Original Article
HBs, HBc and MHBs promote TRAIL-induced apoptosis depending on the STAT1 signaling pathway

Da He1,2, Lu Zhang1,2, Dongbo Wu1,2, Xiangjun Chen3, Hong Tang1,2, Taoyou Zhou1,2

1Center of Infectious Diseases, West China Hospital of Sichuan University, Chengdu 610041, Sichuan Province, China; 2Division of Molecular Biology of Infectious Diseases, State Key Laboratory of Biotherapy, Sichuan University, Chengdu 610041, Sichuan Province, China; 3Department of Medical Quality Control, West China Hospital, Sichuan University, Chengdu, China

Received April 28, 2016; Accepted August 5, 2016; Epub October 15, 2016; Published October 30, 2016

Abstract: The tumor necrosis factor related apoptosis induced ligand (TRAIL) can selectively induce the apoptosis of virus-infected cells. STAT1 plays an important role in modulating cell apoptosis and anti-virus responses. TRAIL and STAT1 may have a crosstalk in modulating the apoptosis of virus-infected cells. Hepatitis B virus (HBV) has several proteins, such as HBV surface protein (HBs), HBV core protein (HBc), middle hepatitis B surface antigen (MHBs), large hepatitis B surface antigen (LHBs). This study was to investigate the role of different HBV proteins in modulating TRAIL and STAT1 signaling pathway. We found that with TRAIL treatment, the HBV-transfected, HBs-expressing, HBc-expressing, MHBs-expressing human fibrosarcoma cell line HT1080 (stat1+/+) had higher apoptosis rates while the STAT1 mutant human fibrosarcoma cell line U3A (stat1-/-) did not. These results suggest that HBV may promote TRAIL-induced apoptosis via HBs, HBc and MHBs relying on the integrity of STAT1 signaling pathway.

Keywords: Tumor necrosis factor related apoptosis inducing ligand, hepatitis B virus protein, STAT1, apoptosis

Introduction

According to the world health organization (WHO), about 2 billion people have been infected with the hepatitis B virus (HBV) in the world [1]. About 65,000 people infected with HBV die every year for the liver failure, cirrhosis and hepatocellular carcinoma (HCC) [2]. To date, the pathogenic mechanism of HBV is not fully understood. But studies indicate cell apoptosis may contribute to the development of the disease that can be mediated by Fas ligand (FasL), TNF and TRAIL, etc [3, 4].

TRAIL firstly found by Wiley, is a member of the tumor necrosis factor (TNF) superfamily. Unlike FasL or TNF, TRAIL can specifically induce the apoptosis of virus-infected cells and tumor cells without any effect on the normal ones [5]. It can eliminate the virus by inducing cell apoptosis through caspase way binding the death receptors DR1 and DR2 which express on the surface of the virus-infected cells.

STAT1 is one member of the signal transducers and activators of transcription (STAT) family. It participate in many physiological process such as inflammation, immune adjustments and anti-infection responses making it play an important role in the anti-virus response. STAT1 can induce cell apoptosis by promoting the expression of apoptosis genes such as caspase, Fas, etc. and inhibiting the expression of anti-apoptosis proteins such as Bcl-XL [6]. Some studies had suggested that STAT1 may promote TRAIL to induce cell apoptosis by increasing the expression of TRAIL and its receptors [7, 8]. So there may be some relationships between STAT1 and TRAIL to promote cell apoptosis.

The HBV virus can express several antigen proteins such as HBV surface protein (HBs), HBV core protein (HBc), middle hepatitis B surface antigen (MHBs), large hepatitis B surface antigen (LHBs), hepatitis B X protein (HBx), etc. Different HBV proteins have different functions during the development of the disease. HBc can inhibit Fas-mediated apoptosis of infected hepatocytes via regulation of Fas and its receptors expression [9]. Other studies suggested that HBx can enhance the sensitivity of hepato-
Apoptosis induced by HBV proteins

carcinoma cells to TRAIL-induced apoptosis [10, 11]. However, the regulation mechanisms of HBV proteins in cell apoptosis are not fully aware of.

To study the role of HBV proteins in modulating TRAIL-induced apoptosis and whether it was relied on STAT1 signaling pathway, we transfected HT1080 and U3A cells with the plasmids pHBV4.1 (containing HBV complete genome), pcDNA3.1/HBs (expressing HBs), pcDNA3.1/HBc (expressing HBc), pcDNA3.1/MHBs (expressing MHBs) and pcDNA3.1/LHBs (expressing LHBs) and treated them with the low concentration of TRAIL. Cell apoptosis may be observed and the molecular modulation of HBV proteins in TRAIL-induced apoptosis mediated by STAT1 signaling pathway may be revealed.

Materials and methods

Cell culture

Human fibrosarcoma cell line HT1080 (stat1+/+) was provided by the Laboratory of Transplantation and Immunology, West China Hospital of Sichuan University. STAT1 mutant human fibrosarcoma cell line U3A (stat1-/-) was a gift from Professor Jianhua Tong of the affiliated Ruijin Hospital of Shanghai Jiao Tong University.

HT1080 cells and U3A cells were cultured in Dulbecco’s Modified Eagle’s Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Gibco) at 37°C in a 5% CO₂ incubator. The cells were passaged at a density of 70% to 80%.

Antibodies and reagents

Western blot primary antibody mouse monoclonal anti-STAT1 (BD, America); The mouse monoclonal anti-GAPDH antibody (Zheng Neng, China); the secondary antibody HRP-conjugated anti-mouse IgG (Zheng Neng, China).

Construction and Identification of pcDNA3.1/HBs

The primers of S gene (expressing HBs) were designed according to the HBV-ayw subtype sequence. S gene forward: 5’-GGAGAGCACAACATCAGGA-3’; S gene reverse: 5’-GCAGCGGCCGCTCAAATGTATACCCAAAGACA-3’. S gene fragments were amplified by PCR from the template, pHBV4.1. Both S gene fragments and pcDNA3.1 were digested with restriction enzymes HindIII and NotI (Thermo, America) and purified using gel extraction kit (Qiagen, German). The recombinant plasmid was successfully constructed after connection, conversion and positive monoclonal selection.

pcDNA3.1/HBs was identified by digestion of HindIII and NotI and was sequence-confirmed. Cell supernatant of pcDNA3.1/HBs-transfected cells was collected and HBs was detected by enzyme-linked immunosorbent assay (ELISA) using HBs ELISA kit (Ke Hua, China), according to the instructions.

Plasmids transfection

Plasmid pHBV4.1 was got from Professor Alan Mclachlan of the American Scripps Institute. Plasmids pcDNA3.1, pcDNA3.1/HBc, pcDNA3.1/MHBs and pcDNA3.1/LHBs were provided by the Laboratory of Infectious Disease Center, West China Hospital of Sichuan University.

Cells were seeded into 6-well plates (1x10⁵ cells/well) and were transfected at 50% to 60% density. 2 ug of Plasmids were added into each well and were incubated with Opti-MEM (Gibco, America) and transfection reagent (X-tremeGENE HP DNA Transfection Reagent, Roche, Germany) at room temperature for 15 minutes. Then the mixture was added into the wells respectively and mixed gently.

The survival rates of TRAIL-treated cells were detected by MTT method

Cells were seeded into 96-well plates (1x10⁴ cells/well). Different concentration of TRAIL (0, 25, 50, 75, 100, 200, 300, 400, 500 ng/mL) (Di Ao, China) were added to the cells. MTT reagent (Bo Shi De, China) was used to detected the cytotoxicity of TRAIL. the optical density (OD) was detected at 570 nm wavelength with 630 nm as control. The relative survival rates were calculated according to the formula: relative survival rate (%) = (experimental well OD-blank well OD)/(control well OD-blank well OD) * 100%. The low concentration of TRAIL was determined according to the MTT results that had little effect on the survival rates of cells. The cells with transfections of pHBV4.1, pcDNA3.1/HBs, pcDNA3.1/HBc, pcDNA3.1/MHB, pcDNA3.1/LHBs were treated with the low concentration of TRAIL for 48 h.
Apoptosis induced by HBV proteins

Cells transfected with pHBV4.1, pcDNA3.1/HBs, pcDNA3.1/HBc, pcDNA3.1/MHB, pcDNA3.1/LHBs were treated with low concentration of TRAIL or PBS for 48 h. The cells were collected at a concentration of 5×10⁵ to 10×10⁵ cells/mL and were washed by PBS at 4°C, 2000 rpm for twice. The cells were suspended in 150 ul binding buffer, 5 ul Annexin V-FITC and 5 ul propidium iodide (PI). The apoptotic cells were detected by Annexin V-PI kit (Kai Ji, China), according to the instructions.

Detection of STAT1 expression by western blot

The cells with different transfections as before were treated with the low concentration of TRAIL. The cells were collected into the lysis buffer (50 mM Tris-HCl, 1% NP40, 150 mM NaCl, 0.5% sodium deoxycholate, 0.7 ug/ml pepstatin) added with protease inhibitor cocktail (Roche, German) and phosphatase inhibitor cocktail (Roche, German) for 1 h. Protein supernatant was got from the lysed cells after 12000 rpm centrifugation for 15 min at 4°C. Equal amount of protein was electrophoresed and transferred to a PVDF membrane (Millipore, America) which was then blocked by 5% non-fat milk (BD, America) for 1 h. The membrane was incubated with STAT1 primary antibody overnight at 4°C, then washed by Tris-buffered saline containing Tween-20 (TBST) and incubated with the secondary antibody for an hour. After washing, the membrane was added to the ECL reagent (Millipore, America) to be visualized by the gel-imaging instrument (Royal, German). The relative expression of STAT1 was analysed by the Image Lab 5.0 software. Its expression was calculated according to the formula: (the value of STAT1 in experimental group/the value of STAT1 in PBS group)/(the value of GAPDH in experimental group/the value of GAPDH in PBS group).

Statistical analysis

Results were statistically analysed by the spss 20.0 software and were presented as means ± standard deviation. The Mann-Whitney U test was used to compare the variables between the rates of two groups. The t test was used to compare the variables between two quantitative datas. A P-value <0.05 was considered statistically significant.

Results

PCR amplification of S gene and the identification of recombinant plasmid pcDNA3.1/HBs

S gene was amplified by PCR from the template, pHBV4.1. The PCR products analyzed on 1% agarose gels were consistent with the expected fragment size, about 680 bp (Figure 1A).
The recombinant plasmid pcDNA3.1/HBs was digested with restriction enzymes HindIII and NotI. The nucleic acid fragments were got from 1% agarose gel consistent with the size of S gene (about 680 bp) and pcDNA3.1 (about 5400 bp) (Figure 1B). And the sequencing confirmed the correct collection of S gene.

The supernatant of HT1080 and U3A cells transfected with pcDNA3.1/HBs was detected by ELISA to observe the expression of HBs. It suggested that pcDNA3.1/HBs could be successfully expressed in both the two cells as with the positive ELISA results (S/COV=8.57±0.98).

The effect of different concentration of TRAIL on HT1080 and U3A cells

HT1080 and U3A cells were treated with different concentration of TRAIL (0, 25, 50, 75, 100, 200, 300, 400, 500 ng/mL) for 48 h. The relative survival rates of HT1080 and U3A cells were detected by MTT assay.

The relative survival rates of HT1080 cell had no statistical difference when comparing the 25, 50, 75 ng/mL groups with 0 ng/mL group (P>0.05). However, the relative survival rates of 100, 200, 300, 400, 500 ng/mL groups decreased comparing with the 0 ng/mL group (P<0.05) (Figure 2A).

The relative survival rates of U3A cell had no statistically difference when comparing the 25, 50, 75, 100 ng/mL groups with the group of 0 ng/mL (P>0.05). However, the relative survival rates of 200, 300, 400, 500 ng/mL groups decreased comparing with the 0 ng/mL group (P<0.05) (Figure 2B).
Apoptosis induced by HBV proteins

![Graphs showing apoptosis induced by HBV proteins](image)
Apoptosis induced by HBV proteins

Figure 4. The apoptotic cells detected by flow cytometry. HT1080 and U3A cells transfected with HBV, HBs, HBC, MHBs and LHBs were treated with TRAIL (50 ng/mL) for 48 h. The apoptotic cells were stained by AnnexinV-PI and detected by flow cytometry; the horizontal axis was FITC staining and the vertical axis was PI staining (A and B).
Apoptosis induced by HBV proteins

TRAIL concentration of 50 ng/mL was considered to have little effect on the survival rates of both cells according to the MTT results and was made to treat cells in the later experiment.

The relative survival rates of cells transfected with pHBV4.1, pcDNA3.1/HBs, pcDNA3.1/HBc, pcDNA3.1/MHBs, pcDNA3.1/LHBs were measured by MTT assay after TRAIL treatment.

The transfected HT1080 and U3A cells had been treated with TRAIL (50 ng/mL) for 48 h and MTT assay was performed to detect survival rates.

For HT1080 cells, the relative survival rates of 4.1+T group (65.22±0.75), S+T group (67.60±1.11), C+T group (65.40±2.21), MHBs+T group (87.19±1.38) decreased significantly compared with the PBS group (100.00±0.00) (P<0.05); however, there were no statistical differences between the transfected groups and the PBS group (P>0.05); nor was there any statistical difference between the TRAIL treated group and the PBS group (P>0.05) (Figures 3A).

There was no statistical difference in relative survival rates between the experimental groups and the PBS group of U3A cells (P>0.05) (Figures 3B).

TRAIL could inhibit the relative survival rates of HBV-transfected, HBs-expressing, HBc-expressing, MHBs-expressing HT1080 cells, but not the LHBs-expressing ones. However, HBV, HBs, HBc, MHBs and LHBs could not mediate TRAIL to inhibit the relative survival rates of U3A cells.

The apoptosis rates of cells transfected with pHBV4.1, pcDNA3.1/HBs, pcDNA3.1/HBc, pcDNA3.1/MHBs, pcDNA3.1/LHBs were measured by flow cytometry after TRAIL treatment.

The apoptosis of transfected HT1080 and U3A cells treated with TRAIL (50 ng/mL) were detected by flow cytometry.

The apoptosis rates of 4.1+T (22.61±1.75), S+T (24.27±0.30), C+T (19.59±0.53), MHBs+T (22.52±1.88) groups of HT1080 cells increased significantly compared with the PBS group (5.70±0.52) (P<0.05); however, there were no statistical differences between the transfected groups and the PBS group (P>0.05); nor was there any statistical difference between the TRAIL treated group and the PBS group (P>0.05) (Figures 4A, 5A).

There was no statistical difference in apoptosis rates between the experimental groups and the PBS group of U3A cells (P>0.05) (Figures 4B, 5B).

It suggested that HBV, HBs, HBC, MHBs but not LHBs could promote HT1080 cells to TRAIL-induced apoptosis. However, the HBV proteins could not increase the sensitivity of U3A cells to TRAIL-induced apoptosis.

The relative STAT1 expression of cells transfected with pHBV4.1, pcDNA3.1/HBs, pcDNA3.1/HBc, pcDNA3.1/MHBs, pcDNA3.1/LHBs were detected by western blot after TRAIL treatment.

The relative STAT1 expressions of HT1080 cells treated with TRAIL (50 ng/mL) were analysed by western blot. STAT1 expression of 4.1+T group (16.13±0.86) was higher than that of 4.1+PBS group (11.61±0.55) (P<0.05), S+T group (13.50±0.57) higher than S+PBS group (8.48±0.51) (P<0.05) and C+T group (12.61±0.53) higher than C+PBS group (5.75±0.07) (P<0.05); however, no statistical differences of the expression were found between MHBs+T group and MHBs+PBS group, LHBs+T group and LHBs+PBS group (P>0.05) (Figure 6A, 6C).

The STAT1 bands were not observed in U3A cells after TRAIL treatment (Figure 6B).

Discussion

The level of TRAIL in the peripheral blood and liver tissue of patients with HBV is much higher than that of the normal people [12, 13]. Higher TRAIL expression was also found on the CD4+ and CD8+ T cells of patients with chronic infection of HBV comparing with the healthy persons [14]. This suggests that TRAIL may participate in the anti-HBV process. TRAIL is more likely to induce apoptosis of virus-infected cells while without any effect on the normal ones, possibly due to the viral proteins. For example, HBx can enhance the sensitivity of HCC cells to TRAIL and lead them to apoptosis [10]. So other HBV proteins may also take part in the modulation of TRAIL-induced apoptosis. HBV infects the target cells and expresses kinds of proteins. We found that HBV transfection could increase the sensitivity of HT1080 cells to TRAIL-induced apoptosis.
Apoptosis induced by HBV proteins

The mechanisms that HBV proteins can promote TRAIL-induced apoptosis remain to be clarified, probably related with the high expression of TRAIL death receptors on the infected cells [15]. However, it was reported that preS2 protein was found to increase the sensitivity of HepG2.2.15 to the apoptosis mediated by TRAIL and this was not related with TRAIL receptor expression [16]. Other studies suggested that Hbc can inhibit hepatocyte apoptosis by blocking the TRAIL death receptor, DR5 [17]. However, opposite result that Hbc could promote TRAIL-induced apoptosis was found in the HT1080 cells in our study. Except for the change of TRAIL receptor expression, it may be explained by other mechanisms, for example, the increase of anti-apoptosis protein, Bax [18]. Activation of other signaling pathways may also be involved. It was reported that the truncated middle hepatitis B surface antigen MHBs(t) can increase the TRAIL-mediated apoptosis by activating ERK2 signaling pathway and by the deg-

Figure 5. The apoptosis rates of transfected cells treated with TRAIL. The apoptosis rates of HT1080 and U3A cells transfected with HBV, HBs, Hbc, MHBs and LHBs after TRAIL treatment (50 ng/mL) for 48 h were compared with the PBS groups (A and B); the datas were obtained from three independent experiments.

Figure 6. STAT1 expression detected by western blot. STAT1 expressions of HT1080 and U3A cells were detected by western blot, GAPDH was used as a loading control (A and B); STAT1 relative expressions of HT1080 cells were compared statistically (C); the datas were obtained from three independent experiments.
Apoptosis induced by HBV proteins

radiation of procaspases-3 and procaspases-9 [19]. We hypothesized that the modulation of HBV proteins in TRAIL-induced apoptosis may depend on the cellular context of infected cells and the regulation of related signal pathway.

We also observed that low concentration of TRAIL could not induce HBV-transfected U3A cell which could not express STAT1. So promotion of TRAIL-induced apoptosis by HBV proteins may depend on the integrity of STAT1 signaling pathway. Further study suggested that when treated with TRAIL, transfection of HBV, HBs, HBc could increase STAT1 expression, indicating that HBV proteins were modulated by STAT1 to promote TRAIL-induced apoptosis.

Interferons (IFN) are produced by the cells after the virus infection and exert antiviral effect by activating STAT1 signaling pathway. On the one hand, STAT1 can directly promote caspase expression. On the other hand, IFN is also regulated by STAT1 to produce caspase and increase the expression of TRAIL and its receptors in order to induce cell apoptosis [6]. Hepatitis B viruses infect cells and express HBV proteins which probably activate IFN and STAT1 signaling pathway. As both STAT1 and TRAIL can promote caspase-mediated apoptosis, there may be a crosstalk between STAT1 and TRAIL in apoptotic pathway that makes it possible to promote each other in inducing cell apoptosis. Thus, whether TRAIL-induced apoptosis promoted by HBV proteins is regulated by STAT1 and IFN signaling pathway needs further study.

In conclusion, this study indicated that HBV can promote TRAIL-induced apoptosis possibly contributed to HBs, HBc and MHBs, but not LHBs, and it was depended on the integrity of STAT1 signaling pathway. However, the mechanisms of TRAIL-mediated apoptosis promoted by HBV proteins and modulated by STAT1 remained to be elucidated.

Acknowledgements

This study was financially supported by the National Natural Science Foundation of China (No. 81170371).

Disclosure of conflict of interest

None.

References

Apoptosis induced by HBV proteins


