EBioMedicine 2 (2015) 1751–1758

Contents lists available at ScienceDirect

EBioMedicine

journal homepage: www.ebiomedicine.com

Research Article

Inhibition of Cyclic Adenosine Monophosphate (cAMP)-response Element-binding Protein (CREB)-binding Protein (CBP)/β-Catenin Reduces Liver Fibrosis in Mice

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ARTICLE INFO

Article history:
Received 14 April 2015
Received in revised form 3 October 2015
Accepted 6 October 2015
Available online 8 October 2015

Keywords:
Liver
Fibrosis
Beta-catenin
Hepatic stellate cell
Macrophage

ABSTRACT

Wnt/β-catenin is involved in every aspect of embryonic development and in the pathogenesis of many human diseases, and is also implicated in organ fibrosis. However, the role of β-catenin-mediated signaling on liver fibrosis remains unclear. To explore this issue, the effects of PRI-724, a selective inhibitor of the cAMP-response element-binding protein-binding protein (CBP)/β-catenin interaction, on liver fibrosis were examined using carbon tetrachloride (CCl_4)- or bile duct ligation (BDL)-induced mouse liver fibrosis models. Following repetitive CCl_4 administrations, the nuclear translocation of β-catenin was observed only in the non-parenchymal cells in the liver. PRI-724 treatment reduced the fibrosis induced by CCl_4 or BDL. C-82, an active form of PRI-724, inhibited the activation of isolated primary mouse quiescent hepatic stellate cells (HSCs) and promoted cell death in culture-activated HSCs. During the fibrosis resolution period, an increase in F4/80$^+$ CD11b$^+$ and Ly6C^{low} CD11b$^+$ macrophages was induced by CCl_4 and was sustained for two weeks thereafter, even after having stopped CCl_4 treatment. PRI-724 accelerated the resolution of CCl_4-induced liver fibrosis, and this was accompanied by increased matrix metalloproteinase (MMP)-9, MMP-2, and MMP-8 expression in intrahepatic leukocytes. In conclusion, targeting the CBP/β-catenin interaction may become a new therapeutic strategy in treating liver fibrosis.

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1. Introduction

Chronic tissue injury leads to fibrosis in many organs, including the liver, lungs, kidneys, and heart. In chronic liver disease, the development of fibrosis is the first step toward the progression to cirrhosis and its complications (such as organ failure, esophageal variceal bleeding, and hepatocellular carcinoma), irrespective of the underlying etiology (Bataller and Brenner, 2005), and there is currently no effective therapy. Although many pathways and cytokines such as transforming growth factor (TGF)-β, platelet-derived growth factor (PDGF), Toll-like receptor (TLR) 4, sphingosine-1-phosphate, AKT, and p38 mitogen-activated protein kinase (MAPK) have been characterized as mediators of liver fibrosis, the underlying molecular mechanism is still not well defined.

Wnt/β-catenin is involved in virtually every aspect of embryonic development and in the pathogenesis of many human diseases (Clevers, 2006), and is also involved in homeostatic self-renewal in adult tissues, such as liver and lung repair following an injury (Monga, 2011; Beers and Morrisey, 2011). Recently, Wnt/β-catenin has been reported to be associated with organ fibrosis (Dees and Distler, 2013; Chilosi et al., 2003) suggesting that they may be new therapeutic targets in liver fibrosis (Cheng et al., 2008). Hepatic stellate cells (HSCs) represent a major fibrogenic cell type in the liver (Bataller and Brenner, 2005). Following a liver injury, HSCs undergo an activation process and change their phenotype from quiescent retinoid storing HSCs to collagen-

Abbreviations: CREB, cAMP-response element-binding protein; CBP, CREB-binding protein; CCl_4, carbon tetrachloride; BDL, bile duct ligation; PBDL, partial BDL; HSC, hepatic stellate cell; MMP, matrix metalloproteinase; TGF-β, transforming growth factor; HCV, hepatitis C virus; αSMA, α-smooth muscle actin,; H–E, hematoxylin and eosin; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; TIMP-1, tissue inhibitor of metalloproteinase; CXCL, c–x–c motif ligand; CCL, c–c motif ligand; SPARC, secreted protein acidic and rich in cysteine.

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http://dx.doi.org/10.1016/j.ebiom.2015.10.010
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producing and contractile myofibroblast-like cells (Friedman, 2000). Wnt signaling is stimulated in activated HSCs compared to quiescent cells, and the inhibition of Wnt signaling by the transduction of the adenoviral Wnt co-receptor antagonist Dickkopf-1 restores HSC quiescence and increases apoptosis in cultured HSCs (Cheng et al., 2008). Macrophages may perform both injury-inducing and repair-promoting tasks simultaneously in an injured organ. The depletion of liver macrophages aggravates hepatocellular damage, while suppressing liver fibrosis following bile duct ligation (BDL) (Osawa et al., 2010). Moreover, the depletion of macrophages decreases myofibroblasts in a liver tumor (Osawa et al., 2013b). By contrast, macrophage depletion during the fibrosis resolution period induces the failure of matrix degradation (Duffield et al., 2005), suggesting that hepatic macrophages are involved in the regression of hepatic fibrosis (Friedman, 2005). The roles of β-catenin in macrophages have been reported. Macrophage-specific knockdown of β-catenin causes insufficient skin wound healing due to defects in migration, in the adhesion to fibroblasts, and in TGF-β production (Amini-Nik et al., 2014).

Following activation by upstream signaling from Wnt, β-catenin translocates to the nucleus. Nuclear β-catenin recruits the Kat3 transcriptional co-activators, cAMP-response element-binding protein (CREB)-binding protein (CBP) (Takemaru and Moon, 2000) or EP300 (p300) (Hecht et al., 2000), to stimulate the transcription of its target genes, and distinct roles have been reported for CBP and p300. CBP/β-catenin-mediated transcription is critical for proliferation/non-differentiation, whereas p300/β-catenin-mediated transcription initiates differentiation (Teo and Kahn, 2010; Lenz and Kahn, 2014). ICG-001, a selective inhibitor of the CBP/β-catenin interaction, attenuates bleomycin-induced lung fibrosis and reverses the established fibrosis (Henderson et al., 2010). ICG-001 also ameliorates renal interstitial fibrosis induced by unilateral ureteral obstruction (Hao et al., 2011). C-82 is a second-generation specific CBP/β-catenin antagonist developed by Prism Pharma, which inhibits the binding between β-catenin and CBP and increases the binding between β-catenin and p300 similar to ICG-001. PRI-724 is phosphorylated-C-82 and is rapidly hydrolyzed to its active form C-82 *in vivo*, and pre-clinical studies have shown a very acceptable toxicity profile (Lenz and Kahn, 2014). A phase I safety study for hepatitis C virus (HCV)-related cirrhosis patients has been ongoing in our hospital since September 2014. To attempt to clarify the precise roles of CBP/β-catenin on liver fibrosis, in this study, we investigated the effects of PRI-724 and of its active form, C-82, on chronic liver injury mouse models and primary isolated mouse HSCs.

2. Material and Methods

2.1. Animals and Treatments

Male wild-type (C57BL/6 and Balb/c) mice aged 8 to 11 weeks or 6 to 9 months were obtained from Japan SLC (Shizuoka, Japan). CCl_4 administration or BDL induced liver fibrosis model was used for this study. The animals were intraperitoneally injected with 1 ml/kg body weight of CCl_4 (1:4 v/v in mineral oil) (Sigma-Aldrich, St. Louis, MO, USA) twice a week. As a BDL model, common BDL is the most established model. However, the mortality of mice operated with common BDL was high at 5 weeks after the surgery. Thus, our established model, partial bile duct ligation (PBDL) was performed as previously reported (Osawa et al., 2010; Osawa et al., 2006). The left hepatic duct was ligated with 6-0 silk after anesthesia. The animals were intraperitoneally injected with or without 0.4 mg/mouse PRI-724 (Prism Pharma, Tokyo, Japan) dissolved in PBS 4 times a week. The experiments were conducted in accordance with the institutional guidelines (Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences) and the protocol was approved by the Research Committee of Komagome Hospital. All surgery was performed under anesthesia, and all efforts were made to minimize suffering.

2.2. Isolation of Mouse Primary HSCs

Mouse primary HSCs were isolated as previously described with slight modifications (Osawa et al., 2013a). The liver of C57BL/6 male wild-type or GFP mouse expressing EGFP ubiquitously under the CAG promoter (chicken β-actin promoter, rabbit β-globin poly A, CMV-IE enhancer) (8–11 week-old or 6–9 month-old) was perfused via the inferior vena cava with collagenase (Wako, Osaka, Japan) and pronase E (EMD Chemicals, Gibbstown, NJ, USA). After digestion, the cell suspension was filtered through nylon mesh and purified via 8.2% Nycodenz (Axis-Shield, Oslo, Norway) gradient centrifugation. The quiescent HSCs were obtained from normal liver and the *in vivo* stimulated HSCs were from fibrotic liver induced by eight week treatment of CCl_4. The isolated HSCs were cultured in uncoated plastic dishes or thin layer matrigel (Corning, corning, NY, USA) coated dishes with DMEM high glucose (Wako) supplemented with 10% fetal bovine serum, 10 mM sodium pyruvate (Invitrogen, Carlsbad, CA, USA), MEM Non-essential amino acids (Invitrogen), 15 mM HEPES, and antibiotic solution at 37 °C in 5% CO_2. After plating for 12 h (day 0), the quiescent HSCs were treated with or without C-82 (10 μM, Prism Pharma) dissolved in dimethyl sulfoxide (DMSO), and replacement of medium (C-82 or DMSO) was performed in every 5 days. The purity of HSCs was always 95% as determined by their typical shape and abundant lipid droplets. As necessary, the cells were fixed with ice-cold methanol and cell number per low magnification field was determined by nuclear counting with 4′,6-diamidino-2-phenylindole (DAPI) staining. Activated HSCs were obtained by continuous culture of quiescent HSCs for more than 20 days with replacement of medium in every 5 days. The culture-activated HSCs were treated with or without C-82. Medium change was not performed after C-82 treatment. Cell viability was examined using XTT based Cell Proliferation Kit (Biological Industries, Kibbutz Beit Haemek, Israel). Cell death was detected by propidium iodide staining, and morphological changes in the nuclei of cells undergoing apoptosis were determined by DAPI staining.

2.3. Isolation of Mouse Intrahepatic Leukocytes (IHLs)

IHL isolation was performed as previously reported (Kimura et al., 2011). Briefly, single-cell suspensions were prepared from the median lobe of the liver by digestion in RPMI 1640 (Wako) containing 0.02% collagenase IV and 0.002% DNase I (Sigma-Aldrich) for 40 min at 37 °C. The cells were overlaid on Lympholyte M (Cedarlane, Westbury, NY, USA) in PBS. After density separation, the isolated IHLs were used for fluorescence activated cell sorting (FACS) analysis and RNA extraction.

2.4. Gelatin Zymography

Gelatin zymography was performed with extracted proteins from the liver as described previously (Wielockx et al., 2001).

2.5. Statistical Analysis

Data are expressed as the mean ± SD of data collected from at least 3 independent experiments. Data between groups were analyzed by the 2-tailed Student's t-test. A P value of less than 0.05 was an indication of statistical significance.

2.6. Other Experimental Procedures

Other experimental procedures are described in the Supplementary experimental procedures. These include histological analysis, Western blot, hydroxyproline measurement, quantitative real time RT-PCR, FACS analysis, microarray analysis, measurement of serum MMP-9 and TIMP-1, and structure information of PRI-724 and C-82.

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