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Activated Notch signaling is required for hepatitis B virus X protein to promote proliferation and survival of human hepatic cells

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ABSTRACT

Hepatitis B virus X protein (HBx) is a multifunctional oncoprotein which plays a crucial role in the pathogenesis of hepatocellular carcinoma (HCC). However, the exact mechanisms remain controversial. Here we show that HBx strongly stimulated cell growth, promoted cell cycle progression and inhibited apoptosis of human non-tumor hepatic cell line L02 cells. It also accelerated tumor formation of L02 cells in BALB/c nude mice. Furthermore, Notch signaling components were upregulated in HBx-expressing L02 cells compared to normal L02 cells. However, blocking Notch signaling with a γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) attenuated cell growth, shortened the S phase of cell cycle and promoted apoptosis of HBx-expressing L02 cell in a dose- and time-dependent manner, but normal L02 cells were not significantly affected by Notch signaling blocking. Therefore, our findings demonstrate that HBx could promote the growth of human non-tumor hepatic cell line L02 cells both *in vitro* and *in vivo*, which may require the activation of Notch signaling pathway.

1. Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the third most common cause of death from cancer [1]. Epidemiological evidence has clearly shown that chronic infection with hepatitis B virus (HBV) is the major risk factor for the development of HCC [2,3]. In the last two decades, abundant *in vivo* and *in vitro* studies on HBV have further demonstrated the importance of HBV in HCC development [4,5]. However, the exact mechanism(s) concerning the pathogenesis of HBV-induced HCC have not been fully elucidated. Accumulating evidence suggests that the HBV X protein (HBx), which is a multifunctional oncoprotein, plays a crucial role in the pathogenesis of HCC [6,7]. As a trans-activating protein, by binding to the nuclear transcription factors, HBx alters gene expression including the upregulation of proto-oncogenes (c-myc, N-myc and c-jun), transcription factors (AP-1, NF-κB, and ATF/CREB), HBV enhancers and the human immunodeficiency virus long terminal repeat [8–10]. It can also affects the regulation of apoptosis and cell cycle through its interaction with caspases, mitochondria, survivin, CDK (cyclin-dependent kinase) and CKI (CDK inhibitor) [11–13]. In addition, HBx regulates a variety of cellular signaling pathways such as Ras-Raf-MAPK pathway, SAPK/JNK pathway, PI3 K-Akt/PKB pathway and Janus kinase/STAT pathway [14–16]. Take these mechanisms together, HBx favors cell survival and probably initiates hepatocarcinogenesis in most instances [17].

Abbreviations: HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HBx, HBV X protein; Notch-IC or NICD, Notch intracellular domain; CSL, mammalian CBF1, Drosophila Su(H), *C. elegans* LAG-1; Hes, hairy and enhancer of split family; DAPT, γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester; hTERT, human telomerase reverse transcriptase; qRT-PCR, quantitative real-time RT-PCR.

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Notch signaling is a highly evolutionarily conserved pathway that plays a critical role in the normal development of tissues and cells through diverse effects on cell fate decision, stem cell renewal, differentiation, proliferation and apoptosis [18]. In mammals, the Notch family consists of four transmembrane receptors (Notch1–Notch4) and five ligands (Jagged-1, Jagged-2, Dll-1, Dll-3, and Dll-4). Upon receptor-ligand binding, Notch receptors are proteolytically cleaved by metalloproteinase and γ-secretase complex in two steps, which in turn results in the liberation of the Notch intracellular domain (Notch-IC or NICD) from the plasma membrane. Subsequently, Notch-IC translocates into the nucleus, and binds to the transcription factor CSL(mammalian CBF1, *Drosophila* Su(H), *C. elegans* LAG-1), which displaces co-repressors (CoR) and recruits co-activators (CoA), leading to the transcriptional activation of Notch target genes, including basic helix-loop-helix transcription factors of the hairy and enhancer of split (Hes) family and the Hes-related repressor protein (Herp, also known as Hrt/Hey) family [19,20]. Mounting data indicate that dysregulated Notch activity is linked to a wide variety of tumors [21]. Depending on the cell type and context, aberrant Notch signaling functions as an oncogene in numerous human cancers including T-cell leukemia, medulloblastoma, pancreatic cancer, prostate cancer and breast cancer, it also plays a tumor suppressor role in small-cell lung cancer, B-cell malignancies, and skin cancer [22–24].

However, little is known about the role of Notch signaling in the development of HCC, and its relationship with HBx is even less documented. To address this, we investigated the role of HBx in the development of HCC *in vitro* and *in vivo* and the role of Notch signaling in HBx-expressing hepatic cells. We found that HBx can promote the growth and survival of the human non-tumor hepatic cell line L02 both *in vitro* and *in vivo*, which was impaired by inhibition of Notch signaling with a γ-secretase inhibitor DAPT, while normal L02 cells were almost independent of Notch signaling to survive and propagate. Our results indicate that Notch signaling may be one of the downstream targets through which HBx functions as an oncoprotein. Moreover, these data will define a novel role of HBx and its relationship with Notch signaling in HCC development, and may justify the Notch pathway as a potential therapeutic target.

2. Materials and methods

2.1. Cell culture

The human non-tumor hepatic cell line L02 was a generous gift from Dr. Xinyuan Liu (Shanghai Institutes for Biological Sciences, Shanghai, China). This cell line originated from normal human liver tissue immortalized by stable transfection with the hTERT gene, which has been used previously [25,26]. L02/HBx and L02/pcDNA3.1 cell lines, which derived from L02 cells by transfection with HBx expression plasmid or its empty plasmid (pcDNA3.1 (+)/V5-HisB), respectively, had been successfully established previously [27]. All cell lines were cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and maintained in humidified incubator at 37 °C under a 5% CO2 atmosphere.

2.2. DAPT treatment

The γ-secretase inhibitor *N*-[*N*-(3,5-difluorophenacetyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) was purchased from the Sigma–Aldrich Company (Sigma, St. Louis, MO, USA). DAPT was dissolved in 100% dimethylsulphoxide (DMSO; Sigma) to make a stock solution of 10 mM, which was then diluted in culture medium to obtain the desired concentrations of 1, 5, 10 and 20 μM. DMSO diluted in culture medium at the final percentage of 0.05% without DAPT was designated as 0 μM. Untreated cells were incubated in the culture medium without any additives. Cells with or without DAPT were cultured for 48 h, after which total RNA or protein was extracted and flow cytometry was carried out.

2.3. Cell proliferation and viability assay

Cell proliferation assays were performed by using a Cell Counting Kit-8 (Dojindo, Kumamoto, Japan) according to the manufacturer's instructions. Briefly, 1×10^4 cells per well were plated in 96-well plates and cultured in growth medium. At the indicated time points, medium was aspirated. Then each well was added with 100 μl serum-free DMEM and 10 μl WST-8 (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H tetrazolium, monosodium salt) and incubated at 37 °C for 1.5 h. Absorbance was measured at 450 nm with a reference wavelength of 630 nm on a spectrophotometer (Molecular Devices, Sunnyvale, CA). To evaluate the viability of L02/HBx cells, 1×10^4 cells per well were plated in 96-well plates followed 12 h later by the addition of DAPT at the concentration of 0, 1, 5, 10 or 20 μM. Cell viability was assessed as percent cell viability in terms of untreated control cells, which were determined for each concentration by use of the following equation: $\%\text{viability} = OD_{experiment}/OD_{control} \times 100\%$. Control cells were considered as 100% viable. All experiments were repeated five times.

2.4. Cell cycle and apoptosis analysis by flow cytometry

After treatment with or without DAPT for 48 h, cells were harvested and immediately fixed in 75% ethanol at 4 °C overnight, then treated with 50 mg/L RNase A (Sigma, St. Louis, MO, USA) for 30 min at 37 °C, and stained with 50 mg/L PI (Sigma) for 10 min. Samples were then analyzed for their DNA content by FACSAria Cell Cytometer (BD Biosciences, San Jose, CA, USA). The data were analyzed with CellQuest software (BD Biosciences). Apoptosis analysis was performed by using an annexin V-FITC KIT (Bender, Burlingame, CA, USA) according to the manufacturer's instructions. The percentage of cells that were annexin V positive but PI negative was compared among the different treatment groups.