A role for MHBs1167/HBx in hepatitis B virus-induced renal tubular cell apoptosis

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Abstract

Background. The pathogenesis of hepatitis B virus (HBV)-associated glomerulonephritis (HBVGN) is generally believed to be immune complex deposition. However, the presence of HBV-DNA and -RNA in HBVGN renal tissues suggested a direct virally induced injury. We previously showed that nuclear factor κB (NF-κB) was activated in HBVGN renal tissues, especially in tubular cells. We therefore investigated the role of NF-κB in tubular epithelial cells with HBV infection.

Methods. Nuclear translocation of NF-κB and alpha subunit of NF-κB inhibitor (IκBα) phosphorylation were assessed by immunodetection following transfection of HK-2 cells with mhbs1167 and/or hbx. Electrophoretic mobility shift assays (EMSA) and dual luciferase reporter assays (DLR) were used to further examine NF-κB activation following transfection. Hochest 33258 and NF-κB terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) double staining were used to detect apoptosis and the correlation between NF-κB activation and apoptosis. Protein kinase C (PKC) assay and ERK phosphorylation were assayed for a possible mechanism of NF-κB activation.

Results. Cells transfected with mhbs1167 and/or hbx increased NF-κB nuclear translocation, phosphor-IκBα, κB-DNA binding activity, κB-dependent transcription and apoptotic index compared to controls (P < 0.05). The nuclear distribution of NF-κB strongly correlated to cellular apoptosis. PKC activity and phosphor-ERK were also increased (P < 0.05) during the NF-κB activation process. However, all above parameters were diminished after pyrrolidine dithiocarbamate (PDTC)-incubation, a NF-κB inhibitor (P < 0.05).

Conclusion. MHBs1167/HBx-induced NF-κB activation via the PKC/ERK pathway in renal tubular cells undergoing apoptosis may be involved in virally induced pathogenesis.

Keywords: apoptosis; HBx; MHBs1167; NF-κB; renal tubular cells

Introduction

Hepatitis B virus (HBV) infections are prevalent in China, and this type of infection can result in the development of HBV-associated glomerulonephritis (HBVGN), which has become one of the most common causes of renal damage in Chinese children [1]. Although the pathogenesis of HBVGN is generally believed to be immune complex deposition [2], some reports that have identified HBV-DNA and -RNA in renal tubular cells suggested a direct virally induced mechanism may also play a part in the disease process [3,4].

HBV has two transcriptional activators, HBV X protein (HBx) [5] and C-terminal truncation of the middle surface antigen (MHBs) [6]. The full-length middle surface antigen (MHBs) is composed of a 55-amino acid (aa) preS2 domain and a 226-aa S domain. The generation of functional MHBs requires to truncate at least 70 aa from the C-terminal of preS2/S to the hydrophobic region III of the S domain [7]. Here, the MHBs1167 is truncated at 167 aa and belongs to the functional MHBs. Because MHBs and HBx proteins have no intrinsic DNA binding activity [8,9], they are believed to transactivate genes by recruiting host transcription factors such as nuclear factor κB (NF-κB) [10–12]. Although the mechanisms associated with the MHBs5- or HBx-induced NF-κB induction have not been fully described, some studies demonstrated that it may be related to PKC activation and the Ras/Raf/MEK/ERK pathways [13,14].

NF-κB is composed of the Rel/NF-κB protein family [15]. The prototype isoform of NF-κB consists of the p50–p65 subunits. NF-κB dimers are present in the cytosol in an inactive form bound to the inhibitory protein IκB. Cell activation by stimuli, e.g. cytokines, viruses or oxidants, promotes degradation of IκB and release of active NF-κB, resulting in targeted gene transcription [16]. Therefore, NF-κB is involved in multiple cellular processes, including immune cell activation, the stress response, expression of inflammatory cytokines and the control of apoptosis [17]. Apoptosis is associated with
cellular loss during both acute and chronic renal damage [18,19]. Our previous studies demonstrated that IκBα was degraded, and there was nuclear translocation of NF-κB in HBVGN renal tissues, especially in tubular cells [20].

However, in renal tubular epithelial cells, NF-κB transactivation and its effect on apoptosis are still unknown. To address this, the apoptotic index, NF-κB translocation and c-B control-transcription were studied after renal tubular cells had been transfected with the genes encoding for MHBst167 and/or HBx. The potential role of PKC and the extracellular-related kinase (ERK) in mediating NF-κB activation were also examined.

**Subjects and Methods**

**Plasmid construction**

For the construction of the plasmids pcDNA3.1 (+) mhbst167 and pcDNA3.1 (+) hbx, the genes were respectively amplified from the p1.2II plasmid (HBV adv genome). The primers were: mhbst167 forward primer, 5′-cccaagcttgctagggtgtgct3′; mhbst167 reverse primer, 5′-agggtctctctgctttagggc3′; hbx forward primer, 5′-gaggattcagagcgttgtgcc3′; and hbx reverse primer, 5′-tctgctctctgctttagggc3′. pGL3-promoter-(+β) contained five repeated β motifs (5′-gttcgagttcgc3′). The gel was photographed on a transilluminator and quantified. Enhanced chemiluminescence (ECL) detection solutions were applied. The scanned images were quantified using QuantaSoft (Pierce) were applied. The scanned images were quantified using QuantaSoft (Pierce). Nuclear extracts (3 μg) were incubated with the binding reaction mixture and the biotin-labelled NF-κB double-stranded probe (5′-agttgaggcttcgccg-3′). Following a 20-min incubation, the DNA–protein complexes were electrophoresed and transferred onto a Hybond N nylon membrane. Membranes were cross-linked under an UV light instrument for 1 min and incubated with stabilized Streptavidin–horseradish peroxidase conjugate (1:300) at 37°C for 30 min. The shift-binding bands were visualized using enhanced chemiluminescence, and the bands were quantified.

**Dual-luciferase reporter assays**

HK-2 cells were seeded in 24-well plates and transfected. Briefly, 90 ng of the pGL3-promoter-(+β) was co-transfected with different amounts (9, 18, 90, 450 and 900 ng) of plasmids mhbst167', hbx, mhbst167' + hbx or the empty vector pcDNA3.1(+) (the total amount was normalized with empty vector). Ten nanograms pRL-TK was co-transfected to monitor transfection efficiency. The relative luciferase activity (‘firefly’ luciferase/‘Renilla’ luciferase) was analysed 24 or 48 h later with the Dual Luciferase Kit (Promega).

**Hochest 33258 staining**

Following transfection for 24 h, HK-2 cells were fixed, stained with Hochest 33258 and observed using fluorescence microscope (Olympus, Tokyo, Japan). Cells were designated as apoptosis with highly condensed, brightly staining nuclei and non-apoptosis with light blue staining. The apoptotic index was defined as the ratio of the apoptotic cell number to total cell number.

**Preparation of cell extracts and western blot analysis**

The whole cellular lysates were prepared with radio-immunoprecipitation assay (RIPA) lysis buffer, and the cytosolic and nuclear fractions were separated according to the manufacturer's instruction (Pierce, Rockford, USA). Cellular extracts (30 μg) were separated by 10% SDS–PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The blots were probed with various antibodies (Santa Cruz Biotechnology): NF-κB p65 (1:600), IκBα (1:600), p-IκBα (1:600), β-actin (1:500), Raf-1 (1:200), ERK2 (1:600) and p-ERK1/2 antibodies (1:600). To visualize antibody-bound protein, the proper secondary antibodies (1:5000, Santa Cruz Biotechnology) and ECL detection solutions (Pierce) were applied. The scanned images were quantified using Quantity-One software (BioRad, Hercules, USA).

**Electrophoretic mobility shift assay**

The assays were performed using a Lightshift® Chemiluminescent EMSA Kit (Pierce). Nuclear extracts (3 μg) were incubated with the DNA–protein complexes were electrophoresed and transferred onto a Hybond N nylon membrane. Membranes were cross-linked under an UV light instrument for 1 min and incubated with stabilized Streptavidin–horseradish peroxidase conjugate (1:300) at 37°C for 30 min. The shift-binding bands were visualized using enhanced chemiluminescence, and the bands were quantified.

**Protein kinase C assay**

Cells were suspended in cold extraction buffer and homogenized using a cold dounce homogenizer. The lysate was centrifuged at 14 000 g for 5 min. PKC activity was measured in supernatants by the PepTag® non-radioactive detection kit (Promega) using the fluorescence peptide (PLSRTLSVAAK), which is a highly specific substrate for PKC. All samples were separated on a 0.8% agarose gel 100 V for 15 min. The gel was photographed on a transilluminator and quantified.

**Statistics**

Pearson’s and one-way analysis of variance (ANOVA) for multiple comparisons were carried out using the statistical software SPSS (version 12.0; SPSS, Chicago, USA). Statistical significance level was defined as P < 0.05.

**Results**

**NF-κB nuclear translocation increases following transfection with mhbst167 and/or hbx**

One feature associated with NF-κB activation is nuclear translocation. The effects of MHBst167 and/or HBx on...
NF-κB translocation in HK-2 cells were depicted in Figure 1. The nuclear NF-κB p65-positive ratio was significantly increased for $mhbs^{t167+}$, $hbx-$ and $mhbs^{t167+}hbx$-transfected groups (12.58% ± 0.543, 22% ± 0.477 and 30.76% ± 0.49, respectively) compared to the pcDNA3.1 (+)-transfected group (5% ± 0.358, $P < 0.05$).
(Figure 1A). Moreover, the mhbs<sup>167</sup> + hbx-co-transfected group had significantly increased nuclear NF-κB p65 levels compared to the mhbs<sup>167</sup>- or hbx-transfected alone groups (P < 0.05), and the nuclear NF-κB p65-positive ratios were inhibited following treatment with PDTC (P < 0.05).

To further investigate NF-κB translocation process, nuclear extracts were assayed for NF-κB p65 by western blot. The nuclear NF-κB p65 levels were increased 1.8- and 1.9-fold following mhbs<sup>167</sup> or hbx transfection, respectively (P < 0.05, Figure 1B). The NF-κB p65 levels of mhbs<sup>167</sup> + hbx-co-transfected cells were increased 2.1-fold which was significantly greater than that of mhbs<sup>167</sup>- or hbx-transfected cells (P < 0.05). As shown in Figure 1B (lane 7–10), the nuclear levels were also decreased significantly with PDTC treatment (P < 0.05), suggesting that MHBs<sup>167</sup> or HBx protein could increase NF-κB nuclear translocation.

IκBα phosphorylation and NF-κB expression levels were increased following transfection with mhbs<sup>167</sup> and/or hbx

No significant differences in the expression of the IκBα protein in whole cell extracts were detected in any treatment groups examined, however, the IκBα proteins were increased slightly after PDTC treatment (Figure 2). In contrast, phosphor-IκBα protein increased 1.44-, 1.52- and 2.03-fold following transfection with mhbs<sup>167</sup>, hbx or mhbs<sup>167</sup>+ hbx when compared to the empty vector-transfected group (P < 0.05, Figure 2). The phospho-IκBα protein was significantly increased in the mhbs<sup>167</sup> + hbx-co-transfected group when compared to the mhbs<sup>167</sup>- or hbx-transfected alone groups (P < 0.05), and phospho-IκBα protein was inhibited following PDTC treatment (P < 0.05). The NF-κB p65 protein level in the mhbs<sup>167</sup> and/or hbx-transfected groups were also significantly increased over the level observed in the pcDNA3.1 (+)-transfected group (P < 0.05, Figure 2), and the mhbs<sup>167</sup> + hbx-co-transfected group had detectable levels of NF-κB p65 greater than that observed for either the mhbs<sup>167</sup>- or hbx-transfected group (P < 0.05). In all groups, the NF-κB p65 levels were inhibited by PDTC (P < 0.05).

Enhancement of κB-specific DNA binding following transfection with mhbs<sup>167</sup> and/or hbx

To observe the DNA-binding activity of NF-κB following mhbs<sup>167</sup> and/or hbx transfection, κB-specific DNA–protein complexes were detected by EMSA. Similarly, the κB-specific binding complexes associated with the mhbs<sup>167</sup>- and hbx-transfected groups were significantly increased (P < 0.05) by 1.7- and 3.4-fold, respectively, compared
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to the pcDNA3.1 (+)-transfected group (Figure 3). The \( \kappa B \)-specific DNA–protein complex levels following \( mhbs \textsuperscript{167}+\ hbx \) co-transfection were significantly higher (5.9-fold, \( P < 0.05 \)) than the levels observed for the \( mhbs \textsuperscript{167} \)- or \( hbx \)-transfected group. PDTC treatment also reduced the complexes in all transfected groups (\( P < 0.05 \)).

Increased transcription of \( \kappa B \)-controlled genes following transfection with \( mhbs \textsuperscript{167} \) and/or \( hbx \)

Transactivation ability of \( \kappa B \) was assessed using plasmid dose-dependent and post-transfection time-dependent parameters. As shown in Figure 4A, the luciferase activity increased in correlation with the plasmid expression levels of \( mhbs \textsuperscript{167} \) and/or \( hbx \) (\( P < 0.05 \)). In Figure 4B, the luciferase activity in the \( mhbs \textsuperscript{167} \)- and/or \( hbx \)-transfected groups increased significantly over that observed for the pcDNA3.1 (+)-transfected group (\( P < 0.05 \)). The luciferase activity of \( mhbs \textsuperscript{167}+\ hbx \)-co-transfected group increased significantly over that of the \( mhbs \textsuperscript{167} \)- or \( hbx \)-transfected alone groups (\( P < 0.05 \)). However, there was no difference in the luciferase activity of the respective groups between 24- and 48-h post-transfection (\( P > 0.05 \)). Finally, the transcription process could also be inhibited by PDTC treatment (\( P < 0.05 \)).

Influence of MHBs\textsuperscript{167} and/or HBx on HK-2 cell apoptosis

In this study, the effect of MHBs\textsuperscript{167} and/or HBx on cellular apoptosis and its correlation with \( \kappa B \)-protein were assessed with Hoechst 33258 staining and \( \kappa B \)-protein/TUNEL double staining (Figure 5). The morphologic changes associated with apoptosis, including cell size reduction and nuclear chromatin condensation, and here, it was shown as bright-ly stained nuclei by Hochest 33258 staining and bright green color by TUNEL staining. In Figure 5A, compared to the pcDNA3.1 (+)-transfected group (3.17% apoptosis), the apoptotic index was significantly increased after \( mhbs \textsuperscript{167} \) (8.18%) or \( hbx \) (10.23%) transfection and the index in \( mhbs \textsuperscript{167}+\ hbx \)-co-transfected group was even greater (13.98%, \( P < 0.05 \)). After PDTC incubation, the apoptotic index of the \( mhbs \textsuperscript{167} \)- and/or \( hbx \)-transfected groups was significantly decreased (\( P < 0.05 \)). To determine whether apoptosis induced by MHBs\textsuperscript{167} and/or HBx was related to \( \kappa B \) activation, a correlation statistic analysis was applied. The apoptotic index of HK-2 cells was consistent with the nuclear \( \kappa B \)-p65 protein level expression (\( r = 0.841, P < 0.05 \)) and the \( \kappa B \)-specific binding activity (\( r = 0.894, P < 0.05 \)). It suggested that the apoptotic process induced by MHBs\textsuperscript{167} and/or HBx might be related to \( \kappa B \) activation.

In Figure 5B, compared to the pcDNA3.1 (+)-transfected group (3.37%), the apoptotic index was significantly increased after \( mhbs \textsuperscript{167} \) (8.48%) or \( hbx \) (12.27%) transfection, and the index in \( mhbs \textsuperscript{167}+\ hbx \)-co-transfected group was even greater (14.78%, \( P < 0.05 \)). After PDTC incubation, the apoptotic index were also significantly decreased (\( P < 0.05 \)). At the same time, the distribution of \( \kappa B \)-protein was detected by immunofluorescence. Figure 5B showed that apoptosis was stained green while \( \kappa B \)-p65 were stained red in cytoplasm and/or nucleus. The yellow staining in dual-labelling experiments indicated overlapping of red and green fluorescent labels, suggesting co-localization of apoptosis and \( \kappa B \) p65 staining in the nucleus. It suggested a possible correlation between \( \kappa B \) activation and the apoptosis process.

Involvement of the PKC/ERK pathway in MHBs\textsuperscript{167} and/or HBx-induced \( \kappa B \) activation

After \( mhbs \textsuperscript{167} \) and/or \( hbx \) transfection, PKC activity was slightly increased in comparison to the empty vector-transfected group (\( P < 0.05 \), Figure 6A); in contrast, the expression of \( \kappa B \)-p65 and phosphor-ERK proteins was also increased (\( P < 0.05 \), Figure 6B), and in the \( mhbs \textsuperscript{167}+\ hbx \)-co-transfected group, the levels were markedly increased compared to the \( mhbs \textsuperscript{167} \)- or \( hbx \)-transfected groups (\( P < 0.05 \)). Furthermore, PKC activity and the levels of phosphor-ERK and \( \kappa B \)-p65 protein were significantly reduced after treatment with the specific PKC inhibitor G66983 (\( P < 0.05 \), Figure 6). The ERK2 protein level in all transfected groups, however, was relatively uniform, and the Raf-1 protein in all groups was undetectable even after PMA treatment.
In hepatic studies, the MHBs<sup>1</sup> and HBx proteins were shown to act as transcriptional transactivators [25]. Both MHBs<sup>1</sup> and HBx appeared to act as pleiotropic transactivators without binding DNA directly [8,26], and a few studies indicated that MHBs<sup>1</sup> [10,14] and HBx [11,12] could affect NF-κB-mediated transactivation. Until now, the relationship between MHBs<sup>1</sup>/HBx and NF-κB activation in renal tubular cells has not been reported.

The transactivation factor NF-κB can be stimulated by various factors that typically result in the phosphorylation and degradation of Ik-B. NF-κB dimmers are then released from the NF-κB/IκB complex, translocate into the nucleus and initiate gene transcription [16,27]. Previously, we showed that there was nuclear translocation of NF-κB in HBVGN renal tissue, especially in renal tubular cells [20]. To investigate whether nuclear translocation of NF-κB was related to MHBs<sup>167</sup>/HBx, the nuclear NF-κB was investigated by immunocytochemistry and western blot analysis after transfection with the genes mhbs<sup>167</sup> and/or hbx. These results suggested that MHBs<sup>167</sup>/HBx could induce NF-κB nuclear translocation in vitro, which was consistent with the increase of NF-κB nuclear translocation in renal tissues from HBVGN patients. The increase in NF-κB nuclear translocation was accompanied by a parallel increase in phosphor-IκB levels, confirming that NF-κB translocation had occurred.

To further identify whether nuclear NF-κB was activated, we detected the NF-κB-specific DNA–protein complex by EMSA and κB–luciferase activity using DLR. EMSA is the most common technique for detecting the transactivation of transcription factors by detecting DNA–protein-binding interactions, and DLR directly shows the transactivation activity of target genes by measuring their relative luciferase intensity. The EMSA results showed that κB-binding ability was significantly higher in mhbs<sup>167</sup> and/or hbx-transfected groups than in empty vector-transfected cells. The DLR results were similar to the EMSA findings, and showed a dose- but not a time-dependent effect of MHBs<sup>167</sup> and/or HBx on NF-κB activation. Our current results indicated that κB-dependent transactivation was increased, and NF-κB could be activated by MHBs<sup>167</sup> or HBx. In addition, since the NF-κB promoter also contained κB-binding sequences [28], NF-κB could be self-upregulated. To some extent, this may explain the increase of NF-κB proteins in whole cellular extracts.

Recently, a report showed that the levels of serum HBV-DNA might have a significant effect on renal tubular cellular apoptosis [29]. Uncontrolled apoptosis of renal tubular cells might be associated with renal injury which might contribute to renal interstitial fibrosis [30]. Here, we further demonstrated that MHBs<sup>167</sup> and HBx could induce apoptosis in HK-2 cells, and the apoptotic rate was correlated with NF-κB activation. Although the idiographic mechanism remains somewhat undefined, our data suggested that some κB-controlled genes might be involved in the process. The upregulation of MHBs<sup>167</sup> or HBx during HBV infection could lead to cellular apoptosis of renal tubular cells and might cause pathological alteration in the affected renal tissue. Recently, some [31,32] studies showed that cell density or confluent con-
dition might affect diverse cellular process and response, including apoptosis behaviour, cell–matrix interactions and adhesion-dependent signalling. Here, all experiments were performed in sub-confluent conditions (nearly 70% coverage), and the density-dependent apoptosis might be involved, but the conclusion was drawn compared to appropriate control (empty vector-transfected group), and the results were believable.

To further identify that NF-κB activation was involved in the apoptotic process, a known NF-κB inhibitor PDTC was used [33]. The apoptotic index of HK-2 cells transfected with mhbs"167 and/or pcDNA3.1(+)
hbx, in the presence or absence of PDTC. (A) Cells were stained with Hochest 33258 and observed by fluorescence microscopy (bar = 50 μm). (B) Cells were observed with NF-κB p65/TUNEL double staining (bar = 50 μm). Cell apoptosis was stained green where NF-κB p65 stained red in cytoplasm and nucleus. The yellow staining in dual-labelling experiments indicated colocalization of DNA fragmentation and NF-κB p65. The data are one representative experiment. The apoptotic index was shown as the mean ± SD. *P < 0.05 versus the pcDNA3.1 (+)-transfected group; #P < 0.05 versus the pcDNA3.1 (+) mhbs"167+pcDNA3.1 (+) hbx-co-transfected group; **P < 0.05 versus the groups not treated with PDTC. △P < 0.05 is consistent with the EMSA results of NF-κB activation (r = 0.894) and the nuclear NF-κB p65 proteins (r = 0.841).

Fig. 5. Influence of MHBS"167 and/or HBx on HK-2 cells apoptosis. HK-2 cells were transfected with pcDNA3.1 (+) mhbs"167 and/or pcDNA3.1 (+) hbx, in the presence or absence of PDTC. (A) Cells were stained with Hochest 33258 and observed by fluorescence microscopy (bar = 50 μm). (B) Cells were observed with NF-κB p65/TUNEL double staining (bar = 50 μm). Cell apoptosis was stained green where NF-κB p65 stained red in cytoplasm and nucleus. The yellow staining in dual-labelling experiments indicated colocalization of DNA fragmentation and NF-κB p65. The data are one representative experiment. The apoptotic index was shown as the mean ± SD. *P < 0.05 versus the pcDNA3.1 (+)-transfected group; #P < 0.05 versus the pcDNA3.1 (+) mhbs"167+pcDNA3.1 (+) hbx-co-transfected group; **P < 0.05 versus the groups not treated with PDTC. △P < 0.05 is consistent with the EMSA results of NF-κB activation (r = 0.894) and the nuclear NF-κB p65 proteins (r = 0.841).
and/or hbx, PKC activity and phosphor-ERK were increased with NF-κB activation and were significantly reduced following Gö6983 treatment, a specific PKC inhibitor. However, the Raf-1 protein was not detected even in the PMA-treated group. It suggested that PKC might trigger ERK signalling that might mediate MHBs\textsuperscript{t167}/HBx-induced NF-κB activation in a Raf-independent manner in tubular cells.

Another interesting finding was the synergistic effect of MHBs\textsuperscript{t167} and HBx. After mhbst167+hbx co-transfection, the NF-κB activation levels and the apoptotic index were significantly increased compared to either mhbst167 or hbx transfection alone.

In summary, our data showed that the NF-κB activation could be induced by MHBs\textsuperscript{t167}/HBx via PKC/ERK pathway and closely correlated with renal tubular cell apoptosis. Furthermore, there might be a synergistic effect between MHBs\textsuperscript{t167} and HBx in the processes. Our studies suggested that MHBs\textsuperscript{t167} and HBx might be involved in a mechanism whereby HBV directly induces tubular cell injury. Since it was only studied in the immortalized cell line, further study might be still needed.

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Conflict of interest statement. None declared.

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