Journal of Ethnopharmacology 145 (2013) 175–181

Contents lists available at SciVerse ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jep

Fuzheng Huayu inhibits carbon tetrachloride-induced liver fibrosis in mice through activating hepatic NK cells

Qi Cheng [a], Ning Li [a], Mingquan Chen [a], Jianming Zheng [a], Zhiping Qian [b], Xinyu Wang [a], Chong Huang [a], Qian Li [a], Qingxia Lin [a], Guangfeng Shi [a,*]

[a] Department of Infectious Diseases, Huashan Hospital of Fudan University, Shanghai 200040, China
[b] Intensive Care Unit, Shanghai Public Health Clinical Center, Fudan University, Shanghai 201508, China

ARTICLE INFO

Article history:
Received 18 May 2012
Received in revised form
14 October 2012
Accepted 25 October 2012
Available online 2 November 2012

Keywords:
Liver fibrosis
Natural killer cells
Chinese medicine
Fuzheng Huayu

ABSTRACT

Aim of the study: Fuzheng Huayu (FZHY) is a Chinese compound herbal preparation which consists of six Chinese herbs. This study examines the preventative effects of FZHY on liver fibrosis induced by carbon tetrachloride (CCl_4) and explores its possible mechanisms of action.

Materials and methods: Liver fibrosis was induced in male C57BL/6N mice by injecting a 10% CCl_4 solution intraperitoneal twice a week for six weeks. After 6 weeks of treatment, serum ALT and AST assay, liver tissue histological examination and immunostaining were carried out to examine the liver function and fibrosis degree. The expression levels of alpha-smooth muscle actin (SMA) were measured by quantitative real-time PCR and western blot. Hepatic natural killer (NK) cells were isolated from liver and evaluated by FACS.

Results: Upon pathological examination, the FZHY-treated mice showed significantly reduced liver damage. The expression of α-SMA increased markedly upon treatment with CCl_4 and the increase was reversed by FZHY treatment. FZHY treatment also enhanced the activation of hepatic NK cells and the production of interferon-gamma (IFN-γ). The protective effects of FZHY were reversed in the mice that were depleted of NK cells by anti-ASGM-1 Ab treatment.

Conclusions: FZHY can efficiently inhibit CCl_4-induced liver fibrosis. Furthermore, the depletion of NK cells attenuates the protective effects of FZHY. We conclude that FZHY could be an effective drug for liver fibrosis, and its mechanism of action involves the activation of hepatic NK cells.

1. Introduction

Liver fibrosis, a precursor of cirrhosis, is a consequence of chronic liver disease (Achliya et al., 2004) and involves an increased production of extracellular matrix (ECM) components, particularly collagens (Ballin et al., 1988). Hepatic stellate cells (HSCs) play a central role in the pathogenesis of hepatic fibrosis (Baroni et al., 1996) as they secrete the vast majority of ECM components (Bataller and Brenner, 2005). The activation of HSCs is controlled by a wide variety of cytokines, growth factors, and other molecules such as reactive oxygen species. Among the HSC-activating molecules, transforming growth factor-beta (TGF-β) and platelet-derived growth factor (PDGF) are the two most important cytokines responsible for HSC activation and proliferation, respectively. IFN-γ is also one of the more important cytokines to inhibit stellate cell activation (Friedman, 2008; Friedman et al., 2007; Masuhara et al., 1996). It has been shown that natural killer (NK) cells possess antifibrotic activity through direct killing HSCs (Radaeva et al., 2006). Clinically, it has also been observed that NK cell activity is negatively correlated with liver fibrosis in patients with chronic hepatitis C infections, suggesting that NK cells may prevent liver fibrosis in patients (Morishima et al., 2006).

Currently, some Chinese herbal medicines, such as Fuzheng Huayu (FZHY), might provide alternative treatment options for hepatic fibrosis. FZHY has been State Food and Drug Administration (SFDA)-approved as an antifibrotic medicine in China (Zhao et al., 2006). FZHY consists of six Chinese medicinal herbs, namely Semen Persicae, Radix Salvia Miltiorrhizae, Gynostemma Pentaphyllammak, Cordyceps, Pollen Pini and Fructus Schisandrae Chinensis. Traditional Chinese Medicine (TCM) theory has proposed that this concoction function of Chinese herbs can promote blood flow, dissolve blood clots, fortify the spirit, and nourish the liver.

Abbreviations: FZHY, Fuzheng Huayu; CCl_4, Carbon tetrachloride; NK, Natural killer; SMA, Smooth muscle actin; IFN, Interferon; ECM, Extracellular matrix; HSCs, Hepatic stellate cells; TGF-β, transforming growth factor-beta; PDGF, Platelet-derived growth factor; TCM, Traditional Chinese Medicine; SFDA, State Food and Drug Administration; ASGM-1, Anti-asialoGM-1; AST, Asparate transaminase; ALT, Alanine transaminase; MNCs, Mononuclear cells

* Corresponding author. Tel.: +86 21 52888124; fax: +86 21 62480170.
E-mail addresses: gfshi@shmu.edu.cn, gigich@yeah.net (G. Shi).

0378-8741/$ - see front matter © 2012 Elsevier Ireland Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.jep.2012.10.047

Pharmacological studies and clinical trials have demonstrated that FZHY is an effective antagonist of liver fibrosis and that many of the effects are attributable to the pharmacological actions of the herbs chemicals (Wang et al., 2010). Mechanistic studies have shown that FZHY decreases α-SMA protein expression in fibrotic livers, as examined by Western blot and immunohistochemical stain, and also attenuates ECM deposition in the liver (Liu et al., 2009). However, complete picture of mechanism by which FZHY inhibits liver fibrosis remains unclear.

The aim of this study was to investigate the therapeutic effects of FZHY in a mouse model of CCl_4-induced liver fibrosis. Furthermore this study aims to assess whether FZHY could affect the activation of liver NK cells in mice with liver fibrosis, and to evaluate hepatic NK cells activation as the potential mechanism through which FZHY prevents liver fibrosis.

2. Materials and methods

2.1. Drugs and chemicals

FZHY was prepared and provided by Pearl Ocean Pharmaceutical Holdings Limited, China (SFDA approval no: Z20050546) (Shanghai, China). The following crude herbs were combined to make the FZHY extract powder: 8.0 g of Danshen, 4.0 g of Dongchongxiacao, 2.0 g of Wuweizi, 2.0 g of Taoren, 2.0 g of Songhuafen, and 6.0 g of Jiaogulan. The quality control and preparing stardardization of FZYH is established and enforced strictly by Shanghai Huanghai Medicine Co., Ltd. In this study, FZHY powder was suspended in distilled water.

Carbon tetrachloride, paraformaldehyde, formalin, Sirius Red and picric acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Trypan blue was obtained from Gibco Life Technology (USA). Phosphate-buffered saline (PBS) was purchased from Disheng Biotech Co. (Shanghai, China).

2.2. Animals and treatments

Forty male C57BL/6N mice weighing 18–25 g were used for in vivo experiments. The mice were purchased from the Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China) and met the approval of the Ethical Committee of Fudan University. The mice were randomly divided into 5 treatment groups: control ($n=8$), model ($n=8$), FZHY treatment (FZHY) ($n=8$), FZHY plus anti-asialoGM-1 antibody (Ab) treatment ($n=8$), FZHY plus placebo Ab treatment ($n=8$). Liver fibrosis was induced by intraperitoneal (IP) injection with CCl_4 (suspended at 10% in olive oil, 2 mL/kg, twice a week) for 6 weeks. After 6 weeks of CCl_4 treatment, the mice in the FZHY treatment groups were administered with FZHY at 4.0 g (crude drug)/kg body weight once a day for 4 weeks.

2.3. Depletion of natural killer cells by anti-asialo GM-1 antibodies

To chronically deplete NK cells (NK1.1+CD3−), anti-asialoGM-1 (ASGM-1) antibody (Ab; 100 μL per mouse; catalog no. 986-10001;Wako, Richmond, VA) was injected IP into the appropriate mice. The mice administered FZHY were treated with anti-ASGM-1 ($n=8$) or placebo ($n=8$) Ab every 48 h for 4 weeks. Depletion of the NK (NK1.1+CD3−) cells was confirmed by flow cytometry. The mice were sacrificed 4 weeks after treatment, after that the livers were surgically excised. A portion of each liver was fixed in 10% phosphate-buffered formalin and embedded in paraffin for histological study. The remainder of the sample was snap-frozen in liquid nitrogen and stored at −80 °C for RNA isolation and protein extractions. All experimental procedures were conducted in accordance with internationally accepted standards for laboratory. All animals received humane care during the study with unlimited access to chow and water.

2.4. Histological assessment of liver injury

The posterior one-third of each liver was fixed in 10% formalin for 24 h and then paraffin-embedded using an automated tissue processor. 7 μm sections were cut from tissue blocks constructed from the livers of each animal. The sections were dewaxed in xylene, rehydrated in serial alcohols and washed in tap water. The slides were incubated in filtered hematoxylin for 1 min, washed in tap water, blued in Scott's tap water and then counter stained with eosin for 30 s. They were dehydrated in serial alcohols and xylene and finally mounted with cover slips. The samples were also stained in 0.1% Sirius Red in saturated picric acid to detect collagen.

2.5. Fibrosis quantitation

The relative fibrosis area (expressed as a percentage of the total liver area) was assessed based on 36 fields from 9 Sirius Red-stained liver sections per animal. Each field was acquired at 10 × magnification and then was analyzed using a computerized Bioquant at morphometry system. To evaluate the relative fibrosis area, the area of collagen staining was divided by the net field area and multiplied by 100%. Subtraction of the vascular luminal area from the total field area yielded the final calculation of the net fibrosis area.

2.6. RNA isolation and quantitative real-time PCR (qPCR)

Total RNA was extracted from whole liver samples using TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Hepatic complementary DNA (cDNA) was used to quantify α-SMA, TGF-β, PDGF-β and IFN-γ messenger RNA (mRNA) levels by qPCR using the SYBRGreen Master Mix (Applied Biosystems, Foster, CA). Mice GAPDH mRNA levels served as an internal control to assess the overall cDNA content. The primers were as follows: α-SMA: forward, 5′- CCCAGGTATTGTGCTGGACT-3′; reverse, 5′-GAAGGAATAGCCACGCTCAG-3′,TGF-β: forward, 5′-CCTGTAGCCCACGTCGTAGC-3′; reverse, 5′-TTGACCTCAGCGCTGAGTTG -3′, PDGF-β: forward, 5′-GTACAAATGACACGCCATGC-3′; reverse, 5′-ACCAGAGCAGAACAGGGAGA-3′; IFN-γ: forward, 5′-TGGGCTTTGATGATGAATGA -3′; reverse, 5′-TGGAAAGGCAGAAGCAAAGT-3′; and β-actin: forward, 5′-AGAGGGAAATCGTGCGTGAC-3′, reverse, 5′-CAATAGTGATGACCTGGCCGT-3′. The cycling parameters for the PCR program were as follows: initial denaturation step for 10 min at 95 °C and then 40 cycles consisting of 10 s at 95 °C, 5 s at 58 °C, and 30 s at 70 °C. The data were analyzed using the comparative cycle threshold method, and these values were normalized to the β-actin expression levels.

2.7. Cell preparation

Mice livers were removed and pressed through 200-gauge stainless steel wire mesh. The liver cell suspension was collected, suspended in RPMI 1640 medium (Invitrogen, Carlsbad, CA) and centrifuged at 50g for 5 min. The supernatants containing mononuclear cells (MNCs) were collected, washed in PBS, and resuspended in 40% Percoll (Sigma) in PBS. The cell suspension was gently overlaid onto 70% Percoll and centrifuged for 20 min at 750g. MNCs were collected from the Percoll interface, washed twice in PBS, and resuspended in PBS containing 2% fetal bovine serum for FACS analysis.