Emodin protects rat liver from CCl₄-induced fibrogenesis via inhibition of hepatic stellate cells activation

Miao-Xian Dong, Yan Jia, Ying-Bo Zhang, Cheng-Chong Li, Yu-Tao Geng, Li Zhou, Xue-Yan Li, Ji-Cheng Liu, Ying-Cai Niu

AIM: To investigate the role of emodin in protecting the liver against fibrogenesis caused by carbon tetrachloride (CCl₄) in rats and to further explore the underlying mechanisms.

METHODS: Rat models of experimental hepatic fibrosis were established by injection with CCl₄; the treated rats received emodin via oral administration at a dosage of 20 mg/kg twice a week at the same time. Rats injected with olive oil served as a normal group. Histopathological changes were observed by hematoxylin and eosin staining. The activities of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum and hepatic hydroxyproline content were assayed by biochemical analyses. The mRNA and protein relevant to hepatic stellate cell (HSC) activation in the liver were assessed using real-time reverse transcription-polymerase chain reaction (RT-PCR), immunohistochemistry, western blotting and enzyme-linked immunosorbert assay.

RESULTS: The degree of hepatic fibrosis increased markedly in the CCl₄ group compared to the normal group (P < 0.01), and decreased markedly in the emodin group compared to the CCl₄ group according to METAVIR scale (P < 0.01) compared with those in the normal control group (51.02 ± 10.64 IU/L and 132.28 ± 18.14 IU/L). The activities of serum ALT and AST were significantly higher in rats injected with CCl₄ (289.25 ± 68.84 IU/L and 423.89 ± 35.67 IU/L, both P < 0.05). Compared with the normal controls (54.53 ± 13.46 mg/g), hepatic hydroxyproline content was significantly higher in rats injected with CCl₄ (120.27 ± 28.47 mg/g, P < 0.05). Hepatic hydroxyproline content was significantly reduced in the rats treated with emodin at 20 mg/kg (71.25 ± 17.02 mg/g, P < 0.05). Emodin significantly protected the liver from injury by reducing serum AST and ALT activities and reducing hepatic hydroxyproline content. The mRNA levels of transforming growth factor-β1 (TGF-β1), Smad4 and α-SMA in liver tissues were significantly down-regulated in SD rats that received emodin treatment. Furthermore, significant down-regulation of serum TGF-β1 protein levels and protein expression of Smad4 and α-SMA in liver tissues was also observed in the rats. Emodin inhibited HSC activation by reducing the abundance of TGF-β1 and Smad4.

CONCLUSION: Emodin protects the rat liver from CCl₄-induced fibrogenesis by inhibiting HSC activation. Emodin might be a therapeutic antifibrotic agent for the treatment of hepatic fibrosis.

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Key words: Emodin; Hepatic fibrosis; Transforming growth factor-β1; Smad4; Hepatic stellate cell; α-smooth muscle actin

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Dong MX, Jia Y, Zhang YB, Li CC, Geng YT, Zhou L, Li XY, Liu JC, Niu YC. Emodin protects rat liver from CCl₄-induced fibrogenesis via inhibition of hepatic stellate cells activation.
The animals were obtained from the Beijing Vital River (weight range: 200-240 g) were employed in the study. Of 50 pathogen-free male Sprague-Dawley (SD) rats this study was approved by the Animal Care and Use Committee of the Chinese Medical Association Committee of Fatty Liver Disease in 2006 and Nouchi et al. Steatosis was determined according to the criteria described by Proctor et al. with minor modifications. Fifty male SD rats were randomLy divided into three groups: the normal control (n = 10) in which rats were not administrated CCl4 or emodin, but they were injected with olive oil and orally given sodium carboxymethylcellulose (CMC); the CCl4 group (n = 20) in which rats were subcutaneously injected with CCl4, without emodin treatment; the emodin group in which rats were injected with CCl4 and treated with emodin at 20 mg/kg. Rats from the emodin group and the CCl4 group were subcutaneously injected with a mixture of 40% CCl4 (a mixture of pure CCl4 and sterile olive oil) at 200 µL/100 g body weight twice weekly for 12 wk. Emodin was dissolved in 0.5% sodium CMC and given once daily by gavage at 20 mg/kg. The rats in the normal group were similarly handled, including subcutaneous injections with the same volume of olive oil and oral administration of the same volume of CMC without emodin. At the end of the experiment, the survivors in the normal group, CCl4 group and emodin group were 10/10, 9/20 and 11/20, respectively.

Forty-eight hours after the last CCl4 injection, rats were sacrificed after being anesthetized by i.p. pentobarbital (50 mg/kg). A small portion of the liver was removed for hematoxylin and eosin (HE) staining and treated with formalin. The remaining liver was cut in pieces and rapidly frozen with liquid nitrogen for extraction of total RNA and protein. Blood was collected directly from the rats when they were sacrificed. Serum was separated by centrifugation within 1 h of blood collection and stored at -20°C until analyzed.

**Light microscopy**

Midsections of the liver lobe a few mm thick were taken from each rat and processed for observation by light microscopy. The process involved fixing the tissue specimen in 10% neutral buffered formalin solution, preparing the block in paraffin, cutting into 5-6 µm thick sections, and staining the sections with HE. The sections were scanned and analyzed by a pathologist who was blinded to the different treatments in the experiment.

The histological changes were measured on HE stained sections. Lobular inflammatory activity and severity of liver steatosis were determined according to the criteria of the Chinese Medical Association Committee of Fatty Liver Disease in 2006 and Nouchi et al. Steatosis was

**INTRODUCTION**

Hepatic fibrosis occurs in advanced liver disease, where normal hepatic tissue is replaced with collagen-rich extracellular matrix (ECM) and, if left untreated, results in cirrhosis. Several studies have shown that hepatic fibrosis is a reversible disease, therefore an effective treatment would probably prevent or reverse the fibrotic process in the liver[1,2]. Transforming growth factor β1 (TGF-β1) is one of the strongest profibrotic cytokines[3,4], and TGF-β1/Smad signaling is the cardinal signal transduction pathway involved in fibrosis which has been verified by several related studies[5]. The down regulation of TGF-β1 expression and modulation of TGF-β/Smad signaling may be effective in preventing liver fibrosis[6].

In the last decade, advances in the understanding of genes promoting hepatic stellate cell (HSC) activation are impressive[7]. However, there are few breakthroughs in therapeutic intervention of hepatic fibrogenesis. Efficient and well-tolerated antifibrotic drugs are lacking and current treatment of hepatic fibrosis is limited to withdrawal of the noxious agent[8]. Therefore, research identifying innocuous antifibrotic agents is of high priority and urgently needed.

Emodin (1,3,8-trihydroxy-6-methylanthraquinone), isolated from the rhizome of the Giant Knotweed Rhizome, has been used for centuries in Asia as a treatment for inflammation, gastrointestinal, pulmonary, and liver disorders. Emodin is regarded as the most active constituent in Giant Knotweed Rhizome and exerts many potent biological effects[9,10], such as anticancer[11], antimicrobial[12], and anti-inflammatory effects[13]. Several studies have revealed that emodin is efficacious in the management of hepatic fibrosis[14,15]. However, the mechanisms underlying remain to be elucidated.

The current study evaluates the in vivo role of emodin in the protection of the liver from fibrogenesis caused by carbon tetrachloride (CCl4) in a rat model and further explores the underlying mechanisms. We hypothesize that emodin might protect the liver from CCl4-induced fibrogenesis by inhibiting activation of HSC via modulating TGF-β1/Smad signaling pathways. Results in this study support our hypothesis and provide novel insight into the mechanisms of emodin in the protection of the liver.

**MATERIALS AND METHODS**

**Animals**

This study was approved by the Animal Care and Use Committee of Qiqihar Medical University. A total of 50 pathogen-free male Sprague-Dawley (SD) rats (weight range: 200-240 g) were employed in the study. The animals were obtained from the Beijing Vital River Experimental Animals Technology (Beijing, China), and were housed in sterile cages under laminar airflow hoods in a specific pathogen-free room with a 12 h light and 12 h dark schedule and fed autoclaved chow and water ad libitum. The animals were weighed every 7 d for the adjustment of the CCl4 and emodin doses. Emodin was purchased from Xi’an Sino-Herb Bio-Technology CO., LTD (Purity: 98% by HPLC).

**Establishment of a rat model with hepatic fibrogenesis caused by CCl4**

The rat model was established using the method originally described by Proctor et al. and since used by many others[17], with minor modifications. Fifty male SD rats were randomly divided into three groups: the normal control (n = 10) in which rats were not administrated CCl4 or emodin, but they were injected with olive oil and orally given sodium carboxymethylcellulose (CMC); the CCl4 group (n = 20) in which rats were subcutaneously injected with CCl4, without emodin treatment; the emodin group in which rats were injected with CCl4 and treated with emodin at 20 mg/kg. Rats from the emodin group and the CCl4 group were subcutaneously injected with a mixture of 40% CCl4 (a mixture of pure CCl4 and sterile olive oil) at 200 µL/100 g body weight twice weekly for 12 wk. Emodin was dissolved in 0.5% sodium CMC and given once daily by gavage at 20 mg/kg. The rats in the normal group were similarly handled, including subcutaneous injections with the same volume of olive oil and oral administration of the same volume of CMC without emodin. At the end of the experiment, the survivors in the normal group, CCl4 group and emodin group were 10/10, 9/20 and 11/20, respectively.

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The histological changes were measured on HE stained sections. Lobular inflammatory activity and severity of liver steatosis were determined according to the criteria of the Chinese Medical Association Committee of Fatty Liver Disease in 2006 and Nouchi et al. Steatosis was
graded on the basis of the extent of parenchyma involved as Grade 0, no hepatocytes were involved; Grade 1, < 30% of hepatocytes were involved; Grade 2, 30% to 50% of hepatocytes were involved; Grade 3, 51% to 75% of hepatocytes were involved; Grade 4, > 75% of hepatocytes were involved. Inflammation was graded as Grade 1, focal collections of mononuclear inflammatory cells; Grade 2, diffuse infiltrates of mononuclear inflammatory cells; Grade 3, focal collections of polymononuclear cells in addition to mononuclear cell infiltrates; and Grade 4, diffuse infiltrates of polymononuclear cells in the parenchymal area or lobular area. The stage of liver fibrosis was graded with the METAVIR scale [20], which grades fibrosis on a five-point scale: Grade 0, no fibrosis; Grade 1, portal fibrosis without septa; Grade 2, portal fibrosis with a few septa; Grade 3, numerous septa without cirrhosis; and Grade 4, cirrhosis.

Biochemical parameters
Activities of alanine transaminase (ALT) and aspartate aminotransferase (AST) in serum were measured by routine laboratory methods using a 7170-automatic biochemistry analyzer (Tokyo, Japan).

Determination of the hepatic hydroxyproline content
The hydroxyproline kit was purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The content of hepatic hydroxyproline was determined by using the hydroxyproline kit following the protocol provided by the manufacturer. Results were expressed as micrograms of hydroxyproline per gram of hepatic tissue.

Enzyme-linked immunosorbent assay (ELISA)
The TGF-β1 ELISA kit was obtained from Boster Biotechnology Co. Ltd. (Wuhan, China). The levels of TGF-β1 in serum were determined by using the TGF-β1 ELISA kit according to the manufacturer’s protocol. In brief, 100 µL of a serum sample was added to each well of the plate, followed by incubation for 2 h at 37°C. A Working Detector (100 µL; Boster Biotechnology Co. Ltd) was loaded into each well, and the plate was incubated for an additional 1 h at room temperature (RT) before the addition of substrate solution (100 µL; Boster Biotechnology Co. Ltd). The reaction was stopped by adding stop solution (1 drop; Boster Biotechnology Co. Ltd). The absorbance was read at 492 nm using a Microplate reader (LabSystems Multiskan Ascent 354, Finland). Calculation of the concentrations of TGF-β1 was performed in a log-log linear regression according to the instructions in the protocol.

IHC analysis
Liver tissues were fixed in 10% neutral buffered formalin solution, embedded in paraffin, and stained for routine histology. The sections were incubated at 4°C overnight with primary antibody (Boster Biotechnology, Wuhan, China) in concentrations of 1:100 (Smad4) and 1:200 (α-SMA). As a secondary antibody, horseradish peroxidase-conjugated immunoglobulin G (Boster Biotechnology), was used for 30 min at 37°C. After further washing with Tris-buffered saline, sections were incubated with complex/horseradish peroxidase (1:200 dilution) for 30 min at 37°C. Immunolocalization was performed by immersion in 0.05% 3,3′-diaminobenzidine tetrahydrochloride as chromagen. Slides were counterstained with hematoxylin before dehydration and mounting. Incubation without the primary antibody was performed as a control for the background staining. Histological evaluation was performed by a pathologist who was blind to the pharmacological characteristics of the drugs.

RNA isolation and real-time reverse transcription polymerase chain reaction (RT-PCR)
Total RNA was extracted from 100 mg of frozen liver tissues using RNAiso Reagent kit (Takara Biotechnology, Dalian, China) according to the protocol provided by the manufacturer. cDNA was synthesized with SYBR ExScript™ RT-PCR kit (Takara Biotechnology, Dalian, China) according to the protocol provided by the manufacturer. Reverse transcription was carried out as follows: 42°C for 15 min, 95°C for 2 min (one cycle). cDNA was stored at -20°C for PCR. Real-time PCR was performed in 50 µL of reaction solution containing 2 × SYBR Premix Ex Taq polymerase, deoxynucleoside triphosphates, ROX Reverser Dye and the corresponding primers. The cycles for PCR were as follows: 1 cycle of 95°C for 10 s, 40 cycles of 5 s at 95°C, 5 s at 60°C, 31 s at 60°C and a final 7 min at 72°C. Melting curve analysis was always included to validate the specificity of the PCR products. Serial cDNA dilution curves were produced to calculate the amplification efficiency for all genes. A graph of threshold cycle (Ct) versus log 10 relative copy number of the sample from a dilution series was produced. The slope of the curve was used to determine the amplification efficiency. Reactions were performed in an ABI7300 Real-time PCR system (Applied Biosystems, CA) and threshold cycle (Ct) data were collected using the Sequence Detection Software version 1.2.3 (Applied Biosystems, CA). GAPDH was used as an internal control. mRNA -fold change relative to GAPDH was calculated with the comparative Ct method of 2-ΔΔCt[21]. The following primers were used.

**5′-GACAACCTTTGGCATCGTGA-3′ (sense) and 5′-ATGCAAGGATGATGTTCTGG-3′ (antisense) for the GAPDH gene; 5′-CTCTATGCTTCTGCTCCTGCTTC7TCAAA-3′ (sense) and 5′-CCACCTGACGTTTCCGTTATTC-3′ (antisense) for the Smad4 gene; 5′-TTATTCGACACCAATGGGTTGGT-3′ (sense) and 5′-TGCTGTCACAGGTCCGTGAG-3′ (antisense) for the TGF-β1 gene; 5′-CCGAGATCTCACCAGACTAC-3′ (sense) and 5′-TCCAGACCCACATAGCACAG-3′ (antisense) for the α-SMA gene.**

The content of hepatic hydroxyproline was determined by using the hydroxyproline kit following the protocol provided by the manufacturer. Results were expressed as micrograms of hydroxyproline per gram of hepatic tissue. The hydroxyproline kit was purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). The hydroxyproline kit was purchased from Nanjing Jiancheng Bioengineering Research Institute (Nanjing, China). Determination of the hepatic hydroxyproline content

**Western blotting**
Cytoplasmic proteins were isolated from 120 mg of frozen liver tissues using a Cytoplasmic Protein
Extraction kit (Beyotime Biotechnology, Haimen, China) according to the protocol provided by the manufacturer. Protein concentrations were determined using the BCA Protein Assay kit according to the protocol provided by the manufacturer (Beyotime Biotechnology, Haimen, China). 100 µL of supernatant was added to an equal volume of 2 × SDS sample buffer and boiled for 5 min at 100°C. The samples were then stored at -80°C until analyzed. The electrophoretic mobility of the proteins analyzed in this study was determined by SDS-polyacrylamide gel electrophoresis using 15% acrylamide concentrations. After electrophoresis, the proteins were transferred electrophoretically to a nitrocellulose filter membrane that was then blocked for 4 h in a solution of 8% nonfat dry milk in Tris-buffered saline containing 0.1% tween (pH 7.6) at RT. The membrane was then incubated overnight at 4°C with Smad4 antibody and GAPDH antibody which are represented on Western blotting by two distinct bands at 65 and 36 kDa. Bands were washed four times, after which they were incubated with Horseradish Peroxidase Labeled Anti-Mouse IgG (Medical Biological Laboratory, Nagoya, Japan) for 2 h and again washed four times. The blots were developed using an ECL Western blotting kit as recommended by the manufacturer. GAPDH was probed as an internal control. GAPDH was used to confirm that an equal amount of protein was loaded in each lane. Band intensities were determined using an AlphaImager™ 2200 using the SpotDenso function of AlphaEaseFC™ Software version 3.1.2 (Witec, Littau, Switzerland).

**Statistical analysis**

All determinations were repeated three times, and results are expressed as the mean ± SD. ANOVA was used to evaluate the difference among multiple groups followed by a post hoc test (Student-Newman-Keuls) for quantitative data, and RIDIT test was used for statistical analysis of qualitative data. The data were analyzed using SPSS 13.0 software (SPSS Inc., Chicago, IL, USA), and *P < 0.05* was considered statistically significant.

**RESULTS**

**Emodin protected the liver against CCl₄-induced injury and suppressed hepatic fibrogenesis in the rat model**

The effects of emodin on the protection of the liver from injury and fibrogenesis were initially evaluated by histological analyses. Representative views of liver sections are shown in Figure 1A. As shown in tissue sections stained with HE, compared with sections from livers in the vehicle controls (normal group), CCl₄ caused prominent hepatic steatosis, necrosis, and formation of regenerative nodules and fibrotic septa between the nodules (CCl₄ group). Oral administration of emodin daily for 12 wk improved the state of steatosis with a significant reduction in the number of macro- and microvesicular steatosis lesions, and it apparently suppressed hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa (emodin group).

**Emodin reduced the content of hepatic hydroxyproline in the CCl₄ rat model**

The efficacy of treatment with emodin on protection of the liver from fibrogenesis was further evaluated by using a quantitative method to determine the content of hepatic hydroxyproline in the rat model. Compared with the normal controls (54.53 ± 13.46 mg/g), the hepatic hydroxyproline content was significantly higher in rats injected with CCl₄ (120.27 ± 28.47 mg/g, *P < 0.05*). The hepatic hydroxyproline content was significantly reduced in rats treated with emodin at 20 mg/kg (71.25 ± 17.02 mg/g, *P < 0.05*).

**Emodin suppresses serum activities of ALT and AST in the CCl₄ rat model**

Biochemical analyses of serum enzymes were performed to verify the role of emodin in the protection of the liver from injury. As shown in Figure 2, compared with those in the normal controls (51.02 ± 10.64 IU/L and 132.28 ± 18.14 IU/L), the activities of serum ALT and AST were significantly higher in rats injected with CCl₄ (289.25 ± 68.84 IU/L and 423.89 ± 35.67 IU/L). The activities of serum ALT and AST were significantly reduced by administration of emodin (176.34 ± 47.29 IU/L and 226.1 ± 44.52 IU/L). These results demonstrated that emodin protected the liver against CCl₄-induced injury.

**Emodin reduces HSC activation in the liver in the CCl₄ rat model**

IHC and real-time PCR experiments were performed to further evaluate the impact of emodin on regulating hepatic stellate cell (HSC) activation. According to METAVIR scale, the degree of hepatic fibrosis increased markedly in the CCl₄ group compared to the normal group, and decreased markedly in the emodin group compared to the CCl₄ group (*P < 0.01*, Table 1). Taken together, emodin reduced hepatic fibrogenesis caused by chronic CCl₄ intoxication.

| Group          | n  | Degree of pathological | U     |
|----------------|----|------------------------|-------|
|                |    | 0  | 1  | 2  | 3  | 4  |
| Steatosis      |    |   |   |    |    |    |
| Normal control | 10 | 0 | 0 | 0 | 0 | 0 | 3.89<sup>a</sup> |
| CCl₄           | 9  | 0 | 0 | 1 | 1 | 7  | 4.21<sup>b</sup> |
| CCl₄ + emodin  | 11 | 0 | 1 | 4 | 5 | 1  | 2.77<sup>c</sup> |
| Inflammation   |    |   |   |    |    |    |    |
| Normal control | 10 | 0 | 0 | 0 | 0 | 0 | 4.21<sup>b</sup> |
| CCl₄           | 9  | 1 | 1 | 3 | 4 | -  | 2.03<sup>c</sup> |
| CCl₄ + emodin  | 11 | 3 | 7 | 1 | 0 | -  | 2.03<sup>c</sup> |
| Fibrosis       |    |   |   |    |    |    |    |
| Normal control | 10 | 0 | 0 | 0 | 0 | 0 | 3.89<sup>a</sup> |
| CCl₄           | 9  | 0 | 0 | 0 | 5 | 4  | 2.81<sup>c</sup> |
| CCl₄ + emodin  | 11 | 0 | 3 | 4 | 3 | 1  | 2.81<sup>c</sup> |

A piece of liver tissue from rats in each group was fixed with formalin, and then it was embedded in paraffin. Thin sections were cut and stained with HE. U represents the RIDIT value of the two groups, *P < 0.05* indicates U > 1.96, *P < 0.01* indicates U > 2.58. *P < 0.05* vs CCl₄ group; *P < 0.01* vs CCl₄ group.

According to METAVIR scale, the degree of hepatic fibrosis increased markedly in the CCl₄ group compared to the normal group, and decreased markedly in the emodin group compared to the CCl₄ group (*P < 0.01*, Table 1). Taken together, emodin reduced hepatic fibrogenesis caused by chronic CCl₄ intoxication.
the expression of α-SMA, the marker of activated HSC. Liver sections from each group were immunolabeled with antibodies against α-SMA. As shown in Figure 1B, as expected, few cells in the liver sections from the normal group were recognized by antibodies against α-SMA, suggesting few activated HSC in the normal livers in the vehicle control rats. Administration of CCl₄ caused a significant increase in the number of cells recognized by antibodies against α-SMA. Emodin treatment significantly reduced the number of cells labeled with α-SMA antibodies, suggesting that emodin might suppress HSC activation in the rat model. The comparative Ct method of 2^ΔΔCt and IHC evaluation result showed that protein and mRNA levels of α-SMA in liver tissues from normal control rats were 8.88 ± 1.26 and 1.01 ± 0.19, respectively while those in the CCl₄ group were 21.97 ± 1.68 and 3.52 ± 0.60, respectively. Treatment of rats with emodin during CCl₄ exposure largely increased expression of α-SMA and resulted in protein and mRNA levels of 14.61 ± 1.67 and 2.46 ± 0.91, respectively (Figure 1C and D).

**Emodin reduces the concentration of TGF-β1 in serum and mRNA levels in liver tissues**

TGF-β1 is the major profibrogenic factor during hepatic fibrogenesis. We examined the effect of emodin on the concentration of TGF-β1 in serum and mRNA levels in liver tissues of the rat model by ELISA and real-time PCR. As shown in Figure 3, compared with those in the normal group (84.89 ± 27.14 pg/mL and 1.01 ± 0.16, respectively), the levels of TGF-β1 in serum (Figure 3A) and mRNA levels of TGF-β1 in liver tissues (Figure 3B) were dramatically increased in the CCl₄ group (313.40 ± 57.75 pg/mL and 3.89 ± 1.00, respectively, both P < 0.05 vs normal group). The levels of TGF-β1 in serum and mRNA levels of TGF-β1 in liver tissues were significantly reduced in the emodin group (151 ± 47.64 pg/mL and
2.16 ± 0.73, respectively, both P < 0.05 vs CCl4 group). Although these were still higher than those of the normal group, these data indicated that emodin significantly reduced the levels of TGF-β1 in serum and mRNA levels in liver tissues in the rat model, which might result in the inhibition of HSC activation stimulated by CCl4.

**Emodin down-regulates the protein and mRNA levels of Smad4 in liver tissues of the CCl4 rat model**

Because TGF-β1 signals within the cell through Smad is involved in fibrosis, the effects of emodin on mRNA and protein levels of Smad4 in liver tissues were demonstrated by real-time PCR (Figure 4C), Western blotting (Figure 4D and E), and IHC analyses (Figure 4A and B). Experiments revealed that exposure of rats to CCl4 significantly increased mRNA and protein levels of Smad4 in liver tissues from 1.00 ± 0.13, 0.54 ± 0.04 and 5.78 ± 1.05, respectively, in the normal group to 4.63 ± 0.86, 13.44 ± 0.64 and 23.95 ± 3.23, respectively, in the CCl4 group. In contrast, protein and mRNA levels of Smad4 in liver tissues from rats treated with emodin during CCl4 exposure were attenuated and were 2.94 ± 0.74, 9.25 ± 0.84 and 17.00 ± 1.88, respectively. Treatment of rats with emodin during CCl4 exposure blunted the increase in protein and mRNA levels of Smad4 significantly.

**DISCUSSION**

In the present study, we confirmed that emodin protects the rat liver from CCl4-induced injury and fibrogenesis. The mechanism for this protective effect may relate to the fact that emodin efficiently inhibits HSC activation in vivo.

Hepatic fibrosis, which may lead to cirrhosis, is associated with most chronic liver diseases[23]. Hepatic fibrosis is thought to be a reversible disease, however, there is no satisfactory method in clinical practice to reverse the pathological process yet[24]. Several drugs, including antisense TGF-β1 receptors, cytokines[25], antioxidants, chemical drugs[26], soluble type II receptor of TGF-β1, and TGF-β1 antibodies[27] have been used in research work to block experimental hepatic fibrosis, but their effects were not as prosperous as we had expected. Some traditional Chinese drugs have been found effective in preventing fibrogenesis and other causes of chronic liver injury[22,28], and this helps to develop a more hopeful future in controlling liver fibrosis and cirrhosis. Emodin is a main active monomer isolated from Giant Knotweed Rhizome, which is widely used in traditional Chinese herb treatment of liver cirrhosis[29]. It is easy to extract, isolate and identify emodin, so it shows excellent prospects in the development of some new drugs for treating hepatic fibrosis.

CCl4, a highly toxic chemical agent, causes hepatic injury including hepatocytic necrosis, steatosis, and inflammation. Research for establishing a model of liver fibrosis caused by CCl4 began in 1936. Since then many methods to establish a model of liver fibrosis have been tried[30]. Among them, hepatic fibrosis caused by CCl4 has been extensively used in experimental models in rats because hepatic responses in rats to chronic CCl4 stimulation are shown to be superficially similar to human cirrhosis[31]. Hepatocyte damage is the initial factor of hepatic fibrogenesis and activities of ALT and AST in serum are the most commonly used biochemical markers of liver injuries[32]. Hydroxyproline is an amino acid found...
almost exclusively in collagens. Determination of the content of hydroxyproline in liver tissue is regarded as a good method to quantify fibrosis and to evaluate the effectiveness of new potentially antifibrotic agents. In this study, the method of subcutaneously injecting \( \text{CCl}_4 \) was used to establish the model of liver fibrosis. Histological analysis showed \( \text{CCl}_4 \) caused prominent hepatic steatosis, necrosis, and formation of regenerative nodules and fibrotic septa between the nodules. Biochemical assay showed serum ALT activities, serum AST activities, and content of hepatic hydroxyproline were markedly increased in rats injected with \( \text{CCl}_4 \) for 12 wk, which are consistent with the histological observations.

Our results suggest that oral administration of emodin daily for 12 wk improved the state of steatosis with a significant reduction in the number of macro- and microvesicular steatosis, and it also apparently suppressed hepatic fibrogenesis by reducing the thickness of bridging fibrotic septa. Emodin could decrease the scores of hepatic fibrosis grading, inhibit the ALT and AST activities in serum and reduced the content of hepatic hydroxyproline. All results confirm that emodin protected the liver from injury and fibrogenesis caused by \( \text{CCl}_4 \) in the rat model.

Chronic liver injury may lead to development of fibrosis, a process in which HSC play a major role. As a result of liver injury, HSC, which in the healthy organ store vitamin A, undergo a process of activation that is mediated by the concerted action of resident hepatic cell types such as Kupffer cells, liver endothelial cells, and hepatocytes. The phenotype of activated HSC is characterized by \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA)
expression. α-SMA expression in the liver tissues is an indicator of hepatic stellate cell activation, which is recognized as being critical in liver fibrogenesis. Thus inhibition of the accumulation of activated HSCs is an important therapeutic strategy. Our results showed the levels of α-SMA in rat liver tissues increased significantly after CCl₄ administration for 12 wk. Emodin reduced α-SMA expression at mRNA and protein levels.

Inflammation is commonly associated with hepatic fibrogenesis during chronic liver diseases. CCl₄ is metabolized in the liver by cytochrome P450 into the free radical CCl₃. The free radical attacks hepatocytes and causes necrosis of parenchymal cells, which promotes inflammatory responses in the liver. Results in this study indicated that emodin suppressed inflammation caused by CCl₄, which might lead to the protection of the liver from injury. It is now widely accepted that the pro-inflammatory cytokine TGF-β1 is a major cytokine in the regulation of the production, degradation, and accumulation of ECM, and it has been suggested that overexpression of TGF-β1 for a prolonged period of time after tissue damage may induce a fibroproliferative response and deposition of ECM, resulting in fibrosis in vital organs. Many studies have detected the presence of TGF-β1, in the form of either protein or message, in the fibrotic tissues of animal models or human samples. Partial inhibition of the accumulation of ECM using either anti-TGF-β1 serum or a TGF-β1-binding protein has been reported in fibrosis models. Our results showed that TGF-β1 mRNA levels and serum TGF-β1 protein levels in normal rat were low. After injection of CCl₄ for 12 wk, mRNA and protein levels of TGF-β1 increased significantly. Emodin down-regulated mRNA levels of TGF-β1 expression in liver tissue. Furthermore, serum TGF-β1 levels in the model rats were also significantly down-regulated by emodin treatment in a manner similar to hepatic fibrosis attenuation. These findings imply that emodin might attenuate hepatic fibrosis through down-regulation of TGF-β1 expression in vivo.

Smad4 is well known to function as one of the downstream effectors of TGF-β1, and it mediates TGF-β1-induced collagen synthesis. Smads are intracellular signal transducing molecules of the TGF-β super family. According to differences in structure and function, nine Smads have been reported and classified into three groups. Smads 2 and 3 are named R-Smads in the pathway and Smad4 Co-Smads for all these pathways. Smads 6, 7, 8 are inhibitory factors of these Smads. When TGF-β1 binds to its receptor, Smad 2/3 is phosphorylated and binds with Smad4 and together they move into the nucleus for translation and expression of the target gene. Smad signal transduction pathways are thought to play a crucial role in the process of liver damage and recovery, as well as liver fibrosis. These transcriptional responses appear to be mediated predominantly through Smad4. The widely held conclusion that Smad4 occupies a central role in transduction of TGF-β1 signals comes from multiple lines of biochemical and genetic evidence. In reconstitution experiments, cell lines that lack Smad4 fail to respond to TGF-β1 signals, transfection of wild-type Smad4 restores the signaling capabilities of these cells. Our study showed that both mRNA and protein expressions of Smad4 were remarkably up-regulated in fibrotic rats. We also observed down-regulation of Smad4 expression in emodin-treated fibrotic rats, suggesting that emodin attenuate hepatic fibrosis by regulating TGF-β1/smad signaling.

In conclusion, the data presented herein provide evidence that emodin is active as an antifibrogenic drug able to reduce the biological effects of TGF-β1 in ongoing fibrogenesis. Giant Knotweed Rhizome, a traditional Chinese herbal medicine, is widely used in clinical practice for treating cirrhosis. Emodin, the main active monomer isolated from Giant Knotweed Rhizome, may be an attractive therapeutic agent for the treatment of fibrotic liver diseases.

COMMENTS

Background
In the last decade, advances in the understanding of genes promoting hepatic stellate cell (HSC) activation are impressive. However, there are few breakthroughs in therapeutic intervention of hepatic fibrogenesis. Efficient and well-tolerated antifibrotic drugs are lacking and current treatment of hepatic fibrosis is limited to withdrawal of the noxious agent. Research identifying innocuous antifibrotic agents is of high priority and urgently needed.

Innovations and breakthroughs
To the best of the authors’ knowledge, this is the first study to report that emodin protects the liver from CCl₄-induced fibrogenesis by inhibiting activation of HSC via modulating transforming growth factor-β1 (TGF-β1)/Smad signaling pathways. Results in this study provide novel insight into the mechanisms of emodin in the protection of the liver.

Applications
By evaluating the role of emodin in protecting the liver against fibrogenesis caused by carbon tetrachloride (CCl₄) in rats via inhibition of hepatic stellate cells activation, emodin might be a therapeutic antifibrotic agent for the treatment of hepatic fibrosis.

Terminology
Smad4 is a protein which in humans is encoded by the SMAD4 gene. SMAD4 is a 552 amino acid protein involved in cell signaling. It is the only known mammalian coSmad. It is a homolog of the Drosophila protein: “Mothers against decapentaplegic”.

Peer review
This study examines the effects of emodin on CCl₄-induced liver fibrosis. The authors show reduced fibrosis, decreased stellate cell smooth muscle actin expression and decreased TGF-β1 expression. The study has been suitably designed and clearly reported.

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