Effects of Shugan-Huayu Powder, A Traditional Chinese Medicine, on Hepatic Fibrosis in Rat Model

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Abstract

Shugan-Huayu powder (SHP) has been administered to outpatients with chronic liver disease without clear anti-fibrosis mechanism. To investigate the anti-fibrotic effects of SHP on liver fibrosis in a rat model and in hepatic stellate cells (HSCs) in vitro, rats were gavaged with CCl₄ at 1.0 g/kg body weight twice a week to induce liver fibrosis and the rats were randomly assigned to one of the three groups: –CCl₄ alone, low-dose SHP and high-dose SHP. SHP was given by gavages 5 times a week for 8 weeks. Serum, livers and HSCs were assayed for serology, pathology, western blot, zymography and quantitative RT-PCR. Hepatic function improved as decreased serum aspartate aminotransferase and alanine transaminase, and collagen deposition and active HSCs were significantly reduced in CCl₄-induced liver by SHP treatment. The expression of matrix metalloproteinase-2 (MMP-2) and transforming growth factor-β₁ (TGF-β₁) mRNA in fibrotic liver showed significant down-regulation after SHP treatment. In vitro, inhibition of α-smooth muscle actin (α-SMA) expression and MMP-2 secretion of active HSCs were also noticed by SHP treatment. SHP has an antifibrotic effect on CCl₄-induced liver fibrosis in rats. Anti-fibrotic mechanisms were probably inhibiting activation of HSCs and decreased expression of MMP-2 and TGF-β₁.

Key Words: liver fibrosis, traditional Chinese herb, hepatic stellate cell, animal model

Introduction

Liver fibrosis is a common outcome of chronic liver diseases such as alcoholic, viral and autoimmune hepatitis. Abnormal accumulation of extracellular matrix (ECM) proteins, especially collagen types I and III, is the main pathologic feature of liver fibrosis (3). Hepatic stellate cells (HSCs), situated in the space of Disse, have been identified as the major cell type involved in fibrogenesis. During liver fibrogenesis, HSCs undergo activation, a process characterized by increased cell proliferation, morphological transformation into myofibroblast-like cells, increased expression of α-smooth muscle actin (α-SMA) and synthesis of excessive ECM components (9). Moreover, HSCs are able to secrete matrix metalloproteinases (MMPs) which can degrade normal matrix proteins in the extracellular space and stimulate migration of HSCs (12). Down-regulation of MMP-1 and up-regulation of MMP-2 and MMP-9 have been
observed in patients with liver fibrosis (21). MMP-2 has reportedly been involved in the mitogenic and promigratory effects of reactive oxygen species (ROS) on HSCs (11). The activities of MMPs are inhibited by a family of tissue inhibitors of metalloproteinases (TIMPs) which control the conversion of MMPs from pro-enzymes to the active catalytic form to result in the inhibition of matrix degradation. Notably, TIMP-1 and -2 are the central inhibitors of MMPs (2).

Traditional herbal medicine has been used over thousands of years and is based on experiences and practices. Sho-saiko-to (TJ-9) (27), Inchinko-to (TJ135) (16, 17, 29) and Han-Dan-Gan-Le (20) have been proved to be valuable in treating liver fibrosis. Shugan-huayu powder (SHP) is extracted from ten medicinal herbs. In Taiwan, although SHP is presently the most commonly administered Chinese herbal medicine to outpatients with chronic liver disease, especially those with chronic hepatitis, the mechanism by which SHP works against liver fibrosis is still unknown. In this study, we investigated the anti-fibrotic effects and mechanisms of SHP on carbon tetrachloride (CCl4)-induced liver fibrosis in a rat model and in vitro in HSC-T6 cells.

**Materials and Methods**

**Preparation of SHP**

The SHP, mainly consisting of ten herbs (Table 1), was boiled in hot-water in 50°C and extracted five times with distilled water. The extract was filtered, freeze-dried and stored at 4°C until used. Clinically, SHP was used at a wide range of dosage, 3 to 9 g/day/70 kg for relieving depressed livers, even at a dosage above 20 g/day/70 kg for severe patients based on doctors’ experiences. Furthermore, the low dose (0.5 g/kg, 0.1 g/200 g BW rat) used in the study was transferred from the human clinical dosage (5.5 g/70 kg BW) by the index of Body Surface Area (200 g BW rat to 70 kg BW adult human is 0.018). The dried extract was dissolved in water at a concentration of 50 mg/ml as a stock solution. In order to maintain the same treated volume, the high-dose SHP was independently prepared as a five-fold concentration stock solution. Quality control was authorized to Sheng Chang Pharmaceutical Co., Ltd and was analyzed by high-performance liquid chromatography. The concentrations of the major active ingredients in freeze-dried SHP were determined to be the following: hesperidin 6.47 mg/g, naringin 4.50 mg/g, ferulic acid 0.03 mg/g, liquiritin 1.18 mg/g, glycyrrhizin 1.81 mg/g and paeoniflorin 6.66 mg/g.

**In Vivo Rat Liver Fibrosis Model**

**Animals and Treatments**

The investigation was conducted in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committees of the Taichung Veterans General Hospital. Twenty-four male Sprague-Dawley rats weighting 250-300 g were randomly divided into four groups of six rats each. Group 1 was orally administered olive oil and saline (control group). The other 18 rats were all given 1.0 g/kg CCl4 orally (prepared in 50% CCl4/olive oil) twice a week for 8 weeks to induce liver fibrosis. Group 2 was treated with CCl4 and orally administered saline (CCl4 alone group). Group

<table>
<thead>
<tr>
<th>Family</th>
<th>Speciesa</th>
<th>Part useda</th>
<th>Components of 28 g SHP (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Asteraceae</td>
<td><em>Atractylodes macrocephala</em> Koidz.</td>
<td>Rhizome</td>
<td>3.0</td>
</tr>
<tr>
<td></td>
<td><em>Carthamus tinctorius</em> L.</td>
<td>Flower</td>
<td>1.0</td>
</tr>
<tr>
<td>Cyperaceae</td>
<td><em>Cyperus rotundus</em> L.</td>
<td>Rhizome</td>
<td>4.0</td>
</tr>
<tr>
<td>Leguminosae</td>
<td><em>Glycyrrhiza uralensis</em> Fisch.</td>
<td>Rhizome</td>
<td>2.0</td>
</tr>
<tr>
<td>Paeoniaceae</td>
<td><em>Paeonia Lactiflora</em> Pall.</td>
<td>Root</td>
<td>4.0</td>
</tr>
<tr>
<td>Rosaceae</td>
<td><em>Prunus persica</em> (L.) Batsch.</td>
<td>Seeds</td>
<td>4.0</td>
</tr>
<tr>
<td>Rutaceae</td>
<td><em>Citrus aurantium</em> L.</td>
<td>Pericarp</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td><em>Citrus reticulate</em> Blanco.</td>
<td>Pericarp</td>
<td>2.0</td>
</tr>
<tr>
<td>Umbellifera</td>
<td><em>Bupleurum chinense</em> DC.</td>
<td>Rhizome</td>
<td>4.0</td>
</tr>
<tr>
<td></td>
<td><em>Ligusticum chuanxiong</em> Hort.</td>
<td>Rhizome</td>
<td>2.0</td>
</tr>
</tbody>
</table>

* All plants were purchased from Mainland China and authenticated by Wei-Chu, Lee who is the leader of Sheng Chang Pharmaceutical Co., Ltd and by Long-Yuan, Wu, Graduate Institute of Chinese Pharmaceutical Sciences. The authentic samples were stored at Sheng Chang Pharmaceutical Co., Ltd.
3 was treated with CCl₄ and orally administered with low-dose SHP (CCl₄ + SHP 0.5 g/kg group). Group 4 was treated with CCl₄ and orally administered with high-dose of SHP (CCl₄ + SHP 2.5 g/kg group). In order to investigate the preventive effect instead of the therapeutic effect of SHP on liver fibrosis, SHP was given at the same time of CCl₄ administration by gavages 5 times a week for 8 weeks. Three days after the last dose, the rats were anesthetized and sacrificed, blood samples were collected, and liver tissues were removed and weighted. Anterior segment of the removed livers was then excised and stored at -80°C until used for further determination. A portion of the liver was rapidly fixed in 10% neutralized formalin.

### Serum Biochemical Measurements

Blood samples for biochemical analysis were collected from the tail vein at the baseline and the end of the third, sixth and eighth week. Blood samples were kept at room temperature for 1 h and centrifuged at 3,500 rpm for 20 min, and the sera were kept at -4°C until further assay. The activity levels of serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were analyzed using an automated-analyzer (Hitachi, model 736-60, Tokyo, Japan) to indicate liver injury (14).

### Histological Examination

The liver tissues, fixed in 10% formalin, were processed by routine histological procedures. Four-μm paraffin sections were prepared to determine histopathological changes in liver fibrosis induced by CCl₄ administration. The samples were stained either with hematoxylin and eosin for hepatocytic degeneration, or with Sirius red stain for fibrosis scores. The criteria used for scoring fibrosis severity were as follows: 0, normal; 1+, fibrosis present (collagen fiber present that extends from portal triad or central vein to peripheral region); 2+, mild fibrosis (mild collagen fiber present with extension without compartment formation); 3+, moderate fibrosis (moderate collagen fiber present with some pseudo lobe formation); and 4+, severe fibrosis (severe collagen fiber present with thickening of the partial compartments, frequent pseudo lobe formation and bile duct proliferation) (19). The criteria used for scoring hepatocytic degeneration severity were as follows: 0, normal; 1+, focal area; 2+, multifocal area; 3+, locally extensive area; and 4+, diffuse area (26).

### Immunohistochemistry

For detection of activated HSCs, α-SMA was assessed immunohistochemically using a specific anti-α-SMA monoclonal antibody by the strepavidin-biotin-peroxidase complex method. Paraffin-embedded sections of each rat liver were deparaffinized in xylene and hydrated in a series of graded alcohol, and subjected to antigen retrieval by microwave at medium power in 0.01 M citrate buffer (pH 6.0). The activity of endogenous peroxidase was blocked with 3% hydrogen peroxide for 10 min. The sections were then incubated with monoclonal anti-α-SMA (Chemicon, Temecula, CA, USA) at 4°C overnight, washed three times with phosphate buffer solution (PBS) between each incubation, and then incubated with goat anti-mouse IgG conjugated with horseradish peroxidase (Santa Cruz Biotech, Santa Cruz, CA, USA). The antigen-antibody complexes were visualized by ABC Immunostain Systems (Santa Cruz Biotech), and exposed to freshly prepared diaminobenzidine (DAB) for 3-5 minutes. Sections were stained lightly with hematoxylin, dehydrated in alcohol series, cleared in xylene, mounted on permount, examined and photographed by light microscopy (Nikon eclipse E800).

### Immuno-Fluorescence Staining (α-SMA and MMP-2 Double Staining)

Tissue sections were incubated with a monoclonal mouse anti-human SMA antibody (1:200 dilution, MU128-UC, Biogenex, San Ramon, CA, USA) overnight at 4°C and 1 h at 37°C and then with a rhodamine-conjugated donkey anti-mouse IgG antibody (Jackson ImmunoResearch Laboratories Inc., USA). All the sections were subsequently incubated with goat anti-human MMP-2 antibody (1:200 dilution, SC-8835, Santa Cruz) for 1 h at 37°C and then with fluorescein-conjugated donkey anti-goat IgG antibody (Jackson ImmunoResearch Laboratories Inc., USA) for 1 h at 37°C. All sections were observed under a laser confocal microscopy (TCS-SP2, Leica, Heidelberg, Germany). The pictures of FITC images and those of rhodamine images were merged using the Leica image analysis software.

### Western Blot Analysis of α-SMA Expression

Frozen liver tissues (0.1 g) from each rat were homogenized in 1 ml of NP-40 lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 7.4, 1% Nonidet P-40) with protease inhibitor (Calbiochem, San Diego, CA, USA). The homogenates were kept on ice for 30 min and then centrifuged at 14,000×g for 10 min at 4°C. The supernatants were collected and assayed for protein concentration by Beckman RU-640 spectrophotometer, with bovine serum albumin (BSA) as standards. Aliquot samples were flash-frozen in liquid nitrogen and stored at -80°C. Fifty μg protein from each tissue sample was diluted with PBS and boiled for 5 min. Samples
were electrophoresed on 10% sodium dodecyl sulfate (SDS)-polyacrylamide gels and the proteins were transferred onto Immobilon-PVDF membranes (Millipore, Bedford, MA, USA) in a transfer buffer (6.2 mM boric acid, pH 8.0). The blots were incubated with blocking buffer (1% nonfat milk, 10 mM Tris, 100 mM NaCl, 0.1% Tween 20, 1% BSA) for 1 h at room temperature, and then with specific primary antibody against mouse anti-α-SMA (MU128-UC, Biogenex) or mouse anti-α-tubulin (Santa Cruz), and thereafter diluted (1:1000) with Tris-buffered saline-Tween 20 (TBS-T) containing 1% BSA and 1% nonfat milk. After incubation, the blots were washed with TBS-T for 1 h and incubated with horseradish peroxidase conjugated anti-mouse IgG (Santa Cruz) for 1 h at room temperature. After washing the secondary antibodies (1:5000) with TBS-T, immunoreactive bands were visualized via Western blotting detection system (an enhanced chemiluminescence kit, Amersham Pharmacia Biotech, Buckinghamshire, England), and were quantified by image analysis with a Gel-pro Analyzer.

Detection of MMP Activity by Gelatin Zymography

Gelatin zymography, modified as previously described (13), was performed to assess the activities of MMP-2 and MMP-9 in liver tissues. Frozen liver tissue (0.1 g) from each rat was homogenized in 1 ml of PBS buffer and centrifuged at 12,000 rpm for 10 min at 4°C. The supernatants were collected and assayed for protein concentration by the Beckman RU-640 spectrophotometer, with BSA as a standard. Aliquot samples were flash-frozen in liquid nitrogen and stored at -80°C. Twenty µg protein from each tissue sample was diluted with PBS and electrophoresed on 8% SDS-polyacrylamide gels containing 1 mg/ml gelatin. The gels were washed twice with 2.5% Triton X-100 for 30 min, then incubated with reaction buffer (0.01% NaN₃, 40 mM Tris-HCl, pH 8.0 and 10 mM CaCl₂) at 37°C for 16 h. The gels were stained with a solution containing 50% methanol, 10% acetic acid and 0.1% Coomassie Brilliant Blue for 10 min, then destained in 10% methanol with 10% acetic acid. The protease bands were quantified by Image Analysis with a Gel-pro Analyzer.

Analysis of Transcripts of TGF-β1 Genes

The mRNA expression levels of TGF-β1 genes in the liver were analyzed by quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR). Total RNA was isolated by a modified method (14). Briefly, total cellular RNA was extracted from liver tissue using the RNeasy Mini kit (Qiagen GmbH, Hilden, Germany). An aliquot of 1.0 µg of total RNA from each liver sample was subjected to assay using TaqMan One-Step RT-PCR Master Mix Reagent kit, and Applied Biosystems TaqMan Gene Expression Assays pre-designed primers and probes were used: The expression levels of the target gene TGF-β1 (Assay ID: Rn_00572010_ml) were normalized with the control housekeeping gene, β-Actin (Assay ID: Rn01759928_g1) according to the manufacturer’s instructions. Real-Time Quantitative RT-PCR was performed on a ABI StepOne Real-Time PCR System (Applied Biosystems, Foster City, CA, USA).

HSC-T6 Cell Lines and In Vitro Assay

Cell Cultures and Cell Viability Assay

The HSC-T6 cell line, a generous gift of Prof. S.L. Friedman, is an immortalized rat HSC cell line transfected by the large T-antigen of SV40 vector containing a Rous sarcoma virus promoter (31). HSC-T6 cells were maintained in Waymouth medium containing 10% FBS, pH 7.0 at 37°C in 5% CO₂.

Alpha-SMA Expression and MMPs Activities in TGF-β1-Treated HSC-T6 Cells

HSCs were seeded in Waymouth medium containing 10% FBS. After 24 h, cells were washed twice with FBS-free Waymouth medium, and replaced by FBS-free Waymouth medium containing TGF-β1 (1 ng/ml) for an hour and washed twice with FBS-free Waymouth medium, then treated with SHP for 24 h, washed with PBS, and collected for the study of α-SMA expression by Western blot analysis or MMPs activities by gelatin-zymography.

Statistical Analysis

All data are expressed as means ± SD. Parametric continuous data between different groups were compared by one-way analysis of variance (ANOVA) followed by Holm-Sidak test. Liver histopathological examination data were analyzed by Kruskal–Wallis nonparametric test. All statistical analysis was conducted using the Scientific Package for the Social Sciences (version 10.1; SPSS, Chicago, IL, USA). A value of P < 0.05 (2-tailed) was considered statistically significant.

Results

In Vivo Effects of SHP on CCl₄ Rats

Serum Biochemistry for Hepatic Injury

Baseline levels of serum AST and ALT showed
no significant differences between groups. At the third week after the CCl4 administration, ALT and AST levels were markedly elevated in all three CCl4-treated groups as compared to the control group (P < 0.001), indicating hepatic injury, but no significant difference was found between the CCl4-alone group and the SHP-treatment groups (Fig. 1). Levels of both ALT and AST in CCl4 rats were significantly decreased by both low-dose and high-dose SHP treatments for 8 weeks (P < 0.05 to 0.001, Fig. 1) suggesting that SHP ameliorated hepatic injury in CCl4 rats.

**Histological Examination and Scoring**

The ratio of liver to body weight in CCl4 group was significant increased as compared to the control group (4.83 ± 0.59 vs. 3.21 ± 0.16 %, P < 0.001). The liver/BW ratio were decreased in low and high dose SHP treatment groups (4.56 ± 0.54 and 3.89 ± 0.24%), but only significantly decreased in the high-dose SHP treatment group (P < 0.01). In the CCl4 alone group, excessive deposition of collagen with strong Sirius-red staining was observed around the portal area expending central veins to form pseudolobuli (Fig. 2b). In addition, hepatocytic degeneration by hematoxylin and eosin staining was also found from multifocal to locally extensive areas. However, the fibrous septa became thinner and interrupted in both low-dose (Fig. 2c) and high-dose (Fig. 2d) SHP treatment groups. Furthermore, the scores of both hepatic fibrosis and degeneration in SHP-treated groups were significantly reduced in a dose-related fashion as compared to the CCl4-alone group (Table 2).

**Qualitative and Quantitative Expression for α-SMA Protein**

We assayed the qualitative expression of α-SMA protein, a marker of HSC activation, in liver sections. Vascular smooth muscle cells were strongly positive for α-SMA, whereas HSCs positive for α-SMA were rarely observed in the control rats (Fig. 3a). In contrast, remarkably increased α-SMA-positive HSCs were detected in centrilobular and pericentral areas in CCl4-alone group (Fig. 3b). However, both low-dose and
high-dose SHP treatments markedly reduced the numbers of α-SMA-positive HSCs in the livers (Figs. 3c and d). Western blotting experiments also revealed quantitative increase of α-SMA expression in CCl4-alone group as compared to the control group ($P < 0.001$), while decrease of α-SMA expression was found in high-dose-SHP treatment group ($P < 0.001$, Fig. 4).

Zymography for MMP-2 and MMP-9 Activity

In CCl4-treated livers, both the proMMP-2 and MMP-2 activities were significantly increased as compared to the control livers in which only the proMMP-2 activity was detected (Fig. 5a). The MMP-9 activity had a similar trend as MMP-2 but was weaker than MMP-2 (Fig. 5a). Both low-dose and high-dose SHP treatments tended to decrease the activities of proMMP-2 and MMP-2 in the liver of CCl4 rats (Fig. 5b), and the high-dose SHP group showed a significant reduction ($P < 0.05$ and 0.01, Fig. 5b).

### Table 2. Histopathological scores of SHP treatment for eight weeks in CCl4 rats

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Severity score of liver fibrosis (incidence)$^a$</th>
<th>Severity score of hepatic degeneration (incidence)$^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>0 1+ 2+ 3+ 4+ Average</td>
<td>0 1+ 2+ 3+ 4+ Average</td>
</tr>
<tr>
<td>Control</td>
<td>6</td>
<td>0 0 0 0 0</td>
<td>0 0 0 0 0</td>
</tr>
<tr>
<td>CCl4 alone</td>
<td>6</td>
<td>0 0 0 4 2</td>
<td>0 2 2 1 1</td>
</tr>
<tr>
<td>CCl4 + SHP 0.5 g/kg</td>
<td>6</td>
<td>0 0 0 2 4 0</td>
<td>2 4 0 0 0</td>
</tr>
<tr>
<td>CCl4 + SHP 2.5 g/kg</td>
<td>6</td>
<td>0 0 3 3 3 #</td>
<td>4 1 1 0 0</td>
</tr>
</tbody>
</table>

$^a$ Data represent the number of rats rated with a given level of liver fibrosis. Categories for liver fibrosis are as follows: 0 = no fibrosis, 1+ = fibrosis present, 2+ = mild fibrosis, 3+ = moderate fibrosis, 4+ = severe fibrosis. See methods for additional details.

$^b$ Data represent the number of rats rated with a given level of hepatic degeneration. Categories for hepatic degeneration are as follows: 0 = normal, 1+ = focal area, 2+ = multifocal area, 3+ = locally extensive area, 4+ = diffuse area. See methods for additional details.

$^#$: $P < 0.05$. $###$: $P < 0.01$ significantly different from CCl4 alone group.

Fig. 3. Expression of α-SMA protein in liver sections of CCl4 rats by immunochemical staining. (a) Control group, α-SMA stains were strongly positive for vascular smooth muscle cells, but absent in hepatic stellate cells; (b) CCl4-alone group, many α-SMA positive hepatic stellate cells were migrated into areas of centrilobular and perportal fibrotic bands; (c) CCl4 + SHP 0.5 g/kg group, and (d) CCl4 + SHP 2.5 g/kg group, liver sections showed markedly reduced numbers of α-SMA-positive hepatic stellate cells (Scale bar = 50 µm).

Fig. 4. Quantitative expression of α-SMA protein in CCl4 rats. The representative pictures of α-SMA and α-tubulin and quantified histograms of Western blotting revealed that high-dose SHP treatment (SHP 2.5 g/kg) significantly inhibited α-SMA expression. Data are means ± SD of six rats. $^{***}: P < 0.001$ between control and CCl4 alone groups; $^{###}: P < 0.001$ vs CCl4 alone group.
Double Immuno-Fluorescence Staining for α-SMA and MMP-2

In the immuno-staining for α-SMA, α-SMA-positive cells (activated HSCs) were observed in the fibrous septa, portal tracts and sinusoid of livers in CCl4 rats (Fig. 6b). Most α-SMA-positive HSCs (red) in CCl4 rats were co-localized with MMP-2 (green) in the merge panel (Fig. 6b, yellow, arrowhead). However, both low-dose (Fig. 6c) and high-dose (Fig. 6d) SHP treatments markedly reduced the number of α-SMA-positive HSCs as shown in Fig. 3, and the MMP-2-positive HSCs in the liver of CCl4 rats (Figs. 6c and d).

Analysis of TGF-β1 Gene Transcripts by qRT-PCR

There was a significant increase in the mRNA expression level of TGF-β1 (P < 0.01) in CCl4-alone group as compared to the control group (Fig. 7). Both low-dose and high-dose SHP treatments tended to decrease the mRNA expression of TGF-β1, and the high-dose SHP group showed a significant reduction (P < 0.05, Fig. 7).

In Vitro Effects of SHP on HSC-T6 Cells

Inhibition of α-SMA Expression in TGF-β1-Treated HSC-T6 Cells

The ratio of α-SMA:α-tubulin protein expression was 1.49 ± 0.04 after TGF-β1 (1 ng/ml) stimulation in HSC-T6 cells. This ratio was concentration-dependently reduced to 0.85 ± 0.04 and 0.33 ± 0.03 by the SHP concentration at the range of 50 to 200 µg/ml (P < 0.001, Fig. 8). The inhibitory effect of SHP on TGF-β1-treated HSC-T6 cells was not due to its cytotoxicity, as SHP induced cytotoxicity only at concentrations above 2.5 mg/ml.

Inhibition of Cellular MMP-2 Secretion in TGF-β1-Treated HSC-T6 Cells

Only MMP-2 was detectable in the medium and cell lysates of TGF-β1-treated HSC-T6 cells by gelatin zymography. (a) A representative picture of zymography for MMP-2 activities; (b) Activities of proMMP-2 and MMP-2 levels were analyzed by computerized densitometry. Data are means ± SD of six rats. **: P < 0.01, ***: P < 0.001 between control and CCl4 alone groups; #: P < 0.05, ##: P < 0.01, CCl4-alone group.
zymography. The relative activity of MMP-2 in the medium and HSC-T6 cell lysate were concentration-dependently decreased by SHP administration in TGF-β1-treated HSC-T6 cells (Fig. 9). Secretory MMP-2 (in the medium) was significantly reduced by SHP at concentrations of 100 to 200 µg/ml (P < 0.01, 0.001, Fig. 9a), and the MMP-2 expression in HST-T6 cells was also significantly inhibited by SHP at concentrations of 50 to 200 µg/ml (P < 0.001, Fig. 9b).

**Discussion**

In the present study, we observed that the traditional Chinese herbal medicine, SHP, effectively reduced CCl4-induced rat hepatic fibrosis. The results showed that hepatic fibrosis/degeneration scores, collagen content, mRNA expression of TGF-β1 gene, protein expression of α-SMA and activity of proMMP-2 and active MMP-2 in CCl4-induced rats were significantly reduced by SHP treatment. The anti-fibrotic effects of SHP were also confirmed in TGF-β1-treated HSC-T6 cells in vitro. Moreover, levels of serum AST and ALT activities in CCl4-induced rats were reduced by SHP treatment. These findings suggest that SHP exerted anti-fibrotic effects on CCl4-induced rat liver fibrosis via inhibition of HSC activation.

Immunohistostaining analysis showed that activated HSC numbers were decreased by SHP...
treatment (Figs. 3 and 6). In the injured liver, HSCs are the main cellular source of MMP-2. Active MMP-2 may contribute to disease progression through degradation of normal liver matrix and pro-proliferative effects on HSCs (10), and over-expression of MMP-2 has been demonstrated in the fibrotic liver (1, 6). In this study, we found that proMMP-2 and activated MMP-2 levels were significantly reduced in SHP treatment group as compared to CCl_4 group in vivo (Figs. 5 and 6) and TGF-β1-treated HSC-T6 cells in vitro (Fig. 9), and this suggests that SHP might down-

regulate the mitogenic and proinvasive effects of HSCs.

In experimental and human hepatic fibrosis, expression of TGF-β1 has been found to be strongly increased, and TGF-β1 induced ECM production by HSCs (7). It has been reported that TGF-β1 stimulates collagen synthesis and pro-collagen I mRNA expression in cultured HSCs (4, 7, 8, 25). In the present study, we also found that high-dose SHP treatment significantly reduced mRNA expression of TGF-β1, and SHP treatment also significantly reduced mRNA expression of procollagen I in CCl_4 rats. Although there are as yet no clinically efficacious anti-fibrotic agents, experimental studies have been constantly conducted to assess potentials of agents targeting the reduction of inflammation, inhibition of HSC activation or proliferation, induction of HSC apoptosis, or promotion of scar matrix degradation (8, 24). There are several traditional herbal medicines, including Sho-saiko-to (TJ-9) (28-30), Inchinko-to (TJ135) (16, 17, 29), silymarin (5, 18), Salvia miltiorrhiza (15, 22, 23) and Han-Dan-Gan-Le (20) with reported anti-fibrotic effects. In the present study, SHP treatment
resulted in decreased levels of serum AST and ALT (Fig. 1). The results demonstrated that SHP administration had hepatoprotective effects in CCl₄-induced rats. Furthermore, SHP treatment led to decreased periportal and bridging fibrosis, and fibrotic septa becoming thinner and interrupted. In addition, the fibrosis score showed a greater improvement in the high-dose SHP group than the CCl₄ group.

In summary, our results showed that an aqueous extract of the traditional Chinese medicine SHP ameliorated CCl₄-induced rat hepatic fibrosis, and the anti-fibrotic effects of SHP were associated with reduction of MMP-2 expression, hepatic collagen content and the number of activated HSCs. These findings might be important in developing a clinically therapeutic strategy for hepatic fibrosis.

Acknowledgments

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References
