Echinacoside promotes the proliferation of human renal tubular epithelial cells by blocking the HBX/TREM2-mediated NF-κB signalling pathway

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Abstract. Hepatitis B virus X (HBX) protein is required for the replication of HBV and plays a role in the progression of hepatitis in humans. However, the underlying function of HBX during HBV-induced chronic glomerulonephritis (HBV-Gn) is unknown. Echinacoside (ECH) is a phenylethanoid glycoside from the Cistanche genus, which possesses strong antiapoptosis and neuroprotective activities. In the present study, the function of HBX and the relationship between HBX and ECH in human renal tubular epithelial cells (RTecs; HK-2 cell line) were explored. Reverse transcription-quantitative PCR and western blot analyses were used to quantify the mRNA and protein expression levels of HBX in HK-2 cells, respectively. The Cell Counting Kit-8 assay was performed to analyse cell proliferation. Flow cytometry analysis was used to determine the rate of apoptosis. HBX showed antiproliferative and proapoptotic effects in HK-2 cells and was positively associated with triggering receptor expressed on myeloid cells 2 (TREM2) expression. Furthermore, ECH disrupted the function of HBX in HK-2 cells, functioning as an HBX suppressor. Moreover, a specific NF-κB inhibitor, PdTc, was used to further examine the relationship between HBX and NF-κB. The results suggested that NF-κB was involved in the HBX/TREM2 signaling pathway and negatively regulated TREM2 expression in RTecs. The present study provided novel insights into the function of HBX, and also indicated the potential value of ECH as a therapeutic agent for HBV-Gn.

Introduction

Hepatitis B virus (HBV) is a Hepadnaviridae virus, which leads to acute and chronic hepatitis in humans (1). HBV, not only causes persistent or transient infection in the liver, but also infects extrahepatic tissue, including the pancreas, bile duct, lymphoid system and kidneys (2). Moreover, HBV infection is closely associated with chronic glomerulonephritis (HBV-GN); however, the molecular mechanisms of HBV during HBV-GN are not completely understood (3,4).

HBV regulatory hepatitis B virus X (HBX) protein is required for HBV replication (5,6). A number of studies have reported that HBX promotes cell proliferation during HBV infection-associated hepatocarcinoma (7-9). Human renal tubular epithelial cells (RTecs) are a major part of the renal tubular interstitium and play an active role in renal inflammation (10). RTEC apoptosis is closely associated with the dysfunction of the tubular interstitium, which subsequently leads to the progression of HBV-GN (10). HBX is primarily expressed in RTEC cells and causes RTEC apoptosis and renal tube lesions in patients with HBV-GN (11,12). Furthermore, HBX has been reported to suppress the proliferation of rat RTecs (13), and HBX overexpression accelerates the cell cycle progression from G1 to S phase in primary RTECs, leading to cell cycle arrest in the S phase (14). Therefore, identifying a HBX inhibitor may provide a novel therapeutic for HBV-GN. However, the precise molecular network of HBX in human RTECs is not completely understood.

Echinacoside (ECH) is a natural compound extracted from Cistanche plants, which shows antiapoptotic and anti-inflammatory effects (15,16). A previous study reported that ECH induces the proliferation and prevents the apoptosis of intestinal epithelial cells (17). Furthermore, ECH has been reported to accelerate bone regeneration by increasing the proliferation of osteoblast cells (18). ECH can also decrease HBV replication, antigen expression and inflammation in rat intestine epithelial cells (16,19). However, the biological effect of ECH on RTECs has not been fully elucidated.

Triggering receptor expressed on myeloid cells 2 (TREM2), a member of the transmembrane receptor family, plays a role in maintaining adequate microglial metabolism during Alzheimer’s disease (20,21). A previous study indicated that...
TREM2 overexpression promoted the proliferation of glioma cells (22). By contrast, TREM2 knockdown decreases the translocation of NF-xB to the nucleus in degenerative human nucleus pulposus cells (23). Furthermore, TREM2 has been identified as a novel therapeutic target for human intervertebral disc degeneration (23). However, the biological function of TREM2 in human RTECs has not been reported.

In the present study, HBX overexpression (oeHBX) was induced in human RTECs (HK-2 cell line) and subsequently, the effect of ECH on transfected oeHBX cells was investigated. The aim of the present study was to investigate the functions of HBX and the potential value of ECH as an effective therapeutic agent for HBV-GN.

Materials and methods

Cell culture. The human RTEC cell line HK-2 was purchased from Shanghai Yaji Biotechnology Co., Ltd. HK-2 cells were cultured in DMEM/F12 media (cat. no. SH30023.01B; HyClone; Cytiva) supplemented with 10% foetal bovine serum (cat. no. 16000-044; Gibco; Thermo Fisher Scientific, Inc.), 2 mM L-glutamine and 1% penicillin/streptomycin (cat. no. P1400-100; Beijing Solarbio Science & Technology Co., Ltd.) at 37°C with 5% CO₂.

RNA isolation and reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from HK-2 cells using TRIzol® (cat. no. 1596-026; Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol.

Total RNA was reverse transcribed into cDNA using the First Strand cDNA Synthesis kit according to the manufacturer's protocol (cat. no. K1622; Thermo Fisher Scientific, Inc.). qPCR was performed using SYBR-Green premix according to the manufacturer's protocol (cat. no. K0223; Thermo Fisher Scientific, Inc.). an ABI-7300 real-time detector (Applied Biosystems; Thermo Fisher Scientific, Inc.). The conditions of PCR were: 95°C for 10 min followed by 40 cycles of 95°C for 15 sec, 60°C for 45 sec. Three repeats were needed for each reaction. The following primer pairs were used for the qPCR: HBX forward, 5'-GGGCTGTAGTGGTACTG-3' and reverse, 5'-CAGAGGTAGCCAGGAGTG-3'; TREM2 forward, 5'-TGGCTCTCCATGATTACG-3' and reverse, 5'-CCTCC ATCATCTCTCTCCTAC-3'; and GAPDH forward, 5'-AAT CCCATACACATCTC-3' and reverse, 5'-AGGCTGTGTG TCATACCTTC-3'. mRNA levels were quantified using the 2⁻ΔΔCq method and normalized to the internal reference gene GAPDH (24).

Overexpression and knockdown. To induce the overexpression of HBX and TREM2, the full-length HBX or TREM2 cDNA sequences were inserted into the pLVX-puro vector (Clontech Laboratories, Inc.) by double digestion (HindIII and EcoRI). Subsequently, the recombined plasmid (1.5 µg) was transfected into HK-2 cells (2x10⁵). The mock plasmid (an empty vector, 1 µg) was transiently transfected into HK-2 cells (2x10⁵) as a negative control (oeNC) using Lipofectamine® 2000 (cat. no. 11668-027, Invitrogen, Thermo Fisher Scientific, Inc.).

ECH (cat. no. 82854-37-3; Biopurify Phytochemicals, Ltd.) was dissolved in DMSO (cat. no. D2650; Sigma-Aldrich; Merck KGaA) at the concentration of 1, 2.5, 5, 10, 20 and 50 mg/l respectively; and added to the culture of transfected oeHBX or oeTREM2 cells. The subsequent experimentation began 48 h later.

To knock down TREM2 expression, three small interfering (si)RNAs (siTREM2-1, siTREM2-2 and siTREM2-3; 20 µM) targeting TREM2 were synthesized (Shanghai Majorbio Bio-Pharm Technology co. ltd.) and subsequently transfected into HK-2 cells (2x10⁵) using Lipofectamine® 2000 according to the manufacturer's protocol (Invitrogen; Thermo Fisher Scientific, Inc.). A non-specific scrambled siRNA sequence (5'UUCUCCGAACGUUCAGUCCG (25 nM) si-negative control (NC) was used as the negative control. The targeted locus and sequences of the TREM2 siRNAs are provided in Table SI. The subsequent experimentation was performed 48 h later.

Western blotting. Total protein was extracted from HK-2 cells using RIPA lysis buffer (cat. no. BYL40825; JRUND Biotech Co., Ltd.). Total protein was quantified using the Bicinchoninic Acid assay kit (cat. no. PICPI23223; Thermo Fisher Scientific, Inc.) according to the manufacturer's protocol and 20 µg protein/lane was separated via SDS-PAGE on a 15% gel. The densitometry of samples were determined using a microplate reader (DNM-9602, Pulongxin). Subsequently, the separated proteins were transferred onto a PVDF membrane. Following blocking with 5% non-fat dry milk for 1 h at room temperature, the membrane was incubated with primary antibodies (Table SII) at 4°C overnight and re-incubated with the secondary antibody anti-mouse IgG (1:1,000, cat. no. A0216, Beyotime Institute of Biotechnology) for 1 h at 37°C. The immunoreactive protein bands were visualized using an ECL system (Cytiva). Blots were performed in triplicate.

Cell proliferation. The Cell Counting Kit-8 (CCK-8; cat. no. CP002; SAB Biotherapeutics, Inc.) assay was performed to quantify cell proliferation. Briefly, a total of 3x10⁴ cells/well were seeded into 96-well plates and CCK-8 reagent was added, according to the manufacturer's protocol. Subsequently, the optical density (OD) 450 value of each well was determined using a microplate reader at 0, 12, 24 and 48 h. The assay was performed in triplicate.

Cell apoptosis. Briefly, an Annexin V-FITC Apoptosis Detection kit (cat. no. C1063; Beyotime Institute of Biotechnology) was used to examine the number of apoptotic HK-2 cells, according to the manufacturer's protocol. After 48 h post-transfection, the cells were performed using a flow cytometer (Accuri C6, BD Biosciences) and analyzed using FlowJo software version 7.6.5 (FlowJo LLC). Three repeats were performed for each sample.

Luciferase reporter assay. The 3'-UTR sequence of TREM2 was inserted into the firefly luciferase reporter plasmid pGL3-Enhancer-luc2 (pGL3-Enhancer-luc2-pTREM2; E1910, Promega Corporation) by double digestion using XhoI and HindIII. Subsequently, the pGL3-Enhancer-luc2-pTREM2 plasmid (1.5 µg) was transfected into oeHBX and oeNC cells (2x10⁵) using Lipofectamine® 2000 (cat. no. 11668-027,
Invitrogen, Thermo Fisher Scientific, Inc.). Additionally, the NF-κB inhibitor PDTC (10 µM; cat. no. S1809; Beyotime institute of Biotechnology) was dissolved in DMSo (cat. no. d2650; Sigma-aldrich; Merck KGaa) and added to the oeHBX cell culture to knock down the expression of NF-κB. Following incubation for 48 h at room temperature, the cells were collected. Subsequently, the firefly and Renilla luciferase activities were determined using the dual-luciferase reporter assay system (cat. no. e1910; Promega corporation), according to the manufacturer’s protocol. Firefly luciferase activity was normalized to Renilla luciferase activity. Three repeats were performed for each sample.

Statistical analysis. Statistical analyses were performed using GraphPad Prism software (version 7.0; GraphPad Software, Inc.). Data are presented as the mean ± SD. Data were analysed using one-way ANOVA followed by Tukey’s post hoc test. P<0.05 was considered to indicate a statistically significant difference.

Results

ECH suppresses the function of HBX in HK-2 cells. To assess the function of HBX, HBX overexpression was induced in HK-2 cells. The relative mRNA and protein levels of HBX were significantly upregulated in oeHBX cells compared with oeNC cells (Fig. 1A and B). Subsequently, oeHBX cells were cultured with different concentrations of echinacoside (1, 2.5, 5, 10, 20 and 50 mg/l; E1, E2.5, E5, E10, E20 and E50, respectively). HBX overexpression significantly reduced the proliferation of HK-2 cells, and this decrease was reversed by echinacoside in a dose-dependent manner at 24 and 48 h (Fig. 1C).

Furthermore, the proliferation rate of oeHBX cells displayed no significant difference compared with E1-treated cells (Fig. 1C). By contrast, E10 treatment significantly increased the proliferation rate of oeHBX cells compared with E20 and E50 treatment which exhibited no significant difference (Fig. 1C). Therefore, the effect of ECH on apoptosis was examined in E2.5, E5 and E10-treated cells. HBX overexpression significantly increased the apoptosis of HK-2 cells, and the apoptosis rate of oeHBX cells decreased with ECH treatment in a dose-dependent manner (Fig. 1D). Overall, the results suggested that ECH inhibited the function of HBX in HK-2 cells in a dose-dependent manner.

TREM2 expression is inhibited by ECH in transfected oeHBX cells. RT-qPCR and western blotting were used to examine the relative mRNA and protein expression levels of TREM2 in oeHBX cells, respectively. TREM2 mRNA and protein expression levels were significantly upregulated in oeHBX cells compared with oeNC cells (Fig. 2A and B). However, ECH decreased the expression levels of TREM2 in transfected oeHBX cells in a dose-dependent manner (Fig. 2A and B). Therefore, the results suggested that HBX expression was positively associated with TREM2 expression, and further indicated the suppressive effect of ECH on HBX in HK-2 cells.

Knockdown and overexpression of TREM2 in HK-2 cells. To further assess the function of TREM2, TREM2 knockdown and overexpression were induced in HK-2 cells using RNA interference and a lentiviral vector, respectively. For the knockdown assay, three siRNAs targeting the human gene TREM2 (siTREM2-1, siTREM2-2 and siTREM2-3) were transfected into HK-2 cells, and a non-specific siRNA was used as a negative control (siNC). All three TREM2 siRNAs successfully knocked down the endogenous expression of TREM2 in HK-2 cells (Fig. 3A and B). However, the lowest relative TREM2 mRNA and protein expression levels were observed in siTREM2-1 and siTREM2-2-transfected cells (Fig. 3A and B), therefore, siTREM2-1 and siTREM2-2-transfected cells were chosen for subsequent experimentation.

For the overexpression experiments, a plasmid containing the full-length human TREM2 cDNA sequence was constructed and subsequently transfected into HK-2 cells (oeTREM2). A
mock plasmid served as a negative control (oeNC). The level of TREM2 expression was significantly increased in oeTREM2 cells compared with oe nc cells (Fig. 3 c and d). Therefore, oeTreM2 cells were used for subsequent experimentation.

TREM2 silencing decreases the apoptosis of oeHBX cells. The apoptosis profile of oeHBX cells transfected with siTREM2 or sinc was assessed. The apoptosis rate of oeHBX + sinc was significantly higher compared with oe nc cells. However, the apoptosis rate was significantly decreased in oeHBX + siTreM2-1 or siT reM2-2 cells compared with oeHBX + si nc cells (Fig. 4 a). The results suggested that TreM2 knockdown inhibited the function of HBX during HK-2 cell apoptosis.

Survivin belongs to the inhibitor of apoptosis protein family, which inhibits apoptotic activity and suppresses cell death (25). Targeting Survivin has been identified as a novel approach for the treatment of renal cell carcinoma (26). NF-κB is also an apoptosis inhibitor that plays a role in antiapoptotic tumor processes (27). Furthermore, caspase3 activation is closely associated with apoptosis (28). In the present study, the protein content of TREM2, Survivin and cleaved caspase3 in HK-2 cells was quantified. The level of TREM2 expression was significantly decreased in oeHBX + siTreM2-1/2 cells compared with oeHBX + sinc cells (Fig. 4B). The level of Survivin expression was significantly increased in oeHBX + siTreM2-1/2 cells compared with oeHBX cells. Additionally, the level of cleaved caspase3 expression was significantly decreased in oeHBX + siTREM2-1/2 cells compared with oeHBX cells. Taken together, the results suggested that TREM2 was a downstream factor of HBX in HK-2 cells, therefore, HBX might promote apoptosis by regulating TREM2 expression in human RTECs.

TREM2 overexpression suppresses the translocation of NF-xB to the nucleus in HK-2 cells. The effect of ECH on oeTREM2-transfected cells was analysed. TREM2 overexpression promoted the apoptosis of HK-2 cells (Fig. 5A). The apoptosis of oeTREM2 cells was significantly decreased by
ECH treatment (E10; Fig. 5A). Furthermore, ECH significantly decreased the expression of TREM2 in oeTREM2 cells. TREM2 overexpression significantly decreased the expression levels of Survivin, however, this decrease in expression was reversed by ECH. Additionally, the protein expression levels of cleaved caspase3 were significantly increased in
oeTREM2 cells compared with oeNC cells; an effect which was significantly decreased by ECH treatment. Furthermore, ECH promoted the translocation of NF-κB to the nucleus in oeTREM2 cells (Fig. 5B). Taken together, the results indicated that ECH also suppressed the function of TREM2 in human RTECs.

**Discussion**

HBX is primarily expressed in RTECs of patients with HBV-Gn, and functions as a determinant of viral pathogenesis (11,29). Therefore, increasing knowledge of the function of the HBX molecule network is a critical step in developing a novel approach for the treatment of HBV-Gn. The aim of the present study was to further examine the function of HBX and to explore its potential molecular network in human RTECs.

HBX inhibits the proliferation of RTECs (11). In the present study, antiproliferation and pro-apoptosis functions of HBX in human RTECs were identified, suggesting that HBX suppressed the development of human RTECs.

A previous study indicated that ECH inhibits HBV replication and antigen expression (19). In the present study, ECH treatment disrupted the function of HBX in HK-2 cells, therefore, HBX may be affected by ECH in HK-2 cells.
Furthermore, the results suggested that ECH may serve as a potential therapeutic agent for HBV-GN.

TREM2 is an innate immune receptor that plays a role in the inflammatory response (30). In the present study, HBX expression was positively associated with TREM2 expression in human RTECs. Moreover, TREM2 knockdown significantly decreased the apoptosis rate of oeHBX cells, and ECH treatment reduced the effect of oeTREM2 on cell apoptosis. Taken together, these results suggested that TREM2 was targeted by HBX in HK-2 cells. Therefore, ECH may inhibit the apoptosis of human RTECs by blocking the activity of the HBX/TREM2 signalling pathway.

A previous report demonstrated that NF-κB, not only plays a role in cell apoptosis, but is also involved in the regulation of immune responses and inflammation (31). Furthermore, TREM2 is mediated by the NF-κB-sensitive microRNA-34a during Alzheimer’s disease and macular degeneration (32,33). In the present study, the apoptotic rate of oeHBX cells was significantly increased in the presence of PTDC. To the best of our knowledge, the present study suggested for the first time that, the NF-κB inhibitor PTDC suppressed the promoter activity of TREM2. Furthermore, the results suggested that TREM2 negatively regulated the translocation of NF-κB to the nucleus in HK-2 cells, but was positively associated with cleaved caspase3 expression levels. TREM2 played an opposite role in HK-2 cells to its role in human degenerative nucleus pulposus and glioma cells (22,23). Therefore, the present study suggested that TREM2 might have different functions in different types of human cells.

Taken together, the present study, not only indicated that NF-κB was a novel component of the HBX/TREM2 signalling pathway, but also suggested that NF-κB negatively regulates TREM2 expression in human RTECs. However, the major limitations of the present study were the lack of in vivo experiments and clinical data. Therefore, further in vivo and clinical studies are required to confirm the findings of the present study.

In the present study, the function of ECH was investigated and the results suggested the potential effects of ECH on the signalling pathways in human RTECs. Furthermore, the present study enhanced the existing knowledge of the biological function of ECH in human RTEC cells and also suggested its potential as a novel therapeutic agent for HBV-GN.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors’ contributions

DG designed this project and wrote the manuscript; YZ and QW performed the experiments; LZ analyzed the data and edited diagrams. LW provided technical assistance. All authors reviewed and approved the final manuscript.

Ethics approval and consent to participate

This research was approved by the ethics committee of Yangpu District KongJiang Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


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