Original Article

Hepatitis B virus X protein maintains hepatic stellate cell activation by regulating peroxisome proliferator-activated receptor γ

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Abstract: Background: Chronic hepatitis B virus (HBV) infection is a major cause of hepatic fibrosis, and the activation of hepatic stellate cells (HSCs) is the main mechanism of fibrosis. However, the mechanism of hepatic fibrosis induced by HBV is not well elucidated. Hepatitis B virus X protein (HBx), one of the HBV-related proteins, induces fibrosis in a paracrine way. Peroxisome proliferator-activated receptor γ (PPARγ) inhibits the activation of HSCs and even switches the cell phenotype from activated to quiescent. The aim of this study was to determine the interaction of HBx and PPARγ in stellate cell activation. Methods: A stable cell line, LX-2-X, which expressed HBx, was established by infecting LX-2 cells with lentivirus. The Cell Counting Kit-8 (CCK-8) assay was used to detect cell proliferation. The expression of PPARγ, transforming growth factor-β1 (TGFβ1), α-smooth muscle actin (α-SMA) and collagen I was measured by quantitative real-time PCR (qRT-PCR), Western blot or ELISA. For the interaction of HBx and PPARγ, co-immunoprecipitation and luciferase reporter assays were performed. Results: LX-2-X cells showed increased proliferation compared to control cells, and the PPARγ ligand troglitazone (0, 5, 10 μmol/L) inhibited LX-2-X cell proliferation in a dose-dependent manner. The expression of TGF-β1, α-SMA, and collagen I increased, while PPARγ decreased in LX-2-X cells. HBx bound to PPARγ and suppressed the transcriptional activity of PPARγ. Conclusions: HBx can maintain stellate cell activation by down-regulating PPARγ in vitro.

Keywords: Hepatitis B virus X protein, peroxisome proliferator-activated receptor γ, hepatic stellate cell, fibrosis

Introduction

Fibrosis, characterized by an excessive deposition of extracellular matrix (ECM), is a wound-healing response to a variety of chronic stimuli [1-3]. Although several other cells are fibrogenic, hepatic stellate cells still have dominated the study of fibrosis [1, 2]. Following a variety of fibrogenic stimuli, hepatic stellate cells (HSCs) are activated, changing from a quiescent vitamin A-storing phenotype to a myofibroblast-like phenotype, and are associated with the expression of smooth muscle α-actin (α-SMA) [3]. Two specific aspects of stellate cell activation are critical in the fibrogenic response: increased cell proliferation and increased synthesis of ECM constituents, of which collagen I predominates [3, 4]. Platelet-derived growth factor (PD-GF) and transforming growth factor-β1 (TGF-β1) are the most potent stellate cell mitogen [5, 6] and profibrogenic cytokine [7-9], respectively.

HBV infection is a major public health problem worldwide and is strongly associated with the development of hepatitis, hepatic fibrosis and hepatocellular carcinoma (HCC) [10, 11]. HBV is a hepatotropic, partially double-stranded DNA virus. The X gene is the smallest fragment among the four overlapping open reading frames (ORFs) of the HBV genome, coding for the X protein [12, 13]. HBx regulates a variety of viral replication and cellular functions through different signaling pathways, both in the cytoplasm and nucleus [14]. Previous studies have suggested that HBx is associated with the development of hepatic fibrosis [8, 15, 16].

Peroxisome proliferator-activated receptor γ (PPARγ) is a transcription factor of the nuclear receptor superfamily. PPARγ plays a critical role in regulating energy homeostasis, especially lipid and glucose metabolism [17]. PPARγ needs to combine with retinoid X receptor (RXR),
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Upon ligand binding, the heterodimer binds to specific DNA regions, peroxisome proliferator response elements (PPREs), to regulate gene transcription [18, 19]. A variety of studies about PPARγ were carried out to uncover the mechanism of hepatic fibrosis because of the antifibrotic effect of this protein [20-24]. Ligands of PPARγ inhibit the proliferation and activation of hepatic stellate cells and may induce a phenotypic switch from activated to quiescent [25]. Troglitazone, one antidiabetic drug of the thiazolidinedione group, could bind and activate PPARγ. HBx inhibits the effect of PPARγ through protein-protein interaction in HCC cells [26], and it can activate hepatic stellate cells through paracrine action [27-29]. Although HBV infects mainly hepatic parenchymal cells, it can also infect stellate cells directly and affect their proliferation and activation [30]. In this study, we examined how HBx affected HSCs via modulating PPARγ, and we also investigated whether ligands of PPARγ could counter the HBx-induced fibrogenesis. Our data may provide novel insight about and suggest potential therapies for hepatic fibrosis.

Material and methods

Plasmids and cell line

To express HBx with a Flag tag, the HBx fragment was subcloned into the Nhe I and Sac I site of the pCMV-N-Flag plasmid (Beyotime technology, Nantong, China). The plasmid TK-PPRE×3-LUC was purchased from Zhongyuan LTD (Beijing, China). It contains PPRE and luciferase gene, and could express luciferase when binded with PPARγ. All the plasmids used for lentivirus construction were purchased from GenePharma (Shanghai, China). We constructed a LV5-X lentivirus containing target gene (HBVgp3 X protein). A stable LX-2-X cell line was established by infecting LX-2 cells with lentivirus LV5-X, and the control cell line was named LX-2-C. The LX-2 cell line was purchased from the Shanghai Cell Bank (Shanghai, China). All the cell lines were cultured in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum, 2 mmol/L streptomycin, 100 U/ml penicillin at 37°C and 5% CO₂.

Cell proliferation assay

The cell proliferation was determined by CCK-8 assay. Briefly, 5×10³ cells were seeded into 96-well plates, treated with or without troglitazone (Sigma, St. Louis, MO, USA) for various times. After treatment, 10 μl CCK-8 solution (Beyotime Technology, Nantong, China) was added to each well, and the cells were incubated for 2 h at 37°C. The optical density (OD) of each well at 450 nm was measured by an ELISA reader.

Real-time quantitative RT-PCR

Total RNA was extracted from cells by Trizol Reagent (Takara, Dalian, China). The reverse transcription for complementary DNA was performed by the PrimeScript RT kit (Takara, Dalian, China). Real-time quantitative PCR was performed by the FastStart Universal SYBR Green Master (ROX) (Roche, USA) on an ABI 7500 (Applied Biosystems, Life Technologies Corporation, CA, USA). The relative mRNA expression was examined as the inverse log of the ΔΔCT, which was normalized to the reference gene GAPDH. The reaction conditions of quantitative RT-PCR were 10 min at 95°C, followed by 40 cycles of 95°C for 15 s and 60 s at 60°C. At the end of the program a melting curve analysis was made. All primers for quantitative RT-PCR were synthesized by Invitrogen (Shanghai, China). The sequences of primers were as follows: GAPDH: 5'-ACCACAGTCCATGCCATCAC-3' (sense); 5'-GCCATCACGACTTATGACAGC-3' (antisense); HBx: 5'-GAGACTCATCCTCCGTGAAC-3' (sense) and 5'-TTGTCTAGCTTACGTTGAAAC-3' (antisense); PPARγ: 5'-GAGATGCATCCGCGACGAC-3' (sense) and 5'-GGGATCAGCTCCGTGGATCT-3' (antisense); TGFβ1: 5'-CTAATGGTGGAAACCCAC-3' (sense) and 5'-TATCGCCAGGAATTGGCTG-3' (antisense); α-SMA: 5'-AAAAGACAGCTACGTGGGTGA-3' (sense) and 5'-GCCATCGTCTGTTGTTG-3' (antisense); collagen I: 5'-GGAGTACGTTGTGTGCTG-3' (sense) and 5'-CGGTTCCTTTTCTCCTC-3' (antisense).

Co-immunoprecipitation and Western blot

To detect the expression of fibrosis-related molecules, cells were lysed with radioimmunoprecipitation assay (RIPA) buffer. Total protein concentration was measured by NanoDrop 2000 protein assay (Thermo Scientific, USA). After boiling for 5 minutes, equal amounts of protein (30 μg) were loaded, and lysates were separated by 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Proteins were transferred to PVDF membranes (Millipore, Bedford, USA), using a trans-blot apparatus (Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked in Tris-buffered sa-
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For the analysis of collagen I, the supernatant was collected from the culture medium. The amount of collagen I was determined by ELISA (Takara, Dalian, China) according to the manufacturer’s instructions.

Statistical analysis

Data were analyzed with SPSS 15.0 (SPSS Inc., Chicago, IL, USA). Each experiment was conducted at least three times. The results were expressed as the mean ± SD. For three or more groups, statistical significance was determined with one-way ANOVA, and for two groups with Student’s t-test. p < 0.05 was considered statistically significant.

Results

HBx promotes the proliferation of HSCs, and troglitazone counters this effect

The LX-2-X cells expressed HBx protein stably (Figure 1). To determine the effect of HBx on the proliferation of LX-2 cells, a CCK-8 assay was performed. LX-2-X cells showed increased proliferation from 72 h compared to LX-2-C cells and LX-2 cells (Figure 2A). However, treated with troglitazone of different folds (0, 5, 10 μM/L), the proliferation of LX-2-X cells was inhibited in a dose-dependent manner (Figure 2B).

HBx-expressing HSCs show increased expression of TGF-β1, α-SMA, and collagen I

Liver fibrosis is characterized by the deposition of collagen I. TGF-β1 is the most potent fibrogenic cytokine, and α-SMA is specifically expressed by activated HSCs [1-4, 7-9]. To determine whether HBx could maintain the stellate cell activation, we detected the expression of these fibrotic markers. RT-PCR and western blotting showed that the LX-2-X cells expressed more TGF-β1 and α-SMA compared to the LX-
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HBx binds to PPARγ in LX-2 cells and suppresses the transcriptional activity of PPARγ in HSCs

HBx can bind to PPARγ, thereby affecting the nuclear localization of PPARγ [26, 31]. We demonstrated that HBx bound to PPARγ in our LX-2-X stellate cell line (Figure 4A), decreasing PPARγ expression at both the gene and protein levels (Figure 4B-D). Furthermore, to determine whether HBx influenced the transcriptional activity of PPARγ, a luciferase activity reporter assay was performed. Compared to the LX-2-C and LX-2 cells, the luciferase activity was decreased in the LX-2-X cells. Although HBx inhibited the transcriptional activity of PPARγ, troglitazone countered this inhibition (Figure 5).

Discussion

Activation of stellate cells from vitamin A-storing cells into contractile myofibroblasts is the dominant pathway leading to hepatic fibrosis. HBV infection, along with HCV infection and ethanol consumption, is a major cause of fibrosis [1-4]. The present hypothesis of HBV-related fibrosis is that HBV infects hepatocytes, which release profibrogenic factors to activate the neighboring HSCs [32]. Among the four proteins of HBV, polymerase, surface, core, and X, HBx has been associated with HBV-related pathogenesis [13]. Several studies have explored the efficacy of HBx in the management of hepatic fibrosis [27-29]. What these studies have in common is that they established a “contacting” system of HSCs and hepatic cell lines infected with HBx to investigate the paracrine function of HBx in the context of fibrosis.

2-C cells and LX-2 cells (Figure 3A-C). Moreover, we found increased secretion of collagen I in the supernatant of the LX-2-X cells using ELISA (Figure 3D), consistent with the mRNA expression in the LX-2-X cells (Figure 3A). However, LX-2-X cells treated with troglitazone (10 μM/L) showed less expression of TGF-β1, α-SMA, and collagen I.

Figure 2. HBx promotes the proliferation of LX-2-X HSCs, and troglitazone counters this effect. A: LX-2, LX-2-C and LX-2-X cells were cultured in 96-well plates for the indicated time, and cell proliferation was determined by CCK-8 assay. *: p < 0.05 (vs. LX-2-C group); #: p < 0.05 (vs. LX-2 group). B: LX-2-X cells were treated with troglitazone (0, 5, 10 μM/L) for the indicated time. *: p < 0.05 (vs. control group); #: p < 0.05 (vs. 5 μM/L group).

Figure 3. HBx increases the mRNA expression of fibrotic markers. A: The mRNA expression of TGF-β1, α-SMA and collagen I in LX-2, LX-2-C and LX-2-X cells was detected by qRT-PCR. B: TGF-β1 and α-SMA were measured by Western blot. C: Grayscale analysis of the Western blot data. D: The concentration of collagen I in the supernatant was detected by ELISA assay. There were no differences between group LX-2 and LX-2-C.*: p < 0.05.
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Initiation and perpetuation are the two major phases of stellate cell activation. Perpetuation results from the effects of stimuli on maintaining the activated phenotype and generating fibrosis. Perpetuation involves autocrine as well as paracrine loops, while initiation mostly results from paracrine stimulation. Proliferation, contractility and fibrogenesis are pivotal and discrete responses of perpetuation [2]. We demonstrated that HBx could promote the growth of HSCs. The PDGF pathway is involved in HBx-stimulated HSC growth. PDGF is the most potent stellate cell mitogen, and the downstream pathways of PDGF signaling have been carefully characterized in stellate cells [5, 6]. Among liver cells, the cytoskeletal protein α-SMA is a specific feature of activated stellate cells and confers increased contractile potential [2]. In our study, α-SMA increased under the stimulation of HBx (Figure 3).

Fibrogenesis is another pivotal event of liver fibrosis [3, 4]. Increasing ECM deposition per
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cell contributes to fibrosis. Collagen I is the
best-studied component of matrix in hepatic
scars. The most potent stimulus for production
of collagen I and other matrix constituents by
stellate cells is TGF-β1, which is derived from
both paracrine and autocrine sources [2, 7-9].
Our results show that HBx increased the expres-
sion of TGF-β1 and collagen I (Figure 3). HBx is
thought to upregulate TGFβ1, activating the
HSCs in a paracrine way [8, 27]. However, in our
study, TGFβ1 maintained the perpetuation of
stellate cell activation more likely in an auto-
crine way in stellate cells.

PPARγ ligands inhibit PDGF-induced prolifera-
tion of hepatic stellate cells [36], and PPARγ
agonists block TGFβ1-mediated activation of
Smad3 and induction of ECM gene expression
in adipocytic human HSCs [20]. Since PPARγ
interacts with the downstream pathways of
stellate cell activation, we proposed that the
interaction of HBx and PPARγ might account
for the HBx-induced fibrosis. PPARα/RXRα can
inhibit the transcription and replication of HBV
[37]. Conversely, the HBx protein can also mod-
ulate the PPARs. Choi demonstrated that HBx
bound to the DNA-binding domain of PPARγ.
This protein-protein interaction interferes with
the nuclear localization of PPARγ and its bind-
ing to PPREs, but the endogenous PPARγ pro-
tein level is unaffected [26]. Kim et al demon-
strated that HBx induces lipid accumulation, and
they proposed that this phenomenon is induced by upregulating the SREBP1 and PPARγ
expression [31]. In this study, we demonstrated that HBx protein bound to PPARγ in LX-2 cell
line (Figure 4A). Interestingly, the expression of
PPARγ in the LX-2-X cells was significantly lower
compared to the control groups (Figure 4), in
contrast to previous studies. Furthermore, the
luciferase activity reporter assay showed that
HBx inhibited the transcriptional activity of
PPARγ (Figure 5). We propose that HBx not only interacted with PPARγ to interfere with its func-
tion but also decreased its expression in some
way. The disruption and the downregulation of
PPARγ removed the protection of the quiescent
phenotype of HSCs. Treatment with the PPARγ
ligand troglitazone made HSCs less activated in the presence of HBx.

Taken together, our data suggest that HBx
could maintain the perpetuation of stellate cell
activation by regulating PPARγ and that trogli-
tazone could attenuate the HBx-induced cell
growth promotion and fibrogenesis, which
might provide a potential therapy for fibrosis.
However, because of the in vitro nature of our
study, these data cannot be generalized to the
situation of in vivo fibrosis. Further studies
should clarify why PPARγ is downregulated by
HBx and investigate the possible interactions
between other HBV proteins and PPARγ.

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Disclosure of conflict of interest

None.

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