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Hepatitis B virus X protein and proinflammatory cytokines synergize to enhance TRAIL-induced apoptosis of renal tubular cells by upregulation of DR4

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ABSTRACT

Persistent infection with hepatitis B virus (HBV) may lead to HBV-associated glomerulonephritis (HBV-GN). Presence of HBV-DNA and -RNA in renal tubular epithelial cells (RTECs) suggests direct virus-induced injury. Increase in proinflammatory cytokines is also observed under these conditions. Apoptosis by tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) plays a significant role in the pathogenesis of HBV-infections. However, the effects of HBV X protein (HBx) on TRAIL-induced apoptosis of RTECs especially under certain inflammatory conditions remain obscure. Here, we show that HBx synergizes with proinflammatory cytokines to significantly increase TRAIL-induced apoptosis of RTECs. HBx markedly up-regulates death receptor-4 (DR4) expression by enhancing the activation of nuclear factor-kappa B (NF-κB) in the presence of proinflammatory cytokines. Dramatic increase in DR4 expression leads to the sensitization of RTECs to TRAIL-induced apoptosis. Furthermore, in patients with HBV-GN, DR4 expression in the kidneys is significantly elevated and is positively correlated with the HBx and proinflammatory cytokines expression. These findings provide a novel insight into the underlying mechanisms of renal tubule lesions induced by HBx in HBV-GN.

1. Introduction

Hepatitis B virus (HBV) infections are prevalent in China. Persistent infection may result in HBV-associated glomerulonephritis (HBV-GN) which has become one of the major secondary renal diseases in China (Zhou et al., 2011). HBV-GN is generally believed to be caused by immune complex deposition (Takekoshi et al., 1991), but previous studies have identified expression of HBV antigens in the kidneys suggesting that virus replication and direct virus-induced pathological alterations are also involved (Chen et al., 2009; Bhimma and Coovadia, 2004). We have previously observed inflammatory cell infiltration and tubulointerstitial injury in the renal biopsies from patients with HBV-GN (Zhou et al., 2013), suggesting that the local inflammatory response may also be involved in this disease process (Lee, 1997).

The HBV protein (HBx) is a soluble protein which can be mainly detected in RTECs of patients with HBV-GN (He et al., 2013) suggesting that HBx is involved in RTECs injury. HBx is the most important determinant of viral pathogenesis (Bouchard and Schneider, 2004) and can modulate HBV replication, cellular transcription, signal transduction, proteasome activity, and cell cycle progression (Kim et al., 1991; Xu et al., 2010). Moreover, HBx can also either directly induce apoptosis or sensitize hepatocytes to a variety of apoptotic stimuli, such as cytokines, TRAIL, and hydrogen peroxide (H_2O_2) (Kim et al., 2005; Liang et al., 2007a,b; Hu et al., 2011; Clippinger et al., 2009) which may also play an important role in the progress of HBV-GN. Our previous studies showed that HBx induces apoptosis of RTECs and causes increased production of proinflammatory cytokines, such as TNF-α and IFN-γ (Wang et al., 2013). However, the specific mechanism (s) by which HBx induces apoptosis of RTECs especially under certain inflammatory conditions remains to be fully understood.

Tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) is a member of the TNF family of death ligands and binds to the four major receptors. Binding to two receptors, TRAIL-R1 (DR4) and TRAIL-R2 (DR5), induces apoptosis signaling, whereas binding to other two, TRAIL-R3 (DcR1) and TRAIL-R4 (DcR2), can resist apoptosis signaling and activate non-apoptotic pathways (Lemke et al., 2014; Azijli et al., 2013). TRAIL binding to DR4 and/or DR5 causes the formation of death-inducing signaling complex (DISC), characterized by recruitment

Abbreviations: Chip, chromatin immunoprecipitation; DR4, death receptor-4; DR5, death receptor-5; DLR, dual luciferase reporter; HBV, hepatitis B virus; HBx, hepatitis B virus X protein; HBV-GN, HBV-associated glomerulonephritis; IFN-γ, interferon gamma; NF-κB, nuclear factor-κB; PDTC, pyrrolidine dithiocarbamate; PGN, primary glomerulonephritis; RTECs, renal tubular epithelial cells; TNF-α, tumour necrosis factor alpha; TRAIL, TNF-related apoptosis inducing ligand

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of the adaptor protein FADD and caspase-8, which then activates downstream effector caspases and leads to apoptosis (Kischkel et al., 2000). Some reports have shown that HBV and/or HBx could enhance TRAIL-induced apoptosis of hepatocytes by increasing DR4, Bax, and DR5 (Liang et al., 2007a,b; Janssen et al., 2003; Kong et al., 2015). However, the role of TRAIL in inducing apoptosis of RTECs transfected with the HBx gene remains obscure.

In this report, we investigated whether TRAIL induces apoptosis of RTECs transfected with the HBx gene especially under proinflammatory cytokines stimulation as a possible underlying mechanism. Our results show that cooperative effects of HBx and proinflammatory cytokines significantly enhanced TRAIL-induced apoptosis of RTECs by upregulating DR4 that is mediated through NF-κB activation, which may contribute to HBV-GN pathogenesis.

2. Materials and methods

2.1. Plasmids

The plasmid pcDNA3.1/myc-HBx was constructed previously (Wang et al., 2016). PGL3-basic luciferase vector (Promega, Madison, WI, USA) was used to clone the promoter of DR4; and NF-κB binding site-specific mutant was generated by PCR. Thus, pGL3-DR4-wt and pGL3-DR4-mut plasmid were separately constructed. Primer sequences are listed in Supplementary Table 1S.

2.2. Cell culture and transfection

HK-2 human proximal tubular epithelial cells (ATCC, Manassas, VA) were cultured as described (Wang et al., 2014). To generate a stable cell line, HK-2 cells were transfected with pcDNA3.1/myc-HBx or pcDNA3.1/myc using lipofectamine 3000 (Invitrogen, Carlsbad, CA, USA) according to manufacturer's instructions. Forty-eight hours after transfection, the transfected cells were selected in a medium containing 500 μg/ml of G418 (Gibco, NY, USA) for about a month. Then the stable cell line was obtained. The cells steadily expressing HBx were named HK-2-X cells, and the cells stably transfected with empty pcDNA3.1/myc was named HK-2-M cells as control cells.

2.3. Cytokines and reagents

Recombinant solution human TRAIL was purchased from PeproTech (Rocky Hill, NJ, USA). TRAIL was used at a concentration of 10 ng/mL except otherwise indicated. Human TNF-α (10 ng/mL) and IFN-γ (300 U/mL) were also from PeproTech. NF-κB pathway inhibitors PDTC (30 μM) and JSH-23 (30 μM) were from Sigma-Aldrich (St Louis, MO, USA).

2.4. Cytotoxicity of TRAIL

The cytotoxicity of TRAIL was detected using the CCK-8 (Dojindo, Kumamoto, Japan). Briefly, HK-2-M and HK-2-X cells were seeded into 96-well plates, and treated with cytokines (TNF-α and IFN-γ) and with different concentrations of TRAIL for 24 h. The absorbance (A) at 450 nm was measured by a microplate reader (Bio-RAD Model 680, USA). Cytotoxicity Rate (%) = (1-mean A value of the experimental group/mean A value of the control group) × 100%.

2.5. Apoptosis assays

Cells were seeded into 6-well plates and transfected with siRNAs for DR4 or scramble siRNAs with TRAIL, cytokines or JSH-23 at concentrations indicated. At time points indicated, above cells were harvested and apoptosis was detected by cleaved caspase-8, caspase-3 and PARP immunoblotting as well as flow cytometry analysis of Annexin-V-FITC/PI (Beckman Coulter, CA, USA)-stained cells using a FACS LSR-II (BD Biosciences, Switzerland). Caspase-3 activity was determined by a Caspase-3 Colorimetric Assay Kit (Clontech, CA, USA) per manufacturer's instructions.

2.6. Semi-quantitative and real-time quantitative RT-PCR

Total RNAs were extracted using Trizol (Invitrogen, Carlsbad, CA, USA) per manufacturer's instructions. After reverse transcription, the products of cDNA were used as template for PCR. Semi-quantitative PCR for HBx, DR4, DR5, DcR1, DcR2 and β-actin were performed as described previously (Wang et al., 2014; Liang et al., 2007a,b). PCR products were subjected to electrophoresis in 1% agarose gel and visualized by ethidiun bromide staining. Each gene mRNA value was normalized to the β-actin mRNA content. Real-time PCR was performed on a LightCycler (Roche Diagnostics, Mannheim, Germany) using SYBR Green Premix (Takara, Otsu, Japan). The expression of DR4, DR5, DcR1, and DcR2 in cells was normalized against the expression of β-actin. The results were analyzed by delta Ct method. Primer sequences are listed in Supplementary Table 1S.

2.7. Western blot

Total protein was extracted and separated by 6%–12% SDS-PAGE and then transferred onto 0.22 μm PVDF membrane (Millipore, Billerica, MA, USA). The following primary antibodies were used in the immunoblotting assays: HBx, DR4, DcR1, DcR2 (Abcam, Cambridge, MA, UK), caspase-8, cleaved caspase-8, caspase-3, cleaved caspase-3, PARP, cleaved PARP, IκBα, p-IκBα, p65 (Cell Signaling Technology, Danvers, MA, USA), DR5 (Novus, Littleton, CO, USA), and β-actin (Santa Cruz Biotechnology, CA, USA).

2.8. Immunofluorescence

Cells cultured on glass coverslips were fixed with 4% paraformaldehyde, blocked with 2% bovine serum albumin and incubated with p65 antibody (1:200, Cell Signaling Technology, MA, USA) overnight at 4 °C. Cells were washed three times and incubated with FITC-conjugated goat anti-rabbit IgG (Sigma) for 2 h at room temperature (RT). Coverslips were mounted using ProLong Gold antifade reagent with DAPI (Invitrogen) and imaged using a LEICA DMI4000B microscope (Leica, Heidelberg, Germany).

2.9. Dual-luciferase reporter assays

Cells were seeded into 24-well plates and transfected. Briefly, the pGL3-DR4-wt and pGL3-DR4-mut plasmid were separately co-transfected with the pRL-TK renilla vector using Lipofectamine 3000 (Invitrogen). Twelve hours after transfection, cells were treated with the indicated cytokines for another 12 h and 24 h. Cells were harvested, and luciferase activity was measured from cell extracts by using a Dual-Luciferase Reporter Assay System (Promega). The ratio of firefly luciferase to renilla luciferase was calculated in each group.

2.10. RNA interference

The small interfering RNAs (siRNAs) targeting p65 and DR4 were purchased from GenePharma (Shanghai, China). Non-specific siRNA duplex served as control. Cells were transfected with p65 siRNA (45 pmol/ml) and DR4 siRNA (50 pmol/ml) by Lipofectamine 3000 (Invitrogen) to knock down p65 and DR4, respectively. siRNAs sequences are listed in Supplementary Table 1S.

2.11. Chromatin immunoprecipitation assay

ChIP assays were performed using the SimpleCHIP enzymatic chromatin immunoprecipitation kit (Cell Signaling Technology, MA,