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15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) promotes apoptosis of HBx-positive liver cells

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ABSTRACT

This study aims to investigate the inflammatory response characteristics of liver cells caused by HBV x protein (HBx) and the unique function of the PGE_2 inhibitor on HBx-positive liver cells. Tetrazolium blue colorimetric method, flow cytometry, and Western blot were performed to detect the proliferation, cycle, and apoptosis protein expression of HBx-positive HL7702 liver and control cells. The effect of the PGE_2 inhibitor 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) on the growth of HL7702-HBx was also observed. HBx induces the PGE_2 accumulation in HL7702 liver cells and promotes their growth and inhibits their apoptosis. HL7702-HBx and HL7702 cells showed increased apoptosis rate, increased apoptosis-promoting protein expression, and reduced apoptosis-inhibiting protein expression under the effect of 15d-PGJ_2, and the changes in HL7702-HBx cells were more significant than in HL7702 cells. HBx expression causes liver cells to be more sensitive to the apoptosis-promoting function of 15d-PGJ_2. Therefore, the use of 15d-PGJ_2 may be a new method for the prevention or treatment of inflammatory changes to cancer caused by HBV infection in liver cells.

1. Introduction

Tumor is a chronic disease whose occurrence has been related to chronic inflammatory process caused by bacteria, virus, and pathogen infection [1]. Hepatitis B virus (HBV) infection has been recognized to be closely related to the occurrence of hepatocellular carcinoma (HCC) [2,3]. HBV x protein (HBx) with various biological functions can be expressed in the nucleus and cytoplasm of liver cells [4]. HBx protein interacts with various transcription factors in the cell nucleus (e.g., AP-1, AP-2, NF-κB, and cAMP) and affects various signal transduction pathways in the cytoplasm (e.g., Akt, Wnt/β-catenin, NF-κB, JAK/STAT, MAPK, Ras, Raf, and SAPK/JNK) [5–8], thereby influencing the proliferation and differentiation of the host cells [9]. The effect of HBx on liver cell cycle and apoptosis is one of the important mechanisms for HBV in causing HCC [10].

Researchers have discovered that the anti-inflammatory painkiller Celecoxib, an inhibitor of PGE_2 (inflammatory mediator), can reduce the incidence of skin cancer [11] and cervical cancer [12]. These studies indicated that the occurrence of PGE_2 and its functional targets have increasingly become important to study inflammation-induced cancers. Xie et al. [13] found that HBx could promote the proliferation of cell by upregulating the expression of PGE_2 in HCC cells. Therefore, in chronic inflammation caused by HBx, inhibiting the synthesis of PGE_2 by using the PGE_2 inhibitor may be helpful in eliminating the inflammation caused by the transition of HBV to cancer [14]. Among various PGE_2 inhibitors, 15d-PGJ_2, an activator of PPARγ, has been proven to inhibit cell proliferation by activating PPARγ and promoting apoptosis [15–17]. However, studies on the effect of 15d-PGJ_2 on the proliferation, apoptosis, and cycle of HBx expressed-positive liver cells are limited. This paper observes the impact of HBx on the HL7702 cell cycle and apoptosis by establishing a liver cell model HL7702-HBx that stably expresses HBx. Moreover, this study investigates the unique function of 15d-PGJ_2 in inhibiting the proliferation of HBx-positive liver cell to provide experimental reference for the clinical treatment of the transformation of liver cells caused by HBx.

2. Materials and methods

2.1. Material

HL7702 cells (Institute of Biochemistry and Cell Biology, Shanghai, China), previously established HL7702-HBx cells (stable HBx expression by transfected with pcDNA3.0-HBx) and HL7702-NC

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cells (by transfected with empty pcDNA3.0 vector) [18] were grown in DMEM containing 10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. The recombinant plasmid pcDNA3.0-HBx was reserved by our laboratory. All major reagents were purchased, including RT-PCR kit (Thermo Scientific, Waltham, MA); 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma, St. Louis, MO); Lipofectamine2000 (Life Technologies, Carlsbad, CA), NS-398, CAY10526, MK-886, 15d-PGJ_2 and Prostaglandin E_2 EIA Kit (Cayman Chemical, Ann Arbor, MI), Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Jose, CA), anti-HBx monoclonal antibody (Chemicon, Temecula, CA), anti-β-actin antibody (Laboratory Vision, Fremont, CA), anti-Bcl2, anti-Bax, anti-pro-Caspase3 antibody (Santa Cruz Biotechnology, CA), anti-NF-kB(p65), anti-p-NF-kB(p65), anti-Akt, anti-p-Akt antibody (Cell Singaling Technology, Danvers, MA).

2.2. RT-PCR analysis

The RNA from the HL7702-HBx and HL7702-NC cell lines were separately extracted and reverse transcribed to cDNA according to the RT-PCR kit instructions. Using 2 μl cDNA as the template, the HBx gene was amplified by polymerase chain reaction (PCR) as follows: predenaturation for 5 min at 94 °C and 35 amplification cycles (denaturation at 94 °C for 45 s, annealing at 61 °C for 30 s, and extension at 72 °C for 1 min). The primers of the HBx gene were: 5′-ATGCAAGCTTATGGCTGCTAGGCTGTACTG-3′ and 5′-TGCGAATTCTTAGGCAGAGGTGAAAAAGTTG-3′. The expected amplification fragment length of the HBx gene was 464 bp.

2.3. PGE_2 analysis

Twenty-four hours after 7×10^4 cells/well were plated onto 24-well plates, the cultures were exposed to the serum media for 7 days. Then the supernatant was removed and centrifuged briefly. The amount of PGE_2 in the supernatant was determined using EIA Kit according to the manufacturer's protocol. Parallel cells were harvested and counted. All assays were performed in three times.

2.4. Proliferation assay

Briefly, 5×10^3 cells were seeded in each well of a 96-well plate and incubated overnight. At the appropriate time (every 24 h), the cells were incubated with 20 μl MTT (5 mg/ml) for 4 h. Formazan crystals were subsequently dissolved in 150 μl dimethylsulfoxide (DMSO). The absorbance of the solution was measured at 490 nm and detected using the Bio-Tek μQuant Universal Microplate Spectrophotometer (Bio-Tek Instruments, Inc., Winooski, VT). All measurements were repeated in triplicate.

2.5. Apoptosis assay

After incubation with each agent for 48 h, cells were washed with phosphate buffered saline (PBS), trypsinized, collected in a 15 mL conical tube, and pelleted by centrifugation (1200 rpm) for 10 min at room temperature. The pellets were washed twice with PBS, and then resuspended in annexin V binding buffer (150 mM NaCl, 18 mM $CaCl_2$, 10 nM HEPES, 5 mM KCl, 1 mM $MgCl_2$). Fifteen minutes after incubation with annexin V-FITC and PI (1 g/mL) in the dark, the cells were analyzed using a CYTOMICS FC 500 flow cytometer (Beckman Coulter Inc, German).

2.6. Cell cycle analysis

In brief, after incubated with each agent for 48 h, cells were collected, re-suspended in cold PBS and fixed with pre-cold 70% ethanol overnight at −20 °C. After the staining using Propidium iodide (PI), samples were then analyzed by CYTOMICS FC 500 flow cytometer (Beckman Coulter Inc, German).

2.7. Western blot

The cells from the two groups were lysed in ice-cold lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 10 g/ml each of aprotinin, leupeptin, and pepstatin, 1% Nonidet P-40, 1 mM sodium orthovanadate (Na3VO4), and 1 mM NaF). The products were centrifuged at 14,000*g* for 15 min to remove cellular debris. Protein concentrations were determined by BCA methods according to the BCA protein assay kit instructions. Equal amounts of sample (50 μg) were subjected to 10% SDS–polyacrylamide gel electrophoresis (PAGE) and then transferred to a polyvinylidene difluoride (PVDF) membrane. The membrane was blocked in TBST (10 mmol/L Tris–Cl, pH 7.4, 150 mmol/L NaCl, and 10 mL/L Tween 20) containing 8% fat-free milk and incubated with the anti-Akt, anti-p-Akt, anti-NF-κB(p65), anti-p-NF-κB(p65) antibody (1:500), anti-HBx antibody (1:200), anti-Bcl2, anti-Bax, anti-pro-Caspase3 antibody (1:500), and anti-β-actin antibody (1:1000) overnight at 4 °C. The membrane was washed three times for 10 min in TBST and TBS, and then incubated with the second antibody at room temperature for 1 h. After washing, the band was visualized by exposure to film with an ECL western blot system according to the manufacturer's instructions. Intensities of band signals were quantified using the densitometric software Quantity One (Biorad, Hercules, CA) and the relative intensity to internal control (β-actin) was calculated and shown at the bottom of the figures.

2.8. Statistical analysis

The data were repeated at least three independent times and expressed with mean ± SD unless otherwise indicated. Assays for characterizing phenotype of cells and expression difference were analyzed by One-way analysis of variance (ANOVA) using SPSS13.0 software package (SPSS, Inc., Chicago, IL). $P < 0.05$ denoted a statistically significant difference.

3. Results

3.1. HBx expression promotes the proliferation of HL7702 cell and PGE_2 secretion

Fig. 1-A shows that the 464 bp HBx gene fragment can be amplified from HL7702-HBx cells, and the 17 kDa HBx protein band can also be observed (Fig. 1-B). The transfected empty vector pcDNA3.0 cells (HL7702-NC) and parent cells (HL7702) are not observed. We compared the proliferation of HL7702-HBx, HL7702-NC, and HL7702 cells. The result shows that from day four, the OD value of the HL7702-HBx cell was obviously higher than those of HL7702-NC and HL7702 cells ($^*P < 0.05$) (Fig. 1-C). This result indicates that the growth rate of HL7702 after HBx gene transfection was obviously faster and that HBx expression could promote the proliferation of HL7702 cells. We collected the cell supernatant on day seven and detected the PGE_2 secretion of three cell lines. The result shows that the PGE_2 level secreted by HL7702-HBx was obviously higher than those of HL7702-NC and HL7702 cells ($^*P < 0.05$) (Fig. 1-D). This result indicates that HBx expression can promote the HL7702 cell to secret PGE_2. No significant difference in the growth and PGE_2 expression was observed between HL7702-NC and the parent cell HL7702; thus, we chose the HL7702-NC cell as the control sample for subsequent experiments.