Full Length Research Paper

Effect of chymase inhibitors on dimethylnitrosamine-induced rat liver fibrosis and on chymase and collagen I expression

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Chymase converts angiotensin (Ang) I to Ang II, which may promote the development of liver fibrosis. In this study, a chymase inhibitor TY-51469 attenuated dimethylnitrosamine (DMN)-induced liver fibrosis was examined. A total of 44 rats were randomly divided into three groups: The model group, the control group, and the chymase inhibitor treatment (Chy-I) group. The rats were given intraperitoneal injections of 1% DMN (1 ml/mg). On the first day after the DMN challenge, the rats in the Chy-I group was given chymase inhibitor (10 mg/kg) by gastric lavage daily for 42 days; the other groups were given sterile saline. Liver tissue samples were collected on days 14, 28 and 56. The chymase levels were determined by enzyme-linked immunosorbent assay (ELISA). Collagen type I (Col-I) expression was evaluated by reverse-transcription polymerase chain reaction (RT-PCR). The Col-I in sinusoidal walls were evidently inhibited after the chymase inhibitor treatment, and they were higher in the liver fibrosis model group than in the control group, and significantly lower in the chymase inhibitor group. The chymase levels in the liver were evidently inhibited after the chymase inhibitor treatment, and were higher in the liver fibrosis model group than in the control group. Chymase expression was significantly lower in the Chy-I group. Chymase inhibitors alleviate DMN-induced liver fibrosis in rats by inhibiting chymase activity and Col-I mRNA expression in liver tissues.

Key words: Liver fibrosis, chymase, angiotensin II, collagen type I.

INTRODUCTION

Chymase is a cell serum protease present in human vascular tissues and mastocytes, and it decomposes angiotensin (Ang) I into Ang II. Ang II induces vascular smooth muscle contractions and promotes the proliferation, hypertrophy, and migration of smooth muscle cells and fibroblasts (Takai et al., 2009). Liver fibrosis is caused by the disruption of the balance of liver fiber formation and degradation during the progression of chronic liver disease, leading to excessive collagen deposits in the liver, often accompanied by inflammation. Liver fibrosis is a necessary stage that develops into all sorts of chronic liver diseases such as cirrhosis and liver cancer. Its key stage is hepatic stellate cell activation, and then transforms into myofibroblast-like cells and fibroblasts. Ang II plays an important role in blood pressure regulation, maintains fluid balance, and participates in the pathologic development of liver fibrosis. The local renin–angiotensin system (RAS) promotes hepatic stellate cell activation, and participates in the development of liver fibrosis through the Ang II type 1 receptor (AT1R) (Yoshiji et al., 2002; Mashall et al., 2000). Ang II also stimulates the expression of transforming growth factor -β1 (TGF-β1) to promote liver fibrosis.

Blocking Ang II activity greatly improves tissue fibrosis (Kagami et al., 1994; Yokohama et al., 2004; Bataller et
In patients with liver cirrhosis, Ang II receptor expression in blood vessels and myofibroblast increases in the local tissue of liver fibrosis (Shimizu et al., 2003). An Ang II receptor blocker, losartan, was shown to reduce portal pressure in patients with hepatic cirrhosis, suggesting that Ang II may induce the contraction and proliferation of hepatic stellate cells (HSC) (Batailler et al., 2000).

In previous studies, accumulation of chymase-positive cells was observed in fibrotic liver regions in chronic cirrhosis patients, suggesting that chymase-dependent Ang II formation pathways may have a close relationship with the occurrence of liver fibrosis (Shimizu et al., 2003; Komeda et al., 2008). In the current study, we established a liver fibrosis rat model, and observed the influence of chymase inhibitors on chymase activity using Col-I expression in liver tissue during different periods of fibrosis.

MATERIALS AND METHODS

Animal grouping

This study was conducted in strict accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The animal using protocol was reviewed and approved by the Institutional Animal Care and Use Committee of Yanbian University. 44 Wistar rats, 22 males and 22 females, 8 weeks old and weighing 180 to 220 g, were provided by the Laboratory Animal Center, Chinese Medical University.

The rats were randomly divided into the normal control group (n = 6), the model group (DMN + physiologic saline, n = 19), and Chy-I group (DMN + chymase inhibitor, n = 19). All groups except the normal control group received 1% dimethylnitrosamine (DMN) (1 ml/kg) via intraperitoneal injection every 3 days during the first week, and every 2 days from the second week for a total of six weeks. The Chy-I group was given an intraperitoneal injection of DMN and 10 mg/kg of chymase inhibitors by gastric lavage every day from day 1 to day 56. In the model group and the Chy-I group, 6 rats were randomly executed on day 14, 6 on day 28, and 7 on day 56 after injection of DMN. The liver tissue was used for further experiment.

Immunohistochemistry

The left hepatic tissue was collected, and stained based on the streptavidin–biotin complex method according to the kit's instruction (Beijing North Institute of Biological Products, China). Col-I expression in the liver tissue in the model and the Chy-I groups was detected during different periods of fibrosis.

ELISA

Chymase activity in the liver tissue was detected by ELISA (Satomura et al., 2002).

Reverse-transcription polymerase chain reaction (RT-PCR)

The PCR primers were designed and synthesized by Dingan Biological Technology Co., Ltd., China. The sequences were as follows: 5'-GACCAGTGGATTCAGTTCG-3', 5'-TGTGACTCGTCGCAGCAGCATC-3', and the PCR product was 468 bp long. The PCR system contained the following: 6.0 μl of cDNA product, 1.5 μl of 10× PCR buffer, 0.2 μl of Taq polymerase, 0.5 μl of primer 1 (1.0 mg/ml), 0.5 μl of primer 2 (1.0 mg/ml), 1 μl of dNTPs (10 mM), and 10.3 μl of water. PCR was performed with 30 cycles of denaturation (95°C, 30 s), annealing (60°C, 45 s), and extension (72°C, 60 s). After PCR, 2.5 μl of reaction mixture was run on 2% agarose gel with ethidium bromide (0.5 mg/ml). The expression levels of type I collagen were measured using densitometric analysis, and standardized to the β-actin control using a digital imaging and analysis software (BiocaptMV).

Statistical analysis

All data are shown as mean ± SD. The mean values were compared by t-test using SPSS 12.0 software (SPSS Inc., Chicago, IL, USA). Differences with P <0.05 were considered statistically significant. All assays were performed three times.

RESULTS

Immunohistochemistry

The immunohistochemistry results show that Col-I was mainly distributed around the liver sinus wall. The Col-I distribution on day 28 was more than that on day 14 after the model was established, and that on day 56 was more than that on day 28. The Col-I distribution in the Chy-I group was clearly less than that of the model group within the same period (Figure 1A to F), suggesting that chymase inhibitors significantly reduce type I collagen expression.

Chymase activity

As shown in Table 1, with the progress of the fibrosis, the liver tissue chymase activity increased gradually. Although the liver tissue chymase activity in the model group and the Chy-I group were higher than that in the control group, but simultaneously, the increase in chymase activity in the Chy-I group was lower than that in the model group, suggesting that chymase inhibitors downregulated the liver tissue chymase activity.

RT-PCR

With the progress of the fibrosis, Col-I mRNA expression in the liver tissue in the model and Chy-I groups increased gradually, whereas that in the Chy-I group was clearly less than that in the model group within the same period (P < 0.05) (Figures 2 and 3), suggesting that chymase inhibitors down regulated the Col-I expression in the liver group.

DISCUSSION

Components independent of the RAS have been
degranulation. Ang II is currently considered closely related with liver fibrosis (Yoshiji et al., 2002). In the liver tissue of patients with chronic liver disease, chymase-positive and Ang II-positive mastocyte expression increased, suggesting that chymase and Ang II might participate in the pathogenesis of common liver fibrosis (Komeda et al., 2008). In the hamster liver fibrosis model, accumulation of Ang II receptor–positive cells was observed in fibrotic liver regions (Yoshiji et al., 2002).

HSC plays an important role in the pathogenesis of liver fibrosis. Ang II can activate hepatic stellate cells through the AT1 receptor on the hepatic stellate cell surface, and promote hepatic stellate cells to produce collagen and extracellular matrix, which can promote the occurrence of liver fibrosis (Scheider et al., 1999). Recently, Ang II blockers have been used to treat chronic hepatitis and liver cirrhosis.

Scheider et al. (1999) reported that portal pressure was significantly reduced when an AT1R antagonist was used to treat patients with liver cirrhosis. Another study reported that liver fibrosis is clearly improved when losartan was used to treat patients with non-alcoholic fatty liver disease (Yokohama et al., 2004). Compared with normal proangiotensin mRNA, the AT1 receptor mRNA expression in local liver tissue in chronic hepatitis B and liver cirrhosis patients increased, suggesting that the angiotensin system is involved in the development of liver fibrosis.

Ang II stimulates TGF-β1 expression on the surface of hepatic stellate cells. Chymase-treated rats also have increased TGF-β1 expression in epithelial cells and endothelial cells, which increase the extracellular matrix (Taipale et al., 1995). Chymase significantly promotes human fibroblast proliferation (Takai et al., 2003), and chymase inhibitors fully suppress the proliferation activation. Chymase increases the expression of TGF-β1 in fibroblasts, and the increased expression is completely inhibited by chymase inhibitors.

Previous studies demonstrated that the chymase inhibitors TY-51469 significantly improves hamsters liver fibrosis in a hamster non-alcoholic fatty liver disease model (Tashiro et al., 2010). In the current study, we demonstrated that: 1) the Col-I distribution in the liver model group increases gradually with the progression of liver fibrosis, whereas in the chymase inhibitors intervention group, Col-I distribution in the liver tissue was significantly

Table 1. Comparison of liver tissue Chymase activity of liver fibrosis in different periods between the model group and Chy-I group.

<table>
<thead>
<tr>
<th>Group</th>
<th>Day 14</th>
<th>Day 28</th>
<th>Day 56</th>
</tr>
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<tbody>
<tr>
<td>Control</td>
<td>1.23 ± 0.78</td>
<td>6.98 ± 4.12</td>
<td>31.37 ± 20.91</td>
</tr>
<tr>
<td>Model</td>
<td>2.04 ± 1.03</td>
<td>2.98 ± 1.84</td>
<td>8.39 ± 4.76</td>
</tr>
</tbody>
</table>

Compared with the control group, a: P < 0.05; b: P < 0.01; compared with the model group, c: P < 0.05.

Figure 1. Immunohistochemistry result of collagen type I. (A) Day 14 after DMN model establishment (×200), Col-I distributed more in the liver sinus wall; (B) Day 14 after DMN model establishment (×200), Col-I of the liver sinus wall in the Chy-I group was significantly reduced than the model group; (C) Day 28 after DMN model establishment (model group), Col-I distributed more in the liver sinus wall; (D) Day 28 after DMN model establishment (Intervention group), Col-I distributed in the liver sinus wall was significantly fewer than that of the model group; (E) Day 56 after DMN model establishment (model group), Col-I distributed more in the liver sinus wall; (F) Day 56 after DMN model establishment (intervention group), Col-I distributed in the liver sinus wall was significantly fewer than that of the model group.

demonstrated in many organs, especially in recent years. Molecular biology experiments have further shown that all RAS components are present in the heart, brain, kidney, and blood vessel tissues, and RAS-independent components have been found in the liver. Yoshiji et al. (2001) showed that Ang II and Col-I are increased at different stages of liver fibrosis using a DMN-injected rat liver fibrosis animal model. Ang II and Col-I gradually increased along with the progression of the fibrosis, whereas AT1R expression decreased, suggesting that the RAS is active during liver fibrosis. In the liver, chymase is the main Ang II-forming enzyme present in mastocytes. It converts Ang I into Ang II when inflammation is stimulated by mastocytes through
less than that in the model group within the same period.
2) In the liver fibrosis rat model, with the progression of liver fibrosis, the chymase activity in the liver tissue increased gradually, whereas that in the Chy-I model group was significantly reduced. 3) The Col-I mRNA expression in the liver tissue in the Chy-I group was significantly reduced compared with that in the model group. The results show that chymase is involved in the pathogenesis of liver fibrosis, and Chy-I down-regulates chymase activity and the expression of Col-I, thereby inhibiting liver fibrosis. The chymase inhibitor Ty-51569 was used to treat hamster liver fibrosis in model induced with carbon tetrachloride, and the activity of chymase, Ang II, and angiotensin-convert enzyme in the liver tissues were observed eight weeks after the model was established. The treatment group was obviously improved compared with that of the model group, and α-smooth muscle antibody-positive cells in the treatment group was significantly reduced compared with that of the model group, suggesting that Ty-51469 improves the liver fibrosis induced by the Ang II (Komeda et al., 2010). This experiment dynamically observed the chymase activity and Col-I mRNA expression in the liver tissue. Chymase inhibitors were used to treat the liver fibrosis for different periods. The results show that with the progression of fibrosis, these indices continued to increase in the model group and the treatment group; all were significantly higher than the control group, and the increase in the Chy-I treatment group was less than that in the model group. This finding suggests that chymase inhibitors obviously improved rat liver fibrosis.

The active Ang II and aldosterone in RAS influence the hepatic stellate cells through the activation of TGF-β1, or directly promote hepatic stellate cell hyperplasia, secretion of collagen types I and III and the extracellular matrix, and promote fibrosis.

ACKNOWLEDGEMENT

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REFERENCES


