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Geniposide alleviates inflammation by suppressing MeCP2 in mice with carbon tetrachloride-induced acute liver injury and LPS-treated THP-1 cells

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ABSTRACT

Geniposide (GP), an iridoid glucoside extracted from *Gardenia jasminoides* Ellis fruits, has been used as a herbal medicine to treat liver and gall bladder disorders for many years. However the mechanism of anti-inflammatory is largely unknown. In this study, GP significantly attenuated inflammation in acute liver injury (ALI) mice model and in lipopolysaccharide (LPS)-induced THP-1 cells. It was demonstrated that GP obviously decreased the expression of Methyl-CpG binding protein 2 (MeCP2) in vivo and in vitro. Knockdown of MeCP2 with siRNA suppressed the expressions of IL-6 and TNF-α, while over-expression of MeCP2 had a proinflammatory effect on the expression of IL-6 and TNF-α in LPS-induced THP-1 cells. Mechanistically, it was indicated that GP had anti-inflammatory effects at least in part, through suppressing MeCP2. Interestingly, GP could attenuate expressions of Sonic hedgehog (Shh) and GLIS family zinc finger 1 (GLIS1) but increase Ptched1 (PTCH1) expression. Similar findings were also demonstrated at the protein level by siRNA MeCP2. Furthermore, over-expression of MeCP2 obviously increased Shh and GLIS1 expressions but reduced PTCH1 expression. Taken together, GP may serve as an effective modulator of MeCP2-hedgehog pathway (Hh)-axis during the pathogenesis of inflammation. Our findings shed light on the potential therapeutic feature of GP in recovering inflammatory diseases.

1. Introduction

Liver injury or dysfunction is well recognized as a serious health problem [1]. Emerging evidence has demonstrated the infiltration of mononuclear phagocytes into the liver correlated with the severity of liver injury [2]. Kupffer cells (KC) are among cells that respond to endotoxins [3,4]. It is now well accepted that high portal level of LPS can lead to a pronounced secretion of pro-inflammatory mediators by KC and ultimately cause endotoxin-induced liver injury [5]. Moreover, pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-6 (IL-6) have been linked to liver injury, and shown to be important mediators in liver injury [6]. Thus, inhibition of KC activity and pro-inflammatory cytokine production would be beneficial to alleviate liver injury [7]. On the basis of these strategies, the use of complementary and alternative medicines, such as natural antioxidants and hepato-protective plant products, has been popular in the last decade [8].

Geniposide, an iridoid glucoside extracted from *Gardenia jasminoides* Ellis fruits, have been used as a herbal medicine to treat liver and gall bladder disorders for many years [9]. Previous study has illustrated the effectiveness of GP in reducing plasma markers of NASH in rats by our group [10]. However, no detailed analysis of the beneficial effects and mechanisms of GP has ever been reported in CCl_4-induced liver injury.

Emerging evidence has demonstrated a central role for hedgehog signaling (Hh) in inflammation. Hh pathway could cross talk with WNT, Notch, FGF, and BMP signals to constitute the cell signaling network [11]. Available evidence suggested that Hh-responsive cell populations expand significantly in many types of adult liver injury and regress [12,13]. These findings demonstrated potential therapeutic targets of Hh pathway in liver disease. Methyl CpG binding protein 2 (MeCP2), a transcriptional repressor, promotes repressed chromatin structure and is detected in liver disease. Previous study by our group demonstrated that MeCP2 played a central role in activation of hepatic stellate cells [14]. Furthermore, MeCP2 was reported to regulate Hh pathway in liver fibrosis [15].

In the current study, it was suggested that GP played a hepato-protective role in regulating Hh pathway and cytokine production through MeCP2-dependant mechanism.

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2. Materials and methods

2.1. Animal and treatment

C57BL/6 J mice were obtained from the Experimental Animal Center, Anhui Medical University. The study and procedures were approved by the Ethic Committee and the Animal Experimental Committee of Anhui Medical University. All mice received human care in compliance with the Animal Experiments Guidelines and Animal Care of Chinese Academy of Sciences. The mice were randomly divided into three groups (n = 10 per group): vehicle group, CCl_4 group and GP treatment group (GP 200 mg/kg). In GP treatment group, 200 mg/kg of GP was given via gavage for three days before the ALI mice model was induced by CCl_4. The mice of CCl_4 group and GP treatment group were injected intraperitoneally with a single dose of 1% CCl_4 (10 ml/kg in oil) on the fourth day, to induce acute liver injury while vehicle groups received the same volume of vehicle (oil) intraperitoneally.

2.2. Reagents

Geniposide (GP) (HPLC ≥ 98%), a yellow powder, was purchased from Biological Products (Nanjing, China). LPS (*Escherichia coli* 055:B5) and PMA(TLC ≥ 99%) were purchased from Sigma Chemical (St. Louis, MO, USA). Mouse monoclonal antibody β-actin was purchased from Bioworld (Minnesota, America). Rabbit MeCP2, PTCH1, Shh and GLIS1 polyclonal antibody were purchased from Bioss (Beijin, China). Rabbit IL-6 and TNF-α polyclonal antibody were purchased from Bioworld (Minnesota, America) and Abcam (Cambridge, UK). ALT and AST activity assay kits were purchased from Jiancheng (Nanjing, China). Enzyme Linked Immunosorbent Assay (ELISA) kit was acquired from Hushang Biotechnology Co. Ltd. (Shanghai, China).

2.3. Isolation of liver macrophages

Liver macrophage cells were isolated following a method established previously [16]. Briefly, the liver was perfused in situ with PB, a kind of buffer solution containing NaCl, KCl, HEPES and NaOH, to purge the liver of blood, followed by a digestion buffer (1 × PBC, supplemented with collagenase (type IV, Sigma, St. Louis, MO, USA, Pronase; Sigma, St. Louis, MO, USA), 4.76 mM CaCl2). After digestion, the liver was destroyed in BSA solution. Then liver macrophage cells were obtained by centrifugation over 25% and 50% Percoll (Sigma, St. Louis, MO, USA) gradient for 30 min at 1350 g.Liver macrophage cells were collected under the interface.

2.4. ALT/AST activity assay

The concentrations of ALT and AST in serum of C57BL/6 J mice with the acute liver injury were determined by ALT and AST activity assay kits according to the manufacturer's instructions. The absorbances at 510 nm were obtained with a Multiskan MK3 (Biotek, USA).

2.5. Histopathology

The liver specimens were fixed with 4% paraformaldehyde for 24 h and embedded in paraffin. Hematoxylin and eosin (H&E) staining was performed according to a standard procedure. The pathological changes were assessed and photographed under an Olympus BX-51 microscope.

2.6. Immunofluorescence staining

Cultured THP-1 cells were induced by PMA (200 ng/ml), then LPS (1 μg/ml) and different concentrations of GP were treated. Immunofluorescence staining was performed with rabbit anti-MeCP2 (Bioss, China). Alexa Fluor 594-conjugated anti-rabbit was used as secondary antibody. Counterstaining of nuclei was performed with 4′,6-diamidino-2-phenylindole (DAPI; Biyuntian biological technology, Shanghai, China).

2.7. Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of IL-6 and TNF-α in serum obtained from mice and THP-1 cells culture supernatant were measured using commercial ELISA kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's instruction. Three duplicate wells were set up for each group.

2.8. Cell culture

THP-1 cells, provided from Cell Bank of Academy of Sciences (Shanghai, China), were cultured in RPMI 1640 medium (HyClone, USA) with supplemented with 10% (vol/vol) heat-inactivated fetal bovine serum (Bovine, China) at 37 °C humidified incubator under 5% CO_2. To induce THP-1 to macrophages, the cells were cultured by adding 200 ng/ml PMA for 24 h, 100 ng/ml LPS for 6 h and different concentrations GP or not for 48 h in the suitable conditions.

2.9. Plasmid construction

Expression plasmid for MeCP2 was generated by amplifying complementary DNA (cDNA) from pancreas cDNA and inserting cDNA coding for MeCP2 into destination vectors by Gateway cloning (Invitrogen, USA). The following primers were used for amplification: forward: 5′-CCGGAATTCCGGATGGT AGCTGGGAT-3′; reverse: 5′-CGCGGATCCGCG TCAGCTA ACTCTCT-3′. The N-terminal region of the MeCP2 coding region containing the predicted CARD domain was cloned into pEGFP-C2 vector by using restriction sites EcoR I and BamH I. Cell transfection was performed with the Lipofectamine TM 2000 according to the manufacturer's manuals.

2.10. RNA interference analysis

Small interfering RNA targeting Mecp2 was purchased from GenaPharma Corporation (Shanghai, China). MeCP2-siRNA (human) 5′-GCUUCCCGAUU AACUGAAAtt-3′ for the sense strand and 5′-UUUC AGUUAAUCGGGAAGCtt-3′ for the antisense strand. THP-1 cells were cultured in 6-well plates with antibiotic-free DMEM (Opti-MEM) for 24 h before operation. Then, the siRNAs were transfected into THP-1 cells using Lipofectamine TM 2000 according to the manufacturer's protocol. Knockdown efficiency was determined by Western blot analysis. Three independent transfection experiments were performed.

2.11. RNA isolation and quantitative real-time PCR

Total RNA was extracted from THP-1 cells with TRIzol (Invitrogen, USA), and reverse transcribed to cDNA using TAKARA kit (TAKARA, Japanese). The reaction mixture was prepared according to the manufacture's instruction using SYBRGreen qPCR Master Mix (QIAGEN, Germany). The primers used were as following: β-actin (forward: 5′-CCCACACTGTGCCCATCTACG-3′ reverse: 5′-GCCATCTCTTGCTCG AAGT CC-3′), MeCP2 (forward: 5′-GCCGAGAGCTATGGACAGCA-3′ reverse: 5′-CC AACCTCAGACAGGTTTCCAG-3′). PCR was performed 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s and at 60 °C about 1 min by using Thermo Step One. Reactions were conducted three times, and threshold cycle values were normalized to β-actin gene expression. Melting curve analysis was used to determine the specificity of the products. Relative mRNA expression of target genes were obtained by normalized to control group and the level of β-actin.

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