Original Paper

Hepatitis B Virus X Protein Modulates Apoptosis in NRK-52E Cells and Activates Fas/FasL Through the MLK3-MKK7-JNK3 Signaling Pathway

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Key Word

HBx • MLK3 • MKK7 • JNK3 • NRK-52E cell • Apoptosis

Abstract

Background/Aims: The hepatitis B virus X protein (HBx) contributes to HBV-induced injury of renal tubular cells and induces apoptosis *via* Fas/FasL up-regulation. However, the mechanism of Fas/FasL activation is unknown. Recent studies indicated that HBx induction of apoptosis in hepatic cells depends on activating the MLK3-MKK7-JNKs signaling module, which then up-regulates FasL expression. In this study, we used NRK-52E cells transfected an HBx expression vector to examine the role of the MLK3-MKK7-JNKs signaling pathway on HBx-induced renal tubular cell injury. **Methods:** NRK-52E cells were transfected with pc-DNA3.1(+)-HBx to establish an HBx over-expression model, and with pc-DNA3.1(+)-HBx and pSilencer3.1-shHBx to establish an HBx low expression model. One control group was not transfected and another control group was transfected with an empty plasmid. Cell proliferation was determined by the formazan dye method (Cell Counting Kit-8) and apoptosis was measured by flow cytometry and fluorescence microscopy. Western blotting was used to measure the expression of Fas, FasL, and MLK3-MKK7-JNKs signaling pathway-related proteins. The activity of caspase-8 was measured by spectrophotometry. **Results:** Transfection of NRK-52E cells with pc-DNA3.1(+)-HBx inhibited cell proliferation and increased apoptosis and caspase-8 activity. The expression of Fas, FasL, and MLK3-MKK7-JNKs signaling pathway-related proteins were also greater in the pc-DNA3.1(+)-HBx group, but lower in RNAi group. Furthermore, the activity of MLK3-MKK7-JNKs signaling pathway, expression of Fas/FasL, and apoptosis were significantly lower in the pc-DNA3.1(+)-HBx group when treated with K252a, a known inhibitor of MLK3. **Conclusions:** Our results show that HBx induces apoptosis in NRK-52E cells and activates Fas/FasL via the MLK3-MKK7-JNK3-c-Jun signaling pathway.

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Introduction

An estimated 350 to 400 million people worldwide have chronic human hepatitis B virus (HBV) infections, and research indicates that China, Southeast Asia, and tropical Africa are highly endemic areas [1]. HBV-associated glomerulonephritis (HBV-GN) is the most common extrahepatic condition caused by HBV infection [2]. HBV-GN has severe consequences and is the primary cause of secondary renal damage among children in China [2]. Although there is evidence that the pathogenesis of HBV-GN is related to immune complex deposition [3], some reports have identified HBV-DNA and HBV-RNA in renal tubular cells, suggesting a direct virus-induced mechanism may contribute to disease progression [4, 5].

The HBV genome contains four 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 HBV replication, cellular transcription, signal transduction pathways, proteasome activity, cell cycle progression, and apoptosis [6]. The mechanism by which HBx regulates apoptosis seems to vary according to cell type and environment [7]. Apoptosis plays a pivotal role in many types of diseases [8-11]. Our previous studies of the pathology of renal tissues of patients with HBV-GN indicated that apoptosis occurs mainly at the proximal and distal ends of the tubular epithelial cells, and only rarely in the glomeruli [12]. We also found that transfection of the HBx eukaryotic expression vector into *in vitro* cultured renal tubular epithelial cells induced renal epithelial apoptosis *via* Fas/FasL up-regulation [13]. However, the upstream signal pathway responsible for Fas/FasL activation in tubular epithelial cells is still unknown.

The key components of apoptosis in neurons include mitogen-activated protein kinase kinase kinases (MAPKKK), mitogen-activated protein kinase kinase (MAPKK), upstream kinases of c-Jun N-terminal protein kinases (JNKs), and downstream substrates [14]. An important component of this pathway is the mixed-lineage kinase (MLK) family of kinases, which activate signal transduction pathways that induce neuronal cell death [15]. This family has three subgroups: MLKs (MLK1-4), dual leucine zipper-bearing kinases (DLKs), and Zipper Sterile-a-Motif Kinases (ZAKs) [16-18]. Studies of ischemic brain injury provide evidence that the ischemia-stimulating factor can activate the MLK3-MKK7-JNKs signaling module to activate the death receptor pathway, leading to apoptosis of neural cells [19, 20]. Similarly, an *in vitro* study of liver cells indicated that HBx induces apoptosis due to activation of the MLK3-MKK7-JNKs signaling module, which up-regulates FasL expression. Our previous research [13] showed that HBx inhibits proliferation and induces apoptosis *via* Fas/FasL up-regulation in rat renal tubular epithelial cells. Therefore, it is important to determine if HBx also activates the MLK3-MKK7-JNKs signaling module and induces apoptosis in renal tubular epithelial cells.

In this study, we performed *in vitro* experiments with renal cells that were transfected with HBx to examine the role of the MLK3-MKK7-JNKs signaling module on the up-regulation of FasL protein expression and apoptosis in renal tubular epithelial cells. K252a is an alkaloid that is a potent inhibitor of tyrosine kinases (Trks), but two K252a analogs (3,9-bis[(alkoxy)methyl]-252a and 3,9-bis[(alkylthio)methyl]-K252a) potent, selective, survival-promoting agents due to their inhibition of MLK activation [21]. Thus, we also examined the effect of K252a on cell apoptosis induced by HBx through inhibition of the MLK3-MKK7-JNKs pathway.

Materials and Methods

Reagents

Rat renal tubular epithelial cells (NRK-52E) were obtained from the China Center for Type Culture Collections (Wuhan, China). All reagents for cell culture (Dulbecco's modified Eagle medium [DMEM], fetal bovine serum [FBS] and so on) were purchased from Invitrogen (Carlsbad, CA, USA). Rabbit polyclonal anti-c-Jun, mouse monoclonal anti-p-JNKs, rabbit polyclonal anti-MLK3, mouse monoclonal anti-p-c-Jun,

rabbit polyclonal anti-Fas and rabbit polyclonal anti-FasL were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Rabbit polyclonal anti-JNKs, rabbit polyclonal anti-p-MLK3, mouse monoclonal anti-p-MKK7 and rabbit polyclonal anti-MKK7 were purchased from Cell Signaling Technology (Danvers, MA, USA). Mouse monoclonal anti-HBx antibody was purchased from Chemicon (Temecula, CA, USA). CCK-8 was purchased from Nanjing KGI Biological Technology Development Co., Ltd (Nanjing, China). Hoechst 33342, Caspase8 activity assay kit and K252a were purchased from Sigma-Aldrich (St. Louis, MO, USA). 20 $\mu\text{mol/L}$ stock solution of K252a were prepared in dimethyl sulfoxide (DMSO) and stored at -20°C . K252a were prepared freshly for each experiment by serial dilution into 10nM/L in DMEM. An Annexin V- FITC (Fluorescein Isothiocyanate) and propidium iodide (PI) double staining kit were purchased from Key Gene (Nanjing, China). K252a was purchased from Cell Signaling Technology (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated secondary antibody and the enhanced chemiluminescence (ECL) Western blotting kit were purchased from Santa Cruz Biotechnology. All other chemicals were obtained from Sinopharm Chemical Reagent Co., Ltd. (Shenyang, China).

Plasmids construction

Full-length HBx was PCR amplified from the p1.2II plasmid (HBV adr genome) as described previously [12, 13]. All ligated vectors were confirmed by sequence analysis.

To construct the expression vector for shRNA targeting HBx, pSilencer3.1-shHBX, two chemically synthesized oligonucleotides encoding HBx specific shRNA with the following sense sequences: 5'-GATCCGGTCTTACATAAGAGGACTTCAAGAG AAGTCCTTATGTAAAGACCTTTTGGAAA-3' (Ambion, Austin, TX).

Cell culture and plasmid transfection

NEK-52E cells were cultured in DMEM containing 4.5 mM glucose, 10% FBS, 100 U/mL penicillin and 100 $\mu\text{g/mL}$ streptomycin at 37°C in a humidified atmosphere of 5% CO_2 and 95% air. The culture medium was changed every day. Cells for assays were detached from the medium with a solution of 0.25% trypsin and 0.02% EDTA. Before treatment, 80-85% of confluent cells were incubated with serum-free media for 12 h to arrest the cell cycle and synchronize growth.

Lipofectamine™ LTX and PLUS™ transfection reagents (Invitrogen, USA) were used to conduct the transfection according to the steps described in the kit instructions. In all experiments, NEK-52E cells were transfected with empty plasmid pcDNA3.1(+), pcDNA3.1-HBx, or cotransfected pcDNA3.1(+)-HBx with pSilencer3.1-shHBx in a ratio of 1:3. And no transfected NEK-52E cells were as control group. In our experiment, 6 wells repetitions were set in each group in every experiment. All experiments were repeated three times.

Cell viability assay

The effect of the inhibition of the HBx gene on the proliferation of NEK-52E cells was determined by CCK-8 (Nanjing KGI Biological Technology Development Co., Ltd.) [13]. After cells in the logarithmic growth phase were digested and collected and prepared into a cell suspension with a concentration of $1 \times 10^5/\text{ml}$, cell suspension was inoculated into three 96-well plates (100 $\mu\text{l/well}$) and incubated overnight. Observations under a microscope confirmed that the cells attached to the wall of the well. Cells in each group were treated accordingly. At 2 h before culture completion CCK-8 (10 μl) was added into each well, and the cells were continuously cultured at 37°C for 2 h. At last, the optical density value (OD) for each well was determined with an ELISA reader at 450 nm. The experiment was conducted in triplicate. The cellular survival rate and proliferation inhibition rate were calculated according to the following formulas: Survival rate (%) = (OD value of the test group/OD value of the control group) $\times$ 100. Inhibition rate (%) = (1 - survival rate) $\times$ 100.

Caspase-8 activity assay

The activity of caspase-8 was measured with the Caspase8 Activity Assay Kit (Sigma, St. Louis, MA, USA) according to the manufacturer's protocol. Briefly, 2×10^6 cells were centrifuged at 1200 rpm for 5 min and were washed two times with PBS (pH 7.4) at 4°C . Cells were re-suspended in 50 μL cell lysis buffer and all subsequent steps were performed on ice. The protein concentration was measured using a micro BCA kit. Each 50 μL cell extract (containing 100 μg protein) was combined with equal volumes of $2 \times$ reaction buffer in a microplate, and 5 μL of peptide substrates of caspase-8 was added. After overnight incubation in the

dark at 37°C, samples were examined by a microplate reader at 405 nm. Caspase-8 activity was calculated as the absorbance ratio of treated/control samples.

Nuclear Extraction

3×10^6 cells were centrifuged at 1200 rpm for 5 min and were washed two times with PBS (pH 7.4) at 4°C. Then, they were re-suspended in a 10× volume of nuclear isolation buffer (15 mM Tris-HCL pH 7.5, 60 mM KCl, 15 mM NaCl, 5 mM MgCl₂, 1 mM CaCl₂, 250 mM Sucrose, 1 mM DTT, 1×HALT phosphatase inhibitor, 20 μM Thiamet G, 10 mM sodium butyrate and 0.3% NP-40), gently pipetted up and down, and incubated on ice for 5 min to disrupt the membranes. The nuclear pellets were extracted in a buffer consisting of 20 mM HEPES (pH 7.5), 20% glycerol, 420 mM NaCl, 0.5 mM MgCl₂, 1 mM EDTA, 1 mM EGTA, and 1 mM 1,4-dithiothreitol. Enzyme inhibitors were then added with 60 min of constant agitation at 4°C. After centrifugation (14,000 g for 15 min at 4°C), nuclear proteins were collected and concentrations were determined.

Western blot analysis

After the cells were harvested, total cell protein was extracted with RIPA lysate containing PMSF. The protein concentrations were determined by the bicinchoninic acid protein method (Biyuntian Company) and each sample was adjusted to a protein content of 40 μg. After an equivalent amount of the sample was added, electrophoretic separation was conducted respectively on SDS-PAGE gel. Subsequently, the proteins were transferred to polyvinylidene difluoride-plus membranes with a semi-dry protein transfer system. The membranes were blocked with 5% non-fat dry milk or 5% bovine serum albumin in Tris-buffered saline (TBS; pH 7.4) with 0.21% Tween-20 (TBST) for 1 h at room temperature, and washed six times (10 min each) in TBST. After mounting, the primary antibodies: HBx (1:1,000), MKK7(1:1000), p-MKK7(1:1000), MLK3(1:500), p-MLK3(1:1000), JNKs(1:1000), p-JNKs(1:500), Fas(1:500), FasL(1:500), c-Jun(1:500) or p-c-Jun(1:500) were respectively added and incubated in a table concentrator at 4°C overnight. The membranes were washed six times (10 min each) in TBST. Next, the secondary antibody was added to carry out the ECL reaction. Then, membranes were washed for 30 min with TBST. Protein levels were visualized with an ECL kit. Membranes were also treated with an anti-b-actin antibody as an internal loading control.

Hoechst 33342 staining

Morphological variations of the apoptotic cells were observed by staining the nuclei with H033342. Cells were stained with Hoechst 33342 (5mg/l) for 8 min in the dark and rewashed with PBS three times. Observations and imaging were immediately conducted under a fluorescence microscope. Two hundred cells were counted on each coverslip and were classified as normal (nuclei with smooth and defined boundaries) or apoptotic (nuclei with condensed or fragmented chromatin). Apoptosis rate (%) = (the number of apoptosis/200) × 100.

Flow cytometry analysis

The percentage of apoptotic cells was determined by flow cytometry using an FITC Annexin V Apoptosis Detection kit. NRK-52E cells were plated into 6-well plates (5×10^5 /ml) and cultured overnight. After transfected with different plasmid, cell apoptosis was analyzed by a FACScan flow cytometer (Becton Dickinson, San Jose, CA, USA). The cells were digested and centrifuged, washed with PBS for three times and suspended in 500 μl binding buffer (1×) to form a cell suspension with a concentration of $\sim 1 \times 10^6$ cells/ml. After 5 μl Annexin V-FITC was added, the cell suspension was gently mixed well and incubated in a refrigerator at 4°C for 30 min (protected from light). Subsequently, 5 μl PI was added, and the resulting cell suspension was gently mixed well and incubated in a refrigerator at 4°C for 15 min (protected from light). Immediately, cell testing was conducted using flow cytometry (within 1 h at latest), and analysis of data was carried out by CellQuest Professional software. 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 showed that the rate of apoptosis was the sum of the early apoptosis rate and the late apoptosis rate.

Statistical analysis

Experimental data are expressed as means ± standard deviations (SDs) and analyzed with SPSS version 13.0. Results were compared using One-way 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

Expression of HBx in NRK-52E cells

We initially transfected NEK-52E cells with pcDNA3.1(+) (an empty plasmid), pcDNA3.1(+)-HBx (HBx group), or pcDNA3.1-HBx with pSilencer3.1-shHBx in a ratio of 1:3 (HBx+RNAi group) (Fig. 1). Western blotting indicated no expression of HBx in cells that were not transfected and in cells transfected with empty plasmids (control groups). However, cells in the HBx group had a prominent protein band at 17 kD, corresponding to the HBx protein. Cells in the HBx+RNAi group had lower expression of HBx than those in the HBx group.

Effect of HBx on activation of the MLK3-MKK7-JNKs signal module and expression of Fas and FasL

We examined the effect of HBx on the phosphorylation of proteins in the MLK3-MKK7-JNKs signaling module (Fig. 2). The results indicate that the HBx group had increasing levels of phosphorylated JNKs, MLK3, and MKK7 over time, whereas cells in the two control groups had no such changes (Fig. 2A, B). The HBx+RNAi group and the HBx+K252a group had lower phosphorylation levels of these 3 proteins than the HBx group (Fig. 2C, D). Furthermore, the HBx+RNAi+K252a group had lower phosphorylation levels of these 3 proteins than the HBx+RNAi group and the HBx+K252a group (Fig. 2C, D).

These treatments had similar effects on the expression of Fas and FasL proteins (Fig. 2E, F). Thus, the HBx group had elevated expression of Fas and FasL, the HBx+RNAi group and the HBx+K252a group had lower levels than the HBx group, and the HBx+RNAi+K252a group had lower levels than the HBx+RNAi group and the HBx+K252a group.

Control experiments indicated that DMSO (the carrier for K252a) had no effect, and that K252a and DMSO treatment had no effect on the protein levels of the non-phosphorylated forms of JNKs, MLK3, and MKK7.

Effect of HBx on c-Jun activation

The c-Jun protein is the nuclear substrate of JNK. Thus, we examined the effect of HBx on phosphorylation and expression of c-Jun using the same treatments described above (Fig. 3A, B). As above, the HBx group had the highest level of phosphorylated c-Jun, the HBx+K252a group and the RNAi+HBx group had lower levels than the HBx group, and the

Fig. 1. Effect of different treatments on expression of the HBx protein in NRK-52E cells. (A) Representative western blots of HBx expression in control cells (no transfection), cells transfected with an empty pc-DNA3.1(+) plasmid, cells transfected with pc-DNA3.1(+)-HBx (HBx group), and cells cotransfected with pc-DNA3.1(+)-HBx and pSilencer3.1-shHBx (HBX+RNAi group). (B) Quantitation of the western blotting results (3 replicates per treatment). ^a $p < 0.05$ relative to control group. ^b $p < 0.05$ relative to HBx group.

HBx+RNAi+K252a group had lower levels than the HBx+K252a group and the RNAi+HBx group. Again, DMSO had no effect, and K252a and DMSO had no effect on the protein levels of non-phosphorylated c-Jun.

Effect of HBx on proliferation of NRK-52E cells

We also examined the effect of HBx on the proliferation of NRK-52E cells (Fig. 4, Tab. 1). There was no significant difference in the survival of non-transfected cells and cells transfected with empty plasmids. Relative to these controls, the HBx group had (27.14 ± 2.14) % inhibition of proliferation, the HBx+RNAi group had (18.21 ± 2.16) % inhibition, and the HBx+RNAi+K252a group had (8.17 ± 1.82) % inhibition.

Mechanism of HBx-induced apoptosis of NRK-52E cells

We further examined the effect of HBx on apoptosis by use of fluorescence microscopy, flow cytometry, and measurement of the activity of caspase-8 (a key protein in apoptosis) (Fig. 5, Table 1). Fluorescence microscopy indicated chromatin condensation and nuclear fragmentation in the HBx group, but fewer such changes in the HBx+RNAi group and the HBx+RNAi+K252a group (Fig. 5A). Quantitation of apoptosis by flow cytometry indicated the apoptosis was (8.41 ± 1.23) % in non-transfected cells and (7.83 ± 1.79) % in cells transfected with empty plasmids. Relative to these controls, the number of apoptotic cells was about 4-fold greater in the HBx group, about 3-fold greater in the HBx+RNAi group, and about 2-fold greater in the HBx+RNAi+K252a group (Fig. 5B-C).

Similar to the results above, the HBx group had a greatly increased caspase-8 activity, and this level was slightly lower in the HBx+RNAi group, and much lower in the HBx+RNAi+K252a group (Fig. 6, Table 1).

Discussion

HBV-GN is the most prevalent extrahepatic lesion caused by HBV infection [13]. Initial descriptions of the pathogenesis of HBV indicated the presence of circular viral genomes and glomerular deposition of *in situ* immune complexes of HBV antigens and antibodies. More recently, observations of direct HBV damage to kidney cells have been attributed to the pantropicity of this virus [22]. HBx is a multifunctional protein that regulates numerous cellular signal transduction pathways, including those that modulate apoptosis. However, the effect of HBx on apoptosis seems to depend on the type of cell and the experimental conditions [23].

Our previous study showed that NRK-52E cells transfected with pc-DNA3.1(+)-HBx underwent increasing apoptosis over time and that HBx induced apoptosis *via* Fas/FasL

Fig. 3. Effect of different treatments on the levels of c-Jun and phosphorylated c-Jun. (A) Representative western blotting results for each group at 48 h after transfection. (B) Quantitation of the western blotting results (3 replicates per treatment). ^a*p* < 0.05 relative to the control group. ^b*p* < 0.05 relative to HBx group. ^c*p* < 0.05 relative to HBx+RNAi group.

Fig. 4. Effect of different treatments on the inhibition of cell proliferation (3 replicates per treatment) at 48 h after transfection. ^a*p* < 0.05 relative to the control group. ^b*p* < 0.05 relative to the HBx group. ^c*p* < 0.05 relative to HBx+RNAi group.

Fig. 5. Effect of different treatments on apoptosis. (A) Representative nuclear staining (Hoechst 33342) and fluorescence microscopy ($\times 400$) results of each group at 48 h after transfection. (B) Flow cytometry analysis of apoptosis in each group at 48 h after transfection. (C) Quantitation (3 replicates per treatment) of the flow cytometry results. ^a $p < 0.05$ relative to the control group. ^b $p < 0.05$ relative to the HBx group. ^c $p < 0.05$ relative to HBx+RNAi group.

upregulation [13]. However, the mechanisms by which HBx activates the Fas/FasL pathway were unclear. Further elucidation of signaling pathway(s) in HBx-induced apoptosis would help us to better understand the role of HBx in the development of HBV-GN. The present study investigated the possible mechanism of HBx-induced apoptosis in rat renal tubular epithelial (NRK-52E) cells. In particular, we established a model in which NRK-52E cells were transfected with the HBx gene, and then examined the effect of HBx on apoptosis and expression of Fas and FasL proteins. Compared with cells transfected with an empty plasmid and untransfected cells, HBx-transfected NRK-52E cells exhibited a significant increase in apoptosis and upregulation of Fas and FasL protein expression. RNA interference which targeted HBx reduced cell

apoptosis and down-regulated the expression of Fas and FasL. These results confirm that HBx can induce apoptosis by up-regulating the Fas/FasL signaling pathway in NRK-52E cells.

We also investigated factors upstream of Fas/FasL in the apoptosis signaling pathway of NRK-52E cells. All MLK family members and ASK1 regulate JNK signaling by phosphorylation of MLK3 and MKK7. MLK3 and MKK7 are dual-specificity kinases that phosphorylate tyrosine and threonine residues in the catalytic domains of JNKs [24]. Recent studies have found

that the MLK3-MKK7-JNKs signaling module can activate the downstream Fas/FasL pathway and induce apoptosis in neuronal cells [19, 20, 25]. These experiments also suggested that K252a may play a neuroprotective role in preventing ischemic injury *via* inhibition of the MLK3-MKK7-JNKs pathway. However, the role of MLK3-MKK7-JNKs signaling in the HBx-induced apoptosis of renal tubular epithelial cells has not been reported.

These previous findings motivated our examination of the expression and phosphorylation levels of MLK3, MKK7, and JNKs following different treatments of NRK-52E cells. In fact, HBx increased the phosphorylation

levels of these proteins and the expression of Fas/FasL. Silencing HBx by RNAi or by K252a treatment lowered the phosphorylation levels of MLK3, MKK7, and JNKs and also lowered the expression of Fas/FasL, and these inhibitory treatments had a stronger effect when given together than separately. The rate of cell apoptosis and caspase-8 activity correlated with the phosphorylation levels of MLK3-MKK7-JNKs module proteins and the expression of Fas/FasL. These results suggest that HBx induces the apoptosis of NRK-52E cells through activating the MLK3-MKK7-JNKs signaling module and its downstream Fas/FasL death receptor pathway. Thus, our results provide a new avenue for research and suggest possible targets for the treatment of HBV-GN.

The downstream mechanism of JNK3-mediated apoptosis may include the induction of proteins in the c-Jun and Bcl-2 families. Apoptosis is associated with alteration of gene expression, and c-Jun is one of the earliest up-regulated genes. Activated JNKs specifically phosphorylate the N-terminal activation domain of transcription factor c-Jun at serine 63 and 73, thereby increasing the transcriptional activity of c-Jun [25-27]. Recent studies reported that MLK3 can induce c-Jun activation in neurons through the MLK3-JNK signaling module [28, 29]. This motivated us to measure the effect of HBx on the levels of phosphorylated c-Jun and c-Jun in NRK-52E cells. Our results demonstrated that HBx activated JNK, and that the activated JNK translocated into the nucleus where it phosphorylated the transcription factor c-Jun, leading to increased tubular epithelial cell apoptosis. Furthermore, the activation of JNK may enhance Fas/FasL expression *via* c-Jun.

In summary, our study demonstrated that HBx induces the apoptosis of NRK-52E cells and activates Fas/FasL through phosphorylation of proteins in the MLK3-MKK7-JNK3-c-Jun signaling pathway. These results provide novel insight into the mechanism of HBx-induced renal tubular epithelial cell apoptosis. We suggest that future work should extend these findings to other cell lines and *in vivo* models.

Table 1. The level of percentage of inhibition, apoptosis rate and caspase-8 activity in every group ($\bar{x} \pm s$)

Group	percentage of inhibition (%)	apoptosis rate (%)		Caspase-8 activity
		Ho333342	FCM	
Control	0.00±0.00	7.24±1.98	8.41±1.23	1.00±0.00
pcDNA3.1(+)	1.97±0.32	8.37±2.89	7.83±1.79	1.21±0.37
HBx	27.14±2.14	29.64±5.52	34.33±4.27	14.16±3.73
RNAi	18.21±2.16	21.04±3.78	20.16±3.18	8.64±2.88
RNAi+252a	8.17±1.82	13.07±3.72	14.80±2.08	4.26±1.26

Fig. 6. Effect of different treatments on the level of caspase-8 activity (3 replicates per treatment) at 48 h after transfection. ^a $p < 0.05$ relative to the control group. ^b $p < 0.05$ relative to the HBx group. ^c $p < 0.05$ relative to HBx+RNAi group.

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Disclosure Statement

None declared.

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