Effects of glycyrrhetinic acid on collagen metabolism of hepatic stellate cells at different stages of liver fibrosis in rats

Ji Yao Wang, Qi Sheng Zhang, Ji Sheng Guo and Mei Yu Hu

INTRODUCTION
Liver fibrosis is a dynamic course leading to cirrhosis from a various chronic liver diseases. The pathological basis of fibrosis is the disturbance of production and degradation of the extracellular matrix (ECM), which causes accumulation of ECM in the liver[1,2]. The deposition of collagen is derived primarily from collagen types I and III in liver fibrosis[3]. The main sources of ECM are the hepatic stellate cells (HSCs)[4,5], especially when HSCs are activated by hepatic injury[6,7]. One of the important methods for preventing liver fibrosis is the inhibition of proliferation and activation of HSCs so as to reduce production of collagen. Glycyrrhetinic acid (GA) has been clinically used in the treatment of liver diseases. It has anti-injury[8-10] and anti-viral[11-13] effects on hepatic diseases. The clinical trials have shown that GA could lower the serum aminotransferase level both in Asian[4,5,11] and European patients[12] with chronic hepatitis. Our previous studies also indicated that GA could down-regulate mRNA expression of types I and III procollagen in NIH3T3 cells[14] and in fibrotic livers of rats induced by alcohol and CCl4[15]. However, the effect of GA on HSCs mRNA expression of types I and III procollagen is unclear. In this study, the effects of GA on HSCs mRNA expression of procollagen types I and III and collagenase were investigated and deposition of types I and III collagen in different stages of fibrotic livers in rats was also observed.

MATERIALS AND METHODS

Animal model of liver fibrosis and drug treatment

Adult SD rats weighing 250-300 g were selected. They were distributed as the normal group, the model group, and the GA group. Each group contained the early (2 weeks), middle (6 weeks), and late stage (9 weeks) subgroups. There were 30 rats in each group and 10 rats in each subgroup. The liver fibrosis model was induced by the administration of CCl4 and alcohol. Potenlini, an injectable compound whose active component is GA, was administered intraperitoneally in the GA group with 3 mL per rat three times a week, beginning at 2 weeks prior to sacrifice. Rats were killed by the end of 2, 6 and 9 weeks respectively.

Isolation and culture of HSCs

HSCs were isolated from rat liver as described by Hu[18] with slight modifications. The HSCs showed a typical stellate-like shape containing fat droplet in cytoplasm. During the culture period, HSCs became larger and contained less amounts of fat droplet. By the end of 2 weeks, HSCs looked like myofibroblast. Cells were seeded in culture flask and maintained in DMEM media supplemented with 20% FCS and antibiotics. The media was changed every 48 h. After 2 weeks, when cell confluence was attained, they were harvested by the trypsinized method and applied to further studies or stored in liquid nitrogen.

Identification of HSCs

Freshly isolated HSCs could be distinguished by its autofluoresence characteristic of vitamin A in the lipid droplets at 328 NM. Immunohistochemistry showed that desmin and α-SMA were positive in 99% of cells after 2 weeks of culture. Besides, transmission electron microscopy confirmed the existence of lipid droplet in cytoplasm and revealed a purification of about 90% in freshly isolated cells.
**3H-TdR and 3H-proline incorporating test**

After two weeks of culture, the HSCs were collected and seeded on 96-well culture plates at a density of 1 × 10^5 cells/mL cultured media. Forty-eight hours later, GA (i.e. potentlini) was added into wells at a final concentration of 1.0, 0.5, 0.25, 0.125 and 0.0625 mg/L, respectively, and incubated for 4 h, 24 h or 48 h, then 3H-TdR or 3H-Proline at a density of 18.5 KBq/per well were added and incubated for 24 h. The cells were then harvested with trypsin and the adhered cells were placed into glass fiber filter by multiple cell collector. The cells were baked at 80°C for 2 h, scintillation fluid was added and the radioactivity in the cells was determined using scintillation counter.

**Plasmid amplification and probe labeling**

Plasmid pUCAU1U (containing procollagen type I cDNA fragment)[20], pHFS3 (containing procollagen type III Cdnna fragment)[21], and pUC19A (containing collagenase cDNA fragment)[22], were amplified in LB culture media. The plasmid DNA was extracted with a plasmid extracting kit (QIAGEN Incorporation, Germany). Plasmid was cleaved with restriction endonuclease and the target cDNA fragment was retrieved and phenolized. The cDNA fragment was labeled with DIG high primer technique (Boehringer Mannheim Incorporation, Germany). The probe was further purified by ethanol precipitation. Finally, the efficiency of probe labeling was determined using pseudohybridization, the optimal probe concentration of procollagen types I and III and collagenase was found to be 25, 35 and 25ug/L respectively.

**RNA extraction and Northern blot**

HSCs RNA was extracted with a Rneasy mini-kit (Boehringer Mannheim Incorporation, Germany). Total RNA 5 μg was electrophorized on a 1% agarose/3% formamide gel. The RNA samples were stained with ethidium bromide and transferred overnight by capillary blotting in 20 × SSC to nylon membrane. The RNA was immobilized by baking for 30 min, at 120°C. Membranes were prehybridized (2 h) and hybridized (overnight) at 60°C in high SDS solution. The membranes were washed at a stringency of 2 × SSC with 0.1% SDS at room temperature for 30 min and 0.1% SDS at 68°C for 30 min. The hybridization band was obtained by the chemoluminemscent method after film exposure for 5-10 min and then quantified by the scanning laser densitomity.

**Dot blots of types I and III collagen**

The types I and III collagens were isolated by limited pepsin digestion. The livers were minced and homogenized thoroughly in cold distilled water and then centrifuged at 12 000rpm for 20 min. The precipitate (5 g) was suspended in 0.5M acetic acid and digested with pepsin for 24 h at 4°C with stirring, and then centrifuged. The supernatant was incubated with NaCl (1.0M) overnight. The precipitate contained types I and III collagen which were purified by salt fractionation and their concentration was estimated by ultraviolet spectroscopy. Twenty μL of each sample was loaded on PVDF membrane and was blocked with 10% BSA for 60 min. The polyclonal antibodies (1:250 dilution) of types I and III collagen were added and incubated overnight. The membrane was then washed with PBST 3 times and blocked again with 10% BSA for 30 min. It was then incubated with the secondary antibody for 2 h at room temperature. After washing with 50mM Tris-HCl, the dot was obtained 5-10 min after incubation with DAB. The dot intensity was quantified by scanning laser densitomity.

**Statistical analysis**

Data were expressed as mean ± SD. One way ANOVA and t test were applied for data analysis.

**RESULTS**

**Histological examination and identification of HSCs**

Histological examination (H&E and collagen specific staining) revealed the successful establishment of the rat liver fibrosis models at different stages. Two weeks after CCl4 and ethanol treatment, denaturation and necrosis were the main microscopic changes in liver. By 6 weeks, except for denaturation, connective tissues began to enlarge and extend. By 9 weeks, pseudo-nodules formed and bands of connective tissues were found in the portal areas. The yield of HSCs was 1.6-1.8 × 10^7 cells per liver. The result of transmission electron microscopy, fluorescence microscopy (Figure 2) and immnohistochemical staining all showed that the purity of HSCs was high, which met the demand of further studies.

**3H-TdR and 3H-proline incorporating test**

Compared with the control group, GA had an inhibitory effect on cultured HSCs on incorporation of 3H-TdR and 3H-proline at 4 h, 24 h, and 48 h (Table 1), with a time dependent relationship. This inhibitory effect was significant in a dose-dependent manner when the concentration of GA was above 0.25 mg/L (3H-TdR), and 0.125 mg/L (3H-proline), respectively (Table 2).

<table>
<thead>
<tr>
<th>Drug</th>
<th>Duration (hrs)</th>
<th>3H-TdR incorporation (cpm)</th>
<th>Inhibition rate(%)</th>
<th>3H-proline incorporation (cpm)</th>
<th>Inhibition rate(%)</th>
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<tbody>
<tr>
<td>None</td>
<td></td>
<td>1540 ± 120</td>
<td>542 ± 102</td>
<td></td>
<td></td>
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<tr>
<td>GA</td>
<td>4</td>
<td>1327 ± 198</td>
<td>15*</td>
<td>421 ± 16</td>
<td>22*</td>
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<tr>
<td></td>
<td>24</td>
<td>1217 ± 254</td>
<td>21*</td>
<td>316 ± 18</td>
<td>42*</td>
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<td></td>
<td>48</td>
<td>1057 ± 121</td>
<td>31*</td>
<td>265 ± 84</td>
<td>51*</td>
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</tbody>
</table>

*Compared with control values, P<0.05; *Compared with control values, P<0.01.
Table 2: Effect of GA of different doses on 3H-TdR and 3H-proline incorporation of HSCs

<table>
<thead>
<tr>
<th>Drug</th>
<th>Duration (hrs)</th>
<th>3H-TdR Incorporation (cpm)</th>
<th>Inhibition Rate (%)</th>
<th>3H-proline Incorporation (cpm)</th>
<th>Inhibition Rate (%)</th>
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<tr>
<td>None</td>
<td>1540 ± 120</td>
<td>542 ± 102</td>
<td></td>
<td></td>
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<tr>
<td>GA</td>
<td>0.0625</td>
<td>1427 ± 175</td>
<td>7</td>
<td>441 ± 76</td>
<td>19</td>
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<tr>
<td></td>
<td>0.125</td>
<td>1321 ± 126</td>
<td>14</td>
<td>327 ± 71</td>
<td>40</td>
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<tr>
<td></td>
<td>0.25</td>
<td>1211 ± 137</td>
<td>21*</td>
<td>316 ± 57</td>
<td>42</td>
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<tr>
<td></td>
<td>0.5</td>
<td>1176 ± 134</td>
<td>24*</td>
<td>295 ± 81</td>
<td>46</td>
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<tr>
<td></td>
<td>1.0</td>
<td>1027 ± 121</td>
<td>33*</td>
<td>220 ± 63</td>
<td>59</td>
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</table>

*aCompared with control values, P<0.05; bCompared with control values, P<0.01.

Plasmid amplification and probe labeling

The purity of extracted plasmid DNA was high, and the ratio OD260/OD280 was over 1.6. The DNA yield of three kinds of plasmid all exceeded 130 µg. Electrophoresis showed that the yield of DNA fragment, which was cleaved with restriction enzyme, was satisfactory. On the other hand, the probe concentration of type I and III procollagen and collagenase was determined to be 15 mg/L, 35 mg/L and 100 mg/L, respectively.

The effect of GA on HSCs mRNA expression of types I and III procollagen and collagenase

At the end of 2, 6 and 9 weeks after the induction of rat liver fibrosis, HSCs mRNA expression of types I and III procollagen in the model group was higher than in normal group (P<0.05). However, HSCs mRNA expression of types I and III procollagen in GA group was lower than that in the model group, but was higher than the normal group (GA group vs model group and GA group vs normal group, P<0.05). HSCs mRNA expression of collagenase in the model group was higher than in normal group at each stage of liver fibrosis (P<0.05). And at the end of 9 weeks, the mRNA expression of HSCs showed a dropping tendency. HSCs mRNA expression of collagenase in GA group was also higher than in normal group, but there was no difference between the GA and the model group (Figure 1).

The effect of GA on liver deposition of types I and III collagen

At the end of the 2nd, 6th and 9th week, the liver deposition of types I and III collagen in the model group was higher than in normal group (P<0.05). The liver deposition of types I and III collagen in GA group was lower than in model group (P<0.05), but was still higher than in the normal group. In addition, the density of type I and III collagen in treatment groups were closer to the model group at the end of the 9th week than at the 2nd and 6th weeks (Figure 2).

Figure 1: Densitometric analysis of collagenase, type III and I procollagen mRNA Northern blot. The result of Densitometric analysis after normalization against hybridization signals for normal groups was expressed as mean percentage ± SD of the control values (n = 5). The level of types I and III procollagen mRNA expression of HSCs in GA groups was lower than in model groups (P<0.05). For the collagenase mRNA expression of HSCs, there was no significant difference between GA groups and model groups.

Figure 2: Dot blot densitometric analysis of types I and III collagen. The result of densitometric analysis after normalization against hybridization signals for normal groups was expressed as mean percentage ± SD of the control values (n = 5). The level of types I and III collagen densitometric values in GA groups was lower than in model groups (P<0.05).
DISCUSSION

In normal livers, HSCs are situated in the Disse’s spaces, separating hepatocytes from sinusoidal endothelium and being rich in fat drips. It has been well known that HSCs are responsible for the excessive production of ECM\cite{23-25}. The central event in liver fibrosis is the activation of HSCs and subsequent transformation from quiescent vitamin A-rich cells to proliferative, fibrogenic and contractile myofibroblasts\cite{26}. In situ hybridization demonstrated the specific transcript of procollagen\cite{27,28}. It is rationale to choose HSCs as a target for pharmacological therapies for anti-fibrosis of liver. In this study, we observed that GA had an inhibitory effect on proliferation and collagen production of HSCs in vitro, which could be associated with the inhibition of activation of HSCs. The activation of HSCs is a pleiotropic process. It involves a series of gene transcriptions. Finally, HSCs display a shape that is similar to myofibroblast-like cells while addition of GA delayed the transformation of HSCs into myofibroblast-like cells. It suggests that GA could serve as a proximal segment modulator to decrease the activation and collagen production of HSCs in culture.

It has been proved that the expression of type I and III procollagen is up-regulated in hepatic fibrosis \cite{29,30}. In this study, we demonstrated that the expression of types I and III procollagen of HSCs increased in hepatic fibrosis of rats. At the end of the 2nd, 6th and 9th week, the expression of types I and III procollagen was down-regulated in GA treated group, but still higher than in normal group, which indicates that GA only decreases the expression of types I and III procollagen partially. We have previously reported that GA inhibits nuclear factor-κB (NF-κB) binding activity\cite{31}. NF-κB is a pleiotropic transcription activator\cite{32} that exists in many kinds of cells\cite{33}. It binds NF-κB inhibitors (IκB) in the cytoplasm as an inactive form. A wide spectrum of cellular stimulating signals, including mitogen, cytokines, bacterial lipopolysaccharides, viruses and viral proteins, and oxidative injury, could induce the activity of NF-κB\cite{34}. Inducers of NF-κB activity resulted in phosphorylation, ubiquitination and degradation of IκB proteins, thus releasing free NF-κB for its translocation into the nucleus to activate transcription. We presume that by this way, GA can down-regulate the expression of types I and III procollagen of HSCs. In normal liver, the production of collagen is relatively static, and only with moderate expression of mRNA of type III and IV procollagen and laminin. The mRNA expression of type I procollagen increased significantly in the formation of hepatic fibrosis. The ratio of type I:III was about 4:1\cite{35} as observed in our study.

Recently, it has been found that the activity of interstitial collagenase is elevated in the early stage of hepatic fibrosis\cite{33}. However, in the development of fibrosis, the activity of collagenase decreased\cite{36-40}. In our study, the activity of collagenase increased at the early and middle stage of fibrosis, i.e. 2 or 6 weeks after CCl4 treatment. At the end of the 9th week (late stage), it dropped to the level similar to that of normal groups. It could be due to the overexpression of tissue inhibitor of metalloproteinase in late stage of hepatic fibrosis\cite{41,42}. GA reduced the mRNA expression of types I and III procollagen of HSCs, but not elevated the mRNA expression of collagenase of HSCs, which indicates that GA decreases the deposition of types I and III collagen by reducing the production of collagen, instead of dissolving the collagen. Therefore, at the end of the 9th week, GA was unable to obviously decrease the deposition of collagen. It suggested that the treatment for hepatic fibrosis with GA should begin at the early stage of fibrosis.

Based on the well-known mechanism of fibrosis, the treatment of fibrosis should include the following elements: removing the injurious stimuli; suppressing the hepatic inflammation; down-regulating the stellate cell activation and promoting the matrix degradation\cite{26}. As we know, the major etiological factor of cirrhosis in patients in China is chronic hepatitis B. Histological improvement was found in the patients responding to antiviral therapy with lamivudine for HBV\cite{63}. The result of a long-term follow-up study suggested that the proliferation of fibrous tissues was reversible\cite{44}. Sun et al\cite{45} indicated that antifibrotic therapy is important even in cirrhotic stage in which the fibrogenesis is still active. The results of our studies indicated that the effects on antifibrosis of GA might be exerted by down-regulating the binding activity of NF-κB and HSC activation, and by suppressing the hepatic inflammation. Our previous study also showed that the effect of GA on serum conversion of HBeAg\cite{49}. Sato et al\cite{66} reported that GA could inhibit the release of HBsAg from the infected hepatocytes. Therefore, GA appears to function at multiple phases of hepatic fibrogenesis. Furthermore, the more exciting report is that GA treatment could inhibit the occurrence of hepatocellular carcinoma\cite{47}. It has been also reported that long term (2-16 years) treatment by GA in chronic hepatitis C patients had no side-effect and was effective in preventing liver carcinogenesis\cite{48}.

Other Chinese herbal recipes have shown their features in antifibrosis. Varieties of recipes or herbal extracts, such as Xiao Chaithu Tang\cite{19}, Recipe 861\cite{50}, Yiganxian\cite{51}, Ganyanping\cite{52} and Matrine\cite{53} have been shown to be effective in prevention and treatment of liver injury and fibrosis with different mechanism and pathway. It implies that the clinical application using the combination of glycyrrhetinic acid with these medicines is an
interesting area for further investigations.

In conclusion, GA inhibits the proliferation and collagen production of HSCs in culture, down-regulates the mRNA expression of type III and I procollagen, and reduces the deposition of type III and I collagen in fibrotic liver. It can be a very useful drug for anti-fibrotic treatment in patients with chronic liver disease.

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