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Curcumin protects against hepatic stellate cells activation and migration by inhibiting the CXCL12/CXCR4 biological axis in liver fibrosis : A study *in vitro* and *in vivo*

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ABSTRACT

The C-X-C motif chemokine 12/C-X-C chemokine receptor type 4 (CXCL12/ CXCR4) biological axis plays an important role in the pathogenesis of liver fibrosis. Curcumin is known to have an anti-fibrosis effect, but the specific mechanism needs to be elucidated. There is currently no evidence illustrating a connection between curcumin and the CXCL12/CXCR4 axis in liver fibrosis. Here, we investigated the contribution of curcumin on CXCL12/ CXCR4 biological axis in liver fibrosis. Our results showed that curcumin remarkably improved hepatic function and liver fibrosis, and the effects are similar as silymarin. The alleviation of liver fibrosis with curcumin treatment was associated with a reduction of CXCL12, CXCR4, α-SMA and RhoA. In addition, curcumin markedly inhibited the proliferation and migration of HSC-T6 cells. This study indicates that curcumin could protect against hepatic stellate cells activation and migration by inhibiting the CXCL12/CXCR4 biological axis in liver fibrosis.

1. Introduction

Liver fibrosis, a repair response of the liver against various injuries, is caused by the excess hyperplasia and deposition of extracellular matrix (ECM) in the liver. Liver fibrosis is a necessary stage by which all chronic liver injury develops into cirrhosis. Previous studies [1,2] have shown that liver fibrosis is a reversible pathological process; therefore, looking for effective drugs to block or reverse the occurrence and progress of liver fibrosis can avoid liver cirrhosis or liver cancer, which can significantly improve the prognosis of patients and save medical resources. Hepatic stellate cells (HSCs) are the cellular source of most of the ECM, and the activation and migration of HSCs is the central link of liver fibrosis. These processes are primarily regulated by cytokines, especially chemokines [3,4].

The C-X-C motif chemokine 12/C-X-C chemokine receptor type 4 (CXCL12/CXCR4) biological axis, mainly composed of chemokine CXCL12 and its receptor CXCR4, has been a hot topic in recent years. The CXCL12/CXCR4 axis is involved in a variety of pathological and physiological processes, such as inflammation, immunity, wound healing, tissue and organ fibrosis, etc.; in particular, it participates in the pathogenesis of liver fibrosis.

Chinese medicine has shown unique advantages and excellent prospects in the prevention and treatment of liver fibrosis, such as salvia, colchicine, silymarin, and so on. They can reverse liver fibrosis or early cirrhosis, and have been widely used in a clinical setting. Curcumin, a monomer extracted from the root of turmeric plants, is a kind of natural phenolic antioxidant. It has been found that curcumin has many pharmacological effects such as anti-inflammatory, anti-lipid peroxidation, anti-tumor and liver protection, etc. [5]. In recent years, curcumin has attracted widespread attention with regard to anti-fibrosis, but its pharmacological mechanism is not entirely clear. There is currently no evidence illustrating a connection between curcumin and the CXCL12/CXCR4 axis in liver fibrosis. This study aims to verify the role of CXCL12/CXCR4 axis in pathogenesis of liver fibrosis and investigate the effect of curcumin on the CXCL12/CXCR4 axis in liver fibrosis *in vitro* and *in vivo*.

2. Materials and methods

2.1. Chemical and reagents

Carbon tetrachloride (CCl_4) and Olive oil were purchased from Aladdin Biochemical Technology Co., Ltd (Shanghai, China). Curcumin (purity: 98%) was purchased from Macklin Biochemical Technology Co., Ltd (Shanghai, China). Silymarin was purchased from MADAUS GmbH (Cologne, Germany). RIPA and PMSF were purchased from

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Beyotime Biotechnology Co., Ltd (Shanghai, China). RNA Extraction Kit and PrimeScript RT Master Mix were purchased from Takara Biotechnology co., Ltd (TaKaRa, Shiga, Japan). All primers were synthesized by Takara Biotechnology co., Ltd (TaKaRa, Shiga, Japan). Primary antibodies against CXCR4, Tubulin and horseradish peroxidase (HRP)-conjugated anti-rabbit secondary antibodies were purchased from Abcam (Cambridge, MA, USA); primary antibodies against CXCL12 were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Primary antibodies against alpha-smooth muscle actin (α-SMA), ras homolog gene family (member A) (RhoA) and biotinylated goat anti-rabbit secondary antibodies were purchased from Boster Biological Technology Co., Ltd (Wuhan, China). Rat Collagen I (COL I), hyaluronic acid (HA), laminin (LN) and procollagen type III (PCIII) ELISA Kit were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd (Shanghai, China). Platelet-derived growth factor-BB (PDGF-BB) and CXCL12α was purchased from R&D Systems, Inc. (Minneapolis, MN, USA).

2.2. Animals and experiment protocols

Sixty 8-week-old, male Sprague-Dawley rats weighing 200–220 g were purchased from the Experimental Animal Center of Guangxi Medical University (Nanning, China) (Animal Use Certificate Number: SYXK Gui 2014-0003), and housed in an environmentally controlled room (23 ± 3 °C, 55 ± 10% humidity) on a 12 h light-dark cycle (06:00–18:00, light), with standard food and water provided *ad libitum*. The animal studies were carried out in strict accordance with the guidelines for the Care and Use of Laboratory Animals of Guangxi Medical University, Nanning, China. All efforts were made to minimize the pain and discomfort of the animals.

After a week-long acclimation period, rats were randomly divided into six groups according to random number table, including the following groups: control, model, silymarin, curcumin low dose ((CUR-L)), curcumin middle dose ((CUR-M)) and curcumin high dose ((CUR-H)) (n = 10/group). Liver fibrosis was induced by the administration of 1.5 ml of CCl_4/olive oil (2:3, v/v) / kg body weight by subcutaneous injection. Rats in all groups except the control group were injected with an olive oil solution of CCl_4 every three days for 8 weeks. Rats in the control group were injected with the same volume of saline for 8 weeks. The treatments were performed simultaneously with the establishment of the fibrosis model. Rats in the silymarin group were given silymarin orally at 50 mg/kg every day for 8 weeks. Rats in the (CUR-L), (CUR-M) and (CUR-H) groups were given curcumin orally at 50 mg/kg, 100 mg/kg and 200 mg/kg, respectively, every day for 8 weeks. Rats in the model group were orally given the same volume of saline as curcumin every day for 8 weeks.

2.3. Measurement of functional liver levels

At 48 h after the last treatment in the experiment, all rats were weighed and killed after being anaesthetized by an intraperitoneal injection of 10% chloral hydrate solution at 3 ml/kg. Immediately after blood sample collection, alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL) and albumin (ALB) levels in serum were measured with an automated analyzer (LX20; Beckman Coulter, Fullerton, United States) at the First Affiliated Hospital of Guangxi Medical University (Nanning, China).

2.4. Histopathology and immunohistochemistry

Harvested liver tissues were fixed in 40 g/L formaldehyde, embedded in paraffin and sectioned at a thickness of 4 μm. Hematoxylin and eosin (HE) staining and Masson's trichrome staining were used for histological structure analysis and fibrosis area analysis, respectively. Five random views of Masson trichrome-stained sections from each sample (n = 10/group) were captured by a light microscope (Olympus, Tokyo, Japan). The fibrotic area was checked with the Image J 1.44s software (National Institutes of Health, Bethesda, MD, United States) [6]. The percentage of the fibrotic area was calculated by comparing the collagen stained area to the total area.

Table 1
Primer sequences for polymerase chain reaction.

Gene name	Direction	Sequences(5′→3′)	Product Length (bp)
CXCL12	Forward	GAGCCAACGTCAAACATCTGAA	147
	Reverse	ACTTGTTTAAGGCTTTGTCCAGGTA	
CXCR4	Forward	ATTGTCCACGCCACCAACAG	119
	Reverse	ACATCGGCGAAGATGATGTCAG	
GAPDH	Forward	GGCACAGTCAAGGCTGAGAATG	143
	Reverse	ATGGTGGTGAAGACGCCAGTA	

The liver tissue sections were de-waxed, hydrated and subjected to 5% H_2O_2 for 10min. Then, slides were transferred to sodium citrate buffer for heat-induced antigen retrieval. Sections were blocked and incubated overnight at 4 °C with rabbit anti-α-SMA antibody (1:200) or rabbit anti–RhoA antibody (1:150), respectively. All antibodies were diluted in tris buffered saline 5% bovine serum albumin, while negative-control sections were incubated with Phosphate Buffered Saline (PBS) instead. After that, the slides were incubated with biotinylated goat anti-rabbit secondary antibodies for 15 min at 37 °C according to the instructions. The immunoreactivities were visualized with 3,3′-diaminobenzidine tetrahydrochloride (DAB) solution, followed by staining the nucleus with hematoxylin, dehydrating, and mounting. The sections were analyzed under a light microscope. The Image-Pro Plus 6.0 system (Media Cybernetics Inc., Bethesda, MD, USA) was used for the semi-quantitative evaluation. Integrated optical density per area (IOD/area) was applied to indicate the level of corresponding protein expression [7].

2.5. Cell culture and cell proliferation assay

The HSC-T6 cell line sample was purchased from Procell Life Science & Technology Co., Ltd (Wuhan, China). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Tianjin Haoyang Biological Manufacture Co., Ltd., Tianjin, China), and incubated at 37 °C in 5% CO_2 air. Following 2 weeks of culture on plastic tissue-culture dishes, the cells were plated at a density of 2×10^4 cells/well in 96-well plates. The HSC-T6 cells were activated with PDGF-BB. The concentration of PDGF-BB used for treatment was 10 ng/ml for 24 h at 37 °C.

Cell proliferation was evaluated using a 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. After HSC-T6 cells were digested by trypsin (0.25%), single suspended state cells were cultured in DMEM medium supplemented with 10% FBS and then seeded in a 96-well plate with 180 μL (5×10^4 cells/mL) per well. After 24–96 h incubation, cells were exposed to curcumin dissolved in 0.1% dimethyl sulfoxide (DMSO) at different concentrations (0, 10, 20, 40, 80, 160 μM) for 24 h. Untreated cells were used as controls. MTT solution (20 μL, 5 mg/mL, diluted in PBS) was added to each well and the plate was incubated at 37 °C in a 5% CO_2 atmosphere for 4 h. Then, the supernatants were removed and 150 μL/well DMSO was added to dissolve formazan crystals. After 5 min of shaking dissolution, the absorbance at 490 nm was read using ELx800 Microplate Reader (BioTek Instruments, Inc., Winooski, VT, USA).

To carry out follow-up experiments, HSC-T6 cells were randomly divided into four groups: the control group, the PDGF-BB group, the PDGF-BB + curcumin (20 μM) group and the PDGF-BB + curcumin (40 μM) group. The concentration and use of PDGF-BB are the same as above. All cells except the control group were activated with PDGF-BB. Cells in the PDGF − BB + curcumin (20 μM) group and the