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

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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 inhibitors 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[6-10], which also can express and secrete matrix metalloproteinase-2 (MMP-2) and tissue inhibitor of metalloproteinases-1 (TIMP-1) in the primary culture[11-16]. 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 CCl₄ 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 Eagle Medium (No. 69K3042), TRIZOL reagent (No. 15596026) and SUPERSCRIPT™ preamplification system for first strand cDNA synthesis were purchased from GIBCO Co. Nycodenz (No. 16H0162), Pronase E, DNase and collagenase I were obtained from Sigma Co; 10×PCR buffer, 25mmol/L MgCl₂, 10×dTTP, 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 GGT CCC CCA CAC TGT GCT GAT CAT-3’, (-) 5’-GTC GAA GCA TTC TGT CAG GAC ATG GAG GG-3’; primer of MMP-2: (+) 5’-GTG CCA TCA TCC TCT TCA AAG TAG GAG G-3’; primer of TIMP-1: (+) 5’-CCA TTC TCT TCA AAG TGT TAG CAC-3’; primer of β-actin: (+) 5’-GCC CTG GTG ATT GAT AAA CTC TTC ACT-3’.

Methods

Development of a CCl₄-induced fibrotic rat model

Test group rats(n=12) received subcutaneous injection of 40% CCl₄ 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 CCl₄[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°C 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
perfusion with 0.1% Pronase E and 0.05% collagenase, added enzymatic solution (67mmol/L NaCl, 6.7mmol/L KCl, 100mmol/L HEPES, 66mmol/L NaOH, and 5mmol/L CaCl2) at 37°C 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°C 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°C. 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 evaluated from the capacity of the cells to exclude trypan 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 5×10⁶-6×10⁶ of HSC. Incubate the homogenized liver tissue of the control and fibrotic rats using 75% ethanol, add at least 1mL of 75% ethanol/mL of TRIZOL reagent. HSC was pelleted by centrifugation at 7500×g for 5min at 4°C. 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°C. 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°C, remove the supernatant, and briefly vacuum-dry the RNA pellet. The RNA precipitate can be stored in RNase-free water at -70°C. Dissolve 2µL RNA sample (1:50) for measuring OD260 and OD280 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°C for 10min with 1µL Oligo (dT)12-18 (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 25mM MgCl2; 1µL of 10mM dTTP 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°C for 5min. One µL (200U) of SUPERSCRIPT II RT was added to each tube and incubated at 42°C for 50min and 70°C for 15min. The sample was chilled on ice bath. One µL RNase H was added and incubated at 37°C for 20min. Then PCR was made or stored at -20°C. The following reagents were added to a 500µL polypropylene microcentrifuge tube: ① 5µL of 10×PCR buffer; ② 3µL of 25mM MgCl2; 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°C 180s initially and 60s during all subsequent cycles), cooled to 56°C to anneal template and primers (60s) each cycle and heated to 72°C to extend for 60s. The final 72°C incubation was extended for additional 10min to maximize strand completion. The samples were then rapidly cooled to 4°C 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 the 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 (±S:E)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 perisinusoid 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 determine 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 level I 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 activated 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 interstitial 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 isolated 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.

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