Apoptosis and its pathway in X gene-transfected HepG2 cells

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INTRODUCTION

Hepatitis B virus (HBV) is a small DNA virus with partial double-stranded DNA genome. HBV contains four opening reading frames (ORF) namely preS1/preS2/S, preC/C, P, and X. X gene is a unique ORF which is well conserved in different mammalian hepadnaviruses, its product consists of 154 amino acids with a molecular weight of 16.7 ku. Based on epidemiical data, HBx is thought to be associated with HBV-related primary hepatocellular carcinoma (HCC), but the molecular basis for the oncogenic activity of HBx remains elusive. HBx is a multiple-functional protein and plays an essential role in viral pathogenesis. HBx can deregulate cell cycle check points, transactivate cells and viral genes, which involve in transcription regulation, single transduction pathway, cell cycle regulation, etc.[10]. It has been shown that HBx can co-ordinate balance between proliferation and programmed cell death, and it is able to induce or block apoptosis. The deregulation of apoptosis is involved in a wide range of pathological processes, including development of HCC.

In the present study, we investigated the effect of HBx expression on human hepatoma cell line HepG2, and its effect on the expression level of apoptosis factors.

MATERIALS AND METHODS

Materials

pDNA(1) expression vector and HBV X gene eukaryon expression vector pDNA(1)-X were previously constructed. Human hepatoma cell line HepG2 was provided by Cell Bank of Chinese Academy of Sciences. Modified Eagle’s medium (MEM) was bought from Hyclone Company, USA. Reverse transcription system, DNA purification system, and TransFast™ transfection reagent were obtained from Promega Biotech (USA). Total RNA isolation kit was purchased from Jingmei Biotech Company (Shanghai, China). PCR primers were synthesized by Shanghai Biotechnology Company. In-site cell apoptosis detection kit was provided by Roche Company.

Methods

Cell culture and DNA transfection HepG2 cells were cultured in MEM supplemented with 10% heat-inactivated FCS. Cultured in MEM supplemented with 10% heat-inactivated FCS. HepG2 cells were transiently transfected into HepG2 cells by lipid-media transfection. Untransfected HepG2 and HepG2 transfected with pDNA(1) were used as controls. Expression of HBx in HepG2 was identified by RT-PCR. MTT and TUNEL were employed to measure proliferation and apoptosis of cells in three groups. Semi-quantified RT-PCR was used to evaluate the expression levels of Fas/FasL, Bax/Bcl-xL, and c-myc in each group.

RESULTS: HBx X gene was transfected into HepG2 cells successfully. RT-PCR showed that HBx was only expressed in HepG2/pDNA(1)-X cells, but not expressed in HepG2 and HepG2/pDNA(1) cells. Analyzed by MTT, cell proliferation capacity was obviously lower in HepG2/pDNA(1)-X cells (0.0891±0.003164) than in HepG2 (0.1441±0.004927) and HepG2/pDNA(1) cells (0.1215±0.007159) (P<0.05 and P<0.01). Analyzed by TUNEL, cell apoptosis was much more in HepG2/pDNA(1)-X cells (980/2,000) than HepG2 (420/2,000), HepG2/pDNA(1) cells (520/2,000) (P<0.05 and P<0.01). Evaluated by semi-quantified RT-PCR, the expression level of Fas/FasL was significantly higher in HepG2 cells transfected with HBx than in HepG2 and HepG2/pDNA(1) cells (P<0.05 and P<0.01). Bax/Bcl-xL expression level was also elevated in HepG2/pDNA(1)-X cells (P<0.05 and P<0.01). Expression of c-myc was markedly higher in HepG2/pDNA(1)-X cells than in HepG2 and HepG2/pDNA(1) cells (P<0.05 and P<0.01).

CONCLUSION: HBx X gene can impair cell proliferation capacity, improve cell apoptosis, and upregulate expression of apoptosis factors. The intervention of HBx X gene on the expression of apoptosis factors may be a possible mechanism responsible for the change in cell apoptosis and proliferation.

Key words: HBx; Transfect; HepG2; Apoptosis; Fas; FasL; Bax; Bcl-xL; c-myc

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fetal bovine serum, 100 IU/mL penicillin and 100 mg/mL streptomycin in a humidified incubator with 50 mL/L CO₂.

A total of 1.5×10⁶ cells/mL were seeded into a 25 cm² cell plate before the experiment. When cells were grown to 80% confluence, pDNA1 or pDNA4-X plasmid was transfected into HepG2 cells by lipofection technique, which were named as HepG2/pcDNA3 cells and HepG2/pcDNA4-X cells. A mixture containing 2 mL serum-free MEM (prewarmed to 37 °C), 5 µg plasmid DNA, 15 µL transfect reagent was added to a cell plate. After incubation for 24 h, 4 mL complete medium was added into cell plate and then incubated for another 48 h. HepG2 cells, untransfected with any plasmid DNA, were used as controls.

**Detection of X gene expression by RT-PCR** Total RNA was extracted from HepG2, HepG2/pcDNA3, and HepG2/pcDNA4-X cells respectively, and reverse transcribed into cDNA. One microliter of RT product was used as template, PCR was carried out. The sequences of X gene primers were: 5'-ATGCAAGCTTATGGCTGCTAGGC-TGTACTG-3' and 5'-TGCGAATTCTTAGGCAGAGGG-TGAAAAAGTGG-3'. The expected amplification fragment was 467 bp. PCR conditions were as follows: pre-denaturation at 95 °C for 5 min, 32 amplification cycles (denaturation at 94 °C for 35 s, annealing at 65 °C for 35 s, and extension at 72 °C for 1 min), and a final extension at 72 °C for 7 min. The PCR products were separated by electrophoresis on 1.5% agarose gel, and detected by ultraviolet radiography.

**Cell viability assay** Cell viability was assayed by MTT. HepG2, HepG2/pcDNA3, and HepG2/pcDNA4-X cells were planted into 96-well plates. Cells in logarithmic growth were used in experiments. One day before the experiment, complete medium was replaced by serum-free medium. During experiment, 75 µL MTT (5 mg/mL, containing in 0.01 mol/L PBS) was added into each well and incubated for 4 h. Then, the medium was replaced by DMSO (75 µL each well) and shaken gently until all crystals were dissolved. Abs was detected to measure the proliferative capacity of each group.

**Cell apoptosis assay** Cell apoptosis was estimated by TUNEL staining. HepG2, HepG2/pcDNA3, and HepG2/pcDNA4-X cells were planted into 96-well plates. Cells at 80% confluence were fixed with 4% paraformaldehyde, and chilled in ice bath for 2 min with permeabilization solution (0.1% Triton X-100 in 0.1% sodium nitrate). Then, 50 µL TUNEL mixture was added, incubated in a humidified chamber at 37 °C for 1 h. TUNEL mixture was removed, 50 µL Converter-AP was added and incubated for another 30 min. The cells were rinsed with PBS, counterstained with NBT/BCIP, and detected by optic microscopy.

**Effect of HBs transient transfection on apoptosis factor mRNA expression** Expressions of Fas/FasL, Bax/Bcl-xl, and c-myc gene were assayed by semi-quantitative RT-PCR. β-Actin was used as internal control. Total RNA was extracted respectively with RNA isolation kit, and reverse transcribed into cDNA. PCR was performed in a 50 µL reaction volume containing 5 µL 10× PCR buffer, 5 µL 2 mmol/L MgCl₂, 1 µL 10 mmol/L dNTP, 1 µL 20 pmol/µL target gene sense and anti-sense primers, 0.5 µL 12.5 pmol/µL β-actin primer pair, 2 µL RT product, 1.5 U Taq DNA polymerase. The sequences of gene primers and amplification conditions are listed in Table 1. The initial denaturation was at 94 °C for 5 min. An additional extension step at 72 °C for 10 min was done finally. About 10 µL PCR products was separated by electrophoresis on 1.5% agarose gel, and detected by ultraviolet radiography. The densities of bands were analyzed by Bio imaging system, the ratio of target gene density to β-actin density was representative of the relative expression level of mRNA. The semi-quantitative detection was analyzed five times.

**Statistical analysis**

All data were expressed as mean±SE. The significance for the difference between groups was assessed with SPSS 10.0 by one-way ANOVA. P<0.05 was considered statistically significant.

**RESULTS**

**Expression of HBV X mRNA in HepG2 cells**

Expressions of HBx mRNA were detected by RT-PCR. The expected band between 400 and 500 bp was found in HepG2/pcDNA4-X cells, but not in HepG2 and HepG2/pcDNA3 cells (Figure 1).
HepG2/pcDNA3-X 5 0.4750±0.015
HepG2/pcDNA3 5 0.2800±0.1000

Figure 1 Expression of HBV X mRNA in HepG2 cells. M: 100-bp DNA ladder; lane 1: HepG2/pcDNA3-X cells; lane 2: HepG2/pcDNA3 cells; lane 3: HepG2 cells.

Cell viability assay
Cell viability was assessed by MTT. All data are shown in Table 2. $A_{590}$ of HepG2/pcDNA3-X cells obviously decreased compared to that in other groups ($P<0.05$ and $P<0.01$), indicating that transient expression of HBx impaired the proliferative capacity of HepG2 cells (Figure 2).

Table 2 $A_{590}$ of HepG2, HepG2/pcDNA3, and HepG2/pcDNA3-X cells (mean±SE)

| Group          | n  | $A_{590}$   |
|---------------|----|------------|
| HepG2         | 10 | 0.14410±0.004927 |
| HepG2/pcDNA3  | 10 | 0.12150±0.007159 |
| HepG2/pcDNA3-X| 10 | 0.08910±0.003164 |

Figure 2 Effect of HBx on cell viability in HepG2 cells.

Cell apoptosis assay
Apoptosis in the three groups was assessed with in-situ cell death detecting kit (TUNEL). A total of 2,000 cells of each group were calculated. The number of apoptosis cells was 980 in HepG2/pcDNA3-X, 520 in HepG2/pcDNA3, and 420 in HepG2. Cell apoptosis markedly increased in HepG2/pcDNA3-X (Table 3 and Figure 3), indicating that transient expression of HBx could promote apoptosis of HepG2 cells (Figure 4).

Table 3 Effect of HBx on cell apoptosis of HepG2 cells (mean±SE)

| Group          | n  | Apoptosis   |
|---------------|----|-------------|
| HepG2         | 5  | 0.2200±0.1000 |
| HepG2/pcDNA3  | 5  | 0.2800±0.1000 |
| HepG2/pcDNA3-X| 5  | 0.4750±0.015  |

Effects of HBx transient transfection on apoptosis factors' mRNA expression
Fas/FasL mRNA mRNA level of Fas and FasL was elevated in HepG2/pcDNA3-X cells (Figures 5A and B, 6A and B), indicating that transient expression of HBx induced expression of Fas and FasL in HepG2 cells.

Bel-xL/Bax mRNA mRNA level of Bel-xL and Bax was enhanced in HepG2/pcDNA3-X cells (Figures 5C and D, 6C and D), indicating that transient expression of HBx induced expression of Bel-xL or Bax in HepG2 cells.

C-myc mRNA mRNA level of c-myc in HepG2/pcDNA3-X cells was the highest (Figures 5E and 6E), indicating that transient expression of HBx induced expression of c-myc in HepG2 cells.

DISCUSSION
In previous studies, it was found that HBx inhibits cell apoptosis in different ways. For example, HBx antagonizes TNF-α-induced apoptosis through activating PI3-kinase signaling pathway[5], and inhibits apoptosis in p53-independent manner[9]. There is evidence that HBx activates NF-κB and induce it to translocate into nuclei, NF-κB acts as an inhibitor of cell apoptosis; HBx also downmodulates expression of Bid and blocks Bid-mediated cell apoptosis[6], inactivates caspase-3 through inhibition of CCP32 enzyme, and blocks caspase pathway[3]. It is thought that anti-apoptosis function of HBX is an important mechanism in the development of HCC.

HBx can either inhibit or promote cell apoptosis in a dose-dependent manner. When HBx expresses at high level, it displays pro-apoptosis effect; whereas it inhibits apoptosis when expressing at physiological level. It was reported that moderate expression level of HBx can inhibit liver regeneration in HBx-expressing transgenic mice after partial hepatectomy[40]. HBx stimulates expression of FasL, which plays an important role in cell’s escaping from immune surveillance by inducing apoptosis of T cell bearing Fas[38]. Some researchers found that HBx can also localize in mitochondria, bind to voltage-dependent anion channel, which results in alteration of the mitochondrial transmembrane potential, promotes cytochrome C and apoptosis-inducing factors to release into cytosol and induces cell apoptosis[13]. In short, HBx has bi-directional function on cell apoptosis regulation. HBx expression levels, availability of survival factors, and stage of infection may profoundly influence.
the fate of cells.

Higher organisms have several mechanisms to eliminate cells by apoptosis. One important role is the signaling pathway mediated by “death factors” including TNFR1, Fas, TNFR2, and their cognate ligand (TNF-α, FasL, and TRAIL) Fas (CD95). The first identified member of “death factors”, is a type I glycoprotein which expresses on cell surface. Crosslinking Fas by binding to the ligand FasL leads to conformational changes of Fas, which results in formation of death induced signal complex (DISC) followed by activation of caspase-8. Activated caspase-8 activates itself and other caspases that switch on apoptosis signal cascade[14,15]. It has been found that Fas and FasL express in hepatocytes and hepatoma cells. Since hepatocytes are highly sensitive to Fas/FasL-mediated apoptosis, Fas/FasL pathway plays an essential role in liver lesion and eliminating virus. In our research, HBx elevated expression of Fas and FasL in HepG2 cells. Although the precise mechanism remains unclear, HBx can activate FasL promoter through binding site for Egr and enhance Egr binding to the co-activator cAMP-response element-binding protein, and induce pro-inflammatory cytokines at transcriptional level such as IL-18 which can amplify the expression of FasL[16]. c-FLIP, a key regulator of the DISC, inhibits the Fas/FasL-mediated death pathway in tumors. HBx abrogates the apoptosis-inhibiting function of c-FLIP and renders cells hypersensitive towards the TNF-α apoptotic signal even below the threshold concentration[17,18].

Members of Bcl-2 family are also involved in apoptosis regulation. Members of this family are divided into three subgroups. One group is composed of anti-apoptosis proteins such as Bcl-2, Bcl-xL, with four Bcl-2 homology domains (BH1, BH2, BH3, and BH4). Another group consists of pro-apoptosis proteins such as Bax, Bak, with BH1, BH2, BH3 domains. The last group includes pro-proteins such as Bid, Bik, with only BH3 domain[4, 19]. As Bcl-2 family members reside in upstream of irreversible cell damage, they play a pivotal role in deciding whether cells die or live. Indeed, the ratio between pro- and anti-apoptosis molecules determines, in part, the susceptibility of cells to death signal[20]. It was reported recently that the anti-apoptosis members lose their ability to inhibit release of pro-apoptosis factors (such as cytochrome C) and trigger apoptosis if they interact with activated pro-apoptosis members[21]. Our data demonstrate that HBx upregulates either pro-apoptosis subset Bax or anti-apoptosis subset Bcl-xL. MTT and TUNEL displayed that apoptosis of HepG2 cells transfected with HBx was enhanced while cell viability was impaired. We postulated that though HBx can upregulate expression of Bax and Bcl-xL, it may promote expression of Bax ever more than Bcl-xL, thus resulting in the predominance of pro-apoptosis protein in the ratio between pro- and anti-apoptosis subsets, then cell apoptosis. On the other hand, interacting with activated Bax, Bcl-xL may lose its anti-apoptosis function and trigger cell death.

C-myc belongs to cell oncogene. HBx accelerates development of primary liver tumors by co-operating with c-myc[22-25]. It was reported that myc can sensitize cells to apoptosis by about two folds in certain conditions such as exposure to TNF-α or other apoptosis factors. In our study, HBx promoted expression of c-myc. Overexpression of c-myc is essential for acute sensitization of cells to be killed.
by HBx plus TNF-α, and may enhance cell apoptosis. This result agrees with the data of MTT and TUNEL in our study.

In hepatocarcinogenesis, preneoplastic, and neoplastic cells display an increased ratio of apoptosis as well as enhanced cell proliferation. It is believed that the anti-apoptosis function of HBx is the major determinant factor for development of HCC. The pro-apoptosis function of HBx, however, is also thought to contribute to hepatocarcinogenesis. Firstly, HBx-induced cell apoptosis results in releasing of hepatocyte growth factor that enhances regeneration of liver cells and accumulation of genetic mutation, thus paving the way for cell malignant transformation. Secondly, increased apoptosis increase the opportunity of mutation, leading cells to grow out of control and develop to HCC. Thirdly, HBx induces expression of FasL in liver cells which can attack T cell bearing Fas and lead to impair of immune defense, which is beneficial for cell bearing HBx to escape from immune detection. Finally, although the accurate mechanism of apoptosis induced by HBx has not been elucidated completely, it may facilitate propagation of viral infection by permitting efficient particle release from cells while minimizing the antiviral inflammation response.

Further study should be focused on protein level. Besides, apoptosis mechanic on stably transfected HBx gene in HepG2 cells is another pivot.

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