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15d-PGJ_2 decreases PGE_2 synthesis in HBx-positive liver cells by interfering EGR1 binding to mPGES-1 promoter

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ABSTRACT

Microsomal prostaglandin E synthase 1 (mPGES-1) is the terminal regulator of PGE_2 synthesis. The expression of mPGES-1 is increased by stimulating inflammatory factors in various human cancers. However, whether hepatitis B virus (HBV) infection affects mPGES-1 and its molecular mechanism in liver cells has not been studied. In this study, we observed that mPGES-1 expression was positively correlated with HBV X protein (HBx) in hepatocellular carcinoma cancerous tissue, and HBx enhanced the mPGES-1 promoter activity in HL7702 liver cells. Mechanistic investigations revealed that HBx can increase the early growth response 1 (EGR1) binding to the transcription site of mPGES-1 promoter. The overexpression and knockdown of EGR1 did not affect cyclooxygenase-2 (COX-2) transcription and expression in HL7702-HBx cells. We also investigated the unique function of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2), a kind of PGE_2 inhibitor, in the regulation of mPGES-1 expression in HBx-positive liver cells. In the presence of 15d-PGJ_2, the expression of COX-2 was unaffected, but that of the EGR1-mPGES-1-PGE_2 axis was inhibited. Moreover, the capacity of EGR1 binding to the mPGES-1 promoter decreased, and the change in HL7702-HBx cells was more significant. The results indicated that EGR1 is a specific transcription factor in the up-regulation of mPGES-1 expression by HBx, and targeting EGR1 may contribute to inhibiting the change from inflammation to HBV-induced cancer.

1. Introduction

Hepatitis B virus (HBV) infection dramatically increases the incidence of hepatocellular carcinoma (HCC), the most common primary malignant cancer of the liver [1]. HBV X protein (HBx) has various important functions in this process, including promoting the secretion of inflammatory factors, cell growth, invasion, and metastasis, and regulating cell cycle and apoptosis [2].

The early inhibition of producing inflammatory mediators [such as prostaglandin E_2 (PGE_2)] by liver cells with HBV infection is particularly important in alleviating malignant lesions of liver cells and the occurrence of liver carcinoma [3,4]. PGE_2 is the only prostaglandin which has a significant role in inflammation. Two enzymes, PGG_2/H_2 synthases, widely known as cycloxygenases or COXs, sequentially convert arachidonic acid to the endoperoxides, PGG_2 and PGH_2, which subsequently are isomerized to the other prostaglandins, PGE_2, PGI_2, thromboxane A_2 (TxA_2), PGD_2, and $PGE_{2\alpha}$ [5]. Several anti-inflammatory drugs, such as non-steroidal compounds, have been currently used to adjuvant anticancer therapy [6,7]. Study of COX-2 inhibitor is the priority in this field of work, but inhibition of COX-2 also affected biosynthesis of other products of the COX-2 pathway, namely PGI_2 and TxA_2. PGI_2 is antithrombotic in nature and TxA_2 is pro-thrombotic. Selective inhibition of COX-2 was found to alter the ratio of these two mediators with a bias toward TxA_2 [8], leading to high incidence of vascular events [9,10]. This set off a world-wide debate with FDA's withdrawal of rofecoxib and valdecoxib from the market. In the search for COX-2 inhibitor substitute, microsomal PGE_2 synthase-1 (mPGES-1) has become an important subject. mPGES-1, the terminal enzyme in the PGE_2 synthesis pathway is induced by

Abbreviations: HBV, Hepatitis B virus; HCC, hepatocellular carcinoma; HBx, HBV X protein; PGE_2, prostaglandin E_2; COX, cyclooxygenase; mPGES-1, microsomal prostaglandin E synthase 1; 15d-PGJ_2, 15-Deoxy-$\Delta^{12,14}$-prostaglandin J_2; EGR1, early growth response 1; PPARγ, peroxisome proliferator-activated receptor γ; RLU, relative luciferase units; EMSA, electrophoretic mobility shift assay; ChIP, chromatin immunoprecipitation assay.

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inflammatory stimuli [11]. An inhibitor of this terminal synthase would specifically inhibit PGE_2 synthesis while sparing the other prostanoids, thus making for a more precise and safer anti-inflammatory therapy [5].

Many scholars have explored the relationship between mPGES-1 and the occurrence and development of tumor. For example, Kamei et al. found that the knockout of mPGES-1 results in decreased PGE_2 synthesis, proliferation, and the invasion of tumor cells [12]. Takii et al. also found that mPGES-1 is involved in the induction of HCC cell proliferation, invasion, and metastasis [13]. However, these studies did not consider HBx. In the present study, we investigated the relationship between HBx and mPGES-1-PGE_2 pathway in liver cells. An in-depth analysis of the effect of the molecular biology mechanism of this pathway was conducted to search for more convincing evidence for the induction of HCC by HBV. We also analyzed the unique function and mechanism of 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2, a kind of PGE_2 inhibitor) as an inhibitor of mPGES-1 in the inhibition of liver cell inflammatory reaction induced by HBx.

2. Materials and methods

2.1. Clinical samples and cell culture

(1) The resected specimens and peripheral blood samples from 65 patients with HCC were obtained from Union Hospital of Fujian Medical University from June 2005 to June 2006 (56 males and 9 females with a median age of 49 years). These patients had no other tumor disease history, had not received any anticancer treatment and use of non-steroidal anti-inflammatory drugs before surgery. The collection of samples for research was approved by the Institution Review Board of the College of Life Sciences, Fujian Medical University in accordance with guidelines for the protection of human subjects. (2) 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 cells (by transfected with empty pcDNA3.0 vector) [14] were maintained in Dulbecco's modified Eagle medium (Life Technologies, Carlsbad, CA) supplemented with 10% (v/v) fetal bovine serum (Life Technologies) at 37 °C in a humidified atmosphere containing 5% CO_2.

2.2. Transfection, RNA interference, and drug intervention testing

(1) Cells were plated at a density of 2×10^5 cells/well. DNA transfection was carried out in 12-well plates by use of Lipofectamine 2000 (Life Technologies), in accordance with the manufacturer's recommendations. (2) The small interfering RNA (siRNA) targets for early growth response 1 (EGR1) (5′-GTGACTGTTTGGCTTATA-3′) were described by Sarra [15]. All siRNA duplexes, which included a negative control that had no homology with known human genes, were chemically synthesized (Beyotime, Shanhai, China); 100 pmol of EGR1-siRNA, the negative control was used for transfection. Total RNA and proteins were isolated at 48 h after transfection. (3) 15d-PGJ_2 (Cayman Chemical, Ann Arbor, MI) was dissolved in culture medium at required concentration and used for the intervention experiment.

2.3. Immunohistochemistry, PGE_2 analysis, RT-qPCR, and western blotting detection

(1) Immunohistochemical testing was assessed by UltraSensitive SP Kit (Maixin-Bio, Fuzhou, China) using anti-HBx (MAB8419, Chemicon, Temecula, CA) and anti-mPGES-1 (sc-12269, Santa Cruz Biotechnology, Santa Cruz, CA) antibody. The method described in the literature was adopted [16]. (2) PGE_2 testing is according to PGE_2 ELISA (No: 514010, Cayman Chemical, USA) manual. The value of PGE_2 in peripheral blood is equal to PGE_2 value of measured specimens (pg/ml). The value of PGE_2 in tissue is equal to (PGE_2 value of measured specimens $\times 2 \times 10^{-3}$)/(the mass of tissue $\times 10^{-3}$). In vitro experiments, 24 h after 7×10^4 cells/well were plated onto 24-well plates, the cultures were exposed to different treatments (as indicated in the Figure legends) for 48 h. Then the supernatant was collected for PGE_2 detection. (3) RT-qPCR was performed with the ABI StepOne Real-Time PCR System (Life Technologies) and the SYBR Premix Ex Taq Kit (Takara, Shiga, Japan). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as an internal control [17]. The primers used for GAPDH, mPGES-1, EGR1, and COX-2 amplification are shown in Table 1. Each sample was analyzed in triplicate. (4) Western blotting. A total of 40 μg protein was subjected to 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and electrophoretic transfer to a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA). Protein blots were incubated separately with a panel of specific antibodies that included anti-HBx (1:1000, MAB8419), anti-mPGES-1 (1:1000, sc-12269) and other antibodies, anti-COX-2 (1:1000, sc-23984, Santa Cruz), anti-EGR1 (1:1000, sc-110, Santa Cruz), anti-peroxisome proliferator-activated receptor γ (PPARγ) (1:1000, sc-7273, Santa Cruz), and anti-β-actin (1:4000, sc-47778, Santa Cruz) overnight at 4 °C, and then incubated with different horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. Visualization of the immunoreactive proteins was performed by chemiluminescence Kit (BeyoECL Plus, Beyotime, Shanhai, China). 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.

2.4. Plasmid constructs

(1) The DNA fragment of mPGES-1 promoter was amplified by PCR with primers containing a HindIII or XhoI restriction site [18], which was connected to the pGL3-Basic vector (Promega). These mPGES-1 promoter luciferase reporter plasmids constructed were named pGL3B-628(−628 to +1), pGL3B-387(−387 to +1), pGL3B-177(−177 to +1, cut from pGL3B-628 by SacI), pGL3B-83(−83 to +1). In the same way, we constructed the COX-2 promoter luciferase reporter plasmids pGL3B-COX-2(−1098 to +122). (2) The pGL3B-177 plasmid was used as a template for the construction of SP1/EGR1-binding sites mutants. These mutant vectors were constructed by overlap PCR technology. The resulting plasmids were pGL3B-177-M1 (with SP1 mutation site), pGL3B-177-M2 (with EGR1 mutation site), and pGL3B-177-M3 (with SP1 and EGR1 mutation site). (3) To obtain full-length cDNA sequence for human EGR1 and PPARγ, PCR was carried out with a SuperScript library (Life Technologies). The PCR-generated *EGR1* and *PPARγ* gene was, respectively, inserted into the XhoI–HindIII and KpnI–XhoI site of pcDNA3.0 (Promega, Madison, WI), and the resultant vector was called pcDNA3.0-EGR1 and pcDNA3.0-PPARγ. All primers used for above amplification are shown in Table 1 and all above constructs were confirmed by DNA sequencing.

2.5. Dual-luciferase reporter assay

Cells with different treatments were transfected with promoter vector. A total of 48 h later, the cell lysates were detected for intracellular luciferase activity using Dual-Luciferase Reporter Assay System (Promega) on a luminometer (Orion II Microplate Luminometer, Berthold Detection Systems, Germany) following the manufacturers' recommendations. The relative luciferase units (RLU) were obtained by comparison with control, which was set to