Hepatitis B virus X protein modifies invasion, proliferation and the inflammatory response in an HTR-8/SVneo cell model

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Abstract. Mother-to-infant transmission of hepatitis B virus (HBV) can occur as an intrauterine, intrapartum or postpartum infection. In the present study, we induced a multifunctional viral regulator of HBV gene products, HBx, and its different fragments to overexpress in a trophoblast cell line, HTR-8/SVneo. We then identified the biological effects of HBx on HTR-8/SVneo cells. Our results indicated that HBx inhibited apoptosis and induced invasion as detected using Annexin V/propidium iodide (PI) double staining and Transwell assay, respectively. Furthermore, we carried out western blot analysis to analyze the possible related signaling pathway. We confirmed that HBx and its different fragments can activate the Smad signaling pathway, accompanied by downregulation of E-cadherin, and upregulation of vimentin and N-cadherin. TGF-β1 was used as a control to activate the Smad signaling pathway in HTR-8/SVneo cells. HBx activated the Smad signaling pathway in the HTR-8/SVneo cells. After the signaling pathway was activated, reduced apoptosis, higher invasive ability and enhanced inflammatory response were observed in the HTR-8/SVneo cells.

Introduction

Hepatitis B virus (HBV) infection is a major global health issue, and more than 350 million individuals are infected by HBV worldwide (1). Approximately 10-15% of pregnant women in China are carriers of the HBV surface antigen (HBsAg) and HBV e antigen (HBeAg) and 5-15% of their babies are infected by intrauterine transmission (2,3). The mechanism of intrauterine HBV infection is not completely understood; transplacental leakage of maternal blood has been suggested as a possible mechanism (4). Trophoblasts, specialized cells of the placenta, mediate the contact between two genetically different individuals, the embryo and the mother, by establishing a transient embryonic organ, the placenta (5). Impaired trophoblast function may result in a range of adverse pregnancy outcomes, such as spontaneous abortion, intrauterine infection and stillbirth (6).

Among the HBV gene products, the hepatitis B virus X protein (HBx) is a multifunctional viral regulator and is considered as one of the most important determinants involved in viral pathogenesis and carcinogenesis (7). HBx, which consists of 154 amino acids, contains four regions important for transcriptional regulation, cell cycle control, cell adhesion and modulation of cytoplasmic signal transduction pathways (8). Different regions of HBx may play different roles in the pathological process. For example, HBxΔ127 (deletion from 382 to 401 bp) was able to upregulate transcriptional activities of nuclear factor-κB, survivin and human telomerase reverse transcriptase, as well as the expression levels of c-Myc and proliferating cell nuclear antigen in hepatoma cells (9).

To our knowledge, the role of HBx in trophoblasts remains unclear. In the present study, we constructed two HBx mutants, including N-terminal and C-terminal mutants, and analyzed the biological activities of HTR-8/SVneo cells with those truncated mutants of the HBx gene. Finally, we identified the underlying mechanism involving the promotion of cell growth and invasion mediated by the HBx mutants.

Materials and methods

Cell lines and culture conditions. The trophoblast cell line HTR-8/SVneo, stored in our laboratory, was maintained in RPMI-1640 medium which was supplemented with 10% heat-inactivated FBS, 2 mM glutamine, penicillin (100 U/ml) and streptomycin (100 mg/ml) at 37°C in 5% CO₂.

Plasmid construction and transfection. The ideograph of HBx protein and its truncated form is shown in Fig. 1A. The plasmid pcDNA3.1-HBx was kindly provided by Mr. Jin-Cheng Li (China Medical University, Shenyang, China). For the preparation of truncated HBx proteins, the fragments were respectively amplified from full length HBx by retro-transcription PCR (RT-PCR) where restriction sites were added to PCR primers (Table I) and cloned into pcDNA3.1. The sequence of the successful clone was confirmed by DNA
sequencing. Transfection of plasmids to the HTR-8/SVneo cells was performed using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Briefly, the cells were plated in RPMI-1640 medium with 10% FBS and cultured until they achieved 70-80% confluency. Culture medium was then replaced with low-serum media (containing 0.5% FBS), and then the cells were transfected with 5 µg/well of the plasmid. Forty-eight hours after transfection, the pcDNA3.1-transfected (mock), pcDNA3.1-HBx-transfected (intact), pcDNA3.1-HBx-∆C-transfected (T1), and pcDNA3.1-HBx-∆N-transfected (T2) HTR-8/SVneo cells were used in the subsequent studies.

Retro-transcription PCR and quantitative real-time PCR. The expression of the relevant HBx in the established cell lines was verified by RT-PCR. Total RNA was extracted from the cell lines (the cells transfected with intact HBx, truncated 1 and 2) using TRizol reagent (Invitrogen) according to the manufacturer's instructions. First-strand cDNAs were generated in reverse transcriptase reactions containing total RNA, poly(dT) oligonucleotides, and SuperScript II reverse transcriptase (Invitrogen). cDNAs were then subjected to RT-PCR analysis. The primers used to generate the intact and truncated forms of HBx are shown in Table I. The mRNA levels of inflammatory factors were detected using the primers that are listed in Table II. GAPDH was used as an internal control. Amplification of the primers as described above was performed with 1 cycle at 95˚C for 10 min and 40 cycles of 95˚C for 15 sec and 60˚C for 60 sec.

TGF-β1 treatment. Recombinant human transforming growth factor-β1 (TGF-β1) was obtained from R&D Systems (Minneapolis, MN, USA). The effect of TGF-β1 on the untransfected HTR-8/SVneo and pcDNA3.1-HBx-transfected cells was determined by adding recombinant TGF-β1 (5 ng/ml) to cell monolayers at 70% confluency. Cells were incubated for an additional 24 h and used in the subsequent studies.

Cell growth assay. Viability of the transfected cells was determined using the 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) assay (Sigma-Aldrich, Carlsbad, CA, USA). The cells were plated in 96-well plates (1,500 cells/well) and incubated under normal culture conditions. After 24 h, the cells were treated with 0.5 mg/ml MTT for 4 h and lysed with dimethyl sulfoxide (DMSO). Absorbance rates were measured at 550-560 nm using a microplate reader (Bio-Rad, Hercules, CA, USA).

Apoptosis detection. Cells were trypsinized, washed twice with cold PBS, and resuspended in 200 µl binding buffer. Annexin V-FITC was added to a final concentration of 0.5 µg/ml, according to the manufacturer's instructions (Keygen, Nanjing, China). After 20 min of incubation at room temperature in the dark, 400 µl of binding buffer containing propidium iodide (PI, 50 µg/µl) was added, and samples were immediately analyzed on a FACSCalibur flow cytometer (Becton-Dickinson Medical Devices, Shanghai, China).

Invasion assays. Transwell chamber (8-µm pore size polycarbonate membrane; Cell Biolabs, San Diego, CA, USA) Matrigel invasion assays were performed as previously described (10). Cells (30,000) were placed in the upper chamber and allowed to invade for 24 h. Twenty fields of cells were acquired at x10 magnification and quantified. Relative invasion was expressed as a ratio to the control cells.
Western blotting. Cells and tissues were washed and lysed and an equal amount of proteins to ensure equal protein loading was subjected to SDS-PAGE and then blotted onto PVDF membranes (GE Healthcare Corp., Piscataway, NJ, USA). The membranes were blocked in 5% milk-TBST and then probed with the primary antibody. TGF-β1 RI (sc-398), p-Smad3 (sc-130218), Smad3 (sc-101154), p-Smad2 (sc-135644), Smad2 (sc-6200), E-cadherin (sc-7870), vimentin (sc-6260), N-cadherin (sc-7939) and β-actin (sc-47778) were purchased from Santa Cruz Biotechnology (Shanghai, China). Anti-HBx (Abcam, Cambridge, UK) was used to identify the results of the transfection. β-actin was used as an internal control. The secondary antibody was anti-mouse IgG, anti-rabbit IgG, or anti-goat IgG (determined by primary antibodies) at a dilution of 1:1,000-2,000 (Amersham Biosciences, Needham, MA, USA). Then the results were detected by enhanced chemiluminescence (Amersham Pharmacia, Piscataway, NJ, USA).

Phyre database was used to generate a predicted structural model. The protein sequence of HBx was obtained from Pubmed (http://www.ncbi.nlm.nih.gov/protein/CAA49453.1) and submitted to Protein Homology/analogY Recognition Engine (Phyre version 2). Based on the homology sequence in the Phyre server, the three-dimensional structure of ING2 protein was predicted.

Physicochemical profiles of HBX. Physicochemical profiles, such as titration curve, hydrophobicity, antigenicity, flexibility, and solvent accessibility, were analyzed using Antheprot 5.0 software.

Statistical analysis. Statistical analyses were carried out using GraphPad Prism 5.0 (GraphPad Software Inc., San Diego, CA, USA). Data are expressed as mean ± standard deviation of three independent experiments performed in triplicate. Data comparisons in relation to the control were performed by one-way ANOVA. Differences were considered statistically significant if a p-value <0.05 was achieved.

Results

mRNA and protein levels of different HBx fragments evaluated in the cell lines. After transfection, the mRNA and protein levels of the different HBx fragments were detected using western blot analysis, RT-PCR and immunofluorescence. As shown in Fig. 1B and C, the levels of HBx protein and mRNA in the HTR-8/SVneo cells following transfection were higher than levels in the cells without transfection. Furthermore, intact HBx protein and truncated HBx fragments were localized in the cytoplasm by immunofluorescence assay (Fig. 1D). The results showed that intact HBx protein and the truncated HBx fragments were successfully transfected into the HTR-8/SVneo cells. Furthermore, 3D structures of intact HBx protein and truncated HBx fragments were confirmed (Fig. 2D).

The roles of different HBx fragments in HTR-8/SVneo cells. Cell viability was monitored using an MTT assay, and Fig. 3A shows that the proliferation rate of the HTR-8/SVneo cells was induced by both intact and truncated HBx (P<0.05). We utilized Annexin V-FITC and PI double staining to detect the
Figure 2. Physicochemical profiles of HBx and the truncated HBx fragments. (A) 3D structure of the intact HBx protein and the truncated HBx fragments. (B) Titration curves of the intact HBx protein or the truncated HBx fragments. (C) Physicochemical profiles of these three proteins. (D) 2D structure of the intact HBx protein and the truncated HBx fragments. HBx, hepatitis B virus X.
apoptotic cells. Our results showed that the apoptotic ratio of the cells after transfection with the intact or truncated HBx was significantly decreased (Fig. 3B, P<0.05). We determined whether there were any changes in the invasive ability in the HBx-expressing cells using the Transwell assay. We found that significantly more HBx-expressing cells migrated to the lower membrane compared with the control cells (Fig. 3C, P<0.05). The mRNA levels of inflammatory factors (IL-6, IL-10 and IFN-γ) were measured using real-time PCR (P<0.05). Untransfected, untransfected HTR-8/SVneo cells; Mock, HTR-8/SVneo cells transfected with pcDNA3.1; Intact, HTR-8/SVneo cells transfected with intact HBx; T1, HTR-8/SVneo cells transfected with truncated 1; T2: HTR-8/SVneo cells transfected with truncated 2. HBx, hepatitis B virus X; PI, propidium iodide.

Figure 3. Biological effects of HBx on HTR-8/SVneo cells. (A) The growth inhibitory ratio of different HBx fragments on HTR-8/SVneo cells was determined by MTT assay (P<0.05). (B) Apoptotic ratio of the HBx-transfected cells was analyzed by double staining with Annexin V/PI (P<0.05). (C) Transwell assays were performed. Cells that migrated to the bottom side of the membrane were stained and counted (P<0.05). (D) The mRNA levels of inflammatory factors (IL-6, IL-10 and IFN-γ) were measured using real-time PCR (P<0.05). Untransfected, untransfected HTR-8/SVneo cells; Mock, HTR-8/SVneo cells transfected with pcDNA3.1; Intact, HTR-8/SVneo cells transfected with intact HBx; T1, HTR-8/SVneo cells transfected with truncated 1; T2: HTR-8/SVneo cells transfected with truncated 2. HBx, hepatitis B virus X; PI, propidium iodide.
In contrast, slightly increased levels of IL-1α, IL-1β and TNF-α were detected in the HTR-8/SVneo cells after HBx treatment (data not shown).

HBx fragments activate the Smad signaling pathway and induce changes in epithelial-mesenchymal transition (EMT). Furthermore, we carried out western blot analysis to identify the mechanism of apoptosis induced by HBx. We found that the levels of phospho-Smad3 and phospho-Smad2 in the transfected cells were higher than the levels in the untransfected cells, while levels of total Smad3 and Smad2 were not altered (Fig. 4). Compared with the untransfected cells, HBx-transfected cells showed a lower expression level of epithelial marker E-cadherin, while higher expression levels of mesenchymal markers vimentin and N-cadherin were noted (Fig. 4).

TGF-β1 decreases HTR-8/SVneo cell proliferation and invasion, while increases HBx-transfected HTR-8/SVneo cell proliferation and invasion. TGF-β1 was used to further clarify the effect of the Smad signaling pathway on HTR-8/SVneo cells. HTR-8/SVneo cells were treated with recombinant human TGF-β1 (5 ng/ml). The proliferation rate of the HTR-8/SVneo cells was inhibited by recombinant human TGF-β1 (Fig. 5A, P<0.05). Annexin V-FITC and PI double staining showed that recombinant human TGF-β1 induced apoptosis in the HTR-8/SVneo cells (Fig. 5B, P<0.05). Matrigel invasion assay showed that treatment with 5 ng/ml of TGF-β1 significantly decreased HTR-8/SVneo cell invasion (Fig. 5C, P<0.05). The mRNA levels of inflammatory factors IL-6 and IL-10 in the HTR-8/SVneo cells treated with TGF-β1 were lower than levels in the control cells (Fig. 5D, P<0.05). Western blot analysis showed that TGF-β1 treatment induced TGF-β1 RI expression, and Smad2 and Smad3 phosphorylation in the HTR-8/SVneo cells (Fig. 5E). Although TGF-β1 also induced Smad2 and Smad3 phosphorylation in the HBx-transfected HTR-8/SVneo cells (Fig. 5E), opposite effects of TGF-β1 on the HBx-transfected HTR-8/SVneo cells were observed when compared with the untransfected cells (Fig. 5). TGF-β1 increased proliferation (Fig. 5A, P<0.05) and invasion (Fig. 5C, P<0.05), inhibited apoptosis (Fig. 5B, P<0.05), and induced the inflammatory response (Fig. 5D, P<0.05) in the HBx-transfected HTR-8/SVneo cells.

Discussion

Mother-to-infant transmission of HBV can occur as an intrauterine, intrapartum or postpartum infection (4). Intrauterine infection by HBV is mainly transmitted through the placenta. HBV can reach the placenta though maternal blood and infect placental trophoblast cells and then survive and replicate in the cells, and functional protein HBxAg is produced (11).

In the present study, we confirmed that HBx inhibited apoptosis and increased invasive ability in the HTR-8/SVneo cells. Bai et al (11) found that the apoptosis index of HBV-infected high replication cells was lower than that of uninfected ones. Liu et al (12) found that HBx could promote hepatoma cell invasion and metastasis. The HBx genome consists of an N-terminal negative regulatory domain and a C-terminal transactivation domain (13). The HBx fragment at the C-terminal can strongly enhance the proliferation and growth of liver cells (14). However, Chau et al (15) found that the first 50 aa NH2 region of HBx has the ability to resist cell death stimulation. Notably, we found that two truncated forms of HBx (N-terminal or C-terminal mutants) had similar effects as intact HBx on the HTR-8/SVneo cells. Based on our present data, we could not explain these contradictory results of HBx in different cells as HBx has been reported to be both pro-apoptotic and anti-apoptotic. We will investigate the contradiction in future research.

The trophoblast is a cell type with endocrine functions that also secretes various cytokines throughout the gestation period (16). IL-10 was found to be a critical molecule for successful pregnancy outcome in both human and mouse pregnancy models (17). Lack of IL-10 expression in trophoblasts may contribute to an increased inflammatory response in the placenta (18). Regulation of IL-6 secretion during pregnancy is essential for maintaining normal gestation. Elevated IL-6 is observed during recurrent miscarriage, pre-eclampsia and preterm delivery (19). Moreover, IFN-γ can be deleterious to pregnancy, as determined by in vivo animal model studies (20). Hu et al (21) provided direct evidence that IFN-γ can influence the mechanism of apoptosis induced by HBx.
extravillous cytotrophoblast (EVT) outgrowth and migration. In the present study, we also found upregulation of IL-6 and IL-10 mRNA levels and downregulation of the IFN-γ mRNA level in the HTR-8/SVneo cells following HBx transfection. EMT is a process whereby epithelial cells change to a mesenchymal phenotype, and this process is important in the progression of human carcinomas to a more invasive, metastatic capacity (22). We confirmed that HBx-induced
invasion of HTR-8/SVneo cells involves EMT. Furthermore, we found that the Smad signaling pathway was activated by HBx in the HTR-8/SVneo cells. The Smad family of proteins (Smad1-8) are classified according to their different functions; Smad1, 2, 3, 5 and 8 are ‘receptor-regulated SMADs’, Smad4 is termed the ‘common-mediator SMAD’, and Smad6 and 7 are ‘inhibitory SMADs’ (23). These proteins regulate numerous cellular processes, such as cell proliferation, differentiation, apoptosis and control of developmental fate (24). Robust activation of Smad2 and Smad3 is through phosphorylation of C-terminal regulatory residues (23). In the present study, we confirmed that p-Smad2 and p-Smad3 were significantly upregulated in the HTR-8/SVneo cells following HBx transfection. To confirm the effect of the Smad signaling pathway on HTR-8/SVneo cells, TGF-β1 was used as a control. TGF-β1 activates TGF-β receptor I (TβR1) and TβRII, which results in the phosphorylation of receptor-regulated SMAD2/3 proteins (25). In the present study, we found that TGF-β1 treatment induced proliferation and invasion, and inhibited apoptosis in the HTR-8/SVneo cells via activation of the Smad signaling pathway. Cheng et al (26) also found that TGF-β1 induced the downregulation of VE-cadherin and decreased cell invasion in human trophoblast cells. Notably, we found that activation of the Smad signaling pathway had opposite roles in the HTR-8/SVneo cells with HBx transfection. HBx plays critical roles in the pathogenesis of hepatocellular carcinoma development, based on its tumorigenic activity in vitro and in vivo (27). According to the results of previous studies, we concluded that HTR-8/SVneo cells with HBx transfection also underwent cancerous transformation. More evidence was provided by Murata et al (28). They found that HBx shifts TGF-β signaling from tumor suppression to oncogenesis in early chronic hepatitis B. However, the mechanism in HTR-8/SVneo cells should be validated in future studies.

Taken together, in all our experiments, we elucidated the biological effects of different HBx fragments on HTR-8/SVneo cells. However, there was no difference among these fragments. We demonstrated that HBx activates the Smad signaling pathway in HTR-8/SVneo cells. After the signaling pathway was activated, a lower apoptotic ratio, higher cell motility, and an enhanced inflammatory response were observed in the HTR-8/SVneo cells (Fig. 6). In further studies, we will truncate other domains of HBx and detect their activities.

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References

20. Liu Z, Chen Y, Yang Y and Peng JP: The effect on MHC class II expression and apoptosis in placenta by IFNgamma administra-