Emodin alleviates CCl4-induced liver fibrosis by suppressing epithelial-mesenchymal transition and transforming growth factor-β1 in rats

FENG LIU1*, JING ZHANG2*, JIANMIN QIAN3, GANG WU3 and ZHENYU MA3

Departments of 1Integrative Medicine, 2Nursing Center and 3General Surgery, Huashan Hospital, Fudan University, Shanghai 200040, P.R. China

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Abstract. Liver fibrosis is a chronic disease that exhibits a complicated pathophysiology. It is characterized by the deposition of the extracellular matrix. Emodin, an active constituent isolated from rhubarb, has antibacterial, immunosuppressive and anti-inflammatory effects. In the present study, the mechanism through which emodin alleviates liver fibrosis in rats was investigated. A rat model of liver fibrosis was generated by administering CCl4 via subcutaneous injection twice a week for 12 weeks. Emodin or sodium carboxymethylcellulose (CMC), as the vehicle, were intragastrically administered daily. After 12 weeks, the liver function index was examined by blood analysis, histopathological scores of fibrosis was determined by hematoxylin and eosin staining and level of collagen deposition was examined by Masson staining. In addition, protein and RNA samples were collected for further analysis. The results of the present study revealed that emodin significantly reduced the level of collagen deposition in a dose-dependent manner. Furthermore, emodin reduced the expression of transforming growth factor-β1 (TGF-β1) and the phosphorylation levels of mothers against decapentaplegic homolog 2/3, and inhibited the CCl4-induced downregulation of E-cadherin and upregulation of the mesenchymal markers, fibronectin and vimentin. The expression levels of TGF-β1, Snail family transcriptional repressor (Snail) 2, Snail, twist-related protein 1 and zinc finger E-box-binding homeobox (ZEB)1 and 2 mRNA were significantly decreased in emodin-treated groups compared with the untreated control. Collectively, the results of the present study suggested that emodin may exert antifibrotic effects via the suppression of TGF-β1 signaling and epithelial-mesenchymal transition.

Introduction

The incidence of liver disease has increased rapidly in recent years. Liver transplantation is an efficient method to treat end-stage liver disease. However, ischemia-reperfusion injury (IRI) of the transplanted liver is inevitable. In addition, the risk of developing an alloimmune response necessitates lifelong oral immunosuppressant treatment, which induces varying degrees of graft fibrosis and thus further affects long-term graft survival (1,2). It has been proposed that the primary factors leading to the development of liver fibrosis following transplantation include the duration of IRI, donor age, the hepatitis virus genotype of recipients and immunosuppressant therapy. Among these, developing an alloimmune response following transplantation is considered to be the primary factor leading to the development of liver fibrosis; however, the specific mechanism through which this occurs remains unclear (3).

Accumulating evidence suggests that epithelial-mesenchymal transition (EMT) leads to tumor metastasis and the occurrence of cancer, including hepatocellular carcinoma (4,5). EMT is a chronic process accompanied by the loss of cell-cell junctions in epithelial cells. The principal characteristic of liver fibrosis is the excessive synthesis of extracellular matrix (ECM) components, predominantly collagen. As the degradation rate of collagen is relatively slow, this leads to a dynamic imbalance and excessive ECM deposition in the liver. This is a recognized mechanism for the development of liver fibrosis (6). However, various other factors are known to be involved in the development of liver fibrosis, including underlying pathological histology, cytokology, cytokines and intracellular signal transduction systems (7-9). Active hepatic stellate cells are the principal source of ECM production during liver fibrosis (10). Growth factors and factors involved in regulating vascular function, and lipid levels are additionally involved in the formation and development of this condition. Among them, transforming growth factor-β1 (TGF-β1), a key fibrogenic cytokine, is reported to be one of the most important cytokines in liver fibrosis (11,12).
Emodin is one of the effective ingredients of the Chinese medicine rhubarb. It has been reported that emodin has a number of pharmacological effects, including antiviral, antibacterial, immunoregulatory and antioxidant functions (13,14). Studies have demonstrated that emodin is able to induce apoptosis via EMT suppression and caspase-dependent signaling (15-17). It has additionally been reported that emodin may alleviate pancreatitis and suppress lung fibrosis by inhibiting TGF-β1 signaling in rats (18,19). Further studies have reported that emodin may reduce CCL_4-induced liver fibrosis in rats; however, the optimal dosage and the specific mechanism involved requires further investigation (20). The present study aimed to further analyze the role of emodin in alleviating CCL_4-induced liver fibrosis by inhibiting EMT and the TGF-β1 signaling pathway.

Materials and methods

**Animals.** A total of 50 adult Sprague-Dawley male rats (6-8 weeks) weighing 240-260 g, were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China). Rats were housed in an a room at 22±0.5˚C with a relative humidity of 60±2% in a 12 h light/dark cycle. In Huashan Hospital affiliated to Fudan University (Shanghai, China), with free access to food and water. All procedures were approved by the Bioethics Committee of Huashan Hospital affiliated to Fudan University.

**Reagents and antibodies.** Emodin was purchased from Shanghai Future Industrial Limited by Share, Ltd. (Shanghai, China). CCl_4 was obtained from Beijing BeiHua Fine Chemicals Co., Ltd. (Beijing, China). Olive oil was purchased from Beyotime Institute of Biotechnology (Haimen, China). TRIzol reagent, superscript II reverse transcriptase and random primer oligonucleotides were purchased from Invitrogen (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The Absolute QPCR SYBR® Green premix was purchased from Takara Bio, Inc. (Otsu, Japan). The thermocycling conditions consisted of: 30 sec at 95˚C, followed by 40 cycles (5 sec at 95˚C, 30 sec at 55˚C and 60 sec at 72˚C). The primers used are listed in Table I. The antibodies against mothers against decapentaplegic homolog 2 (Smad2; 1:1,000; cat. no. 5339; CST Biological Reagents Co., Ltd.), TGF-β1 (1:1,000; cat. no. 3711; CST Biological Reagents Co., Ltd.), E-cadherin (1:1,000; cat. no. 14472; CST Biological Reagents Co., Ltd.), vimentin (1:1,000; cat. no. 5741; CST Biological Reagents Co., Ltd.), TGF-b1 (1:1,000; cat. no. 3711; CST Biological Reagents Co., Ltd.) and GAPDH (1:1,000; cat. no. 5174; CST Biological Reagents Co., Ltd.) and fibronectin (1:500; cat. no. ab2413; Abcam, Cambridge, UK). An ELISA kit to detect TGF-β1 (cat. no. F3766) was purchased from Westang Technology Ltd. (Shanghai, China).

**Histological study.** Liver tissue was embedded with paraffin following 10% formalin fixation at 4˚C for 48 h and sliced to a thickness of ~5 μm. Hematoxylin and eosin (H&E; 3 min each), Sirius red (10 min) and Masson's Trichrome stainings (1 h) were performed post-dewaxing at room temperature. According to the criteria of the Chinese Medical Association Committee of Fatty Liver Disease (21) and Nouchi et al (22), the extent of liver cirrhosis in sections stained with H&E was determined in the present study (22,23). Steatosis was graded on the basis of the extent of parenchyma involved, as described in Table I. The stage of liver fibrosis was graded using the METAVIR five-point scale, additionally described in Table 1 (24). Collagen accumulation was quantified following Sirius red and Masson's Trichrome staining in 10 randomly selected areas per sample, at a magnification of x200 with the image analysis software Image-Pro Plus (version 6.1; Media Cybernetics, Inc., Rockville, MD, USA) under a light microscope (Olympus, Japan). The percentage of the section that was positively stained for interstitial collagen was quantified using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). These photos were collaged using Photoshop software 6.0 (Adobe Systems, Inc., San Jose, CA, USA).

**Reverse transcription-quantitative polymerase chain reaction (RT-qPCR).** Total RNA was extracted from liver tissues using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Inc.). A total of 1 μg total RNA was reverse transcribed into cDNA using a RevertAid™ First Strand cDNA Synthesis kit (Fermentas; Thermo Fisher Scientific, Inc.). RT-qPCR was performed using the SYBR® Premix Ex Taq kit (Takara Bio, Inc.) in a MasterCycler RealPlex4 system (Eppendorf, Hamburg, Germany). The thermocycling conditions consisted of: 30 sec at 95˚C, followed by 40 cycles (5 sec at 95˚C, 30 sec at 55˚C and 60 sec at 72˚C). The primers used are listed in Table I. The expression of mRNA was normalized to GAPDH expression using the 2^-ΔΔCq method (25).

**ELISA analysis.** Liver tissue was collected and processed according to the manufacturer's protocols of the ELISA kit.
The grinding fluid or standard were added to the plate, which was subsequently placed at 37˚C for 40 min following mixing. Primary antibody working fluid, enzyme conjugate and TMB solution were added sequentially subsequent to washing the plate. The absorbance was measured at 450 nm using a microplate reader.

Western blotting. Samples of liver tissues were treated with protein extraction reagent (Beyotime Institute of Biotechnology) and was centrifuged at 12,000 x g at 4˚C for 20 min to obtain the supernatant according to the manufacturer’s protocols. Protein concentration was subsequently quantified with a bicinchoninic acid protein assay. Proteins (20 µg/lane) were subjected to 10% SDS-PAGE prior to transfer onto polyvinylidene fluoride membranes. Following this, membranes were blocked with 5% skim milk for 1 h at room temperature and incubated with primary antibodies at 4˚C for 12 h. Peroxidase-conjugated goat anti-rabbit IgG secondary antibody (1:10,000; cat. no. 111-035-003; Jackson ImmunoResearch) was incubated with the membranes at room temperature for 2 h. Bands were visualized with an enhanced chemiluminescent substrate kit (Amersham Pharmacia). The expression of E-cadherin, vimentin, fibronectin, TGF-β1, Smad2, Smad3, p-Smad2 and p-Smad3 was quantified by normalizing to GAPDH using Image-Pro Plus, version 6.0 (Media Cybernetics, Inc.).

Statistical analysis. Data are expressed as the mean ± standard deviation and analyzed by SPSS 13.0 (SPSS, Inc., Chicago, IL, USA). Statistical analysis was performed using Student’s t-test, or one-way analysis of variance followed by the Student-Newman-Keuls post-hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

Treatment with emodin alleviates CCl4-induced death and improves liver function. The chemical structure of emodin is presented in Fig. 1A. Subcutaneous injection of CCl4 reduced the survival rate of rats to 30%, although administration of emodin at doses of 10, 20 and 40 mg/kg was reported to increase the survival rates at week 12 to 60, 70 and 80%, respectively (Fig. 1B). In order to examine the protective role of emodin, a range of liver function tests were performed in the present study, including measuring ALT, AST, ALP and γ-GGT levels in the peripheral blood serum of animals from each group. The results of the present study demonstrated that the liver function index was significantly reduced following stimulation with CCl4 compared with in the control group (ALT: 28.09±5.86 vs. 96.07±5.37 U/l; AST: 47.59±5.45 vs. 141.07±7.08 U/l; ALP: 200.72±16.13 vs. 500.02±15.71 U/l and γ-GGT: 20.23±1.16 vs. 88.09±4.55 U/l). Conversely, following treatment with emodin, the liver function index increased in a dose-dependent manner, particularly with a dose of 40 mg/kg (ALT: 96.07±5.37 vs. 45.29±3.22 U/l; AST: 141.07±7.08 U/l; ALP: 291.48±13.74 U/l and γ-GGT: 28.42±1.58 U/l; Fig. 1C-F).

Emodin notably attenuates the formation of fatty liver and fibrosis, and reduces the deposition of collagen. Histological analysis notably revealed the structure of the liver lobule in the control group and demonstrated that there were no apparent hepatic cell lesions, no dilation or congestion in the liver sinusoid, and no inflammatory cell infiltration or fibroplasia in the portal area. However, in the CCl4 group, the lobular structure was disordered, and fat degeneration...
and necrosis in liver cells, and infiltration of inflammatory cells, were observed. In addition, liver lobes were divided by collagen fibers and a large amount of collagen deposition was observed in the portal area. Following treatment with emodin, the structure of the liver lobule was observed to be largely complete, no necrotic lesions were detected, and infiltration of inflammatory cells and fibrotic hyperplasia were rare in the portal area. Among all treatment groups, the lightest extent of liver injury was detected in animals administered 40 mg/kg emodin (Fig. 2A and B). Masson’s Trichrome and Sirius staining revealed a significant increase in collagen deposition in rats treated with CCl$_4$ compared with the control group (Masson’s Trichrome: 3.5±1.35 vs. 25.7±2.41%; Sirius: 3.5±1.35 vs. 25.7±2.41%). Following treatment with emodin at various concentrations, collagen deposition was reduced, with the most significant effect observed in the 40 mg/kg group (Masson’s Trichrome: 33.5±4.32 vs. 12.1±2.51%; Sirius: 25.7±2.41 vs. 9±1.76%; Fig. 2A and C).

**Figure 1.** Emodin inhibits CCl$_4$-induced suppression of liver function. (A) Chemical structure of emodin. (B) Survival rates over a 12-week observation period. At 12 weeks post-CCl$_4$ administration, peripheral blood was collected and levels of (C) ALT, (D) AST, (E) ALP and (F) γ-GGT were analyzed by ELISA. Data are presented as the mean ± standard deviation, n=6. *P<0.05; **P<0.01; ***P<0.001. ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; γ-GGT, glutamyl transpeptidase.

Emodin reduces CCl$_4$-induced EMT in rat liver tissue. EMT is an important factor during the process of liver fibrosis. In the present study, western blot analysis and RT-qPCR were employed to detect markers of EMT. Western blotting data revealed that the expression levels of the mesenchymal markers vimentin and fibronectin were upregulated following CCl$_4$ administration. Conversely, the epithelial marker E-cadherin was highly expressed in the control group and downregulated following CCl$_4$ administration. These results suggest that CCl$_4$ stimulation may induce liver fibrosis in rats. Compared with the CCl$_4$ group, emodin decreased the expression of vimentin and fibronectin, and increased the expression of E-cadherin, with most marked effects noted in the 40 mg/kg treatment group (Fig. 3A). This was consistent with the results of the RT-qPCR, investigating the expression of the principal transcriptional repressors of E-cadherin: Snail family transcriptional repressor (Snail) 2 (Slug), Snail, twist-related protein 1 (TWIST1) and zinc finger E-box-binding homeobox 1 (ZEB1), and the expression of collagen types I and III. The
The results of the present study revealed that Slug, Snail, TWIST1, ZEB1, collagen I and collagen III mRNA expression levels were increased in the CCl₄ group. Emodin reduced the expression of all of these EMT-associated markers in a dose-dependent manner, with the most significant effect noted in the 40 mg/kg treatment group. These results therefore indicated that emodin may be able to inhibit CCl₄-induced EMT in the livers of rats (Fig. 3B).
Figure 3. Emolin inhibits CCl₄-induced epithelial-mesenchymal transition in rat liver tissue. (A) Representative western blotting images demonstrating the expression of E-cadherin, fibronectin and vimentin in liver tissues from the control, CCl₄, and CCl₄ + emolin groups. (B) mRNA levels of Slug, Snail, TWIST1, ZEB1, collagen I and III, and SMA were analyzed by reverse transcription-quantitative polymerase chain reaction. Data are presented as mean ± standard deviation, n=6. *P<0.05; **P<0.01, ***P<0.001. Slug, snail family transcriptional repressor 2; Snail, snail family transcriptional repressor; TWIST1, twist-related protein 1; ZEB1, zinc finger E-box binding homeobox 1.
Emodin reduces CCl4-induced TGF-β1 production and the expression of p-Smad2 and Smad3. TGF-β1 serves a critical role in the course of EMT. In order to understand whether TGF-β1 was involved in the emodin-mediated reduction in EMT in the present study, TGF-β1, p-Smad2 and p-Smad3 expression was investigated following treatment with 40 mg/kg emodin (Fig. 4). ELISA results confirmed that the content of liver TGF-β1 increased in CCl4 compared with in the control group (87.98±4.47 vs. 321.97±17.44 pg/ml). Following treatment with emodin, the levels of TGF-β1 decreased significantly (321.97±17.44 vs. 118.72±12.18 ng/ml; Fig. 4A and C). This was in accordance with the findings of the RT-qPCR and western blotting. In order to investigate whether TGF-β1 signaling was inhibited following treatment with emodin, the expression levels of p-Smad2 and p-Smad3 were examined. The results of the present study indicated that CCl4 induced the phosphorylation of Smad2 and 3, which was significantly attenuated by 40 mg/kg emodin (Fig. 4A and C).

Discussion

Liver fibrosis is a common pathological characteristic of the majority of chronic liver diseases. Following liver transplantation, the occurrence of varying degrees of fibrosis is inevitable (26). It characterized by the loss of the normal liver architecture due to structural abnormalities in the nodules. At present, there is a lack of effective clinical treatment for liver fibrosis. Traditional Chinese medicine and natural medicine may offer a promising alternative.

Emodin is an active monomer extracted from the traditional Chinese medicines knotweed and rhubarb root. Emodin has been reported to exert anti-inflammatory, antitumor, antiviral and immunomodulatory effects (27). Previous studies have reported that emodin additionally exhibits an antifibrotic effect in a rat lung fibrosis model, in addition to in heart tissue and other organs (28-30). It has been reported that emodin has a protective effect in liver fibrosis (20). However, its specific mechanism and the optimal dose for antifibrotic treatment in the liver require further investigation. In the present study, CCl4 was employed to induce liver fibrosis in rats. Emodin was administered at doses of 10, 20 and 40 mg/kg, and the effects on rat survival and liver function were observed.

The results of the present study demonstrated an increase in survival rates at doses of 20 and 40 mg/kg. ALT, AST, ALP and γ-GGT enzymes are released from liver cells into the serum when cell damage or necrosis occurs, with the
levels of the enzymes reflecting the amount and type of liver damage (31,32). The biochemical index recorded in the present study suggested that liver function was severely damaged in the CCl4 group. Histological examination confirmed the appearance of degenerated and necrotic liver cells in this group. Masson's Trichrome and Sirius red staining revealed abnormal collagen deposition and a significant increase in the liver fibrosis index. These data indicated that treatment with CCl4 may successfully induce liver fibrosis in rats, and that CCl4-induced liver damage may be prevented by administering emodin via a subcutaneous injection. Indices of liver damage, including ALT, AST, ALP and γ-GGT, were significantly reduced following treatment with emodin and liver cell degeneration and necrosis, and collagen deposition, were ameliorated. The most significant effects were observed following administration of the 40 mg/kg dose. To the best of our knowledge, the present study was the first to demonstrate that emodin may reduce the progression of liver fibrosis via the suppression of EMT and TGF-β signaling, as demonstrated by animal survival rates, together with biochemical, molecular and histological data.

There is accumulating evidence that during damage to hepatic epithelial cells, including hepatocytes and cholangiocytes, phenotypic markers are lost, including N- and E-cadherin; hepatic epithelial cells subsequently transform into fibroblasts, myofibroblasts or mesenchymal cells, with the concomitant expression of markers, including vimentin and α-smooth muscle actin (33-35). This transformation is associated with the progression of liver fibrosis. It has been reported that within a rat model of lung fibrosis, emodin may significantly ameliorate bleomycin-induced EMT (19). In the present study, the expression levels of E-cadherin were significantly lower and the expression of vimentin was significantly higher in the CCl4 group compared with the control, suggesting that CCl4 stimulation may induce EMT in liver cells, which subsequently adopt a mesenchymal cell phenotype. Following treatment with emodin, the expression of E-cadherin was increased and the expression of vimentin was decreased, indicating that CCl4-induced EMT is suppressed by emodin. Furthermore, the most significant effects were observed with the treatment at 40 mg/kg.

TGF-β1 signaling via Smad molecules is a well-documented mechanism of liver fibrosis (36). It serves a critical role in the initiation of fibrosis and the activation of hepatic stellate cell transformation. Activation of Smad2 is reported to be involved in the regulation of cell proliferation, transformation, synthesis, secretion and apoptosis caused by TGF-β1 (37). Smad signaling molecules are considered to be some of the most important intracellular TGF-β1 receptor kinase substrates (38,39). Among them, Smad2 and Smad3 belong to the family of regulatory Smads, which combine with serine/threonine receptors. In mammals, TGF-β1 transmits intracellular signals via Smad2 and Smad3 phosphorylation. In the process of liver fibrosis, TGF-β1 activates Smad2/3, and subsequently phosphorylates Smad3/4 and Smad1/5/8 to form a heteropolymer with Smad4. Finally, Smad 4 translocates to the nucleus to regulate gene transcription by interacting directly with DNA or via coenzyme factors, including Snail, Slug or Twist1 (40,41). Furthermore, Smad4 may inhibit the expression of epithelial genes and promote mesenchymal gene expression (42). Thus, the inhibition of Smad2/3 may block the TGF-β1 signaling pathway. In the present study, the expression of TGF-β1 and phosphorylation Smad2/3 were significantly higher in the liver of animals of the CCl4 group compared with the control group, indicating that this may be a mechanism for CCl4-induced rat liver fibrosis. Additionally, the present study demonstrated that CCl4 induced the production of TGF-β1 and phosphorylation of Smad2/3, which was suppressed via treatment with 40 mg/kg emodin.

Collectively, the results of the present study suggested that the mechanism for emodin-mediated suppression of CCl4-induced liver fibrosis may function via the inhibition of EMT and TGF-β1/Smad signaling. This suggests that emodin may be a potential novel therapeutic agent for the clinical prevention of liver fibrosis.

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Availability of data and materials

All data generated or analyzed during the present study are included in this published article.

Authors' contributions

FL performed the western blot analysis and wrote the manuscript. JZ performed the morphological staining experiments of the liver and analyzed the experimental data. JQ and GW performed the ELISA and revised the manuscript. ZM designed the experiment and funded this research. All authors read and approved the final manuscript.

Ethics approval and consent to participate

The present study was approved by the Bioethics Committee of Huashan Hospital affiliated with Fudan University (Shanghai, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


18: pii: E500.


