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Nonstructural 5A protein activates β-catenin signaling cascades: Implication of hepatitis C virus-induced liver pathogenesis☆

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***Background/Aims*: The nonstructural 5A (NS5A) protein of hepatitis C virus (HCV) has been implicated in HCV-induced liver pathogenesis. Wnt/β-catenin signaling has also been involved in tumorigenesis. To elucidate the molecular mechanism of HCV pathogenesis, we examined the potential effects of HCV NS5A protein on Wnt/β-catenin signal transduction cascades.**

***Methods*: The effects of NS5A protein on β-catenin signaling cascades in hepatic cells were investigated by luciferase reporter gene assay, confocal microscopy, immunoprecipitation assay, and immunoblot analysis.**

***Results*: β-Catenin-mediated transcriptional activity is elevated by NS5A protein, in the context of HCV replication, and by infection of cell culture-produced HCV. NS5A protein directly interacts with endogenous β-catenin and colocalizes with β-catenin in the cytoplasm. NS5A protein inactivates glycogen synthase kinase 3β and increases subsequent accumulation of β-catenin in HepG2 cells. β-Catenin was also accumulated in HCV patients' liver tissues. In addition, the accumulation of β-catenin in HCV replicon cells requires both activation of phosphatidylinositol 3-kinase and inactivation of GSK3β.**

***Conclusions*: NS5A activates β-catenin signaling cascades through increasing the stability of β-catenin. This modulation is accomplished by the protein interplay between viral and cellular signaling transducer. These data suggest that NS5A protein may directly be involved in Wnt/β-catenin-mediated liver pathogenesis.**

***Keywords*: Hepatitis C virus; Liver pathogenesis; NS5A protein; β-Catenin signaling; Tumorigenesis**

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Abbreviations: HCV, hepatitis C virus; NS5A, nonstructural 5A; HCC, hepatocellular carcinoma; GST, glutathione S-transferase; HA, hemaglutinin; IFN, interferon; PBS, phosphate-buffered saline; GFP, green fluorescence protein; TRITC, tetramethylrhodamine isothiocyanate; EGF, epidermal growth factor; PI3K, Phosphatidylinositol 3-kinase; GSK3β, glycogen synthase kinase 3β; LEF/TCF, lymphoid enhancing factor/T-cell factor; Grb2, growth factor receptor-bound protein 2.

1. Introduction

Hepatitis C virus (HCV) is the causative agent of non-A, non-B hepatitis. HCV infection often leads to chronic hepatitis, liver cirrhosis, and ultimately hepatocellular carcinoma (HCC) [1]. However, the molecular events that lead to HCC development during HCV infection are poorly defined. HCV is an enveloped, positive-sense RNA virus belonging to the *Flaviviridae* family. Its genome encodes a single polyprotein precursor of more than 3000 amino acids, which is cleaved by host and viral proteases at the endoplasmic reticulum, yielding structural (core E1

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and E2) and nonstructural (p7, NS2 to NS5B) proteins. The nonstructural 5A (NS5A) protein is generated by the NS3/4A serine protease. NS5A is a phosphoprotein consisting of 447 amino acid residues. NS5A exists in two forms of polypeptide, p56 and p58, which are phosphorylated at serine residues by cellular kinase [2]. Phosphorylation is involved in the HCV life cycle [3]. NS5A protein is localized in the cytoplasm and forms a part of the HCV RNA replication complex [4]. NS5A is a multifunctional protein involved in cellular signal modulations. NS5A modulates interferon (IFN) signaling through protein kinase R (PKR) interaction and also interferes with host cell signaling pathways, including Grb2 and p85 subunits of phosphatidylinositol 3-kinase (PI3K) [5–7]. In addition, NS5A protein has been found to stimulate anchorage-independent growth of murine fibroblast cell lines [8] and induced chromosome instability by mitotic cell cycle dysregulation of HepG2 cell lines [9].

The Wnt/β-catenin signaling pathway was initially discovered by genetic analysis in the wing development of *Drosophila* [10] and has now been implicated in many human cancers. The Wnt family of proteins consists of 350–380 amino acids that serve as the ligands for Frizzled receptors [11]. There are 19 known Wnt ligands in humans [12]. These proteins are highly conserved throughout evolution and play major roles in embryonic patterning, cell polarity, and cell fate determination [12]. β-Catenin was first identified on the basis of its association with cadherin adhesion molecules and is now widely recognized as a key molecule of the Wnt signaling cascade [13]. In the presence of Wnt-1, GSK3β activity is suppressed, which in turn leads to β-catenin stabilization. Abnormalities in the regulation of Wnt/β-catenin signaling have been implicated in various human cancers, including colon cancer, HCC, leukemia, and melanoma [14,15]. It has previously been shown that mutations of β-catenin, specifically stabilizing mutations in exon 3 [16], were detected in approximately 30% of primary HCCs [17,18]. Furthermore, the Wnt/β-catenin signaling pathway has been identified as a common target for perturbation by viruses. For example, hepatitis B virus X protein achieves β-catenin stabilization by suppressing GSK3β activity in a Src-kinase-dependent manner [19]. The Vpu protein of HIV-1 binds to βTrCP and blocks the ubiquitinylation and proteasomal degradation of β-catenin [20]. In addition, HCV NS5A protein is involved in PI3K-mediated β-catenin stabilization [21]. In the present study, we demonstrated that NS5A-activated β-catenin signaling cascades through the stabilization of β-catenin and that this activity may play an important role in HCV pathogenesis.

2. Materials and methods

2.1. Plasmids

Plasmids expressing NS5A (genotype 1b), Myc-tagged NS5A, GST-NS5A, and GFP-NS5A were described elsewhere [22,23]. Either Myc-tagged NS3 or NS4B of HCV (genotype 1b) expression plasmid was generated by PCR and inserted into the EcoRI site in pEF6A/His-Myc vector (Invitrogen, Carlsbad, CA, USA). NS5A mutants were constructed using either the pFlag-CMV-2 or pEF6A/His-Myc vector. Both wild-type and mutants of β-catenin were subcloned into the BamHI site of the pEF6B/His-Myc vector. pTOPFLASH, pFOPFLASH, and TCF-4 expression plasmids were kindly provided by Drs. B. Vogelstein and K. Kinzler (Johns Hopkins University, Baltimore, Maryland, USA). Flag-tagged β-catenin was obtained from Dr. Eric R. Fearon (University of Michigan, Ann Arbor, USA), and HA-human GSK3β was provided by Dr. J. Woodgett (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ont., Canada).

2.2. Cell culture, transfection, and HCV infection

Huh7, HepG2, and Cos7 cells were grown as described previously [24]. Stable cells expressing NS5A were grown as reported previously [23]. HCV subgenomic replicon cells were described elsewhere [23]. RNA prepared from the infectious cDNA clone (JFH-1) was transfected into Huh7 cells, and cell culture-produced HCV was generated as described previously [25].

2.3. Luciferase reporter gene assays

Luciferase and β-galactosidase assays were performed as described previously [24] using 1 μg reporter plasmid (pTOPFLASH or pFOPFLASH) and 0.1 μg of pCH110 reference plasmid (Amersham Biosciences, GE Healthcare UK Ltd., Buckinghamshire, UK) containing the *Escherichia coli lac*Z gene under the control of the Simian virus 40 promoter.

2.4. Immunoblot analysis

Cell lysates were prepared and immunoblotted as described previously [23]. Quantification of the band intensity was determined using a calibrated GS-800 densitometer (Bio-Rad, Hercules, CA, USA).

2.5. Glutathione S-transferase pull-down assay and coimmunoprecipitation

Glutathione S-transferase (GST)-NS5A fusion protein was expressed and purified as described previously [23]. Both in vitro and *in vivo* binding assays between NS5A and β-catenin, and coimmunoprecipitation assay were performed as described elsewhere [24].

2.6. Confocal microscopy

Cos7 cells grown on cover glass were cotransfected with the GFP-NS5A and Flag-catenin expression plasmids, fixed in 4% paraformaldehyde and 0.1% Triton X-100, and analyzed using the LSM 510 laser confocal microscopy system (Carl Zeiss, Inc., Thornwood, New York, USA), as reported previously [22].

2.7. Patient tissues

Human liver tissue specimens were obtained from the Liver Cancer Specimen Bank at Yonsei University in Seoul, South Korea. All patients participating in this study gave informed consent before surgery, and the use of human tissue for this research was authorized by the Institutional Review Board of the College of Medicine at Yonsei University.