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The inhibition of Hippo/Yap signaling pathway is required for magnesium isoglycyrrhizinate to ameliorate hepatic stellate cell inflammation and activation

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ABSTRACT

Liver fibrosis is a reversible pathological process accompanied by abnormal inflammation, and its end-stage cirrhosis is responsible for high morbidity and mortality worldwide. This study was to investigate the effect of Magnesium isoglycyrrhizinate (MgIG) on liver fibrosis and inflammation, and to further clarify molecular mechanism. We found that MgIG treatment significantly alleviated carbon tetrachloride (CCl_4)-induced liver fibrosis and HSC activation by regulating TGF-β signaling and MMP/TIMP systems. In addition, MgIG treatment significantly inhibited the inflammatory response of liver fibrosis in mice characterized by reduced pro-inflammatory factors expression and increased anti-inflammatory factors expression. Interestingly, experiments *in vitro* also showed that MgIG treatment significantly reduced the expression of hepatic stellate cell (HSC) activation markers. Besides, MgIG treatment not only inhibited the expression of pro-inflammatory factors, but also promoted the production of anti-inflammatory factors in activated HSCs. Importantly, treatment with MgIG inhibited Hippo/Yap signaling pathway, which was a potential mechanism for MgIG-induced anti-inflammatory effects. The overexpression of Hippo/Yap signaling effector YAP completely impaired MgIG-induced anti-inflammatory and anti-fibrotic effects. Taken together, these results provide novel implications to reveal the molecular mechanism of the anti-inflammatory properties induced by MgIG, by which points to the possibility of using MgIG to treat liver fibrosis.

1. Introduction

Liver fibrosis is an invertible pathophysiological process associated with intense repair and cicatrization mechanisms [1]. As the pathogenesis progresses without effective management, advanced liver fibrosis can seriously damage the normal function of the liver and give raise to many serious complications, ultimately resulting in hepatocellular carcinoma and liver cirrhosis [2]. Attractively, early cirrhosis and liver fibrosis are dynamic and reversible, thus intervention of fibrogenesis process is necessary for preventive treatment of cirrhosis and hepatic failure [3]. In recent years, natural products have been widely accepted as realistic options for the treatment of liver fibrosis [4]. Novel anti-fibrosis compounds from herbal components represent an attractive alternative for drug development. [4]. Magnesium isoglycyrrhizinate (MgIG), a safe and natural product, shows a good deal of pharmacological activities, including anti-apoptosis, anti-tumor, and anti-oxidant properties [5–7]. However, whether MgIG can ameliorate the inflammatory microenvironment to improve liver fibrosis remains unclear. In the present study, we aimed to assess the effect of MgIG on inflammation of liver fibrosis and further determine the underlying mechanisms.

Inflammation is a central feature of liver fibrosis as suggested by its role in activation of hepatic stellate cells (HSCs) leading to extracellular matrix deposition [8]. Following liver damage, an accumulation of recruited inflammatory cells occurs at the site of injury [9]. A wide repertoire of pro-inflammatory factors and anti-inflammatory compounds, which encompasses cytokines, chemokines, growth factors, and products of oxidative stress, mediates the inflammatory response of immune cells during the fibrosis process [10]. HSCs also take part actively in the inflammation process through interaction with diverse types of immune cells [11]. Furthermore, inflammatory mediators, such as transforming growth factor-beta (TGF-β) and tumor necrosis factor-

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alpha (TNF-α), may induce HSC conversion from a quiescent to an activated state characterized by a myofibroblast-like phenotype responsible for proliferation and excessive extracellular matrix deposition [12]. Therefore, inflammation may be the target of reducing liver fibrosis. Noteworthy, Bian M et al. recently showed that MgIG promoted HSC apoptosis via endoplasmic reticulum stress and ameliorated fibrogenesis *in vitro* and *in vivo* [13]. Attractively, whether MgIG can ameliorate liver fibrosis by controlling inflammatory microenvironment is worth to further research.

Several lines of evidence indicated that the Hippo/Yap signaling played a critical role in fibrous diseases [14,15]. The core components and downstream effectors of the Hippo/Yap signaling are very conservative in mammals and include LATS1/2, MST1/2, MOB1, SAV1, TAZ and YAP. MST1/2 composes a heterodimer by the adaptor protein Salvador 1 (SAV1), which reinforces MST1/2 activity of kinase and boosts MST-LATS interaction [16]. Afterwards, Mob1 homolog (MOB1) and LATS1/2 were phosphorylated by MST1/2 [16]. Phosphorylated MOB1 combines with the autoinhibitory domain of LATS1/2, being capable of the phosphorylation and activation of LATS1/2 [16]. The transcription of the co-factors TAZ and YAP were phosphorylated and inhibited by activated LATS1/2 [16]. Interestingly, Hippo/Yap signaling was related to the development of liver fibrosis, and targeting regulation of YAP can inhibit HSC activation [14,15]. Moreover, mutations in YAP that uncouple its ability to apperceive cell tension result in disorganized tissue and organ development, therefrom offering a paradigm by which YAP activity can coordinate liver size [17]. Importantly, determining the function of Hippo/Yap signaling in pathological conditions will furnish a brand new perspective to open out the pathological mechanism and new ideas about the effective diagnostic signs and therapeutic targets in inflammatory diseases.

In the current study, we first determined the effect of MgIG on inflammation of liver fibrosis, and to further examine the underlying mechanisms in this molecular setting. We showed that MgIG inhibited HSC inflammation and activation by modulating Hippo/Yap signaling. Our results suggest that MgIG as an impactful drug is the remedy for liver fibrosis.

2. Materials and methods

2.1. Reagents and antibodies

MgIG (99.7% pure, #12050617) was bought from the Chia Tai Tianqing Pharmaceutical Group Co., Ltd. (Nanjing, China). Recombinant mouse platelet derived growth factor-BB (PDGF-BB, #CRP138D) was bought from Cell Sciences (Canton, MA). Dimethyl sulfoxide (DMSO, #156914), Carbon tetrachloride (CCl_4, #488488), and Phosphate buffered saline (PBS, #P5368) were bought from Sigma-Aldrich (St Louis, MO). Fetal bovine serum (FBS), Opti MEM medium, trypsin-EDTA, and Dulbecco's modified essential medium (DMEM) were bought from GIBCO BRL (Grand Island, NY). Primary antibodies against β-actin (#ab8226), IL-1β (#ab9722), TNF-α (#ab8348), NF-κB (#ab16502), NLRP3 (#ab4207), α-SMA (#ab5694), Collagen1 (#ab34710), Desmin (#ab32362), Fibronectin (#ab2413), LATS1 (#ab70561), MST1 (#ab124787), YAP (#ab205270), and Lamin B (#ab16048) were bought from Abcam Technology (Abcam, Cambridge, UK). Primary antibodies against phospho-MST1 (#bs-3246R), phospho-MST2 (#bs-4082R), and phospho-LATS1 (#bs-3246R) were purchased from Beijing Biosynthesis Biotechnology Co., Ltd. (Beijing, China). Primary antibody against WWC2 (#sc-515892) was bought from Santa Cruz Biotechnology (Heidelberg, Germany).

2.2. The scheme of animal experiment

The scheme of animal experiment was approved by the institutional and local animal care and use committees of Zhang Zhongjing Traditional Chinese Medicine College (Nanyang, China), and this study was conducted according to the international principles for laboratory animal care and use. Ninety eight-week-old male C57BL/6 mice were bought from Branch of Beijing Weitong Lihua Experimental Animal Technology Co., Ltd. (Beijing, China) and randomly divided into nine groups of ten animals each. The randomization was performed using a freely accessible computer program for scientific randomizations at www.randomizer.org. Liver fibrosis was induced by injecting 10% carbon tetrachloride (CCl_4, 0.5 mL/100 g bodyweight) for 8 weeks (three times a week) in wild type mice [18]. Mice in the control group received olive oil intraperitoneal injection. Mice in the model group received CCl_4 intraperitoneal injection. Mice in the treatment groups (group 3, 4, 5) were i.p. injected by CCl_4, and followed by MgIG with 15, 30 and 60 mg/kg (once daily, weeks 5–8), respectively. Mice in group 6 were injected with control vector (a control adenovirus) by tail vein, and i.p. injected with CCl_4. Mice in group 7 were i.p. injected with MgIG (30 mg/kg) and CCl_4, and injected with control vector by tail vein. Mice in group 8 were injected with YAP plasmid (adenovirus encoding mouse YAP) by tail vein and i.p. injected with CCl_4. Mice in group 9 were injected with YAP plasmid by tail vein, and i.p. injected with MgIG (30 mg/kg) and CCl_4. The endotoxin contents of MgIG with 15, 30 and 60 mg/kg were very low, and were similar to the contents of vehicle control. Adenoviruses (2.5×10^7pfu/g, once per 2 weeks) were injected into mice by tail vein [19]. Blood and liver samples from each group were collected at the end of the experiment for subsequent assy.

2.3. Histological analysis

The liver tissues were embedded in paraffin and sectioned to slices of 4-μm thick. Hematoxylin and eosin (H&E), Sirius Red, and Masson staining were performed for histological studies [19]. The areas of H&E, Sirius Red and Masson staining from 10 random regions were quantified With Image J.

2.4. Liver function assessment

The enzyme-linked immunosorbent assay methods were performed to detect the levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), hydroxyproline, hyaluronic acid (HA), procollagen type III (PC-III), collagen type IV (IV-C), and laminin (LN) according to the kit instructions (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) [19].

2.5. Primary mouse HSC isolation and characterization

Primary mouse HSCs were isolated from the mouse liver according to a previous protocol [20]. Briefly, the livers of the mice were first perfused in situ with DMEM-free including 2 mg/ml pronase (Sigma, #PRON-RO) and 1 mg/ml collagenase IV (Sigma, #V900893) following HBSS including 0.5 mM EDTA (Sigma, #E6758). After a few minutes of perfusion, the digested hepatic cells were dispersed in DMEM-free, and the livers were removed. Subsequently, filamentous gelatinous material was prevented by DNA enzymes, and a filter was used to remove the undigested debris. The filtrates in a centrifuge tube were centrifuged at 50 *g* for 5 min at 4 °C. After gradient centrifugation, the supernatant was collected to isolate primary HSCs with 25% Nycodenz (Sigma, #D2158). Purification and characterization of the obtained HSCs were confirmed by detection of α-SMA and PDGF-Rβ [20].

2.6. Immunofluorescence assay

Immunofluorescence assay of HSCs or liver tissues was conducted as previous description [19]. Celluar nucleus was stained by 4′, 6-Diamidino-2-phenylindole (DAPI, Sigma, #D9542). Fluorescence images were obtained by the fluorescence microscope. The fluorescent intensity of target proteins was calculated using software Image J.