Research Article

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Acyl-CoA:cholesterol acyltransferase 1 mediates liver fibrosis by regulating free cholesterol accumulation in hepatic stellate cells

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Background & Aims: Acyl-coenzyme A: cholesterol acyltransferase (ACAT) catalyzes the conversion of free cholesterol (FC) to cholesterol ester, which prevents excess accumulation of FC. We recently found that FC accumulation in hepatic stellate cells (HSCs) plays a role in progression of liver fibrosis, but the effect of ACAT1 on liver fibrosis has not been clarified. In this study, we aimed to define the role of ACAT1 in the pathogenesis of liver fibrosis.

Methods: ACAT1-deficient and wild-type mice, or Toll-like receptor 4 (*TLR4*)$^{-/-}$*ACAT1*$^{+/+}$ and *TLR4*$^{-/-}$*ACAT1*$^{-/-}$ mice were subjected to bile duct ligation (BDL) for 3 weeks or were given carbon tetrachloride (CCl_4) for 4 weeks to induce liver fibrosis.

Results: ACAT1 was the major isozyme in mice and human primary HSCs, and ACAT2 was the major isozyme in mouse primary hepatocytes and Kupffer cells. ACAT1 deficiency significantly exaggerated liver fibrosis in the mouse models of liver fibrosis, without affecting the degree of hepatocellular injury or liver inflammation, including hepatocyte apoptosis or Kupffer cell activation. ACAT1 deficiency significantly increased FC levels in HSCs, augmenting TLR4 protein and downregulating expression of transforming growth factor-β (TGFβ) pseudoreceptor Bambi (bone morphogenetic protein and activin membrane-bound inhibitor), leading to sensitization of HSCs to TGFβ activation. Exacerbation of liver fibrosis by ACAT1 deficiency was dependent on FC accumulation-induced enhancement of TLR4 signaling.

Conclusions: ACAT1 deficiency exaggerates liver fibrosis mainly through enhanced FC accumulation in HSCs. Regulation of ACAT1 activities in HSCs could be a target for treatment of liver fibrosis.

Keywords: Acyl-coenzyme A:cholesterol acyltransferase; Free cholesterol; Hepatic stellate cell; Toll-like receptor 4; Bone morphogenetic protein and activin membrane-bound inhibitor; Transforming growth factor-β.

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Abbreviations: HSC, hepatic stellate cell; FC, free cholesterol; TLR4, Toll-like receptor 4; Bambi, bone morphogenetic protein and activin membrane-bound inhibitor; TGF, transforming growth factor; CE, cholesterol ester; ACAT, acyl-coenzyme A:cholesterol acyltransferase; MβCD, methyl-β-cyclodextrin; LPS, lipopolysaccharide; CCl_4, carbon tetrachloride; BDL, bile duct ligation; ALT, alanine aminotransferase; HE, hematoxylin-eosin; SMA, smooth muscle actin; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine nick-end labeling; PCR, polymerase chain reaction; TNF, tumor necrosis factor; VCAM-1, vascular cell adhesion molecule-1; ICAM-1, intercellular adhesion molecule-1; MCP-1, monocyte chemoattractant protein-1.

Introduction

Although advanced liver fibrosis results in cirrhosis, liver failure, hepatocellular carcinoma, and portal hypertension and often requires liver transplantation, there is no standard treatment [1]. Hepatic stellate cells (HSCs) are the primary effectors, orchestrating the deposition of extracellular matrix in normal and fibrotic liver, and play a key role in the progression of liver fibrosis [2].

We recently reported that accumulation of free cholesterol (FC) in HSCs promoted Toll-like receptor 4 (TLR4) signal transduction by increasing membrane TLR4 levels, thereby downregulating bone morphogenetic protein and activin membrane-bound inhibitor (Bambi), and consequently sensitizing HSCs to transforming growth factor (TGF) β, resulting in HSC activation and progression of liver fibrosis [3]. These results suggest that the mechanism of FC metabolism in HSCs could play a pivotal role in HSC activation and liver fibrosis.

Excess cellular cholesterol is stored as cholesteryl esters (CE). The conversion of FC to CE is catalyzed by acyl-coenzyme A (CoA):cholesterol acyltransferase (ACAT) [4]. In mammals, isoenzymes ACAT1 and ACAT2, encoded by 2 different genes, mediate cellular cholesterol homeostasis, dietary cholesterol absorption, and lipoprotein assembly [4].

Mammalian ACAT1 is expressed in many different tissues and cell types; ACAT2 is expressed mainly in the liver and intestine. In mice, ACAT2 plays a key role in providing CE to chylomicrons in the intestine and mediates storage and packaging of CE into apoB-containing lipoproteins in the liver [4]. Our study showed that ACAT2 is the major isoenzyme of hepatocytes and Kupffer cells and ACAT1 is the major isoenzyme of HSCs in the mouse liver. We also demonstrated that ACAT1 is the major isoenzyme of human HSCs. These results suggest ACAT1 could play a key role in FC accumulation in HSCs.

We hypothesized that regulation of ACAT1 could play a role in the pathogenesis of liver fibrosis. We used ACAT1-deficient mice and wild-type mice in the two mouse models of liver fibrosis.

Materials and methods

Isolation of human HSCs

Human HSCs were isolated from fragments of normal livers (N = 3) obtained during surgery for colorectal liver metastasis, as described previously [5]. Briefly, after a combined digestion of liver tissue with collagenase and pronase, HSCs were separated from other non-parenchymal cells by centrifugation over a gradient of Nycodenz (9% wt/vol; Sigma). Written informed consent was obtained from all patients. The study protocol was approved by the Ethical Committee of National Defense Medical College Hospital, and followed the ethical guidelines of the Declaration of Helsinki.

Animal model

Male 9-week-old wild-type C57BL/6 mice were purchased from CLEA Japan. *ACAT1*$^{+/-}$ mice were purchased from Jackson Laboratories (Bar Harbor, Maine, USA). *ACAT1*$^{+/+}$ (wild-type) and *ACAT1*$^{-/-}$ (*ACAT1*-deficient) littermates were obtained from crosses of *ACAT1*(+/−) mice with C57BL/6 background. C57BL/6 *TLR4*$^{-/-}$ mice were purchased from Oriental BioService (Kyoto, Japan). *TLR4*$^{-/-}$*ACAT1*$^{+/+}$ and *TLR4*$^{-/-}$*ACAT1*$^{-/-}$ littermates were obtained from crosses of *ACAT1*$^{-/-}$ mice with TLR4$^{-/-}$ mice. In the liver fibrosis experiments, 9-week-old male wild-type and ACAT1-deficient mice, or *TLR4*$^{-/-}$*ACAT1*$^{+/+}$ and *TLR4*$^{-/-}$*ACAT1*$^{-/-}$ mice were given carbon tetrachloride (CCl_4) at 5 μl (10% CCl_4 in corn oil)/g body weight, by intraperitoneal injection twice a week for 4 weeks. For bile duct ligation (BDL), anesthetized mice received midline laparotomy and the common bile duct was ligated twice with silk sutures before abdominal closure. We performed the sham operation similarly, except the bile duct was not ligated. Mice were sacrificed 3 weeks after BDL.

All animals received humane care in compliance with the National Research Council's criteria outlined in the "Guide for the Care and Use of Laboratory Animals," prepared by the US National Academy of Sciences and published by the US National Institutes of Health (Bethesda, MD).

Kupffer cell depletion

Dichloromethylene diphosphonic acid (DMDP, Clodronate)-loaded or PBS-loaded liposomes (Encapsula NanoSciences) were intravenously injected (200 μl per mouse).

Biochemical and histological analysis

Serum concentrations of alanine aminotransferase (ALT) and cholesterol were determined as described [3]. Liver cholesterol levels or the cholesterol content of HSCs were measured using the Cholesterol/Cholesteryl Ester Quantitation Kit (BioVision, Mountain View, CA), following the manufacturer's instructions. Liver tissues were fixed in 4% paraformaldehyde, embedded in paraffin, and stained with hematoxylin-eosin (HE). Liver fibrosis was assessed with Sirius red staining. For protein or RNA analysis, tissues were frozen in liquid nitrogen and stored at −80 °C.

Isolation and culture of mice HSCs

Mouse HSCs were isolated as described [6]. In some experiments, they were cultured for 6 h in DMEM containing 1% or 10% FBS in uncoated plastic tissue culture dishes, and then treated with TGFβ, MβCD, or LPS.

Hepatocyte and Kupffer cell isolation

Hepatocytes and Kupffer cells were isolated from mice as previously described [3,7].

Western blotting

Preparation of whole-cell protein extracts from HSCs, Kupffer cells, and hepatocytes, electrophoresis of whole-cell protein extracts (5 μg), and blotting were performed using antibodies against TLR4, ACAT1 (Abcam, Cambridge, UK), ACAT2 (Santa Cruz Biotechnology, CA, USA), and β-actin (Sigma), as described [3]. Cells were lysed with Complete Lysis-M (Roche Applied Science, Mannheim, Germany).

ACAT activity assay

ACAT activity assay was performed as described [8], using hepatocytes, Kupffer cells, and HSCs immediately isolated from wild-type and ACAT1-deficient mice. The ACAT activity was expressed as pmoles of cholesteryl oleate synthesized per mg cell protein per minute.

Statistical analysis

All data are expressed as the means ± standard errors of the means (s.e.m.). Statistical analyses were performed using the unpaired Student's *t* test or one-way ANOVA ($p < 0.05$ was considered significant).

Results

ACAT1 is the major isoenzyme in human and mouse HSCs

Western blotting showed that ACAT1 was the major isozyme in mouse primary HSCs (Fig. 1A). We detected little ACAT2 expression in these cells. ACAT1 was also the major isozyme in human primary HSCs freshly isolated from normal livers obtained during surgery for colorectal liver metastasis (Fig. 1B). It was also the major isozyme in human HSC cell lines such as LX2 [9] and hTERT-introduced HSCs [10] (Fig. 1B). There was little ACAT2 expression in these cells. In contrast, ACAT2 was the major isozyme in mouse primary hepatocytes and Kupffer cells (Fig. 1A). ACAT1 deficiency did not affect hepatic ACAT2 expression (Supplementary Fig. 1A). We detected very little ACAT activity in ACAT1-deficient HSCs, whereas in ACAT1-deficient Kupffer cells or hepatocytes, substantial ACAT activity remained (Fig. 1C).

ACAT1 deficiency significantly exaggerated liver fibrosis

ACAT1-deficient mice did not spontaneously progress to liver fibrosis (Fig. 1D). Next, to assess the effect of ACAT1 on liver fibrosis, ACAT1-deficient or wild-type mice underwent BDL or sham treatment for 3 weeks (Fig. 1D–F). ACAT1 deficiency significantly exaggerated liver fibrosis, as shown by Sirius red staining in liver tissue (Fig. 1D). Consistent with this, hepatic αSMA