Research Article

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Hic-5 deficiency attenuates the activation of hepatic stellate cells and liver fibrosis through upregulation of Smad7 in mice

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Background & Aim: Hydrogen peroxide-inducible clone-5 (Hic-5), also named as transforming growth factor beta-1-induced transcript 1 protein (Tgfb1i1), was found to be induced by TGF-β. Previous studies have shown that TGF-β is a principal mediator of hepatic stellate cell (HSC) activation in liver fibrosis. However, this process remains elusive. In this study, we aimed to define the role of Hic-5 in HSC activation and liver fibrosis.

Methods: We examined the expression levels of Hic-5 during HSCs activation and in fibrotic liver tissues by quantitative real-time reverse transcriptase polymerase chain reaction, Western blot and immunohistochemistry. *Hic-5* knockout (KO) and wild-type (WT) mice were subjected to bile duct ligation (BDL) or carbon tetrachloride (CCl_4) injection to induce liver fibrosis.

Results: Hic-5 expression was strongly upregulated in activated HSCs of the human fibrotic liver tissue and BDL or CCl_4-induced mouse liver fibrosis. Hic-5 deficiency significantly attenuated mouse liver fibrosis and HSC activation. Furthermore, Hic-5 knockdown by siRNA *in vivo* repressed CCl_4-induced liver fibrosis in mice. Mechanistically, the absence of Hic-5 significantly inhibited the TGF-β/Smad2 signaling pathway, proved by increasing Smad7 expression, resulting in reduced collagen production and α-smooth muscle actin expression in the activated HSCs.

Conclusion: Hic-5 deficiency attenuates the activation of HSCs and liver fibrosis though reducing the TGF-β/Smad2 signaling by upregulation of Smad7. Thus, Hic-5 can be regarded as a potential therapeutic target for liver fibrosis.

Keywords: Hic-5; Tgfb1i1; Smad2; Smad7; Hepatic stellate cells; Liver fibrosis.
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Abbreviations: Ad-Hic-5/flag, adenovirus-mediated flag-tagged Hic-5; Ad-β-gal, adenovirus-mediated β-galactosidase; α-SMA, alpha smooth muscle actin; BDL, bile duct ligation; CCl_4, carbon tetrachloride; COL, collagen; ECM, extracellular matrix; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; Hep, hepatocytes; Hic-5, Hydrogen peroxide-inducible clone-5; *Hic-5* KO, *Hic-5* knockout; HSCs, hepatic stellate cells; LSECs, liver sinusoid endothelial cells; P-Smad2, phosphorylated Smad2; siRNA, small interfering RNA; TGF-β, transforming growth factor-β; Tgfb1i1, Transforming growth factor beta-1-induced transcript 1; WT, Wild-type.

Introduction

Liver fibrosis represents a wound healing process in response to chronic liver damage from a variety of causes and is characterized by the deposition of extracellular matrix (ECM) proteins, which can impair the normal liver function [1]. Following liver injury, quiescent hepatic stellate cells (HSCs) are activated and believed to be the major source of myofibroblasts, which are the main ECM-producing cells during liver fibrosis [2,3]. Increasing evidence has shown that transforming growth factor-β (TGF-β)/Smad signaling pathway plays a critical role in the activation of HSCs [4–6], Smad7, a TGF-β inducible antagonist for TGF-β signaling [7], has been shown to inhibit the activation of HSCs and prevent liver fibrosis [8,9], as well as other fibrotic diseases such as renal and pulmonary fibrosis [10,11]. From these findings, we are aware of the importance of the cellular expression of Smad7 in the regulation of TGF-β/Smad signaling during progression of organ fibrosis. However, the intracellular Smad7 regulatory mechanism in this pathogenic process remains elusive.

Hydrogen peroxide-inducible clone 5 (Hic-5), also known as transforming growth factor beta-1-induced transcript 1 (Tgfb1i1), is originally identified as a gene induced by H_2O_2 as well as TGF-β1 and is a focal adhesion scaffold LIM-containing protein with homology to paxillin [12]. Hic-5 is highly expressed in the vascular smooth muscle cells of different organs [13]. This molecule can shuttle between focal adhesions and the nucleus in response to oxidants [14]. Furthermore, Hic-5 participates in the transcriptional regulation of several genes [15–17]. It was reported that Hic-5 upregulates TGF-β signaling through its ability to directly interact with and neutralize Smad7 in a myofibroblast cell line [18]. In a recent study, we successfully generated mice lacking Hic-5, which developed with no obvious abnormalities [19]. However, in the various types of vascular disorder models, we found that Hic-5 contributes to vascular repair and remodeling [19,20]. Interestingly, Hic-5 also plays an important role in some fibrotic disorders, including scar formation and glomerulosclerosis [21–23]. Currently, no studies have examined

the role of Hic-5 in the liver. Here we hypothesized that Hic-5 may be involved in liver fibrosis. Thus, we first examined Hic-5 expression in normal and fibrotic human and mouse livers. Second, we determined whether Hic-5 is involved in liver fibrosis using the Hic-5 knockout mice. Third, we investigated the mechanisms by which Hic-5 contributes to liver fibrosis.

Materials and methods

Please refer to the Supplementary materials and methods for more detailed descriptions.

Human liver samples

Human liver samples were obtained from patients undergoing surgical hepatectomy. The study protocol was approved by the local ethics committee (The first affiliated Hospital of Sichuan Medical University, Luzhou city, China), and all samples collected were from subjects who provided informed consent for their tissues to be used for research purposes. Patient characteristics were summarized in Supplementary Table 1.

Animal studies

Wild-type (WT) and systemic *Hic-5* knockout (*Hic-5* KO) mice (C57BL/6 background) [19] were maintained under specific pathogen-free conditions in the animal care facility of Showa University School of Medicine. Experiments were performed with age- and sex-matched mice at 8–12 weeks of age. Liver fibrosis was induced by bile duct ligation (BDL) for 2 weeks or injection with carbon tetrachloride (CCl_4) for 12 times (twice a week for six weeks). All experiments were approved by the regional Animal Study Committees and performed according to the institutional guidelines stipulated by Showa University School of Medicine.

Cell isolation and culture

Major cell types in the liver include hepatocytes, Kupffer cells, liver sinusoid endothelial cells (LSECs), and HSCs.

Mouse HSCs were isolated by *in situ* perfusion of livers with pronase/collagenase perfusion digestion followed by subsequent density gradient centrifugation as reported [24]. HSCs were maintained in Dulbecco's modified Eagle medium (WAKO Chemicals, Japan) supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin, and 10 mM HEPES; cells were counted and cell suspensions were incubated in collagen I-coated dishes (IWAKI, Japan) and maintained at 37 °C in a humidified atmosphere of containing 5% CO_2 and 95% air. After culturing for 2 h, the purity of isolated HSCs was checked by identifying the blue fluorescence of vitamin A when excited by ultraviolet light (Supplementary Fig. 1). The culture medium was replaced every 48 h unless otherwise described.

Human primary HSCs were purchased from ScienCell Research Laboratories (SanDiego, CA, USA; Catalog #5300) and cultured in stellate cell medium (Catalog #5301) according to the manufacturer's instructions.

Refer to the Supplementary materials and methods section for isolation of other cell types.

Statistical analysis

Animals were randomly allocated to control and treatment groups. At least three samples were tested in each assay. All data were expressed as means ± the standard error of the mean. Statistical analyses were performed using Mann-Whitney test (GraphPad Prism, version 5.0 for MAC) for comparisons of parameters between the two groups and differences were considered statistically significant at p value <0.05.

Results

Hic-5 expression is enhanced in activated HSCs and fibrotic liver

We first assessed Hic-5 expression in normal and fibrotic human livers. Real-time reverse transcriptase polymerase chain reaction (RT-PCR) and Western blot analyses confirmed that the mRNA or protein expression of *Hic-5*, α-smooth muscle actin (*ACTA2*) and collagen I (*COL1A1*) was markedly increased in human fibrotic livers *vs.* normal livers (Fig. 1A, B; Supplementary Fig. 2A). Using immunostaining, Hic-5 expression was only found in vascular smooth muscle cells of Glisson's sheath in normal livers, whereas the enhanced expression of Hic-5 was found in human fibrotic livers (Fig. 1C; Supplementary Fig. 2B). Furthermore, we analyzed colocalization of Hic-5 with each of α-SMA, LYVE1, and Iba1, the cell type marker for activated HSCs, LSECs, and Kupffer cells, respectively. Hic-5 expression overlapped with α-SMA-positive HSCs as seen by the yellow fluorescence in human fibrotic liver (Fig. 1C, enlarged), but neither LSECs, including endothelial cells of liver central vein, nor Kupffer cells expressed Hic-5 (Supplementary Fig. 3A–C). In addition, immunofluorescence staining of commercially available primary human HSCs showed significant Hic-5 expression (Supplementary Fig. 4A). We then analyzed Hic-5 levels in mouse fibrotic livers that were generated by two different methods, BDL and intraperitoneal CCl_4 injection. Similar to the human results, both mouse models of liver fibrosis showed a significant increase in Hic-5 protein expression: a 5.5-fold increase with BDL and a 4.7-fold increase with CCl_4 injection compared with controls (Fig. 1D). Immunofluorescent microscopy revealed the colocalization of Hic-5 and α-SMA in both the BDL and the CCl_4 fibrosis models evidenced by yellow fluorescence (Fig. 1E). We next examined Hic-5 expression in isolated mouse primary liver cells such as HSCs, LSECs, Kupffer cells, and hepatocytes. After culturing for 2 h, Hic-5 expression was detected only in HSCs, but not in other cell types (Fig. 1F). We comparatively analyzed the expression of Hic-5 and α-SMA in cultured primary mouse HSCs undergoing differentiation from quiescent to activated HSCs at different times (from 2 h to 15 days) by Western blot analysis. Both of these proteins gradually increased during differentiation. Meanwhile, we detected Hic-5 at an earlier stage of differentiation compared with α-SMA (Fig. 2H). Similarly, immunofluorescence analysis confirmed that Hic-5 and α-SMA were increased during differentiation. Hic-5 was detected at focal adhesions of cells from 2 h of culture, while α-SMA was observed at day 3 of culture (Fig. 2G, Supplementary Fig. 4B). Of note, vitamin A lipid droplets were depleted during HSC differentiation as previously reported.

Hic-5 deficiency reduces BDL- and CCl_4-induced liver fibrosis

To investigate the contributing roles of Hic-5 in liver fibrogenesis, we induced liver fibrosis using two different ways, BDL or CCl_4 injection, in WT and *Hic-5* KO mice. Sirius Red and Masson's trichrome stainings were used for morphometric analysis of liver fibrosis. After treatment of mice with BDL or CCl_4, we found significant reduction in both kinds of staining in *Hic-5* KO mice compared with WT mice (Fig. 2A, B and Fig. 2C, D left panels). Histological examination of hematoxylin and eosin-stained liver sections indicated that necrotic area was significantly decreased in *Hic-5* KO mice compared with WT mice after BDL (Supplementary Fig. 5). Moreover, the liver from *Hic-5* KO mice displayed a marked decrease in hydroxyproline, a specific amino acid component of collagen (Fig. 2C, D right panels). In addition, RT-PCR analysis confirmed significantly lower expression of fibrosis-related genes, including collagen I (*Col1a1*, *Col1a2*) and collagen III (*Col3α1*) in *Hic-5* KO mouse livers compared with WT mouse livers after BDL (Fig. 2E) or treatment with CCl_4 (Fig. 2F). We also