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Hepatitis B virus X increases immune cell recruitment by induction of chemokine SDF-1

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ABSTRACT

Hepatitis B virus X protein is a major factor in the HBV-induced disease developments. Stromal cell-derived factor-1 is a small cytokine that is strongly chemotactic for lymphocytes. We explored the role of HBx on recruitment of HBV-induced virus-nonspecific immune cells into liver. Immune cell recruitment and SDF-1 expression level significantly increased in livers of HBx-transgenic mice and X-box binding protein-1 significantly increased *SDF-1* gene expression. Finally, we confirmed that immune cell recruitment into liver tissues of HBx-TG mice was diminished by a chemokine receptor antagonist. Therefore, HBx increases ER stress-dependent SDF-1 expression and induces HBV-induced immune cell recruitment into liver.

1. Introduction

Hepatitis B virus (HBV) is a non-cytopathic virus that causes acute and chronic necroinflammatory liver diseases [1–3], often leading to the development of cirrhosis and hepatocellular carcinoma [1,2]. HBV-specific cytotoxic T lymphocytes (CTLs) play a major role in viral clearance during acute infection or the early stages of liver disease [4,5]. However, depletion of CTLs delays the onset of biochemical, histological, and clinical evidence of hepatitis [5]. Therefore, HBV-specific CTLs may play additional functions during HBV infection. For instance, activated CTLs are known to produce cytokines such as interferon-γ, which recruit large numbers of virus-nonspecific inflammatory cells into the liver via chemokine induction [6]. These chemokine-recruited virus-nonspecific mononuclear cells (i.e., natural killer (NK) cells, natural killer T (NKT) cells, T and B lymphocytes, monocytes, macrophages, and dendritic cells) can contribute to the formation of necroinflammatory foci and virus-specific CTLs are outnumbered by host-derived mononuclear and polymorphonuclear inflammatory cells [7].

Chemokines are 8–14 kDa peptides that function as chemoattractant cytokines in cell activation, immune cell differentiation, and leukocyte trafficking. Chemokines bind to specific G protein-coupled receptors, also known as seven-transmembrane domain receptors [8]. The chemokine stromal cell-derived factor-1 (SDF-1) is constitutively expressed in several tissues, including the liver, lung, kidney, bone marrow, *etc*., and exhibits chemoattractive activity for T cells, precursor B cells, monocytes, and neutrophils [9]. SDF-1 exerts pleiotropic effects, regulating embryonic life, tissue homeostasis, immune surveillance, inflammatory responses, and cancer development [10], and it is also associated with tissue damage, especially liver damage, heart infarct, limb ischemia, *etc.* [11]. Furthermore, SDF-1 plays a proinflammatory role in several autoimmune diseases, particularly rheumatoid arthritis and nephritis in murine lupus [12,13].

The endoplasmic reticulum (ER) is involved in sequestration of calcium, lipid synthesis, facilitation of protein folding, and transport of synthesized proteins [14]. ER stress is induced by the accumulation of unfolded or misfolded proteins in the ER lumen. A plethora of evidence suggests that there is an extensive cross-talk between the ER stress response and inflammatory response [15]. In support of this, we previously reported that HBx induces ER stress via reduction of the cellular ATP level, thereby disrupting proper folding of many secretary proteins, and that activating transcription factor-4 (ATF4) induced by ER stress elevates inflammatory cytokine production [16]. In this study, we report that HBx induces production of chemoattractant cytokines via ER stress, resulting in recruitment of HBV-induced virus-nonspecific immune cells into the liver.

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2. Materials and methods

2.1. Plasmid constructs and reagents

pGL3B-SDF-1-luc (−1914 to +1) was described previously [17]. AP-1 luc was generously provided by J.W. Lee (Chungbuk National University, Korea). pcDNA3-HBx, pcDNA3-HBV 1.2mer, pcDNA3-HBV(-x) 1.2mer, pCMV5-myc-ATF4 and pcDNA-HA-CREBH(n) were described previously [16]. pcDNA3-HA-ATF6α(n), pcDNA3-flag-XBP1(s) and pAdTrack-CMV were kindly gifted from Dr. K.H. Kim (Sungkyunkwan University School of Medicine, Korea). Trimethylamine N-oxide (TMO), thapsigargin and tunicamycin were purchased from Sigma (Steinheim, Germany). The transfection reagent jetPEI and jetPRIME were purchased PolyPlus Transfection (Strasbourg, France).

2.2. Cell culture and HBx transgenic mice

XBP1 +/+ and *XBP1* −/− *MEF* were kindly gifted from Dr. S.H. Back (Ulsan University, Korea). The MEF cells were maintained in DMEM containing 10% FBS, 1% PS and 1% non-essential amino acid (Gibco) at 37 °C in a humid atmosphere of 5% CO_2. B6 transgenic mice liver tissues expressing HBx (n = 19) and their non-transgenic sibs (C57BL/6, n = 12) were described previously [18].

2.3. Construction of recombinant adenovirus carrying HBx

To construct a recombinant adenovirus Ad-HBx, we performed sub-cloning from pcDNA3-HBx to pAdTrack-CMV. pcDNA3-HBx was digested with *Eco*RI and *Xho*I, and the products were cloned into the *Eco*RI/*Xho*I sites of pAdTrack-CMV. pAdTrack-CMV or pAdTrack-CMV-HBx digested with *Pme*I (New England Biolabs, Hitchin, Hertfordshire, UK) was transformed into BJ5183-AD-1 (New England Biolabs) competent cell for homologous recombination with pAdEasy-1. The purified recombinant adenoviral plasmids was digested with *Pac*I (New England Biolabs), and then it was transfected into 293-AD cells. Recombinant Adenovirus was obtained through lysis of the transfected 293-AD cells, and then the adenovirus was amplified. Recombinant adenovirus with a titer of 2.3×10^8 pfu/ml was collected, and then we added recombinant adenovirus at the concentration of 7×10^4 or 7×10^5 pfu/ml to the medium.

2.4. Luciferase assay

Cells were seeded in 24-well culture plate and transfected with reporter vector and β-galactosidase expression plasmid, along with each indicated expression plasmids using JetPEI. Total amounts of expression vectors were kept constant by pcDNA3.1 (Invitorgen, Carlsbad, CA, USA). After 24 h of transfection, cells were incubated in the presence or absence of TMO, thapsigargin or tunicamycin for 12 h. After 36 h of transfection, the cells were lysed in the cell culture lysis buffer (Promega, Madison, WI, USA). Luciferase activity was determined using an analytical luminescence luminometer according to the manufacturer's instructions. Luciferase activity was normalized for transfection efficiency using the corresponding β-galactosidase activity. All assays were performed at least in triplicate.

2.5. RNA isolation, RT (reverse transcriptase)-PCR and real-time PCR analysis

Total RNA was prepared from cell lines or tissues using Trizol reagent (Invitrogen) following the manufacturer's instructions. Total RNA (1 μg) was converted into single strand cDNA by MMLV reverse transcriptase (Takara, Tokyo, Japan) with oligo-dT. A one-tenth aliquot of the cDNA was subjected to PCR amplification using gene specific primers. The PCR products were examined by electrophoresis on a 1.2% agarose gel. Real-time PCR was performed with an SYBR Green I LightCycler-based real-time PCR assay (Roche, Mannheim, Germany). The reaction mixtures were prepared using LightCycler Fast DNA master mixture for SYBR Green I, 0.5 μM of each primer and 4 mM $MgCl_2$. PCR was carried out using the following forward and reverse primers: SDF-1 F, 5′-GCC AGA GCC AAC GTC AAG CAT-3′; SDF-1 R, 5′-CTT GTT TAA AGC TTT CTC CAG GTA CTC-3′. The primers of actin, BIP, HBx, and XBP1 were described previously [16]. The number of wild type and HBx-TG used in RT-PCR was twelve mice, respectively.

2.6. Western blot analysis

The cells were prepared by washing with cold-PBS and lysed. The protein concentration was determined using the BSA as standard and Bradford reagent (Bio-Rad). Equal amount of proteins was loaded and separated by SDS–PAGE and the gels were transferred to polyvinylidene fluoride membrane (Millipore, Billerica, MA, USA). For western blotting, the membranes were incubated with anti-HBx (Chemicon, Temecula, CA, USA), anti-SDF-1 (R&D system, Minneapolis, MN, USA), anti-flag (Cell signaling, Beverly, MA, USA), anti-XBP1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA) and anti-actin (Sigma) in TBST supplemented with 3% non-fat dry skim milk for overnight at 4 °C. After washing three times with cold TBST, the blotted membranes were incubated with peroxidase conjugated secondary antibody (Santa Cruz Biotechnology) for 30 min at room temperature. After washing three times with cold TBST, the proteins were visualized by the enhanced chemiluminescent development reagent (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The number of wild type and HBx-TG used in Western blotting was twelve mice, respectively.

2.7. Chromatin immunoprecipitation (ChIP) assay

ChIP assays were performed as described by the manufacturer (Upstate Biotechnology, Lake Placid, NY) with some modifications. Chromatin solutions were sonicated and incubated with anti-flag, or with control IgG, and rotated overnight at 4 °C. Chromatin DNA was purified and subjected to PCR analysis. To amplify the human SDF-1 promoter region containing TRE site, the following primer sets were used; p-803, 5′-ACG ATG CAG AGA ATT TCG CGG CGT-3′; p-613, 5′-ACC AGC CGA GCC TCA GTT TCC TCG-3′. As a negative control, human SDF-1 promoter region non-containing TRE site was used, the following primer sets were used; p-1139, 5′-GCG GTC ACA AAG CGA GGC CCA AA-3′; p-953, 5′-CTG GCG TCT GCC TTC TGG GTC CAG-3′. After amplification, PCR products were resolved on a 1.5% agarose gel and visualized by ethidium bromide staining.

2.8. AMD 3100 injection

Male HBx transgenic mice (12-weeks old, n = 7) were injected subcutaneously with AMD 3100 (1.25 mg/kg in 200 μl of PBS, n = 4) or PBS (n = 3) twice per day for 10 days.

2.9. H&E stain

The liver tissues of wild-type (n = 4) or HBx transgenic mice (not injected mice, n = 4; AMD-injected mice, n = 4; PBS-injected mice, n = 3) were fixed in 4% prarformaldehyde. The paraffin-embedded tissue sections were mounted on 3-aminopropyltriethoxylsilane-coated slides. Dewaxed paraffin sections were rehydrated in each 100%, 95% and 70% ethanol during