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Role of β-Catenin in regulating the balance between TNF-α- and Fas-induced acute liver injury

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ABSTRACT

β-Catenin plays many critical roles during various liver physiological and pathological processes. However, the role of β-Catenin in acute liver failure remains unclear. Using hepatocyte specific β-Catenin knockout mice, we found that loss of β-Catenin in hepatocyte significantly reduced GalN/LPS-induced liver damage and hepatocyte apoptosis, but exacerbated Jo2-mediated liver injury. Mechanistically, the dual effects of β-Catenin attributes on its function of inhibiting NF-κB signaling, which aggravates oxidative stress but decreases Fas expression under injury conditions. In conclusion, β-Catenin plays an important role in regulating the balance between TNF-α and Fas-induced liver injury via its effect on NF-κB.

1. Introduction

Acute liver failure is a life threatening clinical syndrome associated with devastating consequences [1]. It is one of the most challenging gastrointestinal emergencies encountered in clinical practice and carries a high mortality rate worldwide [2]. Massive hepatocyte necrosis and apoptosis is an important pathologic feature in acute liver injury. Autoimmune hepatitis, viral hepatitis, alcohol consumption and hepatotoxins have been identified as trigger factors of acute liver failure. Orthotopic liver transplantation remains the main effective option of treatment for acute liver failure. However, approximately 37% of the patients die in the long waiting period because of organ shortage [3]. Therefore, increasing the anti-apoptotic properties of hepatocytes is a new way to treating acute liver failure.

β-Catenin is the chief downstream effector of the canonical Wnt signaling pathway, which plays an essential role in regulating cell growth, regeneration, differentiation, and apoptosis [4]. In the absence of Wnt signals, cytoplasmic β-Catenin is phosphorylated by glycogen synthase kinase β (GSK3β) at the serine or threonine residues located at the NH2 terminus. Phosphorylated β-Catenin then undergo degradation by ubiquitination [5]. Upon binding to Wnt ligand, GSK3β is inactivated and cytoplasmic β-Catenin dissociates from a degradation complex and translocates into nucleus to formulate transcription complex with Tcf4 and Lef. Nuclear β-Catenin regulates cell growth and apoptosis by switching on transcription and expression of downstream target genes such as *c-myc*, *cyclin D1* and *survivin* [6]. Meanwhile, β-Catenin also interacts with transmembrane proteins of the cadherin family to promote cell–cell adhesion [7].

In the liver, β-Catenin plays many critical roles during hepatic development and regeneration. It contributes to liver physiology and pathology by regulating various basic cellular events, including

Abbreviations: ALT, serum alanine aminotransferase; AST, aspartate aminotransferase; CHX, cycloheximide; GalN, D-galactosamine; GSH, serum glutathione; GSK3β, glycogen synthase kinase β; HCC, hepatocellular carcinoma; LPS, lipopolysaccharide; MDA, malondialdehyde; NO, Nitric Oxide; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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cell proliferation, apoptosis, oxidative stress, differentiation, zonation, xenobiotic metabolism and other metabolic processes [6]. The aberrant alternations of Wnt/β-Catenin pathway, especially activation of β-Catenin have been described in various human cancers, including hepatocellular carcinoma (HCC). However, due to its complicated role in regulating apoptosis, there has been no functional research addressing the biological function of β-Catenin in acute liver failure.

D-galactosamine (GalN) plus lipopolysaccharide (LPS) treatment of mice is a well-established experimental model of acute liver injury. GalN is a specific hepatotoxic transcriptional inhibitor and sensitizes liver to develop lethal liver injury mimicking fulminant hepatitis [8]. TNF-α is the predominant mediator that induces hepatocyte apoptosis and liver injury in the GalN/LPS model [9]. Fas-regulated apoptosis also plays a major role in the pathogenesis of immuno-mediated liver diseases [10,11]. The liver is very sensitive to Fas-mediated apoptosis because Fas antigen is highly and constitutively expressed on hepatocytes[11]. When mice are injected with anti-Fas antibody (Jo2), the cause of death is secondary to liver failure.

To illustrate the role of β-Catenin in regulating hepatocyte apoptosis and liver injury, we investigated the function of β-Catenin in GalN/LPS- or Fas-induced acute liver injury using hepatocyte-specific β-Catenin knockout mice. Herein, we show that hepatocyte-specific knockout of β-Catenin attenuates D-GalN/LPS induced liver injury, resulting from stronger activation of NF-κB and reduced hepatocyte oxidative stress. Conversely, hepatocyte-specific knockout of β-Catenin aggravates Fas-induced acute liver failure through the increased Fas expression on hepatocytes. We propose that β-Catenin controls the balance between Fas- and TNF-α-induced hepatocytes apoptosis via its regulation of NF-κB.

2. Materials and methods

2.1. Mice

Albumin promoter-driven Cre recombinase transgenic mice (*Alb-Cre* mice) and mice carrying the floxed allele of β-Catenin (*β-catenin*$^{loxP/loxP}$ mice), purchased from the Jackson Laboratory (USA), were crossed to obtain hepatocyte-specific β-Catenin knockout mice (*Alb-Cre;β-catenin*$^{loxP/loxP}$ mice). Transgenic mice were genotyped by polymerase chain reaction (PCR) analysis using genomic DNA isolated from the tail tip. Mice with heterozygous β-Catenin hepatocytes (*Alb-Cre;β-catenin*$^{loxP/+}$) and mice lacking the Cre recombinase (*β-catenin*$^{loxP/loxP}$ and *β-catenin*$^{loxP/+}$) were grouped together as control littermates. Mice used in this study were maintained in the barrier facility at 25 °C, 55% humidity and 12 h light/dark rhythm. The handling of the mice and experimental procedures were conducted according to the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Sciences and published by NIH (publication 86–23 revised 1985).

2.2. Experimental protocol

Hepatocyte-specific β-Catenin knockout mice and control mice were used for experiments at the age of 8–10 weeks. In GalN/LPS model, LPS (80 μg/g bodyweight; Sigma, USA) and GalN (800 μg/g bodyweight; Sigma, USA) were simultaneously injected intraperitonealy into hepatocyte-specific β-Catenin knockout mice and control mice. In Fas induced liver injury model, the mice were intraperitonealy administered Jo2 (0.5 μg/g bodyweight; BD Pharmingen, CA) to induce acute fulminant hepatic failure. Mice were sacrificed at the indicated time points to obtain blood samples and liver tissues.

2.3. Serum transaminases assay

ALT and AST activity were assayed using an automated procedure in the Department of Inspection, Eastern Hepatobiliary Surgery Hospital.

2.4. Histological analysis

Liver tissue was divided and fixed in phosphate-buffered neutral formalin, embedded in paraffin, and cut into 5-μm-thick sections. The following primary antibodies were used: anti-β-Catenin, and anti-P65 (Santa Cruz Biotech, CA). Vector ABC kit (Vector Laboratories, CA) and DAB reagent (Dako Comp, Japan) were employed in the detection procedure. Apoptosis was detected by TUNEL staining of paraffin-embedded slides (Calbiochem, La Jolla, CA), according to the manufacturer's instructions. For immunofluorescent staining, cells were cultured in 12-well plates and fixed with 4% paraformaldehyde. After permeabilization with 0.5% Triton X-100, and block with 10% BSA buffer, cells were incubated with diluted primary antibody and visualized with fluorescein-conjugated secondary antibodies (Invitrogen, USA). All the slides were observed and photographed with an Olympus microscope (IX-70 OLYMPUS, Japan).

2.5. Cell culture

Murine HCC cell lines Hepa1-6 was purchased from the Shanghai Cell Bank (Shanghai, China). Cells were cultured at 37 °C in a humidified incubator containing 5% CO_2, and in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, 100 units/mL penicillin and 100 μg/mL streptomycin. Hepa1-6 cells were infected with mutant active β-Catenin (Ad-S37A) overexpression adenovirus or control blank adenovirus (Ad-blank) for 24 h. Infected cells were stimulated with a cytokine mixture consisting of recombinant murine TNF-α (40 ng/mL; Invitrogen, USA) and cycloheximide (CHX, 10 mg/mL; Sigma, USA). Cell proliferation rate was assessed using CCK-8 assay kit. The relatively cell death rate was derived from cell proliferation rate.

2.6. Luciferase assay

The luciferase reporter construct containing six NF-κB consensus sequences (pNF-κB-Luc) was used as previously reported [12]. A Dual Luciferase reporter assay was carried out according to the manufacturer's suggestions (Promega, USA). pRL-TK (Promega, USA) was co-transfected with each reporter construct to normalize for transfection. Luciferase activity was determined 48 h after transfection. The histograms are presented as the mean ± s.d. from at least three independent experiments.

2.7. Real-time polymerase chain reaction

Total RNA was prepared from liver tissue samples using Trizol (Invitrogen, USA) according to the manufacturer's protocol. Reverse transcriptase PCR was performed using Superscript III RT (Invitrogen, USA) in the presence of random primers. Amplification of the generated cDNA was carried out in SYBR Green PCR Master Mix (Takara, Japan) with ABI PRISM 7300HT Sequence Detection System. Primer sequences are listed in Supplementary Table 1. Each measurement was performed in triplicate and the results were normalized by the expression of the *β-actin* reference gene.

2.8. Oxidative stress analysis

Serum glutathione (GSH), malondialdehyde (MDA), and Nitric Oxide (NO) levels were measured using commercialized assay kits (Jiancheng Co., Nanjing) according to manufacturer's instructions.

2.9. Western blotting

Whole liver tissue or cultured cells were homogenized in Triton lysis buffer (20 mM Tris, pH 7.4, 137 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM PMSF, 10 mM NaF, 5 mg/mL aprotinin, 20 mM leupeptin, and 1 mM sodium orthovanadate) and centrifuged at 12,000 rpm for 15 min. Protein extracts were subjected to SDS–PAGE and analyzed using the following primary antibodies: anti-β-Catenin, anti-P65, anti-GAPDH (Santa Cruz Biotech), anti-PARP, anti-Cleaved Caspase3 (cell signaling technology), and anti-Fas (BD Pharmingen, CA). Protein levels were detected by the Odyssey system (Li-Cor, Lincoln, NE).

2.10. siRNA transfections

SiRNA for β-Catenin and control siRNAs were purchased from Ruibo Biotech (Guangzhou, China). Transfection of the siRNAs was performed with Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The effects of siRNA were validated by quantitative real-time polymerase chain reaction (qRT-PCR) and western blot for the level of β-Catenin and target genes expression.

2.11. Statistic analysis

Data are expressed as mean ± s.d. Difference between experimental and control groups were analyzed by the Student's *t*-test. A value of $p < 0.05$ was considered significant.