PO Box 2345, Beijing 100023, China
Fax: +86-10-85381893
E-mail: wjg@wjgnet.com www.wjgnet.com

World J Gastroentero, 2000; 6(6):881-884
World Journal of Gastroenterology
 ISSN 1007-9327

Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression in fibrotic rat liver

Hai Lin Liu[1], Xuan Hai Li[2], Dan Yi Wang[2] and Shao Ping Yang[2]

Subject heading matrix metalloproteinase;iver cirrhosis; polymerase chain reaction; extracellular matrix; hepatic stellate cells;rats

Liu HL, Li XH, Wang DY, Yang SP. Matrix metalloproteinase-2 and tissue inhibitor of metalloproteinase-1 expression in fibrotic rat liver. ***World J Gastroentero***, 2000;6(6):881-884

INTRODUCTION

Liver fibrosis is an excessive deposition of extracellular matrix (ECM) resulted from both increased synthesis and decreased degradation[1-3]. Matrix metalloproteinases (MMPs) represent a group of neutral proteinases with variable substrate spectra. Their activity may be regulated at the level of gene transcription, proenzyme activation and inhibition of active enzyme by specific inhibit ors such as the tissue inhibitor of metalloproteinases (TIMPs). The remodeling of extracellular matrix during chronic liver disease may partially be attributed to the altered activity of matrix metalloproteinases and their tissue inhibitors (such as TIMPs)[4,5]. Hepatic stellate cell (HSC) (fat storing cell, Ito cell, lipocyte) is the main source of ECM production in liver fibrosis[6-13], which also can express and secrete matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in the primary culture[14-16]. The alteration of MMP-2 and TIMP-1 expression may be implicated in the hepatic fibrogenesis.

In this study, we examined the expression of MMP-2 and TIMP-1 in liver tissue and HSC isolated from normal and CCl_4 induced fibrotic rat respectively, in order to explore the role of MMP-2 and TIMP-1 during hepatic fibrogenesis.

MATERIALS AND METHODS

Materials

Animals Male Wistar rats, body weight exceeding 300g (n=24), purchased from Shanghai Experimental Animal Center, Chinese Academy of Sciences, were randomly divided into test group and control group.

Reagents Dulbecco's Modified Eegle Medium (No. 69K3042), TRIZOL reagent (No. 15596026) and SUPERSCRIPT™ preamplification system for first strand cDNA synthesis (No. JDQ702) were purchased from GIBCO Co. Nycodenz (No. 16H0162), Pronase E, DNase and collagenase I were from Sigma Co; 10×PCR buffer, 25mmol/L $MgCl_2$, 10×dNTP, *Taq* DNA polymerase (No. 6923214, 65595010), RNasin and pGEM-72-f (+)/ *Hae*-III markers were obtained from Promega Co. Primers of MMP-2 and TIMP-1 and β-actin were synthesized by Tumor Molecular Biology Institute, Chinese Academy of Sciences. β-actin: (+) 5'-TGA CGG GGT CAC CCA CAC TGT GCC CAT CTA-3', (-) 5'-CTA GAA GCA TTG CGG TGG ACG ATG GAG GG-3'; primer of MMP-2: (+) 5 '-GTG CTG AAG GAC ACA CTA AAG AAG A-3',(-) 5'-TTG CCA TCC TTC TCA AAG TTG TAG G-3'; primer of TIMP-1: (+) 5'-GAC CTG GTC ATA AGG GCT AAA-3', (-) 5'-GCC CGT GAT GAG AAA CTC TTC ACT-3'.

Methods

Development of a CCl_4-induced fibrotic rat model Test group rats(n=12) received subcutaneous injection of 40% CCl_4 diluted with olive oil at a dose of 0.3mL/100g of body weight twice a week for 6 -8 weeks. Hepatic fibrosis was induced by CCl_4[17].

Separation, purification and identification of rat HSC We modified the procedures of Friedman's methods[18] to improve the yield and viability of HSC. HSC was isolated from control and experimental animals by colleganase perfusion and density gradient centrifugation[19-21]. Rats were anesthetized with 1% sodium pentabarbital 0.2mL/kg of body weight. The liver was perfused through the portal vein *in situ* with heperinized calcium-free solution (142mmol/L NaCl, 6.7mmol/L KCl, 10mmol/L HEPES, 5.5mmol/L NaOH, pH 7.4) at 37℃ for 10min at a flow rate of 30-40 mL/min and the inferior vena cava cut at the same time. Until the perfusion solution from inferior vena cava turned colorless and the liver became white, the liver was carefully excised and placed on a special appliance, and then

[1]Department of Gastroenterology and Hepatology, Ninth People's Hospital, Shanghai Second Medical University, Shanghai 200011, China
[2]Shanghai Second Medical University
Dr. Hai Lin Liu, obtained M.D. from Shanghai Second Medical University in 1994, associate professor of internal medicine, major in digestive disease, having 26 papers published, and deputy editor of *Tumor markers and clinical significance*.
Supported by Natural Science Foundation of Committee of Science and Technology of Shanghai Municipality (952**B**14003**), Science and Technology Development Foundation of Educational Committee of Shanghai Municipality** (96**B**06).
Correspondence to: Dr. Hai Lin Liu, Department of Gastroenterology and Hepatology, Ninth People's Hospital, Shanghai Second Medical University, 639 Zhizaoju Road, Shanghai 200011, China
Tel 0086-21-63138341
Received 2000-06-06 **Accepted** 2000-06-29

perfused with 0.1% Pronase E and 0.05% collegenase added enzymatic solution (67mmol/L NaCl, 6.7mmol/L KCl, 100mmo l/L HEPES, 66mmol/L NaOH and 5mmol/L $CaCl_2$) at 37℃ for 20min at the same flow rate. After the liver turned yellow and became soft, it was cut into pieces and incubated in 30mL enzymatic solution with 0.05% DNase on a magnetic stirrer at 37℃ for 30min. At the end of the incubation period, the suspension was filtered through screen mesh and the filtrate in Hank's solution was centrifuged at 450×g for 7min. The cell pellets were repeatedly resuspended in Hank's solution and centrifuged for three times to further dissociate the cells. The cells were separated by density gradient centrifugation with 1:2 v/v 18% Nycodenz at 1450 ×g for 17min at 4℃. HSC, which remained at the upper and middle interface, was collected and washed twice in DEME followed by centrifugation at 450×g for 8min to remove hepatocyte debris. The pellet was resuspended in a small amount of DEME and used for further studies. Cell viability was eval uated from the capacity of the cells to exclude typan blue. Desmin positive cell was identified by immunohistochemistry.

Total RNA isolation Total RNA was extracted from liver tissue of the control and fibrotic rats using TRIZOL reagent. HSC was pelleted by centrifugation, lysed with TRIZOL reagent by repetitive pipetting and diluted as 1mL of the reagent per 5×10^6-6×10^6 of HSC. Incubate the homogenized samples for 5min to permit the complete dissociation of nucleoprotein complexes, add 0.2mL of chloroform per 1mL of TRIZOL reagent, cap sample tubes securely, shake tubes vigorously by hand for 15s and incubate for 2-3 min at room temperature. Centrifuge the samples at 12 000×g for 15min at 4℃. After centrifugation, the mixture was separated into a lower red phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. Transfer the aqueous phase to another tube, add 0.5mL of isopropanol per 1mL of TRIZOL reagent according to the initial homogenization, incubate samples for 10min at room temperature and centrifuge at 12 000×g for 10min at 4℃. Remove the supernatant, wash the RNA pellet once with 75% ethanol, add at least 1mL of 75% ethanol/mL of TRIZOL reagent according to the initial homogenization. Mix the sample by vortexing and centrifuge at 7 500×g for 5min at 4℃, remove the supernatant, and briefly vaccum-dry the RNA pellet. The RNA precipitate can be stored in RNase-free water at -70℃. Dissolve 2μL RNA sample (1:50) for measuring OD_{260} and OD_{280} by UV spectrophotometer. Three μL RNA sample was electrophoresed through a 1.1% denaturing agarose gel with ethidium bromide.

RT-PCR Four μg of total RNA in 1μL water was reversely transcribed. The RNA sample was incubated at 70℃ for 10 min with 1μL Oligo $(dT)_{12\text{-}18}$ (0.5μg/μL) and 10μL 0.1% DEPC, then placed on ice bath for at least 1min. Then the reagents including 2μL of 10×PCR buffer; 2μL of 25mmol/L $MgCl_2$; 1μL of 10mmol/L dNTP mix; 2μL of 0.1mmol/L DTT and 0.5μL of 40U RNasin were added to each RNA/primer mixture. After mixing, the tubes were incubated at 42℃ for 5 min. One μL (200U) of SUPERSCRIPT II RT was added to each tube and incubated at 42℃ for 50min and 70℃ for 15min. The sample was chilled on ice bath. One μL RNase H was added and incubated at 37℃ for 20min. Then PCR was made or stored at -20℃. The following reagents were added to a 500μL polypropylene microcentrifuge tube: ① 5μL of 10×PCR buffer; ② 3μL of 25mmol/L $MgCl_2$; ③ 5μL of 10×dNTP; ④ 0.5μL of 5U/ μL TaqE; ⑤ two pairs primer mixture (βactin primer was used for internal control); ⑥ 2μL room temperature sample and ⑦ water to 50μL. The components were concentrated at the bottom of the tube by centrifugation and covered with mineral oil. PCR incubation was carried out in a programmable thermal controller . During each PCR cycle (40 cycles), the samples were heated to denature template complexes (94℃ 180s initially and 60s during all subsequent cycles), cooled to 56℃ to anneal template and primers (60s) each cycle and heated to 72℃ to extend for 60s. The final 72℃ incubation was extended for additional 10min to maximize strand completion. The samples were then rapidly cooled to 4℃ and kept on ice bath or frozen until analysis. After amplification, 10μL of each PCR reaction mixture was electrophoresed through a 2.0% agarose gel with ethidium bromide (0.1%). The size of the markers are 1000bp, 750bp, 500bp and 300bp. The gel was photographed over UV light at the same exposure and development time for all gels photographed. The bands on the film were scanned by densitometry for quantitation. The PCR products were electrophoresed in the same gel and ratios for MMP-2/β-actin and TIMP-1/β-actin were determined to eliminate gel -to-gel or film-to-film variance.

Statistical analysis Data were expressed as mean ± SD ($\bar{x}\pm s$)[22].

RESULTS

Expressions of MMP-2 and TIMP-1 in HSC

MMP-2 mRNA was undetectable in HSC isolated from normal rat liver, but it was detected in HSC from fibrotic rat liver. TIMP-1 mRNA was detected both in HSC from normal and fibrotic rat liver (0.25±0.16 and 0.56±0.09), and the expression was increased markedly in liver fibrosis (P<0.02).

Levels of MMP-2 and TIMP-1 mRNA in liver tissue

MMP-2 mRNA was detected both in normal and fibrotic rat liver tissue (0.86±0.09 and 0.99±0.05). Although the level of MMP-2 mRNA was increased in fibrotic liver tissue, there were no significant differences between them (P>0.05). TIMP-1 mRNA was detected in normal and fibrotic liver tissue (0.46±0.03 and 1.36±0.62). TIMP-1 expression was enhanced remarkably in fibrotic liver tissue (P<0.05).

DISCUSSION

MMP-2 degrades collagen IV, V, VII and X, as well as elastic, fibronectin, and denatured collagen type I[23]. Collagen type IV is the primary component of basement membrane. Therefore, the increment of MMP-2 can result in the damage of membrane in the space of Disse, which will activate the perisinunoid cells including HSC and promote hepatic fibrogenesis. Our results showed that the MMP-2 mRNA was only detected in HSC isolated from fibrotic liver, not in HSC from normal rat liver. This provided evidence for MMP-2 expression involving in liver fibrosis[24]. Comparing with cell culture, isolating HSC from liver and extracting RNA directly to determinate the level of MMP-2 mRNA can more exactly reflect the situation *in vivo*, because the HSC has been act ivated in the process of culture *in vitro*. The MMP-2 mRNA was detectable in normal liver tissue. It indicated that there were sources other than HSC. In consistency with our findings, *in situ* hybridization showed low levels of MMP-2 gene transcripts in some mesenchymal cells of portal tracts, central veins and sinusoids of normal human liver tissue[25]. Although the leve l of MMP-2 mRNA was increased in fibrotic liver tissue[26], there were no significant differences between fibrotic and normal liver tissue (P>0.05). We inferred that the increased MMP-2 mRNA was mainly from activat ed HSC and exerted effect at local area. This suggested that the expression of MMP-2 by HSC might be important in liver fibrogenesis[24,26].

Liver fibrosis is associated with excessive accumulation of extracellular matrix , paticular collagen type I and III[27-29]. Collagen type I may comprise over 70% of extracellular matrix as compared to 40%-50% in normal liver. Besides an increase of collagen synthesis, decrease of fibrolysis may be an important factor responsible for the preferential accumulation of intersititial collagens[30]. MMP-1 has a substrate specificity for native type I and III collagens. Reduced MMP-1 activity may therefore contribute to the patterns of extracellular matrix constituents in fibrotic liver. TIMP-1 is a major inhibitor of MMP-1 by combining with the active enzymes[31-34]. TIMP-1 expression was enhanced markedly both in liver tissue and HSC during liver fibrogenesis. It indicated that the increase of TIMP-1 expression is a main cause possibly for the reduced collagen degradation[35-40].

In conclusion, there were no detectable gene transcripts of MMP-2 in HSC isolat ed from normal rat liver, and it was expressed by HSC during liver fibrogenesis. This suggested that the expression of MMP-2 is determined by the state of activation of HSC. The marked increase of TIMP-1 expression may result in the prominent deposition of interstitial collagens by inhibition of matrix metalloproteinases (especially MMP-1). Both MMP-2 and TIMP-1 make contributions to the liver remodeling and the progression of liver fibrosis.

REFERENCES

1 Walsh KM, Timms P, Campbell S, MacSween RN, Morris AJ. Plasma levels of matrix metalloproteinase-2 (MMP-2) and tissue inhibitors of metalloproteinases 1 and 2 (TIMP-1 and TIMP-2) as noninvasive markers of liver disease in chronic hepatitis C: comparison using ROC analysis. *Dig Dis Sci*, 1999;44:624-630
2 Arthur MJ. Role of Ito cells in the degradation of matrix in liver. *J Gastroenterol Hepatol*, 1995;10(Suppl 1): S57-S62
3 Arthur MJ. Collagenases and liver fibrosis. J Hepatol, 1995;22 (Suppl 2):43-48
4 Arthur MJ, Mann DA, Iredale JP. Tissue inhibitors of metalloproteinases, hepatic stellate cells and liver fibrosis. *J Gastroenterol Hepatol*, 1998;13(Suppl):S33-S38
5 Arthur MJ. Fibrogenesis II. Metalloproteinases and their inhibitors in liver fibrosis. *Am J Physiol Gastrointest Liver Physiol*, 2000;279:G245-249
6 Friedman SL. Cellular sources of collagen and regulation of collagen production in liver. *Semin Liver Dis*, 1990;10:20-23
7 Levy MT, Trojanowska M, Reuben A. Oncostatin M: a cytokine upregulated in human cirrhosis, increases collagen production by human hepatic stellate cells. *J Hepatol*, 2000;32:218-226
8 Iredale JP, Benyon RC, Pickering J, McCullen M, Northrop M, Pawley S, Hovell C, Arthur MJ. Mechanisms of spontaneous resolution of rat liver fibrosis. Hepatic stellate cell apoptosis and reduced hepatic expression of metalloproteinase inhibitors. *J Clin Invest*, 1998;102:538-549
9 Iredale JP. Tissue inhibitors of metalloproteinases in liver fibrosis. *Int J Biochem Cell Biol*, 1997;29:43-54
10 Iredale JP, Goddard S, Murphy G, Benyon RC, Arthur MJP. Tissue inhibitor of metalloproteinase-I and interstitial collagenase expression in autoimmune chronic active hepatitis and activated human hepatic lipocytes. *Clin Sci*, 1995;89:75-81
11 Arthur MJ,Iredale JP,Mann DA. Tissue inhibitors of metalloproteinases:role in liver fibrosis and alcoholic liver disease. *Alcohol Clin Exp Res*,1999;23:940-943
12 Huang ZG, Zhai WR, Zhang YE, Zhang XR. Study of heteroserum-induced rat liver fibrosis model and its mechanism. *World J Gastroentero*, 1998;4:206-209
13 Du WD, Zhang YE, Zhai WR, Zhou XM. Dynamic changes of type I, III and IV collagen synthesis and distribution of collagen-producing cells in carbon tetrachloride induced rat liver fibrosis. *World J Gastroentero*, 1999;5:397-403
14 Arthur MJP, Stanley A, Iredale JP, Rafferty JA, Hembry RM, Friedman SL. Secretion of 72 kDa type IV collagenase/gelatinase by cultured human lipocytes. *Biochem J*, 1992;287:701-707
15 Iredale JP, Murphy G, Hembry RM, Friedman SL, Arthur MJP. Human hepatic lipocytes synthesize tissue inhibitor of metalloproteinase-1. *J Clin Invest*, 1992;90:282-287
16 Iredale JP, Benyon RC, Arthur MJP, Ferris WF, Alcolado R, Winwood PJ, Clark N, Murphy G. Tissue inhibitor of metalloproteinases-1 messenger RNA expression is enhanced relative to interstitial collagenase messenger RNA in experimental liver injury and fibrosis. *Hepatology*, 1996;24:176-184
17 Senoo H, Wake K. Suppression of experimental hepatic fibrosis by administration of Vitamin A. *Lab Invest*, 1985;52:182
18 Knook DL, Seffelaar AM, Leeuw AM. Fat storing cells of the rat liver: their isolation and purification. *Exp Cell Res*, 1982;139:468
19 Ji F, Wang WL, Yang ZL, Li YM, Huang HD, Chen WD. Study on the expression of matrix metalloproteinase-2 mRNA in human gastric cancer. *World J Gastroentero*, 1999;5:455-457

20 Lu LG, Zeng MD, Li JQ, Hua J, Fan JG, Fan ZP, Qiu DK. Effect of lipid on proliferation and activation of rat hepatic stellate cells (I). *World J Gastroentero*, 1998;4:497-499
21 Lu LG, Zeng MD, Li JQ, Hua J, Fan JG, Qiu DK. Study on the role of free fatty acids in proliferation of rat hepatic stellate cells (II). *World J Gastroentero*, 1998;4:500-502
22 Nakayama H, Yokoi H, Fujita J. Quantification of mRNA by non radioactive RT-PCR and CCD imaging system. *Nucleic Acid Res*, 1992;20:4939-4944
23 Takahara T, Furui K, Funaki J, Nakayama Y, Itoh H, Miyabayashi C, Sato H, Seiki M, Ooshima A, Watanabe A. Increased expression of matrix metalloproteinase-II in experimental liver fibrosis in rats. *Hepatology*, 1995;21:787-795
24 Preaux AM, Mallat A, Nhieu JTV, D'Ortho MP, Hembry RM, Mavier P. Matrix metalloproteinase-2 activation in human hepatic fibrosis regulation by cell-matrix interactions. *Hepatology*, 1999;30:944-950
25 Milani S, Herbst H, Schuppan D, Grappone C, Pellegrini G, Pinzani M, Casini A, Calabro A, Ciancio G, Stefanini F, Burroughs AK, Surrenti C. Differential expression of matrix metalloproteinase-1 and 2 genes in normal and fibrotic human liver. *Am J Pathol*, 1994;144:528-537
26 Takahara T, Furui K, Yata Y, Jin B, Zhang LP, Nambu S, Sato H, Seiki M, Watanabe A.Dual expression of matrix metallo proteinase-2 and membrane-type 1-matrix metalloproteinase in fibrotic human livers. *Hepatology*, 1997;26:1521-1529
27 Schuppan D. Structure of the extracellular matrix in normal and fibrotic liver: collagens and glycoproteins. *Semin Liver Dis*, 1990; 10:1-10
28 Shiba M, Shimizu I, Yasuda M, Ii K, Ito S. Expression of type I and type III collagens during the course of dimethylnitrosa mine induced hepatic fibrosis in rats. *Liver*, 1998;18:196-204
29 Arthur MJ. Degradation of matrix proteins in liver fibrosis. *Pathol Res Pract*, 1994;190:825-833
30 Roeb E, Purucker E, Breuer B, Nguyen H, Heinrich PC, Rose-John S, Matern S. TIMP expression in toxic and cholestatic liver injury in rat. *J Hepatol*, 1997;27:535-544
31 Benyon RC, Iredale JP, Goddard S, Winwood PJ, Arthur MJP. Expression of tissue inhibitor of metalloproteinases 1 and 2 is increased in fibrotic humanliver. *Gastroenterology*, 1996;110:821-831
32 Kossakowska AE, Edwards DR, Lee SS,Urbanski LS,Stabbler AL, Zhang CL,Phillips BW,Zhang YK,Urbanski SJ.Altered balance between matrix metalloproteinases and their inhibitors in experimental biliary fibrosis. *Am J Pathol*, 1998;153:1895-1902
33 Roeb E, Graeve L, Mullberg J, Matern S, Rose-John S. TIMP-1 protein expression is stimulated by IL-1 beta and IL-6 in primary rat hepatocytes. *FEBS Lett*, 1994;349:45-49
34 Arthur MJ, Iredale JP. Hepatic lipocytes, TIMP-1 and liver fibrosis. *J R Coll Physicians Lond*, 1994;28:200-208
35 Lichtinghagen R, Breitenstein K, Arndt B, Kuhbacher T, B-ker KHW. Comparison of matrix metalloproteinase expression in normal and cirrhotic human liver. *Virchows Arch*, 1998;432:153-158
36 Murphy G, Hembry RM, Hughes CE, Fosang AJ, Hardingham TE. Role and regulation of metalloproteinases in connective tissue turnover. *Biochem Soc Trans*, 1990;18:812-815
37 Herbst H, Wege T, Milani S, Pellegrini G, Orzechowski HD, Bechstein WO, Neuhaus P, Gressner AM, Schuppan D. Tissue inhibitor of metalloproteinase-1 and 2 RNA expression in rat and human liver fibrosis. *Am J Pathol*, 1997;150:1647-1659
38 Murawaki Y, Ikuta Y, Kawasaki H. Clinical usefulness of serum tissue inhibitor of metalloproteinases (TIMP)-2 assay in patients with chronic liver disease in comparison with serum TIMP-1. *Clin Chim Acta*, 1999;281:109-120
39 Murawaki Y, Ikuta Y, Idobe Y, Kitamura Y, Kawasaki H. Tissue inhibitor of metalloproteinase 1 in the liver of patients with chronic liver disease. *J Hepatol*, 1997;26:1213-1219
40 Casini A, Ceni E, Salzano R, Milani S, Schuppan D, Surrenti C. Acetaldehyde regulates the gene expression of matrix-metalloproteinase-1 and 2 in human fat storing cells. *Life Sci*, 1994;55:1311-1316

Edited by You DY and Ma JY