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Cav-1 deficiency promotes liver fibrosis in carbon tetrachloride (CCl_4)-induced mice by regulation of oxidative stress and inflammation responses

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ABSTRACT

Caveolin-1 (Cav-1), as a membrane protein involved in the formation of caveolae, binds steroid receptors and endothelial nitric oxide synthase, limiting its translocation and activation. In the present study, we investigated the role of Cav-1 in the progression of hepatic fibrosis induced by carbon tetrachloride (CCl_4) in murine animals. Therefore, the wild type (WT) and Cav-1-knockout (Cav-$1^{-/-}$) mice were used in our study and subjected to CCl_4. The results indicated that CCl_4 induced the decrease of Cav-1 expression in liver tissue samples. And Cav-$1^{-/-}$ intensified CCl_4-triggered hepatic injury, evidenced by the stronger hepatic histological alterations, serum aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels and liver terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)-positive cells. CCl_4 led to oxidative stress, supported by the reduced superoxide dismutase (SOD) activity and glutathione (GSH) levels, as well as enhanced malondialdehyde (MDA) and O_2^- levels in liver samples. And the process was intensified by Cav-$1^{-/-}$. Additionally, CCl_4-caused hepatic inflammation was aggregated by Cav-$1^{-/-}$ via further increasing the secretion of pro-inflammatory cytokines. Moreover, CCl_4-caused fibrosis was strengthened by Cav-$1^{-/-}$, which was evidenced by the up-regulation of α-smooth muscle actin (α-SMA), collagen alpha 1 type 1 (Col1A1), lysyl oxidase (Lox) and transforming growth factor-β1 (TGF-β1) in liver tissues. Similar results were observed in TGF-β1-stimulated hepatic stellate cells (HSCs) and LX-2 cells without Cav-1 expressions that in vitro, suppressing Cav-1 further accelerated TGF-β1-induced oxidative stress, inflammation and fibrosis development. In conclusion, our results indicated that Cav-1 played an important role in CCl_4-induced hepatic injury, which may be used as potential therapeutic target for hepatic fibrosis treatment.

1. Introduction

The prevalence of chronic hepatic disease is increasing globally. Despite the underlying cause, such as metabolic disease, alcohol, or the non-alcoholic steatohepatitis, hepatic injury leads to fibrosis, which is a dynamic process, characterized by the accumulation of extracellular matrix [1,2]. Presently, there is no effective regimen to prevent or treat fibrosis without adversely affecting repair [3]. Herein, novel and new disease-modifying anti-fibrotic therapeutic strategies are required.

Caveolin-1 (Cav-1) is a membrane protein included in the formation of caveolae [4]. Numerous studies before have indicated that Cav-1 is essential for protection against myocardial I/R injury, which is associated with oxidative stress [5]. In addition, Cav-1 has been reported to be elevated by tumor necrosis factor-α (TNF-α) in endothelial cells during heart failure, demonstrating that there is a possible relationship between inflammation and Cav-1 [6]. Recently, it was suggested that liver regeneration results from the coordination of lipid metabolism, cell activation, as well as cell division. Cav-1 has been linked with the regulation of each one of these processes [7]. Cav-1 has been shown to play a significant role in tissue repair and fibrosis in different studies [8,9]. For example, previous studies reported decrease of Cav-1 in a bleomycin-induced lung fibrosis model and also in other fibrotic

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diseases [10]. However, its role in hepatic fibrosis formation is still not be understood, and further study is required to reveal that if Cav-1 is involved in fibrotic progression in liver.

Here, in the present study, the wild type (WT) and Cav-1-knockout (Cav-1$^{-/-}$) mice were used to explore the role of Cav-1 in CCl_4-induced liver injury, especially its link with fibrosis. We found that Cav-1$^{-/-}$ could aggregate the liver injury triggered by CCl_4 through enhancing oxidative stress, inflammatory response and fibrosis, associated with various related molecules. Our study provides a possible mechanism underlying liver injury and targeting Cav-1 would have therapeutic potential for hepatic fibrosis.

2. Materials and methods

2.1. Animals and treatments

All procedures were performed in accordance with the Regulations of Experimental Animal Administration issued by the Ministry of Science and Technology of the People's Republic of China (http://www.most.gov.cn), and were approved by the Huai'an First People's Hospital, Nanjing Medical University. 40 male WT C57BL/6J mice and 40 male Cav-1$^{-/-}$ C57BL/6J mice (6–8 weeks old, weighed 20–22 g) were purchased from Jackson Laboratory (Bar Harbor, ME). Mice were housed in a specific pathogen-free, temperature and humidity-controlled environment (25 ± 2 °C, 50 ± 5% humidity) with a standard 12 h light/12 h dark cycle with free access to food and water. Before experiment, all mice were required to adapt to the environment for 7 days. All mice were randomly divided into four groups (n = 10/each): 1) the WT vehicle group (WT/Veh); 2) CCl_4-treated WT group (WT/CCl_4); 3) the Cav-1$^{-/-}$ vehicle group (Cav-1$^{-/-}$/Veh) and 4) CCl_4-treated Cav-1$^{-/-}$ group (Cav-1$^{-/-}$/CCl_4). Animals were subjected to 1 μL/g body weight of CCl_4 diluted (1:9 v/v) in olive oil through intraperitoneal (i.p.) injections twice a week for 12 weeks. Animals were then sacrificed at 48 h, 72 h, 1 week and 1 month after the last dose of CCl_4 administration. Finally, the blood samples and hepatic tissues were collected. One part of the liver was immediately fixed in 10% formaldehyde for histopathology observation and the rest was frozen with liquid nitrogen and stored at −80 °C until its use for biochemical, histopathological, cytokine and gene expression examinations, respectively.

2.2. Cells and culture

Primary HSCs were prepared from mice using a two-step collagenase-pronase perfusion of mouse livers as described previously [11]. Human LX-2 HSCs were purchased from Shanghai Cell Bank of the Chinese Academy of Sciences (China). Cells were cultured in DMEM (Gibco, USA) containing 10% fetal bovine serum (FBS; Sigma, USA) with penicillin/streptomycin (Sigma) at 37 °C in a humidified 5% CO_2 atmosphere. Transient transfection of siRNAs (40 nmol/L) was carried out by use of Invitrogen Lipofectamine RNAiMAX (Invitrogen, USA) according to the manufacturer's protocols. The control cells were treated with RNAi Negative Control Duplexes (40 nmol/L). After transfection for 24 h, cells were collected and used for further assay.

2.3. Biochemical measurements

Serum ALS and AST levels were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) following the manufacture's instructions. The levels of MDA, SOD, GSH and O_2^- in liver and total reactive oxygen species (ROS) in cells were also measured using commercial kits (Beyotime, China) following manufacturer's instructions. TNF-α, interleukin-1β (IL-1β), cyclooxygenase-2 (COX-2) and IL-6 levels in liver or cell homogenates were determined using ELISA kits (R&D Systems, USA) following the manufacturer's guidelines. Cav-1 ELISA kit was obtained from BOSTER (Wuhan, China).

2.4. TUNEL analysis

Hepatic sections were dried and permeabilized, followed by incubation with TUNEL reaction mixture at 37 °C for 60 min using In situ Cell Death Detection kit (Roche Molecular Biochemicals, USA) following the manufacturer's guidelines. The percentage of apoptotic cells was calculated by dividing the number of TUNEL-positive cells by the total number of cells visualized in the same field. Three digitized images with similar total cell numbers were selected from each cover slip for counting and averaging and were considered as one independent experiment. Three independent experiments were then averaged and statistically analyzed.

2.5. Western blot analysis

The total protein samples of liver tissues or cells were extracted using cold lysis buffer containing 1 mM PMSF, and the protein contents were determined using BCA Protein Assay Kit (Thermo Fisher Scientific, USA). The proteins were separated by SDS-PAGE (10–12%), transferred onto PVDF membranes (Millipore, USA), then blocked and incubated with the primary antibodies overnight at 4 °C listed in Supplementary Table 1, followed by detection using an HRP-conjugated secondary antibody (Abcam, USA). Protein bands were visualized with the enhanced chemiluminescence (ECL, Thermo Fisher Scientific), and exposed to Kodak X-ray film (Kodak Company, USA). The densitometry of each protein band was quantified using ImageJ 1.38 software (National Institutes of Health, USA) and standardized to housekeeping gene of β-actin and expressed as a fold of control.

2.6. Reverse-transcription real-time PCR (RT-qPCR) assays

Total RNA samples from liver tissues or cells were extracted using RNAiso Plus (Transgen Biotech, Beijing, China) following the manufacturer's protocol. cDNA was synthesized using a PrimeScript® RT reagent kit (Transgen Biotech, Beijing, China) following the manufacturer's instructions. For Real-time PCR, SYBR® Premix Ex TaqTM II (Transgen Biotech) was used and subjected to qPCR in an ABI 7500 Real Time PCR System, and the data was analyzed using System SDS software (Applied Biosystems, USA). The primers used in the study are listed in Supplementary Tables 2 and 3.

2.7. Histology analysis

The liver tissues were fixed in 10% formalin and embedded in paraffin, and 5 μm- thick sections were stained with hematoxylin-eosin (H&E). To examine the degree of necrosis of liver tissues, an injury grading score (Grade 0–4) based on severity of necrotic lesions in the liver parenchyma was carried out [12]. The scoring system was as follows: Grade 0, no pathological change; Grade 1, presence of degenerated hepatocytes with only rare foci of necrosis; Grade 2, small area of mild centrilobular necrosis around the central vein; Grade 3, area of mild centrilobular necrosis severer than Grade 2; and Grade 4, centrilobular necrosis severer than Grade 3. Twenty images were randomly selected from each slide of the sample and scored by two independent pathologists, and the values were analyzed. For quantitation of fibrosis, the relative area of fibrosis was expressed as a % of total hepatic area, which was analyzed based on 35 visual fields of Sirius red-stained liver sections per mouse. For Masson's trichrome staining, the liver tissues in sections were subjected to alcoholic dehydration and embedded in paraffin. 3 μm serial sections were subjected to Masson's trichrome staining.