Hepatitis B Virus X Protein Sensitizes Primary Mouse Hepatocytes to Ethanol- and TNF-α-Induced Apoptosis by a Caspase-3-Dependent Mechanism

Won-Ho Kim¹, 4, Feng Hong¹, Barbara Jaruga¹, Zhengsheng Zhang², Saijun Fan³, T. Jake Liang² and Bin Gao¹

It is well-documented that alcohol drinking together with hepatitis viral infection accelerates liver injury; however, the underlying mechanisms remain unknown. In this paper, we demonstrated that primary hepatocytes from transgenic mice overexpressing hepatitis B virus X protein (HBX) were more susceptible to ethanol- and TNF-α-induced apoptotic killing. Compared to normal control mouse hepatocytes, ethanol and/or TNF-α treatment led to a significant increase in reactive oxygen species, mitochondrial permeability transition, cytochrome C release, caspase-3 activity, and poly (ADP-ribose) polymerase degradation in hepatocytes from HBX transgenic mice. Blocking caspase-3 activity antagonized ethanol- and TNF-α-induced apoptosis in primary hepatocytes from HBX transgenic mice. Taken together, our findings suggest that HBX sensitizes primary mouse hepatocytes to ethanol- and TNF-α-induced apoptosis by a caspase-3-dependent mechanism, which may partly explain the synergistic effects of alcohol consumption and hepatitis B virus infection on liver injury. *Cellular & Molecular Immunology*. 2005;2(1):40-48.

Key Words: HBV X protein, ethanol, apoptosis, TNF-α, caspase-3

Introduction

The two major causes of liver disease worldwide, hepatitis viral infection and alcohol drinking, frequently coexist in patients with chronic liver disease. Together, they synergistically promote the development of liver damage, cirrhosis, and hepatocellular carcinoma (1-4). However, the molecular and cellular mechanisms underlying the synergistic effect of alcohol consumption and hepatitis viral infection on liver disease remain obscure (1-4). Recent studies have shown that several hepatitis viral proteins are involved in the regulation of hepatocyte apoptosis. Among them, the effects of hepatitis C virus core protein (HCV core) and hepatitis B virus X protein (HBX) on cell apoptosis have been extensively investigated in transformed hepatoma or nonhepatic cell lines (5-11), but discordant findings have been reported. HBX has been shown to promote apoptosis through several mechanisms, including a p53-independent mechanism (12), prolonged stimulation of the N-Myc and stress-mediated mitogen-activated-protein kinase kinase 1 (MEKK1) pathway (13), alteration of mitochondrial membrane and activation of caspase 3 (14), interaction with c-FLIP and enhancement of death-inducing signal (15), and other mechanisms (16, 17). Conversely, HBX has also been shown to inhibit apoptosis through activating protein kinase B (18, 19), c-Jun N-terminal kinase (20), inhibiting caspase-3 (21), or through binding p53 (22). Here, we examined ethanol- and tumor necrosis factor α (TNF-α)-mediated apoptosis in primary hepatocytes isolated from HBX transgenic mice and

¹Section on Liver Biology, Laboratory of Physiologic Studies, National Institute on Alcohol Abuse and Alcoholism, National Institutes of Health, Bethesda, MD 20892, USA.
²Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD 20892, USA.
³Department of Oncology, Lombardi Cancer Center, Georgetown University, 3970 Reservoir Road, NW, Washington DC 20057, USA.
⁴Corresponding to: Dr. Won-Ho Kim, Division of Metabolic Disease, National Institutes of Health, #5 Nokbun-Dong, Eunpyung-gu, Seoul 122-701, South Korea. E-mail: jhkwh@hanmail.net.

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Abbreviations: HBX, hepatitis B virus X protein; HCV core protein, hepatitis C virus core protein; NF-κB, nuclear factor-κB; TNF-α, tumor necrosis factor α; PARP, poly (ADP-ribose) polymerase; ROS, reactive oxygen species; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling; MPT, mitochondrial permeability transition.
wild-type control mice. Our data showed that primary hepatocytes from HBX transgenic mice were more susceptible to TNF-α- and ethanol-induced apoptotic cell death by a caspase-3-dependent mechanism.

Materials and Methods

Animals
HBX transgenic mice were originally provided by Dr. Francis Chisari (The Scripps Research Institute, La Jolla, CA) and bred at the NIDDK animal facility. Details of the generation of this mouse strain were described previously (23). The HBX gene is controlled by the developmentally regulated liver-specific mouse major urinary protein (MUP) promoter. High levels of HBX mRNA expression in the livers of HBX transgenic mice were confirmed by reverse transcriptase-polymerase chain reaction. Male C57BL/6 background control mice were purchased from the Jackson Laboratory (Bar Harbor, Maine).

Materials
Caspase-3 inhibitor I (DEVD-CHO) was purchased from Calbiochem (San Diego, CA). Murine TNF-α was purchased from Bioscience International (Camarillo, CA, USA). Ethanol was obtained from Sigma Chemicals (St. Louis, MO, USA).

Isolation and culture of primary mouse hepatocytes
Male C57BL/6 background HBX transgenic mice and control C57BL/6 mice weighing 30-35 g were anesthetized with sodium pentobarbital (30 mg/kg intraperitoneally), and the portal vein was cannulated under aseptic conditions. The liver was perfused with EGTA solution (5.4 mM KCl, 0.44 mM KH₂PO₄, 140 mM NaCl, 0.34 mM Na₂HPO₄, 0.5 mM EGTA, 25 mM Tricine, pH 7.2) and Dulbecco’s Modified Eagle Medium (GIBCO BRL, Gaithersburg, MD), and digested with 0.075% collagenase solution. Isolated mouse hepatocytes were then cultured in Hepato-ZYME-SFM media (GIBCO BRL) in rat-tail collagen coated plates for 24 h, followed by drug treatment.

Western blotting
Liver tissues were lysed in lysis buffer (30 mM Tris, pH 7.5, 150 mM sodium chloride, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium orthovanadate, 1% Nonidet P-40, 10% glycerol) for 15 min at 4°C, vortexed and centrifuged at 16,000 rpm at 4°C for 10 min. The supernatants were mixed in Laemmli running buffer, boiled for 4 min, and then subjected to SDS-PAGE. After electrophoresis, proteins were transferred onto nitrocellulose membranes and blotted against primary antibodies for 16 h. Membranes were washed with TPBS (0.05% [vol/vol] Tween 20 in phosphate-buffered saline [pH 7.4]) and incubated with a 1:4,000 dilution of horseradish peroxidase-conjugated secondary antibodies for 45 min. Protein bands were visualized by an enhanced chemiluminescence reaction (Amersham Pharmacia Biotech, Piscataway, NJ) and quantified using a PhospholImager (Amersham Pharmacia Biotech).

Flow cytometric analysis of apoptosis
After trypsinization, approximately 10⁶ cells were collected by centrifugation at 1,000 g for 5 min. Cells were then washed in phosphate-buffered saline (PBS) followed by re-suspension and fixation in 70% ethanol for approximately 2 h. Next, cells were washed with PBS and re-suspended in 500 µL BS containing 100 µg RNase, followed by a 30-min incubation at 37°C. Cellular DNA was then stained by the addition of 50 µg propidium iodide, and cells were analyzed on FACScan utilizing Cellquest software (Becton Dickinson, Franklin Lakes, NJ).

DNA fragmentation in cultured hepatocytes
Cultured hepatocytes were washed twice with PBS and lysed in buffer (100 mM NaCl, 10 mM Tris-HCl, pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 mg/mL proteinase K) at 37°C for 18 h. DNA was extracted with an equal volume of phenol/chloroform (1:1) and precipitated at -70°C. DNA pellets were re-suspended in 10 µl of 10 mM Tris (pH 7.8), 1 mM EDTA buffer and incubated for 1 h at 37°C with 1 µg/ml RNase (Roche Molecular Biochemicals, Indianapolis, IN) to remove RNA. DNA pellets were electrophoresed for 2-3 h at 90 V on 1.8% agarose gels. The gel was stained with ethidium bromide and the DNA fragments were visualized under ultraviolet light.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL)
Apoptotic primary mouse hepatocytes were detected using Apop Tag (Oncor, Gaithersburg, MD), an in situ apoptosis detection kit. Briefly, cells were fixed and incubated with terminal transferase and avidine-labelled-dUTP, followed by further incubation with FITC-labeled anti-avidine antibody. The slides were then examined under fluorescence microscope.

Measurement of reactive oxygen species (ROS)
Intracellular ROS generation was quantified using flow cytometric measurement of the metabolite fluorescein 2′,7′-dichlorofluorescein (Molecular Probes, Inc., Eugene, OR). ROS produced by cells oxidizes DCFH-DA into the highly fluorescent green compound (2′,7′-dichlorofluorescein). The fluorescence intensity is directly proportional to ROS production (24). Cultured hepatocytes or freshly isolated hepatocytes (2 × 10⁶) were loaded for 1 h with CM-DCF at a final concentration of 10 µmol/L. Fluorescence 2′,7′-dichlorofluorescein was measured by flow cytometry. Results were normalized to control cells and expressed as relative fluorescence intensity.

Measurement of mitochondrial permeability membrane transition (MPT)
MPT was measured using DiOC6 (Molecular Probes Inc., Eugene, OR), which is electrophoretically re-distributed across the mitochondrial membrane according to the membrane potential. Cultured hepatocytes (2 × 10⁶) were incubated for 1 h at 37°C in DMEM medium containing 100 nM DiOC6. Cells were then washed, re-suspended in DMEM...
medium. The MPT was analyzed using a FACScan flow cytometer (Becton Dickinson) equipped with a 488 nm argon laser. Each sample containing a minimum of 10^4 cells was analyzed through the FL1-H channel. Data were acquired in list mode and analyzed using the CellQuest software.

Isolation of cytosol and mitochondrial fractions

Primary hepatocytes isolated from wild-type mice and HBX transgenic mice were plated and treated with ethanol or TNF-α for various time points. Following treatments, cells were re-suspended and homogenized in isolation buffer (20 mmol/L Hepes, pH 7.4, 10 mmol/L KCl, 1.5 mmol/L MgCl₂, 1 mmol/L sodium-EDTA, 1 mmol/L dithiothreitol, 10 mmol/L phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, and 10 μg/ml aprotinin in 250 mmol/L sucrose). Unbroken cells and nuclei were removed by two rounds of centrifugation at 2,500 g at 4°C. The mitochondria were then pelleted by centrifugation at 9,000 g at 4°C for 30 min to obtain the heavy membrane (HM) fraction enriched with mitochondria. The supernatant from the first 9,000 g centrifugation was subsequently centrifuged at 100,000 g to obtain the cytosolic supernatant and light membrane (LM) fractions.

Enzymatic assay of caspase-3

Cleavage of the caspase-3 substrate I (Ac-DEVD-pNA) (Calbiochem, San Diego, CA) was used as a measure of caspase-3 activity. The p-Nitroaniline was used as the standard.

Statistical analysis

For comparing values obtained in three or more groups, one-factor analysis of variance (ANOVA) was used, followed by Tukey’s post hoc test; *p < 0.05 was taken to imply statistical significance.

Results

Hepatocytes from HBX transgenic mice are more susceptible to ethanol- and TNF-α-mediated apoptotic killing

To understand the synergistic effect of alcohol drinking and hepatitis virus infection on liver injury, ethanol- and TNF-α-mediated apoptosis were examined in hepatocytes isolated from HBX transgenic mice and wild-type control mice. As shown in Figure 1, treatment of primary hepatocytes with 25-50 mM ethanol or 100 mM ethanol plus TNF-α only slightly induced DNA fragmentation. In contrast, treatment of HBX transgenic mouse cells with 25 or 50 mM ethanol and 10 ng/ml TNF-α for 24 h did not significantly induce DNA fragmentation, and treatment with 100 mM ethanol or ethanol plus TNF-α only slightly induced DNA fragmentation. In contrast, treatment of HBX transgenic mouse cells with 25 or 50 mM ethanol and 10 ng/ml TNF-α for 24 h did not significantly induce DNA fragmentation, and treatment with 100 mM ethanol or ethanol plus TNF-α only slightly induced DNA fragmentation.
Mouse hepatocytes from wild-type control (Ctrl) and HBX transgenic mice were cultured in serum-free media in the absence or presence of 100 mM ethanol for various time points. Apoptosis was examined by propidium iodide staining as described under “Materials and Methods.”

As shown in Figure 2, a slight increase (about 1.5-2.0 fold) in apoptotic cells was observed in HBX transgenic mouse hepatocytes compared to normal mouse hepatocytes. Ethanol and TNF-α treatment for 24 h caused, respectively, a 1.8 ± 0.2 and 1.6 ± 0.5 fold increase in TUNEL-positive cells in normal primary mouse hepatocytes, whereas the same treatments, respectively, caused a 5.6 ± 0.6 and 4.7 ± 0.6 fold increase in TUNEL-positive cells in HBX transgenic mouse hepatocytes. Flow cytometric analyses showed that treatment with 100 mM ethanol for 24 h slightly induced apoptosis in normal primary hepatocytes but markedly induced apoptosis in HBX transgenic mouse hepatocytes (M1, sub-G1 phase, 13.04% in ethanol-treated wild-type hepatocytes vs. 33.31% in ethanol-treated HBX hepatocytes at 24 h) (Figure 3). Collectively, these findings suggest that HBX sensitizes primary mouse hepatocytes to ethanol- and TNF-α-mediated apoptotic cell death.

**Hepatocytes from HBX transgenic mice are more susceptible to ethanol- or TNF-α-mediated induction of ROS and MPT**

To understand the underlying mechanism by which HBX sensitizes hepatocytes to ethanol- and TNF-α-induced apoptosis, we examined ethanol- and TNF-α-mediated induction of ROS and MPT in normal mouse hepatocytes and HBX transgenic mouse hepatocytes. As shown in Figure 4, exposure to ethanol or TNF-α alone did not cause significant ROS induction in hepatocytes from wild-type C57BL/6 mice, but significantly enhanced ROS production in HBX transgenic mouse hepatocytes (ROS peak shifted to the right). Treatment with ethanol and TNF-α together caused a...
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The effects of ethanol and TNF-α on the activation of caspase-3 were examined in normal and HBX transgenic mouse hepatocytes. As shown in Figure 6A, treatment of normal mouse hepatocytes with 50 mM ethanol or TNF-α did not significantly induce caspase-3 activation, whereas the same treatments caused a 5-fold (ethanol) and 16-fold (TNF-α) increase in caspase 3 activity in HBX transgenic mouse hepatocytes. Moreover, 100 mM ethanol treatment induced a 3-fold activation of caspase-3 in normal mouse hepatocytes, but induced a 9-fold activation in HBX transgenic mouse hepatocytes.

To determine whether ethanol-activated caspase-3 is functionally active in HBX transgenic mouse hepatocytes, cleavage of poly (ADP-ribose) polymerase (PARP) was examined by Western blotting analysis. As shown in Figure 6B, treatment of cells with ethanol rapidly caused PARP cleavage in HBX transgenic mouse hepatocytes, but not in normal hepatocytes. PARP cleavage in HBX transgenic hepatocytes was blocked by pretreatment with the caspase-3 inhibitor DEVD-CHO.

HBX sensitizes primary mouse hepatocytes to ethanol apoptotic killing by a caspase-3-dependent mechanism

To further confirm the involvement of caspase-3 in ethanol- and TNF-α-mediated apoptosis, the caspase-3 inhibitor DEVD-CHO was used. As shown in Figure 7A, treatment of HBX transgenic mouse hepatocytes with ethanol caused significant DNA fragmentation, which was markedly suppressed by pretreatment with DEVD-CHO. Flow cytometric analyses showed that treatment of HBX transgenic mouse hepatocytes with ethanol for 24 h induced apoptosis, which was markedly attenuated by DEVD-CHO (33.31% M1 sub-G1 peak in ethanol-treated group vs. 12.68% DEVD-CHO- plus ethanol-treated group) (Figure 7B). Similarly, TNF-α-mediated induction of apoptosis in HBX transgenic mouse hepatocytes was also blocked by the caspase-3 inhibitor DEVD-CHO (data not shown). These findings suggest that HBX sensitizes primary mouse hepatocytes to ethanol and TNF-α apoptosis by a caspase-3-dependent mechanism.
Discussion

The combined effects of alcohol drinking and hepatitis viral infection on liver injury are well documented, but the mechanisms by which ethanol accelerates progression of liver disease in patients with hepatitis viral infection are not fully understood (1-4). Several mechanisms have been considered (1), including ethanol suppression of interferon antiviral immune response (25-27), promotion of viral replication (28, 29), inhibition of the immune system (30), attenuation of liver regeneration (31), and potentiation of hepatitis viral protein-mediated activation of inflammatory signals (32). Here we demonstrate for the first time that HBX sensitizes primary hepatocytes to ethanol- and TNF-α-induced ROS, MPT, and apoptosis, which may contribute to the synergistic effects of alcohol drinking and viral hepatitis infection on liver injury. Furthermore, we demonstrate that a caspase-3-dependent mechanism may be involved in HBX sensitization of hepatocytes to ethanol- and TNF-α-induced apoptosis.

Although the effects of HBX on cell apoptosis have been extensively investigated (12, 18-22, 33-36), contradictory findings have been reported. It has been shown that HBX can either promote or inhibit apoptosis. The opposing effects by HBX in these studies could be due to differences in HBX expression levels, transient and stable expression of HBX, cell types, and apoptotic stimuli used. For example, in weakly expressing cells, HBX is exclusively or predominantly localized in the nuclei, but in highly expressing cells it accumulates in the cytoplasm (37). Also, HBX is typically expressed in distinctive granular patterns in human hepatoma, Huh-7 cells, but in squamous carcinoma A431 cells, HBX is expressed in dispersed, non-granular patterns (37). Cell specific HBX expression patterns may be responsible for opposing effects on cell survival. Thus, the pro-apoptotic effect of HBX demonstrated in primary mouse hepatocytes in this paper is likely more physiological than studies previously conducted in transformed hepatoma and non-hepatic cell lines (12, 18-22, 33-36).

Several mechanisms may be involved in the sensitization of HBX hepatocytes to apoptosis induced by ethanol and TNF-α. First, ethanol and TNF-α treatment induced significant caspase-3 activation in HBX transgenic mouse hepatocytes, but not in normal control primary hepatocytes. Moreover, blocking caspase-3 activation with a specific inhibitor abolished ethanol- and TNF-α-induced apoptosis in HBX transgenic mouse hepatocytes. These findings suggest that a caspase-3-dependent mechanism is involved in ethanol- and TNF-α-mediated apoptosis in HBX transgenic mouse hepatocytes. Second, HBX transgenic mouse hepatocytes are more susceptible to ethanol- and TNF-α-induced ROS and MPT. Induction of ROS and MPT has been shown...
to play an important role in hepatocyte apoptosis (38-40). Thus, higher induction of ROS and MPT may partly contribute to ethanol- and TNF-α-induced cell death in HBX transgenic mouse hepatocytes. Currently, the underlying mechanism by which HBX sensitizes primary mouse hepatocytes to ethanol- and TNF-α-induction of ROS and MPT is not clear. Co-localization and interaction of HBX protein with several mitochondrial proteins may play an important role in HBX sensitization of hepatocytes to ethanol- and TNF-α-induced ROS and MPT (37, 41, 42).

In summary, this paper demonstrates that HBX sensitizes primary hepatocytes to ethanol- and TNF-α-induced ROS, MPT, and apoptosis, which contributes to the synergistic effect of hepatitis B virus infection and alcohol drinking on liver injury. Excessive hepatocyte apoptosis and replication have been implicated in the development of hepatocellular carcinoma (43, 44). Thus HBX sensitization of hepatocytes to ethanol- and TNF-α-induced apoptosis could also contribute to the high incidence of hepatocellular carcinoma in alcoholics with HBV infection (45, 46).

References


