Original Paper

Hepatitis B Virus X Protein Reduces Podocyte Adhesion via Downregulation of $\alpha 3 \beta 1$ Integrin

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Key Words

HBx • Podocyte • Cell adhesion • Apoptosis • $\alpha 3 \beta 1$ integrin

Abstract

Background/Aims: Hepatitis B virus (HBV)-associated glomerulonephritis (HBV-GN) is characterized by a reduced number of podocytes due to apoptosis and shedding from the basement membrane. However, the pathological mechanism of HBV-GN is unclear. We previously showed that hepatitis B virus X protein (HBx) promotes apoptosis in tubular epithelial cells. In this study, we transfected podocytes with HBx and examined the effects on adhesion and apoptosis of these cells. **Methods:** Podocytes were transfected with pc-DNA3.1 (+)-HBx. One control group was not transfected and another control group was transfected with empty plasmids. Podocyte adhesion was assessed by a fluorescence assay, apoptosis was measured by flow cytometry and fluorescence microscopy, and expression of $\alpha 3 \beta 1$ integrin was determined by western blotting and the reverse transcription polymerase chain reaction (RT-PCR). Activity of caspase-8 was measured by a spectrophotometric assay. **Results:** Relative to controls, podocytes with pc-DNA3.1(+)-HBx had reduced cell adhesion, increased apoptosis, reduced expression of $\alpha 3 \beta 1$ integrin, and increased caspase-8 activity. $\beta 1$ integrin blockage reduced podocyte adhesion, but increased apoptosis and caspase-8 activity. Treatment of transfected podocytes with a caspase-8 inhibitor (Z-IETD-FMK) had no effect on the HBx-mediated integrin downregulation and reduced podocyte adhesion, suggesting that $\alpha 3 \beta 1$ integrin downregulation is sufficient to alter cell adhesion. **Conclusions:** Our *in vitro* results indicate that HBx reduced podocyte adhesion and expression of $\alpha 3 \beta 1$ integrin, and increased apoptosis. Moreover, HBx-mediated downregulation of $\alpha 3 \beta 1$ integrin expression is sufficient to reduce podocyte adhesion. HBx-induced apoptosis of podocytes may contribute to HBV-GN.

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Introduction

Approximately 350 million individuals worldwide have chronic hepatitis B virus (HBV) infections [1]. HBV infection rates vary remarkably by geographical area, and the prevalence is particularly high in China, Southeast Asia, and Africa. HBV infection is currently a

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significant public health problem in China [2]. A variety of extrahepatic manifestations, one of the commonest being HBV-associated nephropathy, may develop in individuals with chronic HBV infections [3]. HBV is also a well known cause of membranous nephropathy (MN) [3]. In addition, several other forms of renal disease, including membranoproliferative glomerulonephritis (MPGN), IgA nephropathy, and rarely focal segmental glomerular sclerosis (FSGS), are associated with HBV infection [3].

Recent studies indicated that the number and density of podocytes decrease significantly in patients with HBV-MN, and that this decrease accompanies an increase in the excretion of urinary protein [4]. Thus, the reduction in the number of podocytes seems to play an important role in the pathogenesis of HBV-MN [4]. Podocytes are highly differentiated terminal cells that extrude foot processes which wrap the glomerular basement membrane (GBM). These foot processes connect *via* tight junctions and form the last barrier of glomerular filtration. Thus, a reduced number of podocytes leads to proteinuria. Apoptosis, compromised adhesion of podocytes, and shedding from the GBM are the major causes of the reduced number of podocytes [5-8]. Integrins are transmembrane receptors that have a major role in the attachment of podocytes to the GBM and in the regulation of cell apoptosis [7, 9]. Downregulation of podocyte $\alpha 3\beta 1$ integrin occurs in diverse glomerular disorders in humans and experimental animal models of renal diseases [10]. However, changes in integrin expression have never been studied in HBV-associated glomerulonephritis (HBV-GN).

Sakai reported a case of focal segmental glomerulosclerosis (FSGS) with HBV infection, in which HBV-DNA occurred in urinary podocytes, based on real-time PCR [3]. After administration of anti-viral therapy, the FSGS resolved and this coincided with a decreased level of HBV DNA in podocytes. This was the first report to document HBV infection of podocytes. These findings indicate that HBV infection can reduce the adhesion of podocytes to the GBM and lead to shedding from the GBM. The HBV genome contains 4 overlapping open reading frames that encode the viral envelope, capsid, polymerase/reverse transcriptase, and nonstructural X (HBx) proteins. HBx is a multifunctional protein that regulates numerous signal transduction pathways, including those that modulate apoptosis [1].

Our previous study showed that HBx plays an important role in the pathogenesis of HBV-GN, in that it activates the JAK2/STAT3 signaling pathway and induces apoptosis of tubular epithelial cells [2]. However, the effects of HBx on the apoptosis and adhesion of podocytes are still uncertain. In the present study, we examined the mechanism of the effects of HBx on podocytes. Thus, we transfected pcDNA3.1(+) HBx into podocytes and evaluated adhesion of podocytes, apoptosis, and $\alpha 3\beta 1$ integrin expression in transfected cells relative to controls.

Materials and Methods

Cell culture

A conditionally immortalized mouse podocyte cell line was kindly provided by Dr. Lin, Dalian Medical University (Dalian, China). Cells were cultured and maintained in RPMI-1640 medium that was supplemented with 10% fetal bovine serum (FBS), 0.1 mg/mL streptomycin, 100 U/mL penicillin, and 10 U/mL interferon- γ at a "permissive" temperature of 33°C with 5% carbon dioxide. When cells reached 80–90% confluence, they were trypsinized, and differentiation was induced under "restrictive" conditions (absence of interferon- γ at 37°C for 2 weeks). Experimental procedures were conducted in the same medium without interferon- γ .

Human lung adenocarcinoma epithelial cells (A549) were used as a source of laminin-10/11, as previously described [9, 11]. These cells were cultured in DMEM medium that was supplemented with 100 U/mL penicillin, 0.1 mg/mL streptomycin, and 10% fetal bovine serum (FBS).

Plasmid construction and transfection

The plasmid pcDNA3.1(+)-HBx was constructed as previously reported [12]. Some experiments had three treatment groups: (i) control (no transfection); (ii) transfection with empty pc-DNA3.1(+) plasmid; and (iii) transfection with pc-DNA3.1(+)-HBx and evaluated after 48 h. Lipofectamine™ LTX and PLUS™ transfection reagents (Invitrogen, USA) were used to conduct the transfection according to the steps

described in the instructions. For transfection experiments, cells were seeded at 2×10^5 /mL, transfected with equal amounts of DNA, and collected at 48 h. All experiments were repeated three times.

Cell adhesion assay

Ninety-six-well plates were coated with human placental collagen IV ($30 \mu\text{g}/\text{cm}^2$) over a 2-h period at 37°C . For laminin coating, A549 cells were seeded (3×10^4 cells/well) to near confluence, maintained in culture at confluence for 3 days, and subsequently lysed with 0.1 mol/L NH_4OH , as previously described [9, 11]. Post-coating, the plates were washed with PBS and blocked for 30 min at 37°C with 1% BSA in PBS. A cell adhesion quantification kit based on fluorescence (Calbiochem, San Diego, CA, USA) was used to detect podocyte adhesion as previously described, with small modifications [9, 11, 13]. After different interventions, podocytes were re-suspended in FBS-free RPMI-1640 at a density of 2×10^5 cells/mL, seeded into 96-well plates ($100 \mu\text{L}$ /well), and then incubated at 37°C with 5% CO_2 for 3 h. The medium was removed, and cells were washed twice with PBS ($200 \mu\text{L}$ /well). Then, a Calcein-AM working solution ($100 \mu\text{L}$) was added, followed by incubation at 37°C with 5% CO_2 for 1 h. The relative fluorescence units (RFUs) were determined with a microplate reader (excitation wavelength: 485 nm, emission wavelength: 520 nm).

A standard curve was run in each experiment as an internal control. Results are expressed as percentage change relative to the control. All experiments were conducted at least 3 times (10 wells per group), and means were calculated for statistical analysis.

Apoptosis

The morphology of apoptotic cells was observed by staining the nuclei with HO33342 (Sigma, USA). Cell slides were taken out, washed 3 times with PBS, fixed with 4% paraformaldehyde for 20 min, and washed 3 times again with PBS. After HO33342 fluorochrome (5 mg/L) was added, the slides were incubated for 8 min at 37°C while protected from light, and then rewashed 3 times with PBS. Observations and imaging were immediately conducted under a fluorescence microscope. Two hundred cells were counted on each coverslip, and all cells were classified as normal (nuclei with smooth and defined boundaries) or apoptotic (nuclei with condensed or fragmented chromatin).

The percentage of apoptotic cells was determined by flow cytometry using an FITC Annexin V Apoptosis Detection kit (Nanjing KGI Biotechnology Development Co., Ltd., China). Cells were stained according to the manufacturer's instructions, and flow cytometry was conducted using a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). Cells in early apoptosis were defined as those with Annexin V-positivity and PI-negativity; cells in late apoptosis were defined as those with Annexin V-positivity and PI-positivity. The results show the sums of early and late apoptosis.

To investigate the relationship of $\alpha\beta 1$ integrin, apoptosis, and cell adhesion, podocytes were exposed to the anti- $\beta 1$ integrin antibody for 30 min, prior to determination of adhesion and measurement of apoptosis.

Measurement of Caspase-8 Activity

The activity of caspase-8 was measured with the Caspase-8 Activity Assay Kit (Sigma, St. Louis, USA), according to the manufacturer's protocol. In some experiments, a caspase-8 inhibitor (Z-IETD-FMK, $10 \mu\text{mol/L}$, [Sigma, St. Louis, USA]) was added into fresh medium with podocytes at 1 h before transfection with HBx.

Western blotting

Cells were collected and washed 3 times in cold phosphate-buffered saline and combined with cell lysis buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% sodium dodecyl sulfate [SDS], 1 mM EDTA, 100 mM NaF, 1 mM Na_3VO_4 , 1 mM phenylmethylsulfonyl fluoride, $2 \mu\text{g}/\text{mL}$ aprotinin) on ice for 30 min. Cell lysates were centrifuged at $12,000 \text{ rpm}$ at 4°C for 20 min, and supernatants were stored at -80°C . Total protein concentration in the supernatant was determined by the bicinchoninic acid (BCA) protein assay. Protein samples were electrophoresed on a graded SDS/polyacrylamide gel under reducing conditions, transferred onto a nitrocellulose membrane, blocked in 5% non-fat dried milk in Tris-buffered saline with Tween, and incubated overnight at 4°C with the primary antibodies (anti-HBx antibody [Chemicon, Temecula, USA], anti-heavy chain of integrin $\alpha 3$ [BD Biosciences, Oxford, UK], anti-integrin $\beta 1$ [Cell Signaling Technology, Beverly, USA]). After washing, a secondary antibody was added and incubated

Table 1. Nucleotide sequences of the primers used for real-time PCR. PCR=polymerase chain reaction

Genes	Forward (5'- 3')	Reverse (5'- 3')
$\alpha 3$ integrin	CCCTCGCTTTGTAGGTTA	GTCCCTGTCAGCCTCCACT
$\beta 1$ integrin	GACCTGCCTTGGTGTCTGTGC	AGCAACCACACCAGCTACAAT
β -actin	GGAGATTACTGCCCTGGCTCCTA	GACTCATCGTACTCCTGCTTGCTG

for 1 h at room temperature. Protein bands were visualized using ECL Plus (Thermo Scientific). Membranes were also treated with an anti- β -actin antibody (Santa Cruz, CA, USA) as an internal loading control.

Real-time reverse transcription polymerase chain reaction

Total RNA was isolated from cells using the Trizol reagent according to the manufacturer's protocol (Takara Biotechnology, Dalian, China). Complementary DNA (cDNA) was synthesized from total RNA by reverse transcription (RT) using a high-capacity cDNA archive kit (Takara Biotechnology, Dalian, China), according to the manufacturer's instructions. Primers for PCR that targeted $\alpha 3$ integrin, $\beta 1$ integrin, and β -actin genes were designed using the corresponding GenBank sequences, and were synthesized by Takara Biotechnology (Dalian, China) (Table 1). Targets were amplified by real-time RT-PCR using a TaKaRa realtime PCR kit on an ABI 7500 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). Quantitation of mRNA was determined from the threshold PCR cycle number at which exponential growth of the PCR product was detectable. The mRNA level in each sample was normalized to that of β -actin.

Statistical analysis

All results are expressed as means $\pm$ standard deviations (SDs) and analyzed with SPSS version 15.0. Results were compared using ANOVA. When ANOVA indicated a statistically significant difference, multiple comparisons was performed using Tukey. A p-value less than 0.05 was considered statistically significant.

Results

Transfected podocytes express HBx

Western blotting indicated the presence of a 17-kD band (corresponding to HBx) in podocytes transfected with pc-DNA3.1(+)-HBx (Fig. 1A, B). Both control groups (no transfection, transfection with empty plasmid) had no HBx expression.

HBx downregulates $\alpha 3\beta 1$ integrin

Next, we used western blotting to examine the expression of $\alpha 3\beta 1$ integrin to assess the possible link between integrins, cell adhesion, and apoptosis. The results indicate that cells transfected with HBx had significantly lower expression of $\alpha 3\beta 1$ integrin than cells in both control groups (Fig. 2A, B). RT-PCR analysis of $\alpha 3\beta 1$ integrin mRNA also indicated lower expression in cells transfected with HBx than in both control groups (Fig. 2C).

HBx induces apoptosis

We also examined the effect of HBx on apoptosis by use of fluorescence microscopy, flow cytometry, and measurement of caspase-8 activity (a key protein in apoptosis). The results indicate chromatin condensation and nuclear fragmentation in cells transfected with HBx (Fig. 3A), but not in both control groups. The flow cytometry results provide quantitation of the effect of HBx on apoptosis (Fig. 3B). These results show low percentages of apoptotic cells in both control groups (no transfection: $3.31\% \pm 1.12$, transfection with empty plasmid: $2.22\% \pm 0.93$). In contrast, $25.36\% (\pm 3.01)$ of cells transfected with HBx were apoptotic at 48 h (Fig. 3B, C). Cells transfected with HBx also had upregulated caspase-8 activity ($38.13\% \pm 2.19$) relative to no transfection cells (Fig. 4).

HBx reduces cell adhesion

We measured podocyte adhesion in the same 3 groups of cells using a fluorescence assay. The results indicate that podocyte adhesion was significantly reduced ($26.2\% \pm 2.48\%$) in cells transfected with HBx relative to no transfection cells (Fig. 5).

Fig. 1. (A) Representative western blots of HBx in the 2 control groups (no transfection, transfection with empty pc-DNA3.1(+) plasmid), and cells transfected with pc-DNA3.1(+)-HBx. (B) Quantitation of western blotting results.[#] $p < 0.05$ relative to the 2 controls. HBx: hepatitis B virus X protein.

Fig. 2. (A) Representative western blotting of α3 integrin and β1 integrin in the 2 control groups (no transfection, transfection with empty plasmid) and cells transfected with HBx. (B) Quantitation of western blotting results. ^{a,b} $p < 0.05$ relative to the 2 controls. (C) Quantitation of RT-PCR results. ^{a,b} $p < 0.05$ relative to the 2 controls.

Inhibition of β1 integrin increases apoptosis and decreases cell adhesion

To confirm the link between β1-integrin, adhesion, and apoptosis, we treated transfected podocytes with a specific β1-integrin antibody. The results indicate that blocking of β1-integrin in cells transfected with HBx led to a significant reduction in podocyte adhesion ($34.3\% \pm 2.35$, $p < 0.05$) (Fig. 6A), a significant increase in apoptosis as measured by flow cytometry ($24.2\% \pm 1.86$, $p < 0.05$), and increased caspase-8 activity (Fig. 6B).

Caspase-8 inhibition does not affect HBx-induced downregulation of integrin and cell adhesion

We treated podocytes with a caspase-8 blocker (Z-IETD-FMK, BioVision, San Francisco, CA, USA) to further elucidate the relationship between increased podocyte apoptosis and reduced podocyte adhesion in cells transfected with HBx. In cells that were not transfected, this inhibitor reduced caspase-8 activity by ~30% ($p < 0.05$), but it did not affect the expression of α3β1 integrin or podocyte adhesion (Fig. 7A–C). This inhibitor also reduced caspase-8 activity and apoptosis in cells transfected with HBx (Fig. 7D–E), but had no effect on the downregulation of α3β1 integrin (Fig. 8A–C). The adhesion of HBx-transfected cells was similar in the presence or absence of the caspase-8 inhibitor (Fig. 9). These findings suggest that the HBx-induced reduction in podocyte adhesion is associated with reduced expression of α3β1 integrin, but not with HBx-induced apoptosis. In other words, inhibition

Fig. 3. (A) Nuclear staining (Hoechst 33342) and fluorescence microscopy ($\times 400$) of the 2 controls (no transfection, transfection with an empty plasmid) and cells transfected with HBx. (B) Flow cytometry of apoptosis in each group. (C) Quantitation of the flow cytometry results. Abscissa presents cell groups and ordinate presents apoptosis rate of the various groups. $^a p < 0.05$ relative to the 2 controls.

Fig. 4. Activity of caspase-8 in control cells (no transfection, transfection with empty plasmid) and cells transfected with HBx. Caspase-8 activity is expressed as a percentage of no transfection cells. $^a p < 0.05$ relative to the 2 controls.

Fig. 5. Adhesion to an extracellular matrix substrate in control cells (no transfection, transfection with an empty plasmid) and cells transfected with HBx. Cell adhesion is expressed as a percentage of no transfection cells. $^a p < 0.05$ relative to the 2 controls.

of HBx-induced apoptosis in podocytes had no effect on the reduced adhesion of these cells.

Discussion

Proteinuria is a well-known symptom of injury to the glomerular filtration barrier. Podocytes play a crucial role in maintaining the permeability of the glomerular filtration

barrier [14], and podocyte injury is present in many types of human and experimental glomerular diseases, including HBV-GN [4, 15-17]. Moreover, a decreased number of podocytes per glomerulus is the strongest predictor of renal disease progression [18]. The reduced number of podocytes may be largely ascribed to their reduced adhesion to the GBM, shedding, and apoptosis. As is well-known, the immune complexes formed by HBV antigens and antibodies have an important role in the pathogenesis of HBV-GN. These immune-complexes for deposits in patients with HBV-GN [19, 20]. Subepithelial immune deposits initiate activation of the complement system of the C3 component, conversion of C5, and subsequent formation of the C5b-9 complex in podocyte membranes [19, 21]. The membrane attack complex (C5b-9) leads to podocyte injury, including calcium influx, oxidative injury, production of arachidonic acid metabolites, cell cycle dysregulation, and endoplasmic reticulum stress [21, 22]. These lead to podocyte apoptosis, reduced podocyte adhesion, and detachment of podocytes from GBM, which may result in proteinuria and renal failure [19, 22]. However, some recent reports that identified HBV-DNA, HBV-RNA, and cccDNA

in the kidneys suggested that direct virally induced renal damage may also play an important role in the disease process [3, 12, 23]. The focus of the present paper is to examine the direct effect of HBV on podocyte injury and to find the reason for the reduced podocyte numbers in HBV-GN.

Integrins are transmembrane glycoprotein surface receptors composed of α and β subunits that are an important component of the extracellular matrix (ECM) [17]. The integrins of the ECM regulate many cell functions, including migration, proliferation, differentiation, cell cycle progression, and survival [24-26]. Recent studies of integrin expression in podocytes demonstrated that $\alpha 3\beta 1$ integrin is the major integrin on the surface of podocytes in humans [27] and rats [28]. This integrin is responsible for cell adhesion and signal transduction between podocytes and the extracellular matrix (ECM) of the glomerular basement membrane (GBM) [14]. Previous studies have shown that the absolute number and relative density of podocytes are reduced in HBV-GN [4], although the precise mechanism of this effect is unknown. In our previous study of tubular epithelial cells [2, 12], we found that HBx inhibited cell proliferation and induced apoptosis. Thus, in the present study, we investigated the effects of HBx on the apoptosis and adhesion of podocytes and the mechanism of this response.

Under normal conditions, we found that $\beta 1$ integrin expression was continuous along the glomerular capillary loop, consistent with previous reports [29, 30]. However, there is reduced expression of $\alpha 3\beta 1$ integrin in the podocytes of humans with FSGS [31], in humans and rats with diabetes [32], and in rats treated with puromycin aminonucleoside (PAN) [33]. However, the function of integrin in podocytes transfected with HBx has not been studied to date. We had no way to get the human immortalized podocytes within a short time for the limitations of resource, funds and technology. And we were pressed for time, so we used mouse podocytes in the present study. We employed real time RT-PCR and immunoblotting

Fig. 6. (A) Effect of anti- $\beta 1$ integrin antibody on podocyte adhesion in cells transfected with HBx. ^a $p < 0.05$ relative to cells transfected with HBx but without anti- $\beta 1$ integrin antibody treatment. (B) Effect of anti- $\beta 1$ integrin antibody on caspase-8 activity and apoptosis in cells transfected with HBx. ^{a,b} $p < 0.05$ relative to cells transfected with HBx but without anti- $\beta 1$ integrin antibody treatment.

Fig. 7. (A) Caspase-8 activity and podocyte adhesion in the presence of vehicle (Veh) or a caspase-8 inhibitor (Inh) in cells with no transfection. ^a*p* < 0.05 relative to Veh. (B, C) Representative western blots of $\alpha 3$ integrin and $\beta 1$ integrin in the presence of Veh or Inh in cells with no transfection, and quantitation of these results. (D, E) Caspase-8 activity and apoptosis in control cells (no transfection, transfection with an empty plasmid), cells transfected with HBx, and cells transfected with HBx and treated with Inh. Data are presented as percentage change from control (no transfection and without a caspase-8 inhibitor). ^a*p* < 0.05 relative to controls (no transfection and without a caspase-8 inhibitor, transfection with an empty plasmid and without a caspase-8 inhibitor). ^b*p* < 0.05 relative to cells transfected with HBx and without a caspase-8 inhibitor.

assays to examine $\alpha 3\beta 1$ integrin expression in mouse podocytes that express HBx. The results indicate that as early as 48 h after transfection, there is decreased expression of $\alpha 3\beta 1$ integrin and reduced podocyte adhesion. These results are consistent with previous studies of podocytes, and suggest a link between integrin expression and cell adhesion. In particular, previous studies reported that downregulation of $\alpha 3\beta 1$ integrin is causally related to the loss of podocytes [31-33]. This is supported by our recent observations (He, Ping et al., unpublished data), in which we found podocytes in the urinary sediment of patients with HBV-GN and a parallel reduction in the number of podocytes in renal biopsy tissue. To elucidate the relationship between $\beta 1$ integrin expression and podocyte adhesion, we treated podocytes with a $\beta 1$ integrin inhibitor. The results showed that this inhibitor significantly reduced podocyte adhesion, and therefore suggest a relationship between $\beta 1$ integrin expression and podocyte adhesion. Apoptosis is another major cause of the reduced number of podocytes in diverse renal diseases. For example, increased apoptosis of podocytes occurs in diseased glomeruli and may be responsible for cell loss in glomerulosclerosis [34, 35].

HBx appears to be a multifunctional protein that can activate signaling pathways in various cell types, regulate cell proliferation, and induce apoptosis [36-38].

Fig. 8. Effect of caspase-8 inhibition (Inh) on integrin expression in control cells (no transfection, transfection with an empty plasmid) and cells transfected with HBx. Inh treatment did not affect HBx-mediated down-regulation of $\alpha 3$ and $\beta 1$ integrin. (A) Representative western blotting of $\alpha 3$ integrin and $\beta 1$ integrin in the presence of vehicle (Veh) or a caspase-8 inhibitor (Inh) in control cells (no transfection, transfection with an empty plasmid) and cells transfected with HBx. (B-C) Quantitation of these results. ^a $p < 0.05$ relative to the 2 controls for Veh-treated cells; ^b $p < 0.05$ relative to the 2 controls for Inh-treated cells.

The mechanism by which HBx regulates apoptosis seems to vary according to cell type and environment [1]. Some research indicates that HBx transfection limits the proliferative capacity of podocytes through cell cycle regulation [39]. However, the effect of HBx on apoptosis of podocytes and the specific underlying mechanism are still poorly understood. Integrin can have pro-survival or proapoptotic effects on cells [40]. A connection between $\alpha 3\beta 1$ integrin and apoptosis of podocytes is supported by the recent work of Chen et al. [7], who showed that $\alpha 3\beta 1$ integrin antagonists increased the apoptosis of podocytes and increased the levels of several markers of apoptosis. Our findings showed that podocytes transfected with HBx had reduced expression of $\alpha 3\beta 1$ integrin, increased apoptosis, and increased caspase-8 activity.

We treated podocytes with an integrin $\beta 1$ inhibitor to elucidate the relationship of downregulation of $\alpha 3\beta 1$ integrin with increased apoptosis and reduced adhesion. The results showed that this inhibitor significantly increased podocyte apoptosis and reduced adhesion relative to the controls. This confirms that reduced $\beta 1$ integrin expression is related to increased apoptosis and reduced adhesion. We further investigated the role of apoptosis in podocyte adhesion by treating podocytes transfected with HBx with a caspase-8 inhibitor. Interestingly, this inhibitor did not affect HBx-induced downregulation of $\alpha 3\beta 1$ integrin or the HBx-induced decline in cell adhesion. This suggests that downregulation of $\alpha 3\beta 1$ integrin by itself is sufficient to alter cell adhesion, and that activation of apoptosis

Fig. 9. Effect of caspase-8 inhibition (Inh) on podocyte adhesion in control cells (no transfection, transfection with an empty plasmid) and cells transfected with HBx. Inh treatment had no effect on HBx-mediated reduction of podocyte adhesion. Data are presented as percentage change from control (no transfection and no Inh). ^a $p < 0.05$ relative to controls (no transfection, transfection with an empty plasmid, and Veh); ^b $p < 0.05$ relative to controls (no transfection, transfection with an empty plasmid, and Inh).

is not necessary for the loss of podocyte adhesion. However, a loss of integrin binding to ECM substrates (complete cell detachment) may favour the process of anoikis and trigger frank apoptosis [9, 41]. Reduced adhesion-induced apoptosis may have a crucial role on the fate of detaching podocytes [9]. In other words, HBx-induced podocyte apoptosis may be downstream of decreased cell adhesion.

In conclusion, this study found that HBx reduces the adhesion and increases the apoptosis of podocytes. HBx also reduced expression of $\alpha 3 \beta 1$ integrin, and the downregulation of $\alpha 3 \beta 1$ integrin is sufficient to reduce podocyte adhesion. HBx-induced podocyte apoptosis may be a downstream effect of decreased cell adhesion, although we cannot exclude the possibility that downregulation of $\alpha 3 \beta 1$ integrin directly induces podocyte apoptosis. We suggest that future work should seek to further understand these relationships. Our results strongly suggest that HBx is involved in the HBV-induced injury of podocytes. A limitation of this study is that we did not directly examine the molecular mechanism underlying the HBx-induced down-regulation of integrin. Our future work will investigate the mechanism by which HBx causes down-regulation of integrin $\alpha 3 \beta 1$, and will attempt to extend these studies to *in vivo* models.

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Disclosure Statement

The results presented in this paper have not been published previously in whole or part, except in abstract format. The authors declare that there are no conflicts of interest.

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