The Effect of Gypenosides on TGF-\(\beta_1\)/Smad Pathway in Liver Fibrosis Induced by Carbon Tetrachloride in Rats

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Abstract

Objective: To investigate the effect of gypenosides on TGF-\(\beta_1\)/Smad pathway in liver fibrosis induced by carbon tetrachloride (CCl4) in rats.

Method: Liver fibrosis in rats was induced by CCl4 subcutaneous injection over nine weeks, using 3 mg/kg of 100% CCl4 for the first injection and then 2 ml/kg of 50% CCl4-olive oil solution, twice per week. At the beginning of the seventh week, the rats stimulated by CCl4 were divided into model group (n=12), gypenosides group (n=11) and colchicine group (n=11). The rats in the gypenosides and colchicines groups were administered with gypenosides (200 mg/kg.d) and colchicine (0.1mg/kg.d), respectively. The rest were administered with sterile water. At the end of the ninth week, hepatic hydroxyproline (Hyp) was tested and the histological changes in liver tissue were observed, as well as hepatic \(\alpha\)-smooth muscle actin (\(\alpha\)-SMA), transforming growth factor-\(\beta_1\) (TGF-\(\beta_1\)), transforming growth factor-\(\beta_1\) receptor (T\(\beta\)R-I), transforming growth factor-\(\beta_2\) receptor(T\(\beta\)R-II), Smad2, phosphorylated Smad2 (p-Smad2), Smad3 and phosphorylated Smad3 (p-Smad3).

Results: At the end of the experiment, two rats had died in the model group but none in the gypenosides and colchicine groups. With the stimulation of CCl4, hepatic Hyp content increased significantly in the model group but clearly decreased in the gypenosides and colchicine groups. Histological detection revealed serious steatosis, inflammation and fibrosis as well as collagen deposition in the liver tissue of the model group, but these were alleviated in the gypenosides and colchicine groups. The protein expressions of hepatic \(\alpha\)-SMA, TGF-\(\beta_1\), T\(\beta\)R-I, T\(\beta\)R-II, Smad2, p-Smad2 and p-Smad3 were all up-regulated in the model group. In the gypenosides-treated group, the protein expressions of hepatic \(\alpha\)-SMA, TGF-\(\beta_1\), Smad2, p-Smad2 and p-Smad3 were all down-regulated. In the colchicine-treated group, hepatic \(\alpha\)-SMA, TGF-\(\beta_1\), Smad2 and p-Smad3 were also down-regulated. The protein expressions of hepatic T\(\beta\)R-I and T\(\beta\)R-II were not inhibited significantly in the gypenosides- or colchicine-treated groups compared to the model group.
Conclusion: Gypenosides alleviated liver fibrosis induced by CCl4 in rats, which is probably related to their inhibitory effects on hepatic stellate cell activation and the protein expression of TGF-β1, Smad2, p-Smad2 and p-Smad3.

Keywords Liver fibrosis, Gypenosides, Hepatic Stellate Cell, Transforming Growth Factor-β.

1. Introduction

Hepatic fibrosis is the common wound-healing response of the liver to chronic injury of any type. It has long been established that injury to the structures and functions of the liver is commonly induced by activated hepatic stellate cells (HSCs), which are responsible for excessive extracellular matrix (ECM) deposition in chronically damaged livers [1]. During the process of chronic liver injury, HSCs, activated by paracrine and autocrine pathways, convert into myofibroblasts (MFb), which are able to secrete and synthesize collagen, an important composition of ECM. Among many known profibrogenic pathways of stellate cell activation, the transforming growth factor-β1 (TGF-β1)/α-smooth muscle actin (a-SMA) pathway is the most potent fibrogenic stimulus to HSCs, resulting in production of ECM. During the process, TGF-β1, derived from both paracrine and autocrine sources, binds to the type II receptor (TβRII) and type I receptor (TβRI), forming a heterotrimer complex. The intrinsic serine/threonine kinase activity of TβRII results in the phosphorylation of TβRI, which in turn leads to downstream signals, via its own kinase activity, to the Smad proteins. Smad2 and Smad3 are phosphorylated by TβRI and combined with Smad4 before migrating into the nucleus where they regulate gene expression of collagen[2-5]. Many studies have shown that inhibition of the binding of the receptor or blocking the activations of Smad proteins such as Smad2 and Smad3, could reduce HSC activation and collagen synthesis, attenuating hepatic fibrosis[6-9].

Gypenosides are active component of the Chinese herb Gynostemma pentaphyllum with a tetracyclic triterpenoid dammarane structure. They are commonly used in the treatment of liver diseases in traditional Chinese medicine. Recent studies have discovered that gypenosides have many potential pharmacological applications, such as anti-inflammatory, anti-tumour, improving the conditions of atherosclerosis, lowering blood pressure and regulating immunity of the liver and hepatic protective activities [10-13]. The effects of gypenosides on liver fibrosis induced by carbon tetrachloride (CCl4) were confirmed in our previous study [14]. The purpose of the present study is to observe the effect of gypenosides on TGF-β1/Smad pathway in liver fibrosis induced by CCl4 in rats.

2. Methods

2.1 Experimental animals and reagents

Animal: Wistar male rats of clean grade, weighing 150 g to 160 g were purchased from the Shanghai Laboratory Animal Centre, Chinese Academy of Sciences (Certificate of Conformity: SCXK [Shanghai] 2007-0005). The experiment was conducted in accordance with the principles for laboratory animal use and care approved by the local ethics committee.

Reagents: Gypenosides of 98% purity, derived from Gynostemma pentaphyllum, were purchased from Hongsheng Biological Co. Ltd., Xi’an, China (Lot: GY060913). Colchicine was produced by the Banna Pharmaceutical Co. Ltd., Xishuangbanna, China (batch number: 06910). CCl4 and olive oil were purchased from Sinoreagent Co. Ltd., China; Hydroxyproline (Hyp) standard, AR, was produced by Wako Pure Chemical Industries, Ltd. Japan; Hematoxylin dye and Protein Quantification Kit (Coomassie Brilliant Blue) were both purchased from Nanjing Jiancheng Bioengineering Institute, Nanjing, China. The antibodies of Western-blot and immunohistochemistry are shown in Table 1.

Antibody Species Company Cat. No. Dilution ratio

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<th>Company</th>
<th>Cat. No.</th>
<th>Dilution ratio</th>
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Table 1. Antibodies of Western-blot and immunohistochemistry

2.2 Animal models and groups

According to the method previously described [15], liver fibrosis in rats was induced by subcutaneous injection over nine weeks, with 3 ml/kg of 100% CCl4 for the first injection and subsequently 2 ml/kg of 50% CCl4-olive oil solution, twice per week. At the beginning of the seventh week, rats stimulated by CCl4 were divided into model group (n=12), gypenosides group (n=11) and colchicine group (n=11). The rats in the gypenosides and colchicine groups were administered with gypenosides (200 mg/kg,d) and colchicine (0.1 mg/kg,d), respectively, and the others with sterile water. Colchicine was used as a positive control.

2.3 Methods and medical indexes

At the end of the ninth week, the rats were anaesthetized by intraperitoneal injection with 2% pentobarbital sodium 0.3 ml/100g and serum, and liver tissue was harvested for testing and analysis. The content of Hyp in hepatic tissue
was determined according to the Jamall method \[16\]. The histopathological changes in the liver tissue were observed by Hematoxylin - eosin staining (H.E. staining), and Sirius red staining was employed for collagen observation. The expression of the proteins in liver tissue, including α-smooth-muscle actin (α-SMA), transforming growth factor-β1 (TGF-β1), TGF-β1 receptor (TβR-I), TβR-II, Smad2/p-Smad2 and Smad3/p-Smad3 were measured by the Western-blot method.

2.4 Statistical Analysis

SPSS 18.0 statistical software was used for the statistical analysis. All the values were expressed as mean ± standard deviation. Comparisons were performed by (One-Way ANOVA) LSD test. Differences were considered statistically significant if the P-value was lower than 0.05.

3. Results

3.1 General conditions of the rats

At the end of the experiment, two rats had died in the model group but none in gypenosides and colchicine groups. Generally, with CCl4 treatment, the body weight and food intake of rats in the model group had decreased compared to the normal group. After treatment with gypenosides and colchicines, the body weight and food intake were partially recovered in the two drug groups.

3.2 The hepatic Hyp content

The content of Hyp of the liver tissues in the model group was significantly increased (524±143 μg/g liver tissue) compared to the normal group (178 ± 21 μg/g liver tissue, \(P < 0.01\)). The Hyp contained in the liver tissues in both the gypenosides (390±71μg/g liver tissue) and colchicine (373±112 μg/g liver tissue) groups was reduced significantly compared with the model group (\(P <0.05\)). There was no remarkable difference between the gypenosides and colchicine groups, suggesting that both the two compounds have similar functions in inhibiting the deposition of collagen in liver fibrosis.

3.3 Pathological changes in hepatic tissues

In the H.E. staining and collagen staining (Sirius red staining) of liver tissue sections, the normal structure of the hepatic lobular of model rats was observed to be destroyed and the fatty degeneration in hepatic parenchymal cells was obvious. There were a lot of fibrous connective tissue proliferations, almost integrated into pseudo-lobules. Compared with the model group, the degree of the proliferation of the hepatic fibrosis in the gypenosides and colchicine groups reduced significantly (Figure 1).

**Figure 1.** Histological observations of liver tissues in the four groups. A: H.E. Staining, \(\times 200\); B: Sirius red staining, \(\times 200\). In the normal group, no morphological abnormality was observed under the microscope and collagen was scarce except around small central venous walls in portal area. Fibrous connective tissue proliferation was obvious in the livers of the model group and almost integrated into pseudo-lobules. There are examples of bridging collagen connections between central veins and neighbouring portal areas; the deposition of collagen fibres was steadily enhanced and the pseudo nodules were formed within liver parenchyma. Compared with the model group, the reduction of hepatocytes, gradual proliferation of fibrosis, continuous infiltration of neutrophils and collagen deposition in the liver were significantly attenuated in the gypenosides group. In the gypenosides group, the collagen deposition of the liver in the colchicine group was similarly attenuated.

3.4 Effects of gypenosides on HSC activation

α-SMA is a biological marker of HSC activation. The result of the Western blot analysis of the liver tissue showed that, compared with the normal group, the expression of α-SMA in the model group was significantly increased (\(P <0.05\)). Compared with the model group, the expression of hepatic α-SMA in the gypenosides and colchicine groups was decreased (\(P <0.05\)), with no significant difference between the two groups (Figure 2).

**Figure 2.** Protein expression by Western-blotting of α-SMA in liver tissue vs. normal group, * \(P <0.05\); vs. model group, # \(P <0.05\)

3.5 Effect of gypenosides on TGF-β1/Smad signal pathway

Western blot analysis (Figure 3) showed the expressions of some key cytokines of TGF-β1/Smad signal pathway
included TGF-β1, TβR-I, TβR-II, Smad2/p-Smad2 and Smad3/p-Smad3.

Figure 3. Protein expressions by Western blotting of TGF-β1, TβR/I/II, Smad2/ p-Smad2 and Smad3/ρ-Smad3 in liver tissue vs. normal group, * P < 0.05, ** P < 0.01; vs. model group, # P < 0.05, ## P < 0.01.

The results revealed that, compared with the normal group, the protein expressions of TGF-β1, TβR-I, TβR-II, Smad2 and p-Smad2 were significantly increased in the rat liver tissue of the model group (P < 0.05 or 0.01). Compared with the model group, the protein expressions of TGF-β1, Smad2 and p-Smad2 was significantly decreased in the liver tissue of the gypenosides group (P < 0.05 or 0.01), while the protein expressions of TβR-I and TβR-II were not significantly different between the model and gypenosides groups. In the colchicine group the protein expressions of TGF-β1 and Smad2 obviously reduced (P < 0.05); compared with the model group, there was no significantly change in the expressions of TβR-I, TβR-II and p-Smad2. The protein expression of Smad3 was similar between the groups. Compared with the normal group, the protein expression of p-Smad3 in the model group increased much more significantly (P < 0.01); it decreased significantly in both the gypenosides (P < 0.05) and the colchicine (P < 0.01) group.

4. Discussion

Fibrosis is a dynamic process and its essence is the excessive deposition of ECM. During the process, TGF-β1 remains the classic fibrogenic cytokine. Signals downstream of TGFβ converge on Smad proteins, which fine-tune and enhance the effects of TGFβ during stellate cell activation; Smads 2 and 3 are stimulatory whereas Smad7 is inhibitory and is antagonized by Id1 (17-19). The response of Smads in stellate cells evolves as injury becomes chronic, further enhancing fibrogenesis.

The anti-fibrotic effect of colchicine has been investigated in a number of studies (20), but clinical application is seriously limited because of side-effects. The results of this study demonstrate that, after treatment with gypenosides, hepatic collagen (Hyp) was significantly decreased compared to the model group, and the histological degree of the liver fibrosis was significantly improved, comparably to colchicine group. This indicates that gypenosides play a significantly role in anti-hepatic-fibrosis.

Early studies on CCl4-induced liver injury confirmed that HSC, the primary source of ECM in the liver which is activated during liver injury, could convert into MFB, which greatly proliferates and synthesizes ECM (21,22). The activation of HSC is therefore the key point in liver fibrosis. In this study the protein expression of the HSC activation marker α-SMA of the CCl4-induced liver fibrosis tissue was significantly higher than for the normal group but decreased obviously in the gypenosides group, indicating that the activation of HSC in the liver tissue was greatly decreased in the gypenosides group compared to the model group. Generally speaking, HSC activation includes two phases, initiation and perpetuation. Initiation refers to early changes in gene expression and phenotype, which render the cells responsive to other cytokines and stimuli, while perpetuation results from the effects of these stimuli in maintaining the activated phenotype and generating fibrosis. Initiation is largely due to paracrine stimulation,
whereas perpetuation involves autocrine and paracrine loops. During the perpetuation of HSC, liver injury mainly causes hepatic inflammatory cells (mainly Kupffer cells) and platelets to secrete large amounts of cytokines such as TGF-β, which further stimulate HSC activation through paracrine loop. During the late stage of the inflammation, the HSC can further promote self-activation by secreting TGF-β; at this stage, even with the removal of the original stimulatory factors, the autocrine and paracrine of MFB will still exist and eventually promote the development of liver fibrosis. The present study reveals that the protein expression of hepatic TGF-β in the CCl4-induced rat was significantly higher compared to the normal group, but decreased obviously in the gypenosides group, which shows the same trend for the HSC activation marker α-SMA.

The signal of TGF-β was delivered into cells through its trans membrane receptors TβR-I and TβR-II. When TGF-β binds to the receptors, TβR-I and TβR-II become activated and catalyse Smad2/3 phosphorylation, entering into the nucleus, binding to transcriptional factors and regulating the expression of target genes (e.g., collagen, tissue inhibitor of matrix metalloproteinases) [24,25]. In this study, the protein expressions of hepatic TGF-β, TβR-I, TβR-II, p-Smad2 and p-Smad3 in CCl4-induced rats were significantly increased compared to the normal group, indicating that the TGF-β/Smad signalling pathway was activated during the liver fibrosis. Through the intervention of gypenosides, the protein expression of TGF-β in liver tissue was significantly reduced; the protein expression of TβR-I and TβR-II shows a downward trend despite no statistically significant difference. Compared with the model group, the protein expression of p-Smad2 in gypenosides was significantly reduced, but with no remarkable difference to the colchicine group. The protein expression of p-Smad3 in both the gypenosides and the colchicine group was significantly reduced, which shows that after the intervention of gypenosides, the TGF-β/Smad signalling pathway in the CCl4-induced liver fibrosis model is inhibited, and that its main anti-fibrosis process is the inhibition of the protein expression of TGF-β, p-Smad2 and p-Smad3.

In conclusion, the present study has confirmed the significant inhibitory effect of gypenosides on CCl4-induced hepatic fibrosis in rats, which probably correlates with the inhibition of hepatic HSC activation, TGF-β secretion and p-Smad2, p-Smad3 related signalling pathways.

5. Acknowledgements

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6. References


