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Asiatic acid attenuates CCl_4-induced liver fibrosis in rats by regulating the PI3K/AKT/mTOR and Bcl-2/Bax signaling pathways

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ABSTRACT

Liver fibrosis is a major pathological feature of chronic liver diseases, and effective therapies are limited at present. Asiatic acid (AA) is a triterpenoid isolated from *Centella asiatica*, which exhibits efficient anti-inflammatory and anti-oxidative activities. In this study, we attempted to evaluate the potential therapeutic effect of AA on CCl_4-induced liver fibrosis in rats and to investigate the underlying molecular mechanisms. Liver fibrosis-related indexes including body weight, biochemical parameters, histological changes, the mRNA expression levels of inflammatory cytokines and biomarkers, and changes in the expression of related proteins in liver tissue were assessed. The results showed that AA treatment effectively ameliorated CCl_4-induced liver injury and fibrosis. Mechanistically, AA treatment attenuated CCl_4-induced oxidative stress, inflammation, and hepatocyte apoptosis and regulated the Bcl-2/Bax signaling pathway in the liver. Additionally, we demonstrated that AA also inhibited hepatic stellate cell activation and extra cellular matrix (ECM) synthesis by regulating the PI3K/AKT/mTOR signaling pathway. In conclusion, these findings suggest that AA prevents the progression of liver fibrosis through multiple mechanisms and indicate that AA might be used for the treatment of liver fibrosis in the future.

1. Introduction

Liver fibrosis is a dynamic wound-healing process that occurs in response to various liver injuries, such as hepatitis viral infection, alcohol consumption, cholestasis, and non-alcoholic steatohepatitis [1]. It is characterized by the excess synthesis and deposition of extracellular matrix (ECM) components, which disrupts the normal liver architecture and function [2–4]. If left untreated, persistent liver fibrosis can lead to liver cirrhosis and hepatic carcinoma. In addition, liver fibrosis causes a significant amount of morbidity and mortality in patients and effective therapy is not yet available. Thus, there is an urgent demand for developing new drugs to prevent or treat liver fibrosis.

In recent years, the mechanisms of liver fibrosis have been widely investigated. It has been demonstrated that hepatic stellate cells (HSCs) play a critical role in the development and progression of liver fibrosis, as activated HSCs secrete amounts of ECM [5,6]. Damaged hepatocytes release inflammatory and fibrogenic cytokines that could induce the activation of HSCs [7]. Several studies have demonstrated that PI3K/AKT/mTOR signaling is closely associated with the proliferation, activation and ECM synthesis of HSCs [8]. Furthermore, these studies also showed that the inhibition of PI3K signaling by pharmacological and genetic methods dramatically suppresses the activation of HSCs, ECM synthesis and experimentally induced liver fibrosis *in vivo* [8]. Therefore, the PI3K/AKT/mTOR signaling pathway has emerged as one of the major therapeutic targets for treating liver fibrosis. In addition to the PI3K/AKT/mTOR pathway, the Bcl-2/Bax signaling pathway also plays an important role in the progression of liver fibrosis by regulating cell apoptosis. The anti-apoptotic members of the Bcl-2 family are the guardians of the mitochondrial pathway of cell apoptosis. Because persistent hepatocyte apoptosis promotes liver fibrosis, regulating the Bcl-2/Bax pathway may inhibit hepatocyte apoptosis and attenuate liver fibrosis [9,10].

Asiatic acid (AA) is one of the triterpenoid components of *Centella asiatica*, which has anti-oxidative, anti-inflammatory and hepatoprotective activities [11–15]. Previous studies have reported that AA alleviates CCl_4-induced liver fibrosis by blocking the TGF-β/Smad signaling pathway *in vivo* and *in vitro* [16]. However, further mechanisms underlying the protective effect of AA against CCl_4-induced liver fibrosis remain unclear, and whether this protective effect is associated with the inhibition of the Bcl-2/Bax and PI3K/AKT/mTOR signaling

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pathways needs to be further clarified. Therefore, the purpose of this study was to evaluate the effects of AA against CCl_4-induced liver fibrosis in rats and to investigate oxidative stress, inflammation, apoptosis, HSC activation and the related signaling pathways to elucidate the possible mechanism of action of AA.

2. Materials and methods

2.1. Chemicals and reagents

AA was purchased from Shanghai Nature Standard R&D and Biotech Co., Ltd. (purity 98.0%; molecular weight 488.70; Shanghai, China). CCl_4 was purchased from Shenzhen Xunye Chemical (Shenzhen, China). Monoclonal anti-Bcl-2, anti-Bax, anti-cleaved caspase-3, anti-caspase-3, and GAPDH antibodies were obtained from Cell Signaling Technology (Danvers, MA). Monoclonal anti-α-SMA and phospho-p70S6K rabbit antibody, and p70S6K was purchased from Abcam (Cambridge, MA). Monoclonal antibodies against AKT, phospho-AKT, mTOR, and phospho-mTOR were purchased from Cell Signaling Technology (Danvers, MA). Secondary antibodies used in western blotting were goat anti-rabbit IgG (H + L) (Bioworld Technology Co., Ltd., Shanghai, China). Secondary antibodies used in immunohistological staining were purchased from Dako (Glostrup, Denmark). Secondary antibodies used in immunofluorescence staining were purchased from Abcam (Cambridge, MA).

2.2. Animals and experimental design

Male Sprague-Dawley rats, aged 10 weeks (200–220 g), were purchased from the B&K Universal Group Ltd. (Shanghai, China). All rats were maintained at 21 ± 2 °C on a 12 h light/dark cycle with free access to food and water. All animal experimental procedures were performed in accordance with the Guide for the Care and Use of Laboratory Animals and were approved by the Animal Experimental Ethics Committee of the Second Military Medical University (Shanghai, China).

Forty-eight rats were divided randomly into four groups ($n = 12$ for each group) as follows: the normal group; the model group; the 5 mg/kg AA group; and the 15 mg/kg AA group. Except those in the normal group, all rats received 1 mL/kg CCl_4 orally (diluted in 50% peanut oil) twice a week for 6 weeks to induce liver fibrosis. Meanwhile, the rats in the 5 mg/kg AA or 15 mg/kg AA groups were orally given AA (5 mg/kg or 15 mg/kg) suspended in a 0.5% carboxymethylcellulose (CMC) mixture once a day for 6 consecutive weeks. The rats in the normal group simultaneously received the same volumes of peanut oil and the 0.5% CMC mixture without AA. At the end of 6 weeks, the surviving rats were weighed and anesthetized with pentobarbital sodium (Shanghai Beizhuo Biochemical & Technological Co., Ltd., Shanghai, China). Blood samples were collected from the abdominal aorta and serum was isolated from blood after centrifugation (1000 × g for 15 min). The liver samples in each group were fixed with 10% formaldehyde, and other parts of the liver were stored at −80 °C until further use.

2.3. Histological analysis

The liver tissues were fixed in 10% formalin, paraffin-embedded and sectioned. The liver sections were stained with haematoxylin/eosin (H&E) for histological examination. Microscopic fields in all liver sections were randomly selected for examination using a light microscope (Olympus, Tokyo, Japan). The scoring of liver fibrosis degree was evaluated following the criteria. 0, no obvious fibrosis; 1, fibrosis present: collagen fibers that extend from the portal triad or central vein to peripheral regions; 2, mild fibrosis: few collagen fibers extending without formation of compartments; 3, moderate fibrosis: collagen fibers with formation of "pseudo leaves"; and 4, severe fibrosis: many collagen fibers with thickening of partial compartments and formation of "pseudo lobes".

Masson's trichrome staining is usually used to discriminate collagen fibers from tissues on histological slides. Besides HE staining, liver sections with Masson staining were to microscopically (Olympus, Tokyo, Japan) estimate collagen deposition. Five different fields were randomly observed in each slice (×100), and three slices were selected in each group. All histological examinations were undertaken by a very experienced pathologist blinded to the study protocol.

Table 1
Effects of AA on serum concentrations of ALT, AST and TBIL.

Group	n	ALT (U/L)	AST (U/L)	TBIL (μmol/L)
Normal	12	27.2 ± 4.8	20.4 ± 5.0	5.6 ± 0.85
Model	10	404.2 ± 27.1*	349.2 ± 34.7*	34.1 ± 5.6*
AA 5 mg/kg	9	322.6 ± 33.9[a]	243.1 ± 27.4[a]	26.1 ± 4.0[a]
AA 15 mg/kg	11	271.0 ± 30.9[a]	186.8 ± 39.0[a]	20.0 ± 3.8[a]

* $p < 0.05$ as compared with normal group.
[a] $p < 0.05$ as compared with model group.

2.4. Biochemical analysis

Serum alanine transaminase (ALT) and aspartate transaminase (AST) were determined by using a clinical automatic analyzer (Hitachi, Japan) and a commercial reagent kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's protocol. Hepatic hydroxyproline (hyp) content was measured with hydroxyproline detection kit (Sigma-Aldrich, St. Louis, Mo, USA) according to the manufacturer's protocol. In addition, hepatic MDA, SOD and GSH-Px activities were determined with corresponding detection kit (Jiancheng Institute of Biotechnology, Nanjing, China) according to the manufacturer's protocol. Liver fibrosis serum markers Hyaluronic acid (HA), Laminin (LN), collagen type IV (IV-C), and procollagen III N-terminal peptide (PIIINP) were measured by radioimmunoassay (RIA) kits (Beifang Inst. of Biotechnology, Beijing, China). All the procedures were performed based on the manufacturers' protocols.

2.5. Immunohistological staining

Immunohistological examinations were carried out to detect the expression of Bcl-2 and Bax. Briefly, liver sections were deparaffinized and treated with 3% H_2O_2 to block endogenous peroxidase activity. Antigen retrieval was performed in citrate buffer. After cooling, the sections were treated with 5% BSA to block non-specific protein binding. The sections were incubated with anti-Bcl-2 (1:200) and anti-Bax (1:200) overnight at 4 °C. Meanwhile, sections incubated with PBS alone were set as negative controls. Finally, the sections were washed with PBS, incubated with a biotinylated secondary antibody (1:1) and then with an avidin-biotin-peroxidase complex, and stained with DAB. All sections were imaged by microscope (Olympus, Tokyo, Japan).

2.6. Immunofluorescence staining

After being deparaffinized and blocked with 1% bovine serum albumin, the liver tissue sections were incubated with primary antibodies including anti-α-SMA (1:100), anti-p-AKT (1:200), anti-p-mTOR (1:50), anti-p-p70S6K1 (1:100), overnight at 4 °C. Meanwhile, sections incubated with PBS alone were set as negative controls. Subsequently, the sections were incubated with the fluorescent secondary conjugated Alexa Fluor-488 or Alexa Fluor-555 at room temperature for 2 h after three PBS washes. The cell nuclei were counter stained with DAPI, and all stained sections were observed and photographed by fluorescence microscope (Olympus, Tokyo, Japan).