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Astragaloside prevents BDL-induced liver fibrosis through inhibition of notch signaling activation

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ABSTRACT

Ethnopharmacological relevance: Huangqi decoction was first described in Prescriptions of the Bureau of Taiping People's Welfare Pharmacy in the Song Dynasty (AD1078). It consists of Radix Astragali (*Astragalus membranceus (Fisch.) Bge.* Root, Huangqi) and Radix Glycyrrhizae (*Glycyrrhiza uralensis Fisch.*, root and rhizome, Gancao), and it is an effective recipe that is usually used to treat consumptive disease and chronic liver diseases. *Astragaloside* (AS) is a main component of Radix Astragali had an effect similar to the Huangqi decoction on hepatic fibrosis.

Aim of the study: Cholestasis is associated with a number of chronic liver diseases and Notch signaling has been demonstrated to be involved in ductular reaction. Previous studies have shown that AS can prevent the progression of cholestatic liver fibrosis, however, whether AS affects the Notch signaling pathway is unclear.

Materials and methods: Cholestatic liver fibrosis was established by common bile duct ligation (BDL) in rats. At first weekend, the rats were randomly divided into a model group (BDL), an AS group, and a Sorafenib positive control group (SORA) and treated for 3 weeks. Bile duct proliferation and liver fibrosis were determined by tissue staining. Activation of the Notch signaling pathway was evaluated by analyzing expressions of Notch-1, -2, -3, -4, Jagged 1 (JAG1), Delta-like (DLL)-1, -3, -4, Hes1, Numb and RBP-Jκ. Activation of the Wnt signaling pathway was evaluated by analyzing expressions of Wnt-4, -5a, -5b, Frizzled (Fzd)-2, -3, -6 and β-catenin.

Results: (1) Compared with the BDL group, AS significantly reduced the deposition of collagen and the Hyp content of liver tissue and inhibited the activation of HSCs. In addition, AS significantly decreased the protein and mRNA expressions of TGF-β1 and α-SMA. In contrast, AS significantly enhanced expression of the Smad 7 protein. AS also reduced biliary epithelial cell proliferation, and reduced the mRNA and protein expressions of CK7, CK8, CK18, CK19, OV6, Sox9 and EpCAM. (2) The mRNA and protein expressions of Notch-2, -3, -4 and JAG1 were significantly reduced in the AS compared to the BDL group. In contrast, the mRNA and protein level of Numb was clearly enhanced after AS treatment.

Conclusion: AS may prevent biliary liver fibrosis via inhibition of the Notch signaling pathway, thereby inhibiting the abnormal proliferation of biliary epithelial cells. Results indicate that AS may be a potential therapeutic drug for cholestatic liver disease.

1. Introduction

Human cholestatic liver disease is characterized by a progressive destruction of biliary epithelial cells (BECs) followed by fibrosis, cirrhosis and liver failure (Choi and Diehl, 2009; Glaser et al., 2009). Multiple signaling pathways, including Notch, Wnt and Hedgehog, are involved in BEC proliferation (Fabris and Strazzabosco, 2011). Proliferating BECs have been shown to secrete platelet-derived growth factor B (PDGF-B), transforming growth factor (TGF)-β1, monocyte chemotactic protein (MCP)-1 and connective tissue growth factor (CTGF),

Abbreviations: α-SMA, alpha-smooth muscle actin; BDL, bile duct ligation; CK7, cytokeratin 7; CK8, cytokeratin 8; CK19, cytokeratin 19; Col(1), collagen type I; Col (4), collagen type IV; DLL, Delta-like; EpCAM, epithelial cell adhesion molecule; HSCs, hepatic stellate cells; Hyp, hydroxyproline; HPC, hepatic progenitor cell; JAG1, Jagged 1; NICD, Notch intracellular domain; RPB-Jκ, recombination signal binding protein for immunoglobulin kappa J region; Sox9, SRY-related high mobility group-box gene 9; TGF-β1, transforming growth factor beta 1; TNF-α, tumor necrosis factor-alpha

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which stimulate the activation, migration and proliferation of hepatic stellate cells (HSCs) and fibroblasts. This leads to excessive generation of extracellular matrix (ECM) and accelerates the progression of fibrosis (Matsumoto et al., 1994; Marra et al., 1998; Harada et al., 2011; Sedlaczek, et al., 2001). Thus, inhibition of abnormal BEC activation and proliferation may reverse biliary fibrosis, partially or even completely. Drugs that inhibit abnormal activation of BECs could be very useful for early treatment of biliary liver fibrosis (Park, 2012); however, until recently, no drugs have been shown to effectively inhibit abnormal BEC activation.

Although ursodeoxycholic acid (UDCA) can even improves survival rate of patients with advanced primary biliary cirrhosis (PBC), up to 40% of patients do not respond satisfactorily to UDCA therapy and adjunctive therapies should be taken into consideration (Momah and Lindor, 2014). A previous study done by our group confirmed that the Huangqi decoction, a classical recipe for treating liver injury that has a long history in traditional Chinese medicine, consists of two medicinal herbs, contains Radix Astragali (*Astragalus membranceus* (*Fisch.*) *Bge*., Root, Huangqi) and Radix Glycyrrhizae (*Glycyrrhiza uralensis Fisch*., root and rhizome, Gancao). It has significant therapeutic effects in the treatment of liver cirrhosis induced by dimethylnitrosamine (DMN) in rats (Du et al., 2012; Liu et al., 2012). Radix Astragali has been routinely used in China for patients with stroke or chronic debilitating diseases, because it can tonifying Qi, strengthen superficial resistance and promote the discharge of pus and the growth of new tissue (Lin et al., 2000; Guo et al., 2012). Further study by this laboratory found that Astragaloside (AS), the main component of Radix Astragali (Ma et al., 2002), had an effect similar to the Huangqi decoction on hepatic fibrosis induced by DMN (Tong et al., 2011). In the present study, AS was found to inhibit hepatic fibrosis induced by BDL via inhibition of Notch signaling pathway activation, thereby reducing the transformation of hepatic progenitor cells (HPCs) to BECs.

2. Materials and methods

2.1. Materials

Astragaloside (AS) (90% purity) was obtained from Xi'an Honson Biotechnology Co., Ltd. (Xi'an, China), Cas:060614. Sorafinib (SORA) was purchased from Bayer Schering Pharma (Leverkusen, Germany) and was used as a positive control.

Mouse monoclonal antibody against α-smooth muscle actin (α-SMA, Clone 1A4) was obtained from Sigma-Aldrich (St Louis, MO, USA). Rabbit polyclonal antibodies against CK7 (Cat: 15539-1-AP) and CK19 (Cat: 10712-1-AP) were purchased from Proteintech Group Inc. (Chicago, IL, USA). Mouse monoclonal antibody against CK8 (sc-101459) and SRY-related high mobility group-box gene 9 (Sox9) (sc-166505) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse monoclonal antibody against TGF-β1 (Cat: MAB240) and OV-6 (Cat: MAB2020) were purchased from R&D Systems, Inc. (Minneapolis, MN, USA). Mouse monoclonal antibody against CD68 (Clone: ED1) was purchased from Hycult biotech (Uden, Netherlands). Rabbit polyclonal antibody against Smad7 (Cat: 3670-100) was purchased from BioVision Inc. (Palo Alto, CA, USA). Rabbit polyclonal antibody Notch2 (sc-5545), Notch3 (sc-5593), Notch4 (sc-8645) and JAG1 (sc-8303) were purchased from Santa Cruz Biotechnology. Rabbit monoclonal antibody against Numb (ab177465) was purchased from Abcam, Inc. (Cambridge, MA, USA). Mouse monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was obtained from Chemicon International (Temecula, CA, USA). Horseradish peroxidase (HRP)-conjugated polyclonal rabbit anti-mouse immunoglobulins, HRP-conjugated polyclonal swine anti-rabbit immunoglobulins were obtained from Dako Denmark A/S (Glostrup, Denmark). Hybond-ECL nitrocellulose membranes and ECL detection reagent were obtained from Amersham Pharmacia Biotech (Buckinghamshire, UK). All other reagents were purchased from Sigma Chemical or Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

2.2. Animals and experimental protocol

Male Sprague–Dawley (SD) rats (7–8 weeks of age, weighing, 220–240 g, $n=36$) were obtained from Shanghai Experimental Animal Center of the Chinese Academy of Sciences (Shanghai, China). Animals were kept in a constant temperature environment and supplied with laboratory chow and water ad libitum. The experimental protocol was approved by the Animal Research Committee of Shanghai University of Traditional Chinese Medicine (No. 20130132).

The rats were randomly divided into a Sham group ($n=6$) and a BDL group ($n=30$). BDL was performed as previously described (Alpini et al., 1988) with modifications. In brief, the rats were anesthetized with 2% pentobarbital sodium. Left and right hepatic ducts and the hepatic portal and duodenum site of the common bile duct were isolated and ligated, respectively. Control rats (sham-operated) underwent laparotomy but no ligation. At first weekend after BDL, BDL rats were randomly divided into a BDL group ($n=10$), an AS group ($n=10$), and a SORA group ($n=10$). The AS and SORA groups were oral administrated at dosage 164 mg/kg and 1.0 mg/kg, respectively, once per day for 3 weeks. Sham and BDL rats were treated orally with same volume of physiological saline.

2.3. Sample harvesting

At the end of the fourth week, rats were euthanized via administration of 2% pentobarbital sodium anesthesia and the portal vein was cannulated using an 18-G Teflon catheter. Blood samples were collected from the inferior vena cava and centrifuged at 3000 rpm for 30 min at 4 °C. The sera were stored at −70 °C for later use. The livers were perfused with 100 ml of phosphate-buffered saline (PBS, pH 7.4) to remove the blood, washed with ice-cold saline, dried using filter paper and weighed. A small portion of the liver was fixed with 4% paraformaldehyde, embedded in paraffin and frozen. Another portion of the liver was fixed with OCT, snap-frozen in liquid nitrogen and stored at −70 °C until reverse transcription-PCR (RT-PCR) and immunoblot testing.

2.4. Serum biochemistry

Serum levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBil), alkaline phosphatase (AKP), gamma-glutamyl transferase (GGT) and total bile acid (TBA) were measured at Special Reference Laboratories (Shanghai, China).

2.5. The hepatic hydroxyproline content

Hepatic hydroxyproline (Hyp) content was measured using a modified version of the method described by Jamall et al. (1981). Briefly, liver samples were homogenized and hydrolyzed in 6 N HCl, at 110 °C, for 18 h. The hydrolysate was filtered through a 0.45-mm Millipore filter (Millipore, Bedford, MA, USA) and chloramine T was then added to a final concentration of 2.5 mM. The mixture was treated with 410 mM N,N-dimethyl-4-amino benzaldehyde and incubated at 60 °C for 30 min. After cooling to room temperature, the samples were read at 560 nm against a reagent blank that contained the complete system without added tissue.