Research Article

JOURNAL OF HEPATOLOGY

Restoration of Wnt/β-catenin signaling attenuates alcoholic liver disease progression in a rat model

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Background & Aims: Alcoholic liver disease (ALD) is characterized by the development of fatty liver, alcoholic hepatitis, fibrosis and cirrhosis. However, the underlying mechanism(s) associated with progression remains elusive. Pro-inflammatory cytokines have been implicated in ALD progression due to pro-apoptotic effects on hepatocytes. Wnt/β-catenin signaling recently has been shown to promote inflammation and apoptosis, suggesting that activation of this signaling pathway may modulate ALD progression. The current study was designed to test whether pharmacological activation of Wnt/β-catenin signaling altered ALD development and progression in a rat model.

Methods: Adult male Long Evans rats were fed with isocaloric liquid diets containing 0% or 37% ethanol for 8 weeks, and also treated with Wnt agonist during the last 3 weeks of the feeding regimen. Liver and blood samples were subjected to histology, TUNEL assay, immunoblot analysis, real-time quantitative PCR, and alanine transaminase (ALT) assay.

Results: Wnt/β-catenin signaling was negatively correlated with Foxo3A expression and reduced steatosis, cellular injury and apoptosis in ALD rats. Mutation experiments demonstrated that Foxo3A was critical for modulating these effects. Activation of Wnt/β-catenin signaling suppressed Foxo3A-induced apoptosis through upregulation of serum/glucocorticoid regulated kinase 1 (SGK1). Moreover, pharmacological restoration of Wnt/β-catenin signaling reduced ALD progression *in vivo*.

Conclusions: Wnt/β-catenin signaling plays a protective role in ALD progression via antagonizing Foxo3A-induced apoptosis, and activation of the Wnt/β-catenin signaling cascade attenuates ALD progression.

Keywords: Wnt; Alcoholic liver disease; Apoptosis; Foxo3A; SGK1.
Received 23 September 2014; received in revised form 14 January 2015; accepted 17 February 2015; available online 24 February 2015

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Abbreviations: Foxo3A, forkhead box O3; Wnt, wingless-type MMTV integration site family; ALD, alcoholic liver disease; SGK1, serum/glucocorticoid regulated kinase 1; Sirt1, sirtuin 1; LiCl, lithium chloride.

Introduction

ALD is associated with heavy consumption and often presents after 20–30 years chronic abuse. ALD progression features three histologic stages, i.e. fatty liver, alcoholic hepatitis, and hepatic fibrosis or cirrhosis. Fatty liver is often reversible with cessation of drinking. On the other hand, chronic alcoholic hepatitis is a more severe form of ALD and 20% of patients develop hepatic cirrhosis, which is the 12th leading cause of death in the United States in 2008 [1,2]. Although the early stages of ALD are usually reversible with abstinence, chronic alcoholic hepatitis is a potentially progressive and difficult disease to control due to inflammation-induced hepatic damage and hepatocyte apoptosis. Pro-inflammatory cytokines that trigger inflammatory responses and apoptosis have been observed in the liver of animal models of ALD as well as in chronic alcoholics [3,4]. Moreover, targeting elevated levels of inflammatory cytokines, such as TNFα and IL1β has been investigated as potential therapeutic approaches to alter ALD progression [5,6]. However, inhibition of pro-inflammatory cytokine signaling by anti-TNFα agents has been shown to increase the risk of bacterial or viral infection [7–14]. Currently, other potential targets that modulate inflammatory responses are being explored in a therapeutic context to arrest ALD progression via suppression of inflammation [4].

Wnt/β-catenin signaling has been evaluated for its role in liver development and regeneration. Conditional depletion of β-catenin in hepatocytes with hepatocyte nuclear factor 3 gamma (HNF3γ) driven by Cre recombinase produced an undeveloped liver at embryonic day 12, and lethality at day 17 in mice [15]. In addition, loss of β-catenin expression in the liver delayed proliferation of hepatocytes in mice following partial hepatectomy [16,17]. However, β-catenin has been recently investigated for its function in modulating inflammatory responses. Knocking-out of β-catenin specifically in dendritic cells has been shown to inhibit the immune response against bacterial infection [18]. Wnt/β-catenin signaling has also been shown to be involved in Foxo3A-mediated apoptosis. Overexpression of β-catenin antagonizes Foxo3A-induced apoptosis in colon cancer cells [19]. Interestingly, liver-specific β-catenin knockout mice show more severe ALD phenotypes than wild-type mice after chronic ethanol

consumption, and the mechanisms appear to involve induction of oxidative stress [20,21].

Although evidence is presented to indicate that Wnt/β-catenin signaling is very important in liver development, regeneration or ALD progression, there is no information on whether activation of Wnt/β-catenin signaling could reduce ALD progression. Herein, the current study was designed to test whether pharmacological activation of Wnt/β-catenin signaling altered ALD development and progression in a rat model.

Materials and methods

Animal experiments

Long Evans (LE) rats were purchased from Harlan Laboratories (South Easton, MA). This ALD model was developed as previously described [22]. Briefly, LE rats were pair-fed with a liquid diet containing either 37% ethanol or an isocaloric liquid diet for 8 weeks. During the last three weeks of feeding, a Wnt agonist (Sigma; 5 mg/kg) was introduced by intra-peritoneal injection twice weekly. Rats were sacrificed with isoflurane and the liver and blood collected for biochemical analysis.

Cells and reagents

Immortalized human hepatocytes, HuH7, and Hep3B cells were grown in DMEM Dulbecco's Modified Eagle Medium with 10% fetal bovine serum, L-glutamine (Life Technologies, Gaithersburg, MD), and MEM non-essential amino acids (Sigma Chemical Co, St Louis, MO). Alanine aminotransferase enzymatic activity assay kit was purchased from Cayman Chemical. TUNEL staining kit (In Situ Cell Death Detection Kit) was purchased from Roche. CID 11210285 hydrochloride (2-Amino-4-(3,4-(methylenedioxy)benzylamino)-6-(3-methoxyphenyl)pyrimidine hydrochloride, AMBMP, N4-(1,3-benzodioxol-5-ylmethyl)-6-(3-methoxyphenyl)-2,4-pyrimidinediamine hydrochloride, Wnt agonist) and LiCl were purchased from Sigma. SGK1 inhibitor was purchased from Santa Cruz Biotechnology. The plasmids of Foxo3A (FLAG-Foxo3A WT, #8360) and Foxo3A without DNA binding domain (FLAG-Foxo3A TM, #8361) [23] were obtained from Addgene.

Immunoblot analysis

Immunoblot analysis (IB) was performed as previously described [24]. In brief, 50 μg of total protein was used for all IB data. Antibodies of pAkt, Akt, pGSK3β, GSK3β, β-catenin, β-actin, pFoxo3A, Foxo3A, pro-caspase 3, cleaved caspase 3, and Bcl-XL were purchased from Cell Signaling Technology and the dilution factor was 1:1000. α-tubulin antibody was purchased from Sigma and the dilution factor was 1:3000. Antibodies of SGK1, cyclin D1, Sirt1, lamin A/C, and GADPH were purchased from Santa Cruz Biotechnology and the dilution factor was 1:500. Relative expression density was quantified using Quantity One software (Bio-Rad).

Real Time quantitative PCR (RT-qPCR)

RT-qPCR was performed as previously described [25]. Briefly, 1 μg of total mRNA was used to reverse transcribe cDNA with iScript kit (Bio-Rad). cDNA was diluted 10 folds before performing qPCR. mRNA expression was quantified relative to internal control and assayed in triplicate.

Immunohistochemical staining

Rat livers were obtained, fixed in 10% neutral buffered formalin, and processed to a paraffin embedded block as previously described [26]. Basically, 5 μm tissue slides were processed for antigen retrieval with 10 mM Sodium Citrate (pH value of 6). The tissue slides were blocked with 5% BSA for 1 h and incubated with indicated 1st antibodies prepared with 5% BSA in PBS overnight at 4 °C. Then slides were washed 5 times with 0.5% PBST and incubated with 1:1000 diluted biotinylated secondary antibody (Vector Laboratories, Burlingame, CA) for another hour. Slides were washed 5 times with 0.5% PBST and incubated with ABC solution (Vector Laboratories). Tissues were visualized by AEC (DAKO, Carpenteria, CA), followed by Mayor's hematoxylin counterstaining. Stained tissue slides were dehydrated and covered with cover slides. Images were obtained with the microscope camera and quantified with the Image-Pro Plus software.

TUNEL staining

TUNEL staining was performed following instruction manual. Basically, liver slides were processed and stained with TUNEL kit. Images were taken and analyzed as previously described [27].

Statistical analysis

All statistical analyses were performed using student *t* test. *p* value was considered significant smaller than 0.05. Results were reported as mean ± SD or SE (n = 3–10).

Results

Chronic ethanol consumption produces injury, apoptosis, and reduced β-catenin signaling

Eight weeks of 37% ethanol consumption reduced body weight in ALD rats starting as early as week 2 (Fig. 1A and Supplementary Fig. 1A). Although body weight gain was reduced in ALD rats, no significant differences in food consumption was measured between control and 37% ethanol fed animals (Fig. 1B). There was no difference in liver to body weight ratio as well (Supplementary Fig. 1B). However, fat to body weight ratio and blood glucose level were decreased (non-fasting) in ethanol fed rats (Supplementary Fig. 1C and D). Significantly elevated ethanol levels were found in ethanol fed rats (Supplementary Fig. 1E). To determine the degree of liver injury produced by chronic ethanol consumption, we performed H&E staining and measured alanine transaminase (ALT) levels to assess lipid accumulation and hepatic injury, respectively. The H&E staining revealed extensive steatosis in ethanol fed rats (Fig. 1C). An ALT enzymatic assay revealed significantly higher ALT activity compared to isocaloric pair-fed controls. Further studies with terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay exhibited robust positive signals in the liver of ethanol fed rats, but only barely detectable signal in isocaloric pair-fed controls, which suggests extensive hepatic DNA damage produced by chronic ethanol. Interestingly, nuclear and cytoplasmic expression of β-catenin was decreased in the livers of ALD rats as well (Fig. 1F), suggesting that Wnt/β-catenin signaling may be disrupted and could place the liver at risk for enhanced apoptosis.

Downregulation of β-catenin hepatic levels negatively correlates with Foxo3A expression and apoptosis in ALD rats

The Wnt/β-catenin signaling pathway has been shown to be regulated by Akt/GSK3β signaling [28]. Accordingly, we tested whether the observed downregulation of hepatocyte nuclear/cytoplasmic β-catenin levels was influenced by the Akt/GSK3β pathway, and measured expression levels of p-Akt, Akt, β-catenin, p-GSK3β, and GSK3β. As shown in Fig. 2A and Supplementary Fig. 2A, levels of pAkt, p-GSK3β, and β-catenin were significantly reduced in the livers of ethanol fed rats, implying that decreased generation of pAkt could reduce phosphorylation of GSK3β, which led to activation of GSK3β and resulted in the degradation of β-catenin. Furthermore, inhibition of cyclin D1 expression (Fig. 2A), which is one of Wnt/β-catenin target genes, supported that impaired β-catenin signaling was associated with Akt/GSK3β pathway in ALD rats.

Foxo3A is one of the pAkt regulated target genes and is highly associated with apoptosis [29,30]. To determine whether pAkt regulates Foxo3A, levels of p-Foxo3A and Foxo3A (Fig. 2B and C)