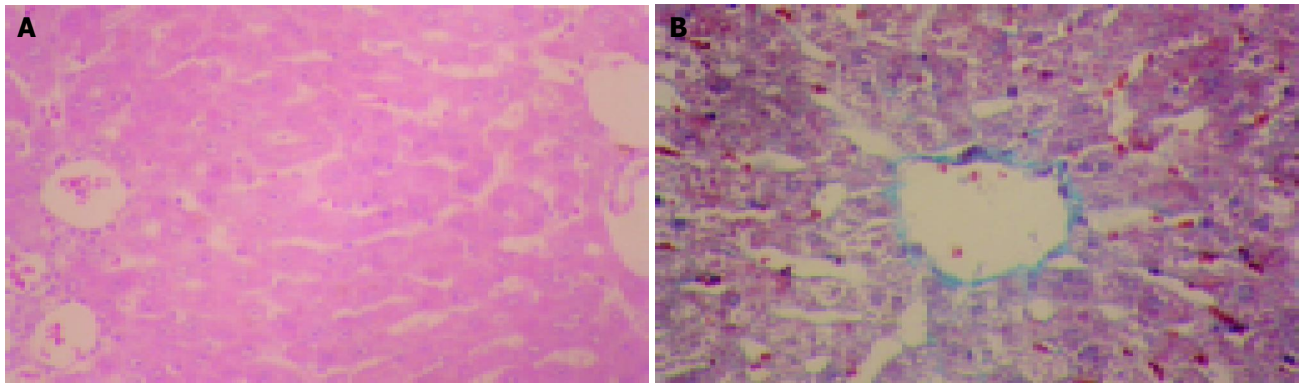
### mRNA expression of TIMP-1, MMP-2 and MMP-9

We examined the mRNA expression of TIMP-1 and MMP-2, MMP-9 in liver from rats of various ages with semi-quantitative RT-PCR (Table 2). The expression of TIMP-1 mRNA tended to increase in the 24-mo-old rats ( $t = 4.61$ ,  $P = 0.002 < 0.05$ , 24- *vs* 10-mo-old rats;  $t = 4.31$ ,  $P = 0.003 < 0.05$ , 24- *vs* 3-mo-old rats). The expression of MMP-2 and MMP-9 mRNA remained unchanged throughout the observation time. The ratios of TIMP-1/MMP-2 and TIMP-1/MMP-9 in aging liver were significantly higher than those in mature and young livers.

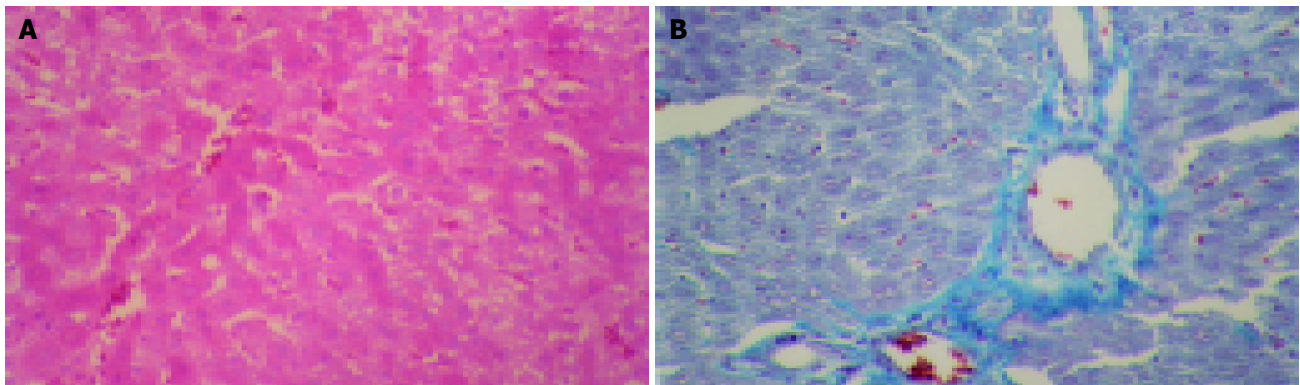
**Table 2** Expression of TIMP-1 and MMP-2, MMP-9 mRNA in rat liver (mean $\pm$ SD)

Age (mo)	TIMP-1/ $\beta$ -actin	MMP-2/ $\beta$ -actin	MMP-9/ $\beta$ -actin
3	0.29 $\pm$ 0.08	0.52 $\pm$ 0.04	1.48 $\pm$ 0.14
10	0.35 $\pm$ 0.01	0.59 $\pm$ 0.08	1.50 $\pm$ 0.09
24	0.89 $\pm$ 0.28 <sup>a</sup>	0.66 $\pm$ 0.02	1.51 $\pm$ 0.05

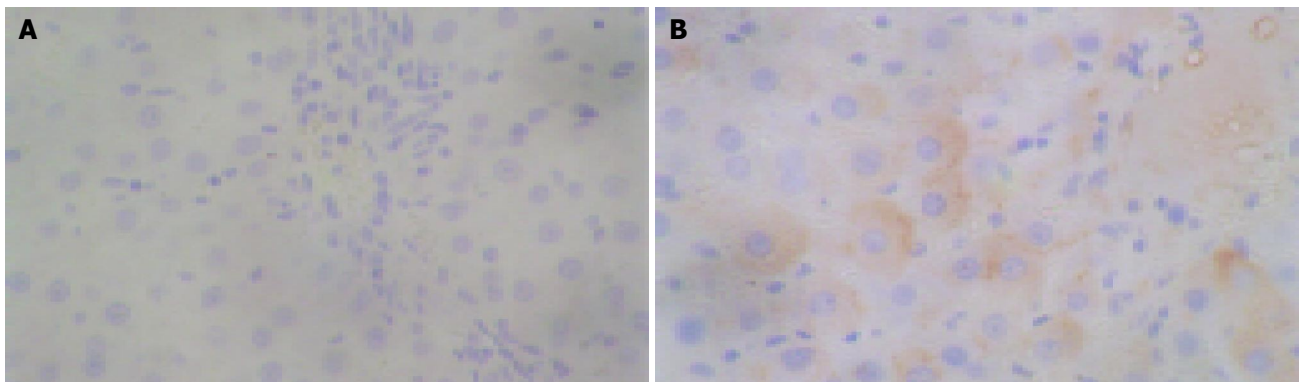
<sup>a</sup> $P < 0.05$  *vs* 3-mo, 10-mo rats.



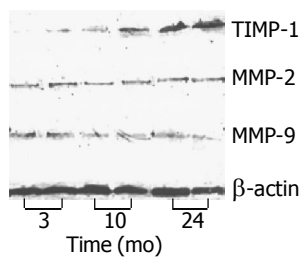
**Figure 1** Normal lobular architecture of mature and young liver (A) and normal distribution of collagen in mature and young liver (B).



**Figure 2** Severe fatty degeneration (A) and collagen deposition (B) in aging liver.



**Figure 3** Expression of TIMP-1 in mature and young liver (A) and aging liver (B).



**Figure 4** Protein expressions of TIMP-1 and MMP-2, MMP-9 in rat livers (Western blot).

#### **Protein expression of TIMP-1, MMP-2 and MMP-9**

Western blot analysis revealed a progressive age-dependent

protein expression of TIMP-1 (Figure 4). TIMP-1 level increased in 24-mo-old rats compared with the mature and young livers ( $P < 0.05$ ). In contrast, MMP-2 and MMP-9 remained almost unchanged at all the considered ages.

#### **DISCUSSION**

Fibrosis is a hallmark of aging of various organs, including heart, kidney and liver<sup>[6-8]</sup>. Fibrosis impairs liver function<sup>[9]</sup> and changes in liver function are important because they may promote susceptibility to adverse drug reactions, neurotoxicity, atherosclerosis and other important diseases in older people<sup>[10]</sup>. Fibrosis is pivotal in the modification of blood flow leading to portal hypertension<sup>[11]</sup>. Fibrosis reflects increased deposition of the physiological components of ECM. The liver ECM is a passive structural

support since it plays key roles in providing a structural framework and maintaining the differentiated phenotype and normal function of hepatocytes, sinusoidal endothelial and stellate cells<sup>[12,13]</sup>.

A major group of enzymes responsible for ECM degradation is the MMP family, including collagenase, gelatinase, and stromelysin<sup>[14,15]</sup>. MMPs are a group of zinc- and calcium-dependent enzymes that regulate cell-matrix composition by degrading components of the ECM. After activation, MMPs are secreted into the extracellular medium except for the membrane type (MT)-MMPs<sup>[16]</sup>. TIMPs, specific inhibitors of matrix metalloproteinases, have been found to be the key regulators of MMP activity and ECM degradation<sup>[17,18]</sup>. MMPs and TIMPs play an important role in various fibrotic diseases<sup>[19,20]</sup>; expression of TIMP-1 is increased in hepatic fibrosis and reflects the changes of liver fibrosis<sup>[21-24]</sup>. Gelatinase is comprised of gelatinase A (MMP-2) and gelatinase B (MMP-9) and can degrade main components of the ECM<sup>[25,26]</sup>. TIMP-1 is a natural tissue inhibitor of gelatinases and can inhibit the gelatinolytic activities of gelatinases<sup>[27]</sup>.

The expression of TIMP-1 is related to aging of many organs. Increase of total fibrillar collagen content is related to increased myocardial TIMP-1 levels<sup>[28]</sup>. Aged human microvascular endothelial cells increase expression of TIMP-1<sup>[29]</sup>. Gagliano provides a comprehensive description of hepatic collagen expression and metabolism during natural aging in rats<sup>[30]</sup>. Interstitial collagen accumulates significantly in the oldest animals and TIMP-1 seems to be a major regulating factor.

Immunohistochemical staining showed that TIMP-1 antigen in livers was located in cytoplasm, and its expression in aging liver was much stronger than that in mature and young livers. We found that TIMP-1 expression was higher in the aging liver and the transcription activity of this gene was upregulated. TIMP-1, TIMP-1/MMP-2, and TIMP-1/MMP-9 significantly increased while no change was observed in expression of MMP-2 and MMP-9, suggesting that MMP inactivation is increased by TIMP-1 in aging liver.

These findings suggest that a disturbed TIMP-1/MMP ratio may reflect the imbalance of extracellular homeostasis. The data further support that age-dependent liver fibrosclerosis is related to TIMP-1.

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