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Hepatitis B virus X protein up-regulates tumor necrosis factor- α expression in cultured mesangial cells via ERKs and NF- κ B pathways

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PEER REVIEW

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Comments

It is a good study in which the author investigated the effect of HBx protein on the expression of TNF- α in glomerular mesangial cells and the underlying intracellular signal pathways. The results are interesting and suggests that HBx protein can up-regulate TNF- α expression.

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ABSTRACT

Objective: To investigate the effects of hepatitis B virus (HBV) X protein (HBx) on the expression of tumor necrosis factor- α (TNF- α) in glomerular mesangial cells (GMCs) and the underlying intracellular signal pathways. **Methods:** The plasmid pCI-neo-X that carries the X gene of hepatitis B virus was transfected into cultured GMCs. HBx expression in the transfected GMCs was assessed by Western-blot. TNF- α protein and mRNA were assessed by ELISA and semi-quantitative RT-PCR, respectively. Three kinase inhibitors-U0126, an inhibitor of extracellular signal-regulated kinases (ERKs); lactacystin, an inhibitor of nuclear factor- κ B (NF- κ B); and SB203580, a selective inhibitor of p38 MAP kinase (p38 MAPK) were used to determine which intracellular signal pathways may underlie the action of HBx on TNF- α expression in transfected GMCs. **Results:** A significant increase in HBx expression in pCI-neo-X transfected GMCs was detected at 36 h and 48 h, which was not affected by any of those kinase inhibitors mentioned above. A similar increase in the expression of both TNF- α protein and mRNA was also observed at 36 h and 48 h, which was significantly decreased in the presence of U0126 or lactacystin, but not SB203580. **Conclusions:** HBx upregulates TNF- α expression in cultured GMCs, possibly through ERKs and NF- κ B pathway, but not p38 MAPK pathway.

KEYWORDS

Heptitis B virus, X protein, Nuclear factor- κ B, Tumor necrosis factor- α . Glomerulonephritis, Extracellular signal-regulated kinase

1. Introduction

Glomerulonephritis is a well-known complication of chronic hepatitis B virus (HBV) infection[1–3]. About 30% hepatitis B patients have progressive glomerulonephritis in the presence or absence of any anti-hepatitis treatment[4,5]. Almost all hepatitis B virus-associated nephropathy (HBV-GN) are membrane nephropathy[6]. Mesangial proliferation and sclerosis are also commonly reported[7–9]. In few cases, membrane proliferative glomerulonephritis is reported

with significant mesangial cell interposition, glomerular basement membrane reduplication, and subendothelial glomerular deposits[10]. Strikingly, HBV DNA can be detected in various proliferative glomerulonephritis, including type I and type III membrane proliferative glomerulonephritis (MPGN) as well as crescentic glomerulonephritis[10], suggesting that HBV infection may contribute to the development of glomerulonephritis. However, the related studies and the underlying mechanisms are largely unclear.

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Previous studies have shown that the HBV X (HBx) gene plays a important role in promoting cell proliferation and blocking apoptosis in human liver normal cells and tumor cells^[11–14]. Given the important role of tumor necrosis factor α (TNF- α), a proinflammatory factor, in the development of various kidney diseases such as hyperproliferation of mesangial cells and renal injury^[15–18], we hypothesized that HBx might increase TNF- α expression, and therefore, contribute to the development of glomerulonephritis.

Therefore, in the present study, we investigated the effects of HBx on TNF- α expression in cultured glomerular mesangial cells (GMCs). To determine the underlying mechanisms, we further observed the effects of several compounds that selectively inhibit extracellular signal-regulated kinases (ERKs), nuclear factor- κ B (NF- κ B) or p38 MAP kinase (p38 MAPK) on HBx-induced changes in TNF- α expression. These kinases play different roles in different cells, promoting cell proliferation or apoptosis.

2. Materials and methods

2.1. Construction of HBV X gene-containing vector

The pCI-neo-X plasmid containing full-length X gene of HBV was constructed. The sense primer is 5'-CCG CTC GAG GTA TAC ATC ATT TCC ATG GC-3', and the antisense primer is 5'-CCG GAA TTC GAG ATG ATT AGG CAG AGG TG-3'. The full-length X gene of HBV fragment was from the wild type HBV and transferred into the *EcoR I/Xho I* site of pCI-neo vector.

2.2. Cell culture and plasmid transfection

GMCs were cultured in Dulbecco's modified Eagle's medium purchased from Sigma Aldrich (USA), containing 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 100 mg/L heated-inactivated fetal bovine serum (FBS) bought from Invitrogen (USA), in a humidified incubator with 5% CO₂ at 37 °C. When the cells were grown to 80% confluence, pCI-neo or pCI-neo-X plasmid was transfected into GMCs by lipofection technique^[16]. The transfected cells were called GMC-pCI-neo and GMC-pCI-neo-X cells, respectively. A mixture solution containing 2 mL serum-free opti-MEM (Invitrogen) (pre-warmed to 37 °C), 4 μ g plasmid DNA, 10 μ L lipofectamine 2000 were added into each well. After incubation for 6 h, 2 mL DMEM-0.5% FBS medium was

added into each well, while untransfected GMCs in serum-free opti-MEM were used as controls.

2.3. Western blot analysis

Proteins extracted from GMCs and GMC-pCI-neo-X were subjected to Western blot analysis using specific antibodies for β -actin (McAb, Boster, China) and HBx (Jingmei, China). In brief, the harvested cells ($\sim 2 \times 10^8$) were resuspended in 200 μ L of PBS and sonicated (5 times). Cellular proteins were separated by SDS-PAGE, and the proteins were electrotransferred onto nitrocellulose membrane. The membrane was then blocked with milk casein (50 mg/L in PBS) for 24 h, and then hybridized with each diluted primary antibody for 24 h at 4 °C. The membrane was then washed 4 times and incubated for additional 15 min with TBST (10 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl and 1 g/L Tween 20). Finally, the membrane was hybridized with HRP-conjugated secondary antibody. Specific immunoblotting bands were visualized by ECM-associated fluorography.

2.4. Semi-quantitative RT-PCR assays

Total RNA extracts obtained from GMCs, GMC-pCI-neo and GMC-pCI-neo-X, respectively, according to the manufacturer's protocol (Invitrogen, USA), were reverse-transcribed at room temperature for 10 min, and then at 42 °C for 60 min. Reaction was stopped by heat inactivation at 90 °C for 10 min. TNF- α and β -actin gene cDNA products were amplified by PCR with TNF- α and β -actin primers (Table 1). The cDNA samples were pre-denatured at 95 °C for 4 min, amplified by 25 cycles at 94 °C for 45 seconds, annealed at 53 °C for 45 seconds, and then extended at 72 °C for 5 min. The RT-PCR products were separated on 1% agarose gels and visualized by ethidium bromide staining.

2.5. ELISA test

TNF- α ELISA kit was purchased from Quantikine (China), TNF- α in supernatants of each well was measured by ELISA test, according to the manufacturer's instruction.

2.6. MTT assay

A MTT kit (Sigma Aldrich) was used to measure the metabolic activities of viable cells and the degree of cell proliferation.

Table 1

PCR primers and amplified base pair.

Cytokine	Primers	Amplified base pair
TNF- α	Sense: 5'-GGA TCA TCT TCT CAA AAC TCG-3'	419 bp
	Antisense: 5'-TCA CAG GAG CAA TGA CTC CAA A-3'	
β -actin	Sense: 5'-CCC ATC TAT GAG GGT TAC GC-3'	150 bp
	Antisense: 5'-TTT AAT GTC ACG CAC GAT TTC-3'	

Subconfluent monolayer GMCs were detached from the cultured dishes by trypsin and centrifuged at 12 000 r/min for 5 min. Cell pellets were re-suspended in fresh media (2.5×10^4 cells/mL), and then 200 μ L suspension was transferred into each well of a flat-bottomed 96-well plate. The cells were incubated at 37°C with 5% CO₂ for different time (6, 24, 36 or 48 h). At the end of incubation, cultured medium was replaced by 200 μ L fresh medium. MTT (2.5 mg dissolved in 50 μ L of dimethylsulfoxide) was then added to each well and incubated for 4 h at 37°C. To remove formed MTT-formazan crystals, 200 μ L DMSO and then 25 μ L glycine buffer (pH 10.5) were added to each well. Viable cells were detected by ELISA analyzer at 570 nm.

2.7. Signal pathway and their inhibitors

We selected three signal pathways (NF- κ B, ERKs and p38 MAPK) that related to TNF- α . The various pathways were inhibited respectively by their specific inhibitors, lactacystin (10 μ mol/L) for NF- κ B, U0126 (10 μ mol/L) for ERKs, and SB203580 for p38 MAPK.

2.8. Statistical analysis

All data were expressed as mean $\pm$ SEM. One-way ANOVA (with SPSS 11.0 software) was used to assess the significance for the differences between different experimental groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Expression of HBx and TNF- α in transfected cells

The pCI-neo-X plasmid was transfected into the GMCs *in vitro* to investigate the effects of HBV X gene on expression of HBx and TNF- α . Figure 1 shows the time course of transfection-induced changes (relative to β -actin) in HBx and TNF- α , demonstrating that both HBx and TNF- α levels increased significantly at 36 h and 48 h after the pCI-neo-X plasmid transfection.

Figure 1. Representative Western blot data, showing the time courses of the changes in the expression of HBx and TNF- α in GMCs after pCI-neo-X plasmid transfection.

3.2. Effects of inhibitors on HBx expression in transfected cells

Figures 2 and 3 show that incubation of the HBV X gene-

transfected GMCs with SB203580, U0126 or lactacystin failed to significantly alter the level of HBx expression in the HBV X gene-transfected GMCs.

Figure 2. Effects of SB203580, U0126 and lactacystin on HBx expression in the HBV X gene-transfected GMCs. -: without inhibitor; +: with inhibitor.

Figure 3. Averaged levels of the HBx expression in the HBV X gene-transfected GMCs (HBx levels are normalized over β -actin, $n=4$).

3.3. Effects of inhibitors on TNF- α mRNA in transfected cells

Figure 4 and 5 show that the HBV X gene transfection induced a significant increase in TNF- α mRNA at 36 h and 48 h after transfection (Lane 2 and Lane 7). This increase was significantly attenuated in the presence of lactacystin or U0126, but not SB203580 in the media. Figure 5 shows the averaged levels in TNF- α mRNA in the presence or absence of different kinase inhibitors at 36 h and 48 h after HBV X gene transfection.

Figure 4. Representative RT-PCR data showing the effects of different kinase inhibitors on TNF- α mRNA level in HBV X gene-transfected GMCs.

-: Without inhibitor; +: with inhibitor. M: Marker. Lane 1 and lane 6 show the GMC+pCI-neo control; the other lanes show TNF- α mRNA levels after successful GMC+pCI-neo-X transfection.

Figure 5. Averaged data of TNF- α mRNA level, indicating that U0126, lactacystin, but not SB203580, significantly attenuated TNF- α mRNA levels in HBV X gene-transfected cells. -: Without inhibitor; +: With inhibitor. Group 1 and group 6 refer to GMC+pCI-neo control. * $P < 0.05$, compared with the data shown in lane 2 or lane 7. TNF- α mRNA levels are normalized over β -actin ($n=4$).

3.4. Effects of inhibitors on TNF- α protein expression in transfected cells

Figure 6 shows both the ERKs inhibitor U0126 and the NF- κ B inhibitor lactacystin, but not the p38 MAPK inhibitor SB203580, significantly attenuated TNF- α level in the supernatants of HBV X gene-transfected MGCs.

Figure 6. Effects of the different kinase inhibitors on TNF- α expression as assessed by ELISA in HBV X gene-transfected cells ($n=4$). * $P < 0.05$, compared with the GMC+pCI-neo-X group.

4. Discussion

The major findings of the present study are that, first, HBV X gene infection (or transfection) significantly increases HBx and TNF- α expression in the transfected GMCs. Since the changes in both HBx and TNF- α expression displayed similar time courses it is suggested that increased TNF- α expression was secondary to an increase in HBx expression in the transfected cells. Second, blockade of intracellular ERK by U0126 or NF- κ B by lactacystin, but p38 MAPK by

SB203580, significantly attenuated HBx-induced TNF- α expression (at both protein and mRNA levels), suggesting that both the intracellular ERK and NF- κ B signal pathways may underlie the action of HBx on TNF- α expression. Given the important role of both ERK and NF- κ B in various inflammatory diseases, the present findings suggest that HBV infection may significantly contribute to the development of many kidney diseases such as glomerulonephritis.

The GMC proliferation can be seen in some HBV-GN. The mechanism underlying GMC proliferation remains unclear. HBV DNA was detected in the glomerular of HBV-GN. Previous studies have shown that HBx can promote cell proliferation or apoptosis under different experimental conditions[17,18]. The present study demonstrated that HBV X gene can be transfected into rat GMCs and functionally express HBx in these cells at 36 h to 48 h after transfection. In addition, transfection of HBV X gene also promoted the expression of TNF- α protein and mRNA as well as GMC proliferations. These data suggest that HBx can promote the expression of TNF- α in the GMCs, and therefore promoting GMC proliferation. This is consistent with our previous report[18].

Previous studies have shown that HBx can also activate many cell signal transduction pathways, including ERKs[19,20], p38[21] and NF- κ B[22,23]. Therefore, we further investigated whether these intracellular signal pathways also underlie HBx-induced TNF- α expression. For this purpose, we used the selective ERK inhibitor U0126, the p38 MAPK inhibitor SB203580, and the NF- κ B inhibitor lactacystin to block each signal pathway, respectively. We found that both U0126 and lactacystin significantly attenuated HBx-induced TNF- α expression and also GMC proliferation (data not shown), suggesting that both ERK and a NF- κ B pathways may underlie the action of HBx on TNF- α expression and the GMC proliferation. However, when SB203580 was used, HBx-induced TNF- α expression did not change, suggesting that p38 MAPK is not involved in HBx action in the present study.

Many kidney cells, including mesangial cells and tubular cells, can increase TNF- α expression under certain pathological conditions, while the basal level of TNF- α mRNA in GMCs is very low[24,25]. For example, administration of lipopolysaccharide or concanavalin A may increase TNF- α mRNA expression and GMC proliferation. It was reported that TNF- α may stimulate GMC proliferation via increasing IL-1 β and IL-6 expression in GMCs[26].

HBx activates Ras and rapidly induces a cytoplasmic signaling cascade linking Ras, Raf, and mitogen-activated protein kinase, leading to transcriptional transactivation. HBx strongly elevates levels of GTP-bound Ras, activated and phosphorylated Raf, and tyrosine-phosphorylated and activated MAP kinase. Transactivation of transcription factor AP-1 by HBx is blocked by inhibition of Ras or Raf

activities but not by inhibition of Ca^{2+} and diacylglycerol-dependent protein kinase C. HBx was also found to stimulate DNA synthesis in serum-starved cells. HBx protein therefore stimulates Ras-GTP complex formation and promotes downstream signaling through Raf and MAPK, and may influence cell proliferation[27].

NF- κ B/Rel constitutes a family of transcriptional factors involved in the regulation of numerous cytokine genes and immune responses in different cell populations. The most abundant form of NF- κ B is the p50-p65 heterodimer. The inactive form of NF- κ B exists in the cytoplasm bound to an inhibitory complex containing I- κ B α . Following cellular stimulation and chemotactic factors, NF- κ B inducible kinase phosphorylates and activates the I- κ B kinase (IKK) complex consisting of IKK-1 and IKK-2, which in turn phosphorylates the I- κ B α subunit. Phosphorylation, ubiquitination, and subsequent degradation of I- κ B α result in NF- κ B nuclear translocation. This allows the transcription factor to bind to promoter regions of specific pro-inflammatory genes and influence transcription. Because a number of potential κ B sites are present in the nucleotide sequences of the TNF- α gene, NF- κ B is considered to be a critical transcriptional factor involved in TNF- α gene expression and protein synthesis[28].

HBx protein stimulates the ERKs and NF- κ B. HBx activation of ERKs, leads to induction and activation of AP-1 DNA binding site and upregulate the TNF- α gene transcriptional activation, resulting in TNF- α high expression[29,30].

From above, we can supposed that GMCs proliferation was contributed to HBx. TNF- α is closely related to the GMCs proliferation[27]. The mechanism of the GMC proliferation by HBx may be associated with high expression of TNF- α through autocrine or paracrine of GMCs. The signaling mechanism of HBx inducing cell proliferation and TNF- α high expression are partly through ERKs and NF- κ B pathway, but not p38 MAPK pathway.

Conflict of interest statement

We declare that we have no conflict of interest.

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Comments

Background

Hepatitis is one of the important infectious diseases in the world. Glomerulonephritis is a complication of chronic hepatitis B virus infection. HBV X gene function involves in promoting cell proliferation and blocking apoptosis. This research investigated the effects of HBV X gene on TNF- α expression in cell culture via ERKs and NF- κ B pathways. These pathways involve many disease mechanisms. It is interesting to study about HBV X gene and TNF- α expression in these pathways.

Research frontiers

From my viewpoint, ERKs and NF- κ B pathways are studied in many important diseases. Study about HBV X gene expression in these pathway and the various kinase inhibitors may lead to know the complication of hepatitis infection and the related diseases. The study suggested that HBx can promote the expression of TNF- α in the GMCs, and therefore promoting GMC proliferation.

Related reports

This is consistent with their previous report. Other studies also have shown that HBx can activate many cell signal transduction pathways, including ERKs, p38 and NF- κ B. The author further investigated these intracellular signal pathways which underlie HBx-induced TNF- α expression. It would be better to assess for further correlations among Hepatitis infection/AIDS co-infected in ERKs and NF- κ B pathways

Innovations and breakthroughs

Data proved that the mechanism of the GMC proliferation by HBx may be associated with high expression of TNF- α through autocrine or paracrine of GMCs. The signaling mechanism of HBx inducing cell proliferation and TNF- α high expression are partly through ERKs and NF- κ B pathway, but not p38 MAPK pathway.

Applications

It's important to explore the mechanism of the GMC proliferation induced by HBx. Understanding the signaling mechanism of HBx inducing cell proliferation and the relationship with TNF- α expression underlie some experiment data for the further associated studies. This knowledge may be useful for drug experiment in the future. This study can be applied for studying about pharmacological HBV mechanism in ERKs and NF- κ B pathways. It may also be applied in drug therapy.

Peer review

This is a good study in which the authors explored the

effects of HBx on TNF- α expression in cultured GMCs, and discussed the underlying intracellular signal pathways. The results are interesting and suggest that HBx protein can up-regulate TNF- α expression.

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