The S protein of hepatitis B virus promotes collagen type I expression in hepatic stellate cells by virtue of hepatocytes

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Abstract. This study was conducted in order to investigate whether hepatitis B surface S protein (HBs) was able to directly or indirectly promote the proliferation and expression of collagen type I (Col I) and α-smooth muscle actin (α-SMA) in hepatic stellate cells (HSCs). The LX-2 human cell line and the HepG2 human hepatocellular carcinoma cell line were employed as HSCs and as hepatocytes, respectively. Recombinant HBs was added to the LX-2 cells for 48 h and the cell proliferation was assessed by the MTT assay. Col I and α-SMA were measured in the supernatant by ELISA, following treatment of the LX-2 and/or HepG2 cells with recombinant HBs. Transforming growth factor-β1 (TGF-β1) was also determined by ELISA in the HepG2 cell supernatants. The data demonstrated that high concentrations of recombinant HBs (10-50 ng/ml) inhibited the proliferation of LX-2 cells, whereas low concentrations (0.5-5 ng/ml) did not affect LX-2 cell proliferation. After treating LX-2 cells alone with recombinant HBs for 48 h, there was no significant increase in the Col I and α-SMA levels. However, Col I was increased ~1.7-fold in co-cultured (LX-2 and HepG2) cell supernatants following treatment with HBs for 24 h (HBs vs. control group: 48.51±3.51 vs. 28.23±2.55 ng/ml, respectively). Furthermore, TGF-β1 was significantly increased in the HepG2 cell supernatants following treatment with recombinant HBs. Therefore, we concluded that HBs directly affected the proliferation of HSCs, but promoted the Col I expression in HSCs possibly by virtue of hepatocytes secreting TGF-β1. This may provide a novel explanation of the fibrogenetic mechanism induced by hepatitis B virus-related proteins.

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Introduction

The infection with hepatitis B virus (HBV) is a major health concern worldwide. It is estimated that ~350 million individuals are carriers of the hepatitis B surface S protein (HBs) and over one million patients eventually succumb to HBV-related chronic liver diseases annually (1-2). Persistent HBV infection confers a high risk of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (HCC) (3). Three forms of viral particles may be detected in the serum of HBV-infected patients: 42-nm diameter mature virion particles, 22-nm diameter spherical particles and 22-nm diameter filamentous particles (4-5). Subviral particles (22 nm), composed of HBs, are unique in that do not contain viral DNA and usually exceed the numbers of virions by ≥1,000-fold in the patient serum (5). A number of individuals reportedly reached a state of non-replicative infection following persistent anti-virus therapy; however, numerous HBs particles were still detected in their serum and the prolonged immunological response to infection may result in the development of fibrosis in the majority of the patients and eventually lead to the development of cirrhosis, liver failure, or HCC in ~40% of the patients (6). During this process, HBs may play an important role. However, the mechanism underlying the hepatic fibrogenesis induced by HBs has not yet been fully elucidated.

It was demonstrated that the progression of hepatic fibrosis requires sustained inflammation, leading to the activation of the hepatic stellate cells (HSCs) into a fibrogenic and proliferative cell type, such as the fibroblast (7). Regardless of the underlying disease, HSCs, the key fibrogenic cells, have been established as the main extracellular matrix (ECM)-producing cells in liver injury (8).

We hypothesized that HBs contributes to the regulation of HSCs activation and ECM deposition during the process of hepatic fibrogenesis. The proliferative activity and the expression of collagen type I (Col I) and α-smooth muscle actin (α-SMA) in HSCs were evaluated.

Materials and methods

Cell lines and cell culture. LX-2, a strain of human hepatic stellate cell line, was obtained from Professor Friedman SL. HepG2, a type of human HCC cell line, was purchased from the Insitute of Biochemistry and Cell Biology of the Chinese

Academy of Sciences (Shanghai, China). The LX-2 and HepG2 cells were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Grand Island, NY, USA) supplemented with 100 U/ml penicillin G, 100 μg/ml streptomycin and 10% fetal bovine serum (Gibco-BRL) in an incubator with 95% humidity and 5% carbon dioxide at 37°C.

Essential reagents. Recombinant HBs (no. 10-251-40733) was purchased from GenWay Biotech Inc. (San Diego, CA, USA). ELISA kits for Col I (CSB-E08082h; detection range, 1.56-100 ng/ml; sensitivity, 0.39 ng/ml), α-SMA (CSB-E09343h; detection range, 3.12-200 ng/ml; sensitivity, 0.78 ng/ml) and transforming growth factor-β1 (TGF-β1) (CSB-E04725h; detection range, 0.78-50 ng/ml; sensitivity, 0.39 ng/ml) were purchased from Cusabio Biotech Co., Ltd. (Wuhan, China).

MTT assay. The LX-2 cell proliferation was determined by the MTT assay. The cells were cultured at a density of 2×10^3 cells/well in flat-bottomed 96-well microplates. After 24 h, the experimental cultures were divided into 6 groups, followed by the addition of 0.5-50 ng/ml recombinant HBs per well. A total of 6 parallel cells were set for each group. After a 24- or 48-h incubation at 37°C, cell viability was determined by the MTT assay. The cells were incubated with 0.5 % MTT for 4 h. Upon removal of the supernatant, 150 μl dimethyl sulfoxide was added and shaken for 5 min until the crystals were dissolved. The optical density value at 492 nm (OD$_{492}$) was measured by ELISA. The negative control well was used as zero point of absorbance. All the experiments were independently performed in triplicate.

ELISA. Col I, α-SMA and TGF-β1 were measured by the standard sandwich ELISA according to the instructions provided by the manufacturer. A total of 6 parallel cells were set for each group. The absorbance was measured at 450 nm using a microplate reader (model 680; Bio-Rad, Hercules, CA, USA).

Statistical analysis. The results are expressed as means ± standard error (SE). The statistical analysis was performed with an analysis of variance and $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effect of HBs on LX-2 cell proliferation. The LX-2 cells were placed in 96-well plates and incubated with various concentrations of HBs. It was demonstrated that high concentrations of HBs (10-50 ng/ml) inhibited the proliferation of LX-2 cells. This inhibitory effect was gradually enhanced with increasing concentration of HBs. Low concentrations of HBs (0.5-5 ng/ml) did not affect cell proliferation (Fig. 1). Although the OD value was increased, no significant difference was observed in LX-2 cell survival when the HBs concentration reached a plateau of 1-5 ng/ml.

Effect of HBs on the secretion of α-SMA and Col I in LX-2 cells and co-culture system of HepG2 and LX-2 cells. As described above, the proliferation of LX-2 cells was significantly inhibited by HBs at concentrations ≥10 ng/ml. Therefore, the concentration
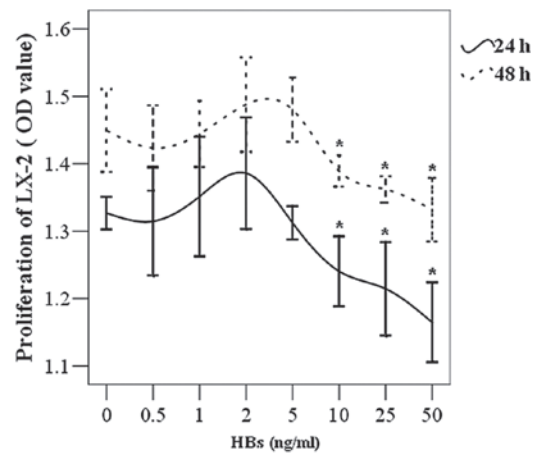

Figure 1. Effects of hepatitis B surface S protein (HBs) on LX-2 cell proliferation. The LX-2 cells were incubated with various concentrations of HBs. High concentrations of HBs (10-50 ng/ml) inhibited the proliferation of LX-2 cells, whereas low concentrations (0.5-5 ng/ml) did not affect LX-2 cell proliferation. No significant difference was observed in LX-2 cell survival, although the optical density (OD) value was increased, when HBs concentration reached a plateau of 1-5 ng/ml. *P<0.05. Error bars, ±2.00 SE.

of 10 ng/ml of HBs was employed in subsequent experiments. Col I is the major content of ECM and α-SMA is an indicator of HSCs transforming into fibroblasts. Col I and α-SMA were used to evaluate the role of HBs in the fibrogenetic process. The cells (2×10^3 cells/well) were cultured for 24 h and incubated with HBs (10 ng/ml) for 48 h. The ELISA results demonstrated that the changes in Col I and α-SMA were no different between the HBs treatment and control groups (Col I: 28.61±3.25 vs. 26.30±3.69 ng/ml, t=0.47, P=0.648 and α-SMA: 25.08±5.33 vs. 24.48±2.62 ng/ml, t=0.101, P=0.962, respectively).

A receptor of HBs exists in hepatocytes, although it has not been definitively determined. We investigated whether the expression of Col I and α-SMA in LX-2 cell supernatants was affected by hepatocytes. HepG2 (2×10^3 cells/well) and LX-2 cells (2×10^3 cells/well) were co-cultured for 24 h, incubated with HBs (10 ng/ml) for 24 h and the supernatants were collected for ELISA. The ELISA demonstrated that the Col I levels were significantly increased following HBs treatment (48.51±3.51 vs. 28.23±2.55 ng/ml, t=4.674, P=0.001), whereas there was no obvious change in α-SMA levels (30.66±2.69 vs. 23.42±3.86 ng/ml, t=1.538, P=0.155). Likewise, the HepG2 cells (2×10^3 cells/well) were cultured alone with HBs for 24 h to eliminate the secretion of Col I and α-SMA. The ELISA demonstrated that Col I was increased in the HepG2 cell supernatants (37.63±3.43 vs. 18.49±3.58 ng/ml, t=3.856, P=0.003), although α-SMA was not (20.70±2.38 vs. 18.46±1.48 ng/ml, t=0.799, P=0.443). However, Col I was significantly lower in the supernatant of HepG2 cells stimulated by HBs than that in the supernatant of the co-culture system (37.63±3.43 vs. 48.51±3.51 ng/ml, t=3.132, P=0.03) (Fig. 2).

Effect of HBs on secretion of TGF-β1 in HepG2 cells. TGF-β1 is considered to be the major cytokine affecting HSCs. To elucidate the mechanism underlying the effect of HepG2 cells on LX-2 cells, we investigated whether HBs promoted TGF-β1 secretion from HepG2 cells. The cells (2×10^3 cells/well) were
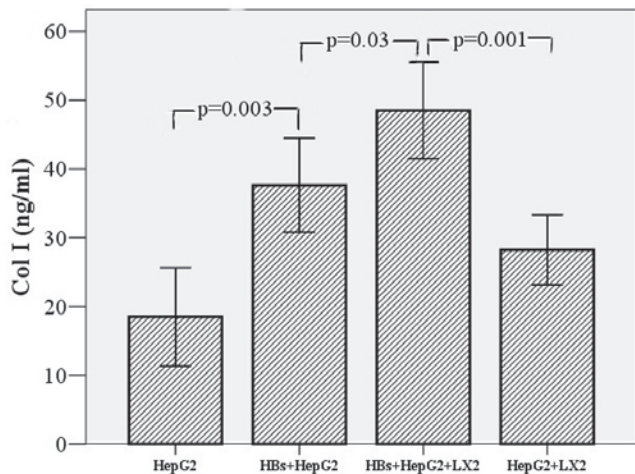
Figure 2. Effect of hepatitis B surface S protein (HBs) on secretion of α-smooth muscle actin (α-SMA) and collagen type I (Col I) in LX-2 cells and the co-culture system of HepG2 and LX-2 cells. To detect the effect of HBs on the expression of Col I and α-SMA in LX-2 cells, the cells ($2x10^3$ cells/well) were cultured for 24 h, then incubated with HBs (10 ng/ml) for 48 h. The change in Col I and α-SMA levels was no different between the HBs treatment and the control groups (data not shown): Col I, 28.61±3.25 vs. 26.30±3.69 ng/ml, t=0.47, P=0.648 and α-SMA, 25.08±5.33 vs. 24.48±2.62 ng/ml, t=0.101, P=0.962, respectively. HepG2 ($2x10^3$ cells/well) and LX-2 cells ($2x10^3$ cells/well) were co-cultured for 24 h, then incubated with HBs (10 ng/ml) for 24 h. Col I was found to be significantly increased following HBs treatment (48.51±3.51 vs. 28.23±2.55 ng/ml, t=4.674, P=0.001), whereas there was no obvious change in α-SMA (data not shown; 30.66±2.69 vs. 23.42±3.86 ng/ml, t=1.538, P=0.155). The HepG2 cells ($2x10^3$ cells/well) were cultured alone with HBs for 24 h to eliminate the secretion of Col I and α-SMA. The results demonstrated that Col I was increased in HepG2 cell supernatants (37.63±3.43 vs. 18.49±3.58 ng/ml, t=3.856, P=0.003), although α-SMA was not (20.70±2.38 vs. 18.46±1.48 ng/ml, t=0.799, P=0.443). However, Col I was significantly lower in the supernatant of HepG2 cells stimulated by HBs than in the supernatant of the co-culture system (37.63±3.43 vs. 48.51±3.51 ng/ml, t=3.132, P=0.03), whereas the data did not reveal similar results for α-SMA. Error bars, ±2.00 SE.

Figure 3. Effect of hepatitis B surface S protein (HBs) on the secretion of transforming growth factor-β1 (TGF-β1) in HepG2 cells. To elucidate the mechanism underlying the effect of HepG2 on LX-2 cells, the HepG2 cells ($2x10^3$ cells/well) were cultured for 24 h, then incubated with HBs (10 ng/ml) for 24 h. The results demonstrated that the TGF-β1 levels were higher in the HBs treatment group compared to those in the control group (9.80±1.89 vs. 6.49±1.41 ng/ml, t=3.635, P=0.005). *P<0.05. Error bars, ±2.00 SE.

cultured for 24 h, incubated with HBs (10 ng/ml) for 24 h and the TGF-β1 in the cell supernatants was measured by ELISA. The results demonstrated that the TGF-β1 levels were higher in the HBs treatment group compared to those in the control group (9.80±1.89 vs. 6.49±1.41 ng/ml, t=3.635, P=0.005) (Fig. 3).

Discussion

All chronic liver diseases may cause liver fibrosis through a similar pathway; however, different causes of liver injury may employ various mechanisms in this process. Among the major causes of chronic liver disease, hepatitis B confers a particularly high risk of fibrosis progression (9). Among the proteins encoded by HBV, it was demonstrated that proteins E and X may activate HSCs and directly promote the expression of collagens (10-15). However, whether HBs leads to fibrosis has not been established. Although HBs was identified as the neutralizing antigen of HBV and has been used as the major component of the preventive vaccine for viral hepatitis B, the persistence of HBs in the serum of patients has been recognized as a high-risk factor for the development of liver cirrhosis and HCC (16-17). One-fourth of the hepatitis B surface antigen-positive patients will eventually develop complications, such as cirrhosis or HCC, which constitute major causes of liver disease-related mortality (18).
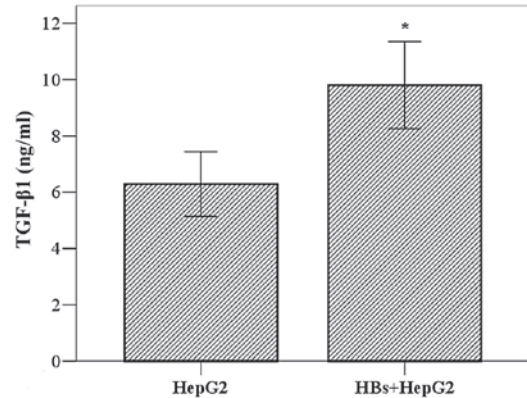
It was previously demonstrated that hepatocytes may be a harbor of refuge for hepatitis C virus (HCV) replication and the hepatocyte medium is stimulated by the HCV envelope protein, promoting HSC activity and production of Col I (19). HBs, similar to the envelope protein, exerts an effect similar to that of the HCV envelope protein. However, the number of available studies on HBs-related liver fibrosis is limited. We first investigated the effect of HBs on the proliferation of HSCs, which are considered to play a central role in hepatic fibrogenesis. There is a 98.7% similarity in gene expression between LX-2 cells and primary HSCs (20); therefore, LX-2 cells were used as substitutes of HSCs in our experiments. No effect on LX-2 cell proliferation was observed by low concentrations of HBs (0.5-5 ng/ml), whereas the proliferation was inhibited by high concentrations (10-50 ng/ml). However, Liu et al (15) reported that HBs (1.25-20 μg/ml) inhibited the proliferation of LX-2 cells, whereas low concentrations of HBs (0.04-0.62 μg/ml) promoted LX-2 cell proliferation. This difference may be attributed to the use of recombinant HBs. In the experiments conducted in that study, the injection vaccine protecting against hepatitis B was used as recombinant HBs and its constitution and purity may have affected the experimental results.

The secretion and expression of Col I and α-SMA at the protein level is an indicator of fibrosis and transformation of HSCs into fibroblasts, respectively (21). We demonstrated that the expression of Col I and α-SMA in LX-2 cell supernatants was not increased following treatment with HBs. Therefore, it was suggested that HBs is not a direct activator of LX-2 cells during the fibrogenetic process.

It was previously demonstrated that ethanol induces TGF-α expression in hepatocytes, leading to the stimulation of collagen synthesis by HSCs (22). Furthermore, toxic iron overload was shown to modulate HSCs proliferation and gene expression by rat hepatocytes (23). Accordingly, we hypothesized that HBs binds to its receptor on hepatocytes and sequentially stimulates the activation of LX-2 cells. Therefore, LX-2 and HepG2 cells were co-cultured in HBs conditioned medium and we observed that the Col I levels were significantly higher compared to those in the control group; however,

there was no significant difference regarding the production of α-SMA between the HBs treatment and the control groups, although the value was higher in the former. We hypothesized that HSCs increased the release and expression of Col I prior to their transformation into fibroblasts. In addition, it was demonstrated that HBs stimulated the enhanced expression of Col I, but not α-SMA, in HepG2 cells. However, the production of Col I was lower in HepG2 cells compared to that in the co-cultured system. Therefore, we concluded that HBs promotes Col I expression in HSCs by virtue of hepatocytes.

The activation of HSCs is triggered by adjacent hepatocytes, Kupffer cells and liver sinus endothelial cells, by a paracrine secretion pathway. TGF-β1, one of the most important cell factors secreted by the above-mentioned cells, significantly promotes collagen expression (8,24). Therefore, TGF-β1 was measured in the HepG2 cell supernatants. As was expected, HBs promoted TGF-β1 expression in HepG2 cells. We concluded that the increased secretion of latent TGF-β1 by hepatocytes is a potential factor affecting the fibrogenic behavior of HSCs. The HBs level is a reflection of the transcriptional activity of covalently closed circular DNA (cccDNA), is an important marker of active hepatitis B infection and may also predict clinical and treatment outcomes. Higher HBs levels indicate a higher risk of cirrhosis (25). The HBs quantification has been used for monitoring natural history and treatment outcome (26). The HBs concentration that inhibited HSCs promoted the expression of Col I in our study and proved the role of HBs in clinical cases. In addition, the presence of peritumoral activated HSCs in HBV-related HCC was recently demonstrated (27). HepG2 HCC cells activated HSCs through HBs in our study, in accordance with the above-mentioned findings. The inhibition of HSCs proliferation is considered to be the most important strategy for anti-fibrotic therapy (28). Our results indicated that inhibiting the HBs receptor expression may be a target for the treatment of the liver fibrosis.

There were certain limitation to our study. Although primary human cells are difficult to obtain, the use of human primary liver cells and primary HSCs may validate our conclusions. In summary, our data suggest that the HBs of HBV is crucial in liver fibrogenesis. Through HBs stimulation, the hepatocytes exhibit increased expression of TGF-β1 and promote Col I production in adjacent HSCs. This may be a novel explanation for the fibrogenetic mechanism induced by HBV-related proteins. However, further investigations of the role of HBs in fibrogenesis are required.

Acknowledgements

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