Berberine Inhibits Hepatic Stellate Cell Proliferation and Prevents Experimental Liver Fibrosis

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Proliferation of hepatic stellate cells (HSCs) is central for the development of fibrosis during liver injury. Our aim in this study was to determine whether berberine could inhibit HSC proliferation in vitro and prevent experimental liver fibrosis in vivo. Activated rat hepatic stellate cells (CFSCs) were incubated with various concentrations (0–20 μg/ml) of berberine. After 48 h incubation, berberine significantly inhibited CFSC proliferation and induced cell cycle arrest in G1 phase. Real-time and Western blotting revealed that both p21 and p27 expression was markedly reduced by berberine. Berberine also decreased Akt phosphorylation and FOXO1 phosphorylation, which led to FOXO1 nuclear translocation. Berberine effectively prevented CCl4-induced liver fibrosis in mice, which was accompanied by a decrease in the number of activated HSCs. Thus, berberine was able to prevent liver fibrosis by inhibition of hepatic stellate cell proliferation.

Key words liver fibrosis; hepatic stellate cell; berberine; cell cycle; cell proliferation

Hepatic fibrosis is a wound-healing response to various chronic liver injuries, including viral, metabolic, genetic, and cholestatic liver diseases. Hepatic stellate cells (HSCs), vitamin A-storing quiescent cells located in the space of Disse, play a crucial role in hepatic fibrosis development. During liver fibrosis, HSCs are activated and changed into myofibroblast-like cells, which are characterized by increased proliferation and extracellular matrix synthesis. It is widely accepted that the suppression of HSC proliferation is an effective therapeutic strategy for the treatment and prevention of hepatic fibrosis.

Berberine is an isoquinoline alkaloid present in a number of important medicinal plant species, such as Berberis aristata and Berberis aquifolium. Berberine exhibits multiple pharmacological properties, including antibacterial, anti-hypertensive, anti-inflammatory, antidiabetic, and anti-hyperlipidemic activities. Previous studies have demonstrated that berberine inhibits the proliferation of several tumour cell lines through the induction of G1 phase arrest or apoptosis. Wang et al. investigated the effect of berberine on rat liver fibrosis induced by multiple hepatotoxic factors, including CCl4, ethanol, and high cholesterol, demonstrating that berberine could prevent experimental liver fibrosis by regulating the anti-oxidant system and lipid peroxidation. However, it remains unclear whether berberine directly inhibits HSC proliferation.

Here, we investigated the effect of berberine on HSC proliferation and the underlying molecular mechanism. We also examined the protective effects of berberine against liver fibrosis in a CCl4-treated mouse model.

MATERIALS AND METHODS

Reagents and Cell Culture The cell line rat hepatic stellate cell (CFSC) is an activated rat HSC clone established from a cirrhotic rat liver induced by repeated CCl4 injections, and was kindly provided by Dr. Shudong Xiao (Renji Hospital, Shanghai). CFSCs were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen, Carlsbad, CA, U.S.A.) with 10% foetal bovine saline (FBS, Invitrogen) at 37 °C in a humidified atmosphere of 95% air and 5% CO2. Highly purified berberine (BBR) was purchased from Sigma (St. Louis, U.S.A.) (Fig. 1A). Berberine was dissolved in dimethyl sulfoxide (DMSO) and was used with a final concentration of 0.1% DMSO in the medium.

MTT Assay for Cell Proliferation The effect of berberine on cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as described previously. Briefly, 5×103 cells/well were plated in 96-well culture plates. After an overnight incubation, the cells were treated with various concentrations of berberine (0, 1, 2.5, 5, 10, or 20 μg/ml) for 48 h. Ten microliters of 5 mg/ml MTT (Genebase Gene-Tech Co. Shanghai, China) was added to each well for an additional 4 h, and the resulting formazan crystals were dissolved in triad solutions [10% sodium dodecyl sulfate (SDS), 5% isopropylcarbinol, 0.01 mol/l HCl (w/v/v)]. The absorbance at 570 nm was read by a Multiscan MK3 (Thermo Fisher Scientific, Waltham, MA, U.S.A.).

Cell Cycle Analysis Cell cycle analysis was performed by flow cytometry. CFSCs were treated with various concentrations of berberine for 48 h. After harvested and washed twice with phosphate buffered saline (PBS), cells were suspended and fixed in 1 ml of 70% ethanol for 2 h at 4 °C. The cells were centrifuged at 1000 rpm for 5 min, washed with cold PBS, resuspended in 500 μl of PBS, and incubated at 37 °C with 5 μl RNase A for 30 min (40 μg/ml final concentration, Watson Biotechnologies Inc., Shanghai, China). Staining was performed with propidium iodide (10 μg/ml final concentration, Sigma) on ice for 30 min in the dark. The cells were then analyzed with a FACS Caliber using Cell Quest software (Becton Dickinson Co., San Jose, CA, U.S.A.).

Real-Time Polymerase Chain Reaction (PCR) The mRNA levels of p21, and p27 were evaluated by quantitative real-time PCR. The total RNA was extracted using TRizol Reagent (Invitrogen) and treated with DNase I at 37 °C for 30 min. The cDNA was synthesized from 1 μg of total RNA.
using 15 U MMLV reverse transcriptase (Invitrogen) in 20 μl of a reaction mixture containing 0.5 μg oligo(dT) 18 primer, 40 U recombinant RNase inhibitor (Takara, Dalian, China), reverse transcription buffer, and 1 mM of each dNTP (Takara). The reaction was incubated at 37 °C for 50 min, followed by 70 °C for 5 min and 4 °C for 5 min. The cDNA samples were diluted 10-fold with nuclease-free water. Real-time PCR was then performed in an Opticon machine (MJ Research, Waltham, MA, U.S.A.) using SYBR Green I Dye (Takara), with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as a reference, and the results were evaluated with Opticon Monitor software. Primer sequences for p21 were upstream: 5'-GCA AAG TAT GCC GTC GTC T-3' and downstream: 5'-CAA AGT TCC ACC GTT CTC G-3'. For p27, the primers were upstream: 5'-GAG GGC AGA TAC GAG TGG CAG -3' and downstream: 5'-CTG GAC ACT GCT CCG CTA ACC -3'. For GAPDH, the primers were upstream: 5'-TCC TGC ACC ACC TGC TTA G-3' and downstream: 5'-AGT GGC AGT GAT GGC ATG GAC T-3'.

**Western Blot**
Protein was collected by lysing cells in RIPA lysis buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 0.1% SDS, 1% TritonX-100, 1% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride (PMSF)). Each sample (30 μg of cellular protein) was size-fractionated using SDS-polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto polyvinylidene difluoride (PVDF) transfer membranes (DuPont, Boston, MA, U.S.A.). Blots were incubated for 1 h at room temperature in 5% BSA for blocking, and proteins were detected with antibodies against pFoxO1 (Ser-256), pAkt (Ser-473), Akt, p21, p27 (Cell Signaling Technology, Beverly, MA, U.S.A.), and FoxO1 (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) overnight. Horseradish peroxidase-conjugated goat anti-rabbit IgG was used as the secondary antibodies at a dilution of 1:1000 (Cell Signaling, Beverly, MA, U.S.A.). The immunoblots were visualized using ECL substrate (GE Healthcare, Bucks, U.K.).

**Subcellular Localization of FoxO1**
The enhanced green fluorescent protein (EGFP)-FoxO1 plasmid was a gift from Professor D. Accili (Columbia University, New York). CFSCs were transfected using FuGENE 6 reagent (Roche Diagnostics, Indianapolis, IN, U.S.A.) according to the manufacturer's instruction. Briefly, CFSCs were grown to 50—60% confluence and then transfected with 1 μg of EGFP-FoxO1 plasmid DNA. After 24 h of transfection, CFSCs were treated with 20 μg/ml berberine for 24 h. EGFP-FoxO1 protein localization was observed using a fluorescence microscope (DP-70, Olympus, Tokyo, Japan).

**Animal Studies**
Six-week-old C57BL/6 male mice were purchased from Shanghai Slac Laboratory Animal Co., Ltd. (Shanghai, China) and housed in a temperature-controlled environment (20—22 °C) with a 12:12 h light:dark cycle. Access to food and tap water was ad libitum throughout the study period. Mice were divided into five groups, with 12 animals in each group: The control group (receiving olive oil only), the CCl4 group, and three treatment groups (receiving 100, 200, or 400 mg/kg of berberine, intragastrically). Mice from the CCl4 group and the berberine treatment groups were injected intraperitoneally with 0.1 ml/kg of CCl4 dissolved in olive oil (v:v=1:10) twice a week for 6 weeks. For the treatment groups, berberine was dissolved in 0.5% sodium carboxymethylcellulose and administered daily during the six weeks of CCl4 administration. After six weeks, blood was collected, centrifuged at 3000 rpm for 10 min at 4 °C, and serum was separated to detect ALT and AST activities using kits from Rongsheng Co. (Shanghai, China). The animals were then sacrificed for histological examination of the liver. All experimental protocols were approved by the Committee for the Care and Use of Laboratory Animals of Shanghai Institute of Materia Medica, Chinese Academy of Sciences.

**Liver Histology and Immunohistochemistry**
The rat livers were routinely fixed in 10% formalin and embedded in paraffin, then 4-μm-thick sections were stained with haematoxylin and eosin (HE) for routine histology and with Van Gieson solution for collagen. The sections were incubated with an α-smooth muscle actin (α-SMA) antibody (1:100 dilution, Boster Biological Technology, Wuhan, China) overnight at 4 °C and then with FITC-conjugated horse anti-mouse IgG antibody (Vector Laboratories, Inc., Burlingame, CA, U.S.A.) for 1 h at 37 °C. Sample was observed under a microscope (DP-70).

**Statistical Analysis**
All values are expressed as the mean±S.D. Statistical analysis was performed by Dunnett's test. Differences were considered significant when the p value was <0.05.

**RESULTS**

**Berberine Inhibited CFSC Proliferation**
The chemical structure of berberine is shown in Fig. 1A. CFSCs were treated with various concentrations of berberine (0, 1, 2.5, 5, 10, or 20 μg/ml) for 48 h. The MTT assay revealed that berberine significantly inhibited CFSC proliferation in a dose-dependent way (Fig. 1B). Cell proliferation was decreased by 31.4±1.1% at 10 μg/ml, or 53.2±0.9% at 20 μg/ml of berberine. The inhibition of cell proliferation by berberine was also observed under the microscope (Fig. 1C).

**Berberine Induced G1 Phase Cell Cycle Arrest in CFSCs**
To clarify the possible mechanism of the anti-proliferative activity of berberine, flow cytometry was used to determine the cell cycle distribution in CFSCs following 0, 5, 10, or 20 μg/ml doses of berberine treatment for 48 h. Berberine induced a significant increase in G1 phase cells (Table 1). This increase in G1 phase cells corresponded to a significant decrease in S phase cells and a minimal increase in G2-M cells. This assay indicates that berberine inhibited CFSC proliferation by inducing G1 phase arrest.

**Berberine Increased Protein and mRNA Levels of p21 and p27 in CFSCs**
We next assessed the influence of berberine on p21 and p27 gene expressions, which regulate the transition from the G1 to S phase. Treatment of CFSCs with berberine for 48 h resulted in a dose-dependent increase in the protein levels of p21 and p27 (Fig. 2A). Real-time PCR revealed that 20 μg/ml of berberine resulted in a 2.1-fold increase of p21 mRNA level and a 1.7-fold increase of p27 mRNA level, respectively (Fig. 2B). These results demonstrated that berberine induced G phase arrest in CFSCs via up-regulation of P21 and P27 expression.

**Berberine Inhibited Protein Phosphorylation of FoxO1 and Akt**
The phosphorylation status of FoxO1 and Akt in response to berberine treatment was determined by Western blot. Compared with GAPDH protein level, both
phospho-FoxO1 (Ser-256) and phospho-Akt (Ser-473) protein levels were markedly decreased by treatment of 20 μg/ml berberine for 24 h, while there was no significant change in the total FoxO1 and Akt levels (Fig. 3B).

Generally, the dephosphorylation of FoxO1 leads to FoxO1 nuclear translocation, which reflects its activation status. Transfecting CFSC cells with the pEGFP-FoxO1 plasmid revealed that most EGFP-FoxO1 fusion proteins were localized in the cytoplasm in control cells (Fig. 3A, 0 h), however, EGFP-FoxO1 proteins predominantly localized to the nuclei in CFSCs treated with 20 μg/ml berberine for 24 h (Fig. 3A, 24 h).

Thus, inhibition of HSC proliferation by berberine may be associated with dephosphorylation of Akt and FoxO1 nuclear translocation.

**Berberine Prevented CCl4-Induced Liver Fibrosis**

CCl4-injected mice had higher serum alanine transaminase (ALT) and aspartate aminotransferase (AST) levels than the control group, indicating hepatic injury (Fig. 4). Levels of both ALT and AST in CCl4-injected mice were significantly decreased in the 200 or 400 mg/kg/d berberine groups. Histopathologic examination of the liver specimens showed an expanded fibrotic septa area in CCl4-injected mice compared with the control group (Figs. 5A, B), while berberine treatment clearly suppressed this expansion (Fig. 5C, 400 mg/kg/d). Van Gieson staining revealed that berberine

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**Table 1. Effect of Berberine on the Cell Cycle Progression of CFSC Cells**

<table>
<thead>
<tr>
<th>Conc. (μg/ml)</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>38.21 ± 1.32</td>
<td>45.51 ± 2.45</td>
<td>16.28 ± 1.76</td>
</tr>
<tr>
<td>5</td>
<td>44.65 ± 2.04*</td>
<td>38.61 ± 1.54*</td>
<td>17.74 ± 2.34</td>
</tr>
<tr>
<td>10</td>
<td>48.47 ± 1.63*</td>
<td>34.53 ± 2.63*</td>
<td>17.00 ± 1.65</td>
</tr>
<tr>
<td>20</td>
<td>51.06 ± 3.14*</td>
<td>31.29 ± 2.54*</td>
<td>17.65 ± 2.40</td>
</tr>
</tbody>
</table>

Results are expressed as mean±S.D. Significant difference from the control group (*p < 0.05).
(400 mg/kg/d) markedly reduced CCl4-induced hepatic collagen deposition (Figs. 5D—F). These results indicate that berberine prevented CCl4-induced liver fibrosis in mice.

Moreover, α-SMA-positive cells (activated HSCs) were observed by in the fibrotic septa, portal tracts, and sinusoid of the livers in CCl4-injected mice by immunohistochemistry (Figs. 5G—I). After treatment of berberine, the number of α-SMA-positive cells was markedly decreased, demonstrating inhibition of activated HSC proliferation in vivo.

**DISCUSSION**

The inhibition of HSC proliferation is considered to be one promising therapeutic strategy to prevent the progression of hepatic fibrosis in chronic liver diseases. In the present study, we found that berberine significantly inhibits the proliferation of the rat stellate cell line CFSC in vitro, and prevents liver fibrosis in CCl4-injected mice in vivo.

Treatment of CFSCs with berberine induced G1 phase arrest, leading to the inhibition of CFSC cell proliferation. Cyclin-dependent kinase (CDK) complexes are serine/threonine kinases that drive cell-cycle progression and proliferation by phosphorylation of key substrates. The CDKs inhibitors p21 and p27 regulate the progression of the cell cycle in the G0—G1 phase. The induction of these proteins causes a blockade of the G1 to S transition, thereby resulting in a G1 phase arrest. The importance of p21 and p27 in the transition from the G1 to S phase in HSCs has been reported. We demonstrated that berberine treatment increased both the mRNA and protein levels of p21 and p27 in CFSCs.

FoxO transcription factors control a variety of target
genes, including antioxidant genes and regulators of metabolism, cell cycle, and cell fate. Several studies have demonstrated that p21 and p27 are specific transcriptional targets of FoxO that link to cell-cycle arrest in the G1 phase. A previous study reported that p27 is the crucial downstream target of the FoxO1 to control HSC proliferation and differentiation. The nuclear localization of FoxO1 is a prerequisite for transcriptional activation, which results in increased transcription of p21 and p27. The Akt kinase pathway inhibits FoxO1 transcriptional activity by phosphorylating FoxO1, since phospho-FoxO1 resides in the cytoplasm. Berberine induced the subcellular redistribution of FoxO1 from the cytoplasm into the nucleus in CFSC cells, which was accompanied by a reduction of both phospho-FoxO1 (Ser-256) and phospho-Akt. These results indicate that inhibition of HSC proliferation by berberine may be associated with FoxO1 nuclear localization, which led to up-regulation of p21 and p27 expression and subsequent G1 phase arrest.

Using a classical mouse model of hepatic fibrosis induced by CCl_4, we demonstrated that berberine dramatically decreased the number of activated HSCs, the amount of fibrotic septa, and the content of hepatic collagen. It is well accepted that HSCs play a central role in the development of liver fibrosis and that suppression of HSC proliferation may therefore be effective in the resolution of liver fibrosis. A previous study showed that berberine could prevent experimental rat liver fibrosis by regulating the anti-oxidant system and the lipid peroxidation induced by multiple hepatotoxic factors. Our data suggest that the anti-proliferative effect of berberine on HSC cells may be another important molecular mechanism of its anti-hepatic fibrosis activity.

In conclusion, we demonstrated that berberine directly inhibits the proliferation of CFSC cells by increasing p21 and p27 expression and inducing G1 arrest, which may be related to regulating Akt/FoxO1 signalling pathway. Moreover, berberine prevents liver fibrosis in experimental models, which was at least partly due to the decreased number of hepatic stellate cells. Further clinical investigations would be necessary to determine whether berberine could be effective for the prevention of liver fibrosis in humans. Also, berberine analogues or other small molecules that share these properties may serve as good drug candidates for liver fibrosis.

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