Biochemical and Biophysical Research Communications 435 (2013) 391–396

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Hepatitis B virus e antigen induces activation of rat hepatic stellate cells

Yanlu Zan [a,b], Yuxia Zhang [a,*], Po Tien [a,*]

[a] Center for Molecular Virology, CAS Key Laboratory of Pathogenic Microbiology and Immunology, Institute of Microbiology, Chinese Academy of Sciences, Beijing 100101, PR China
[b] University of Chinese Academy of Sciences, Beijing 100049, PR China

ARTICLE INFO

Article history:
Received 15 April 2013
Available online 9 May 2013

Keywords:
Hepatic stellate cells
Hepatitis B virus e antigen
Collagen I
TGF-β
α-SMA
TIMP-1

ABSTRACT

Chronic hepatitis B virus infection is a major cause of hepatic fibrosis, leading to liver cirrhosis and hepatocellular carcinoma. Hepatitis B virus e antigen (HBeAg) is an accessory protein of HBV, not required for viral replication but important for natural infection *in vivo*. Hepatic stellate cells (HSCs) are the major producers of excessive extracellular matrix during liver fibrogenesis. Therefore, we examined the influence of HBeAg on HSCs. The rat HSC line HSC-T6 was transfected with HBeAg plasmids, and expression of α-smooth muscle actin, collagen I, transforming growth factor-β1 (TGF-β), and tissue inhibitors of metalloproteinase 1 (TIMP-1) was investigated by quantitative real-time PCR. The proliferation of HSCs was determined by MTS analysis. HBeAg transduction induced up-regulation of these fibrogenic genes and proliferation of HSCs. We found that HBeAg induced TGF-β secretion in HSCs, and the activation of HSCs was prevented by a neutralizing anti-TGF-β antibody. Depletion and addition of HBeAg protein in conditioned medium from HSC-T6 cells transduced with HBeAg indicated that HBeAg directly induced the activation and proliferation of rat primary HSCs. Taken together, HBeAg induces the activation and proliferation of HSCs, mainly mediated by TGF-β, and HBeAg protein purified from cell medium can directly activate HSCs.

1. Introduction

Liver fibrosis is a chronic disorder that results in accumulation of excessive amounts of extracellular matrix (ECM) and distortion of the normal liver architecture [1,2]. The activation and transformation of quiescent hepatic stellate cells (HSCs) into myofibroblast-like cells constitute a major mechanism for the increased production of ECM in the liver. Activated HSCs display increased production of ECM components and profibrotic factors, such as collagen I, connective tissue growth factor (CTGF), and tissue inhibitor of metalloproteinase-1 (TIMP-1). HSCs can be activated by a variety of cytokines and growth factors, including transforming growth factor β (TGF-β), tumor necrosis factor alpha (TNF-α), and platelet-derived growth factor (PDGF) [1,3,4].

In the liver, injury by a variety of means results in a rapid induction of TGF-β synthesis, predominantly in HSCs. Concomitant with increased TGF-β production, HSCs increase the production of collagen. TGF-β plays an important role in the pathogenesis of liver fibrosis (fibrogenesis) due to its multiple effects on HSC activation and transdifferentiation, stimulation of matrix protein expression, depression of matrix metalloproteinase, increase of tissue inhibitors of metalloproteinase (TIMPs), and autoinduction of cytokines [5,6]. HSCs are not only the source, but also the target cells, of TGF-β.

Development of cirrhosis and hepatocellular carcinoma (HCC) is strongly associated with chronic hepatitis B virus (HBV) infection [7,8]. However, the mechanism of hepatic fibrogenesis induced by HBV is not fully unstood. Recent research suggests that some hepatitis C virus (HCV) proteins may contribute to hepatic fibrogenesis via interaction with HSCs [9–12]. Other investigations revealed that hepatitis B virus X protein induces paracrine activation of human HSCs [13]. Further, continuous inhibition of HBV-DNA replication can delay clinical progression in patients with chronic hepatitis B and advanced fibrosis or cirrhosis [14,15]. *In vitro* experiments indicate that HBV affects the proliferation and expression of collagen I in HSCs [7]. The function of the hepatitis B virus e antigen (HBeAg) is largely unknown because it is not required for viral assembly, replication, or infection [16–18]. It is known that there are more HBeAg-positive patients with liver fibrosis than HBeAg-negative patients, especially at the early stage of the disease, but survival is significantly reduced for Chi-

Abbreviations: HCC, hepatocellular carcinoma; HBeAg, hepatitis B virus e antigen; HSC, hepatic stellate cell; α-SMA, α-smooth muscle actin; TIMP, tissue inhibitors of metalloproteinase; TGF-β, transforming growth factor β; ELISA, enzyme-linked immunoabsorbent assay.

* Corresponding authors. Current address: The Walter and Eliza Hall Institute of Medical Research, Parkville, VIC 3052, Australia (Y. Zhang). Fax: +86 10 64807381.
E-mail addresses: yzhang@wehi.edu.au (Y. Zhang), tienpo@sun.im.ac.cn (P. Tien).

0006-291X/$ - see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2013.04.098

nese patients with HBeAg-negative rather than HBeAg-positive cirrhosis [19]. In this work, we describe the ability of HBeAg to induce HSCs activation.

2. Materials and methods

2.1. Cell lines, primary cells, and culture

We grew cell lines 293T, HepG2, and HSC-T6 [20] in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM streptomycin, 5 mM L-glutamine, and 100 U/ml penicillin in a 5% CO_2 incubator (Gibco).

Rat primary HSCs were routinely prepared by collagenase/pronase digestion of rat liver using a perfusion system and subsequent fractionation of the heterogenous cell suspension on continuous density Percoll (Amersham) gradients as described previously [21]. The isolated HSCs were cultured in DMEM, supplemented with 2% FBS (Sciencell, San Diego, CA) and used for four passages.

The HSCs were cultured in serum-depleted DMEM during the activation and conditional medium incubation analysis. After transfection with Lipofectamine 2000 (Invitrogen), HSC-T6 cells were incubated in DMEM supplemented with 10% FBS for 4 h to recover, and medium was then changed to serum-depleted DMEM.

2.2. Lentivirus packaging and cell clone selection

To construct stable HBeAg expression cell lines, we used a lentivirus package system (a gift from Prof. Liu W.J., IMCAS) containing the plenti3.7 vector and three packaging vectors: VSVG, RSV-REV and pMDLg/pRRE [22]. Briefly, we cloned HBeAg (with or without a V5-8His tag) into the plenti3.7 vector and co-transfected it with an appropriate amount of packaging vectors into 293T cells. Forty-eight hours after transfection, virus was harvested and added to HepG2 cells. The following day, the infected cells were selected with puromycin (1 μg/ml, Amresco) for 2 weeks, and the medium was refreshed every 3 days.

2.3. Purification of HBeAg using Ni-NTA resin

Cell medium without FBS from HepG2-HBeAg-8His cells (adjusted to a final concentration of 100 mM Tris–HCl, 150 mM NaCl, pH 8.0) was transferred to a Ni-NTA (Invitrogen) column. After washing the column with two column volumes of wash buffer (100 mM Tris–HCl, 150 mM NaCl, pH 8.0, and 20 mM Imidazol), we eluted the protein complex with elution buffer (100 mM Tris–HCl, 150 mM NaCl, pH 8.0, and 200 mM Imidazol). The eluent was concentrated, and the buffer was changed to PBS.

2.4. RNA isolation and quantitative real-time PCR

Total RNA was extracted using TRIZOL reagent according to the manufacturer's instructions (Invitrogen). The RNA was first reverse-transcribed into cDNA using a PrimScript RT reagent Kit (Takara) and then subjected to qPCR with GoTaq qPCR Master Mix (Promega). Real-time PCR was performed on an Applied Biosystem 7300 real-time PCR system, and data was analyzed using 7300 system SDS software (ABI). The primers used for real-time PCR are listed in Table 1.

Table 1
Primers for amplification of rat genes.

Gene	Forward primer	Reverse primer
α-SMA	5′TGTGCTGGACTCTGGAGATG3′	5′GATCACCTGCCCATCAGG3′
COL1A1	5′CCTGGCAAGAACGGAGATGAT3′	5′ACCGACAGCACCATCGTTACC3′
COL3A1	5′TGGCCAACCAGGAGAGAAGG3′	5′ATCCGTCTCGACCTGGCTGA3′
TGFB1	5′CAAAGACATCACACACAGTA3′	5′AGGTGTTGAGCCCTTTCCAG3′
TIMP-1	5′GATTCGACGCTGTGGGAAAT3′	5′GGCCCGCGATGAGAAACT3′
GAPDH	5′TGCACCACCAACTGCTTAG3′	5′GGATGCAGGGATGATGTTC3′

2.5. Enzyme-linked immunosorbent assay (ELISA)

ELISAs were performed to relatively quantify protein secreted into medium during conditional cell culturing. All of the procedures followed the manufacturer's instructions. A Diagnostic Kit for Hepatitis B e Antigen was purchased from KEHUA BIOENGINEERING CO., LTD. A Rat Transforming Growth Factor β1 (TGF-β1) ELISA Kit and a Rat Collagen Type I (COL1) ELISA Kit were obtained from R&D Systems, Inc.

2.6. Antibodies

The following primary antibodies were used: antibodies to α-SMA, V5, and neutralizing antibodies to TGF-β (Invitrogen) and GAPDH (Santa Cruz Technology). The HRP-conjugated or FITC-conjugated secondary antibody were purchased from ZSGB-BIO, Inc.

2.7. Immunofluorescence staining

To determine the expression of HBeAg in HepG2 clones and HSC-T6, we fixed the cells with 2–4% formaldehyde in PBS for 15 min at room temperature. After rinsing the cells in PBS three times for 5 min, we incubated the cells in ice-cold 100% methanol for 10 min to permeabilize them. We incubated the cells with anti-V5 primary antibody overnight, then FITC-anti-mouse secondary antibody. All of the samples were examined using fluorescence microscopy or a confocal imaging system.

2.8. Western blotting

Proteins were extracted with cell extraction buffer containing 0.05 M Tris–HCl (pH 8.0), 0.15 M NaCl, 5.0 mM EDTA, 1% NP-40, and protease inhibitors cocktail (Sigma) at 4 °C. After centrifugation, soluble protein in the extract was quantified according to the methods supplied with the DC protein assay reagent (BioRad). Briefly, after denaturation at 95 °C for 5 min, proteins were separated by SDS–PAGE using 12% gels and transferred onto PVDF membranes. The membranes were blocked in TBST buffer (50 mM Tris–HCl, 100 mM NaCl, and 0.1% Tween 20, pH 7.4) containing 5% skim milk and incubated with primary antibody for 1 h at room temperature. The membrane was further incubated with secondary antibody for 45 min at room temperature. The signals were visualized by subsequent chemoluminescent reaction with HRP-IgG using the ECL system (Amersham).

2.9. Proliferation assay

Five thousand HSCs per well were seeded into 96-well plates in serum-depleted medium with different treatments, and proliferation was analyzed by the MTS cell proliferation assay (Promega).

2.10. Data presentation and statistical analyses

All experiments were performed at least three times. Student's unpaired *t*-test was used to analyze the results. Data were considered to be significantly different at $P < 0.05$ and are presented as means ± SEM. Statistical calculations were performed using Prism 5.0 software (Graphpad Software, San Diego, CA).

3. Results

3.1. HBeAg induces expression of fibrogenic-relevant genes in HSC-T6

Liver fibrosis is characterized by the production of α-SMA, collagen I, COL3A1, TIMP-1, and other fibrotic markers by activated HSCs [3]. To determine whether HBeAg could activate HSCs [13], we investigated the expression of fibrogenic-relevant genes in HSCs transduced with pHBeAg (pcDNA3.0-HBeAg-V5). Immunofluorescence staining with anti-V5 primary antibody revealed that HBeAg is mostly present in the cytoplasm of HSC-T6 (Fig. 1A). We analyzed the expression of α-SMA, collagen I, COL3A1, and TIMP-1 by western blotting of protein extracts and quantitative real-time PCR (qRT-PCR) using RNA extracted from HSC-T6 [13]. The expression of HBeAg in HSC-T6 resulted in the production of α-SMA as detected by western blotting (Fig. 1B). qRT-PCR also showed that HBeAg expression induced increases in the mRNA levels of α-SMA (1.21 ± 0.03-fold) and collagen I (1.58 ± 0.08-fold), but no significant differences were observed for COL3A1 and TIMP-1 (Fig. 1C). Moreover, we detected an increase in collagen I secretion (1.65 ± 0.12-fold) into the culture medium of HSC-T6 cells (Fig. 1D).

HSC proliferation is another feature of liver fibrosis. To determine whether HBeAg contributes to hepatic fibrogenesis by up-regulation of the proliferation of HSCs, we analyzed the proliferative capacity of HSCs induced by the expression of HBeAg using MTS analysis. We found that pHBeAg transduction increased the proliferation of HSC-T6 as is seen for pHBV1.3 (carrying 1.3 copies of the HBV genome, producing HBV), suggesting a proliferative response in the HSCs to HBeAg expression (Fig. 1E).

3.2. TGF-β mediates the activation of HSC-T6 by HBeAg expression

TGF-β plays a key role in the pathogenesis of liver fibrosis (fibrogenesis) due to its multiple effects on HSC activation and the stimulation of ECM protein expression [23–26]. HSCs are both an important source and the predominant target cell of TGF-β. RNA samples were extracted for qRT-PCR from HSC-T6 cells transfected with control or pHBeAg plasmids. HBeAg expression induced up-regulation of TGF-β mRNA expression (1.5 ± 0.03-fold) (Fig. 2A). Next, the amount of TGF-β secreted into the cell culture supernatant with different treatments was determined by ELISA, and the concentration was up-regulated from 137.7 ± 8.8 pg/ml to 185.3 ± 5.4 pg/ml (Fig. 2B). These results confirmed the up-regulation of TGF-β during the activation of HSCs by HBeAg.

To identify if TGF-β was responsible for the activation of HSCs by HBeAg expression, we incubated transfected HSC-T6 cells with a neutralizing anti-TGF-β antibody to block the TGF-β pathway and compared them with cells exposed to an isotype-matched control antibody [13]. The anti-TGF-β blocking antibody prevented the increase in collagen I expression, both in the HSC-T6 cells transfected with pHBeAg and pHBV1.3 (Fig. 2C).

3.3. HBeAg directly contributes to the activation of rat HSCs

To investigate the function of circulating HBeAg on HSC activation, we subsequently determined the direct function of HBeAg se-

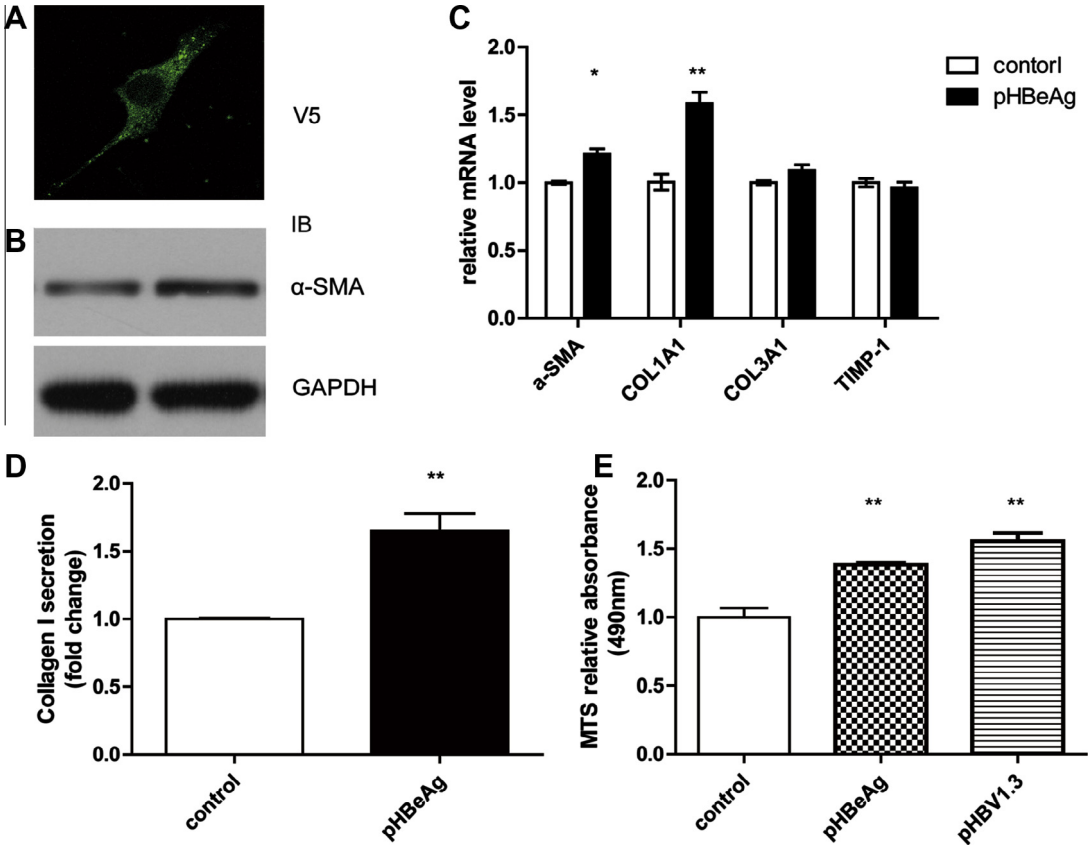

Fig. 1. HBeAg expression resulted in activation of HSC-T6 cells. HSC-T6 cells were transfected with control (pcDNA3.0), pHBeAg (pcDNA3.0-HBeAg-V5), or pHBV1.3 (carrying 1.3 copies of the HBV genome) plasmids. Twenty-four hours post-transfection, the expression of HBeAg in HSC-T6 cells was analyzed by immunofluorescence staining with an anti-V5 antibody (A). The expression of α-SMA was determined by western blotting. The left lane is a sample transfected with pcDNA3.0, and the right is from a pHBeAg-transfected sample (B). The mRNA expression of fibrotic markers α-SMA, collagen I (COL1A1), COL3A1, and TIMP-1 was analyzed by quantitative real-time PCR (C). The concentration of collagen I secreted into the medium after transfection was measured by ELISA (D). MTS assay indicated cell proliferation (E). Results are expressed as the fold induction ± the standard error (SE) over the values of HSC-T6 cells transfected with pcDNA3.0. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ (Student's *t*-test, $n = 3$).

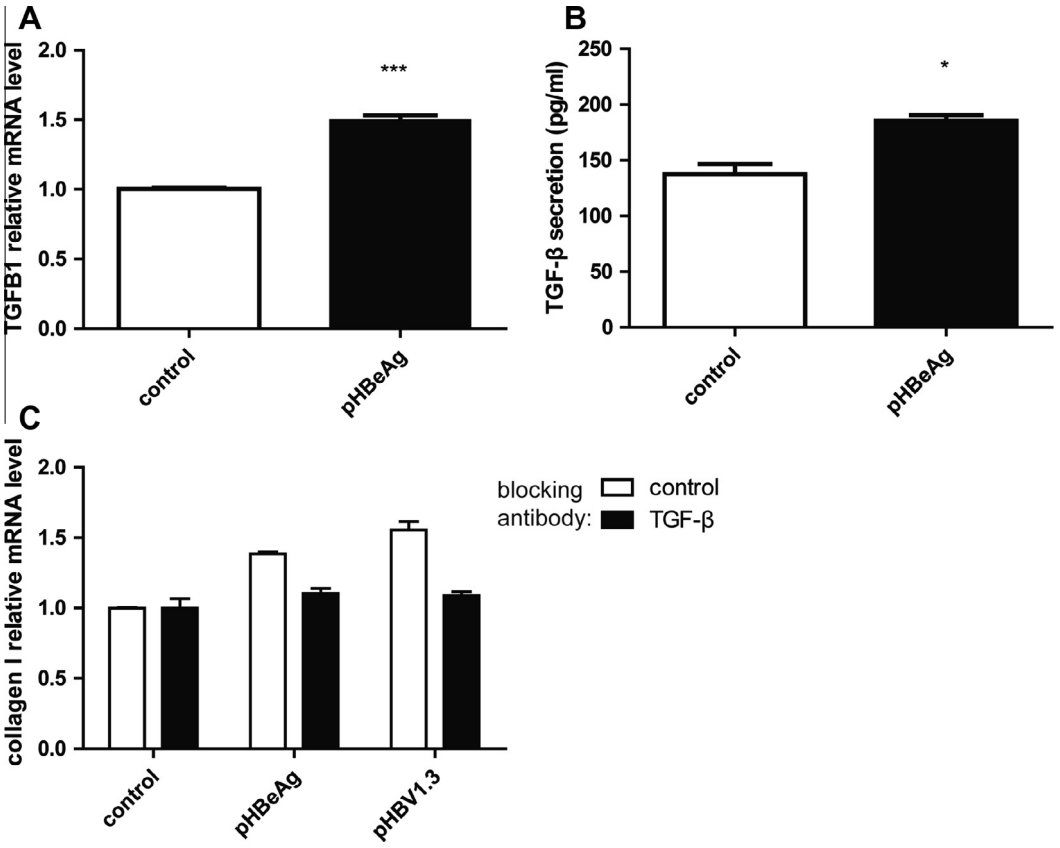

Fig. 2. HBeAg-induced activation of HSC-T6 cells is mediated by the TGF-β pathway. HSC-T6 cells were transfected with control, pHBeAg, or pHBV1.3 plasmids as in Fig. 1. The expression of TGFB1 mRNA was analyzed by qRT-PCR (A). The amount of TGF-β secreted into the cell culture supernatant was determined by ELISA (B). A neutralizing antibody against TGF-β was added to the medium after transfection, while an isotype-matched control antibody was used as a negative control. Collagen I mRNA expression was determined by qRT-PCR to indicate the activation of HSC-T6 cells (C). Results are expressed as the fold induction ± the standard error (SE) over the values of HSC-T6 cells transfected with pcDNA3.0. $^{*}P < 0.05$; $^{**}P < 0.01$; and $^{***}P < 0.001$ (Student's *t*-test, $n = 3$).

creted into the medium. HBeAg secreted into the medium by the stable cell line HepG2-HBeAg-V5-8His (Fig. 3A and B) was purified with Ni-NTA resin as described in the materials and methods. We identified purified HBeAg by SDS–PAGE and western blotting (Fig. 3C and D).

To eliminate the effect of HBeAg, protein-G beads coupled with anti-V5 primary antibody were incubated with medium from serum-starved HSC-T6 24 h post-transfection with control or pHBeAg plasmids. Rat primary HSCs were then exposed to the conditioned medium for 24 h. The mRNA expression of collagen I, representing the activation of HSCs, was analyzed by qRT-PCR (Fig. 4A), and HSC proliferation was measured by MTS assay (Fig. 4B). We found that depleting HBeAg from the conditioned medium decreased the activation of rat HSCs, and this down-regulation could be abrogated after the addition of purified HBeAg (Fig. 3).

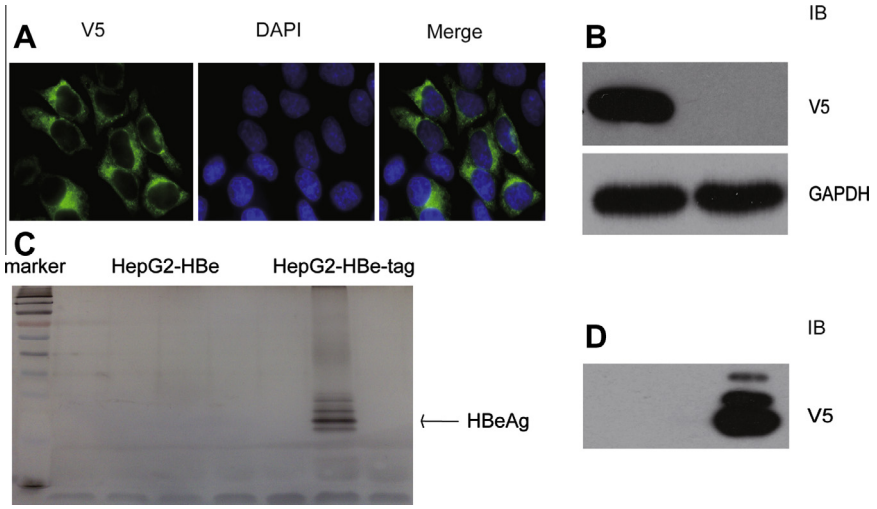

Fig. 3. HBeAg was purified using the Ni-NTA purification system. The stable cell line HepG2-HBe-tag (HepG2-HBe-V5-8His), expressing HBeAg with a V5 peptide tag and an 8xHis tag, was observed by immunofluorescence staining (A). Western blotting also shows HBeAg in HeG2-HBe-tag cells (B). HBeAg secreted into the cell culture was purified by Ni-NTA beads, concentrated, and buffer exchanged by ultrafitration. SDS–PAGE (C) and western blotting (D) were used to detect the purified HBeAg.

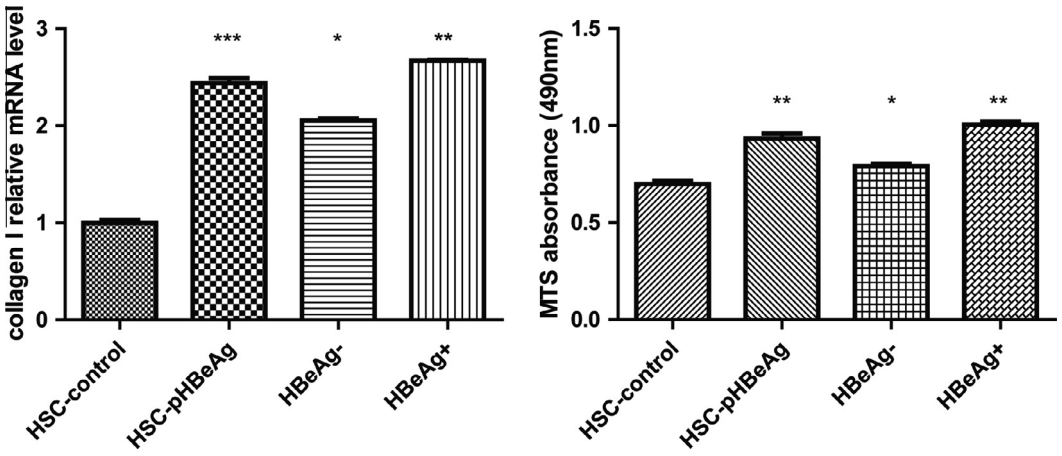

Fig. 4. HBeAg directly contributed to the activation of rat HSCs. HSC-T6 cells were transfected with control or pHBeAg plasmids. Rat primary HSCs were stimulated with conditioned medium from transfected HSC-T6 cells. The mRNA expression of collagen I was detected by qRT-PCR to represent activation of rat HSCs (A). MTS analysis of the proliferation of rat HSCs (B). Lane 1: exposure of rat HSCs to medium from serum-starved HSC-T6 cells transfected with control plasmids; lane 2: medium from serum-starved HSC-T6 cells transfected with pHBeAg plasmids; lane 3: HBeAg was depleted with protein-G beads coupled with anti-V5 antibody from the medium of lane 2; lane 4: HBeAg was added to the medium of lane 3 to a final concentration of 1 μg/ml.

4. Discussion

Advanced liver fibrosis results in cirrhosis, liver failure, and portal hypertension and often requires liver transplantation. Chronic infection by HBV is a major cause of liver fibrosis and eventually leads to the development of liver cirrhosis and HCC [7,8,27]. Clinic-pathologic examinations reveal that secretion of HBeAg, a protein that is not required for viral replication, correlates with the progression of liver disease in patients with chronic HBV infection [18,28]. Here, we focused on the functional involvement of HBeAg in liver fibrogenesis. In this work, we present different lines of evidence supporting the ability of HBeAg to induce autocrine HSC activation and proliferation, suggesting a direct implication for HBV in liver fibrogenesis.

Liver fibrosis is the excessive accumulation of ECM proteins, including α-SMA and collagen I, produced by HSCs that occurs in most types of chronic liver disease and liver injury. In this regard, we observed increased expression of α-SMA and collagen I in HSCs expressing HBeAg. This effect was accompanied by induction of fibrotic marker TGF-β in HSCs, which mediate HSC proliferation, adhesion, and collagen synthesis. Thus, HBeAg promotes the release of soluble mediators capable of activating HSCs to produce ECM components and related factors, thereby contributing to fibrogenesis in chronically HBV-infected patients.

The role of TGF-β as a major inducer of liver fibrosis is well established [5,6,23–26,29]. The transgenic mouse model gives *in vivo* evidence for the important role of TGF-β1 in HSC activation and liver fibrogenesis [30]. Here, we identified TGF-β as the major factor responsible for the autocrine activation of HSCs by HBeAg. HBeAg directly induced expression of TGF-β, and TGF-β in turn mediated the activation and proliferation of HSCs .

HBeAg-positive patients are prominent among those suffering from liver fibrosis caused by chronic HBV infection in China, especially at the early stage of the disease. Because HBeAg is an important virus antigen in sera, the direct interaction between HBeAg and HSCs was determined. Incubation with purified HBeAg directly induced the activation of HSCs.

In summary, our results provide evidence that HBeAg induces autocrine activation of HSCs and suggest that HBeAg can directly contribute to hepatic fibrogenesis in HBV-infected patients. These results highlight a new aspect of HBV biology that is potentially relevant for understanding the pathogenesis of HBV-induced liver fibrosis and a basis for possible new anti-fibrotic therapy strategies.

5. Author contributions

Yanlu Zan performed the experiments, data analysis, and wrote the manuscript; Yuxia Zhang and Po Tien designed the study.

6. Supportive foundations

This work was supported by grants from the National Basic Research Program of China (973 Program) (No. 2011CB504703, 2010CB530102) and the National Natural Science Foundation of China (NSFC, Grant Nos. 81021003, 31270211).

Acknowledgments

We are grateful to Prof. W.J. Liu of IMCAS for kindly providing the lentivirus system. We also thank Weihua Zhuang and Fulian Liao for their kind help with the experiments. This work was supported by grants from the National Basic Research Program of China (973 Program) (No. 2011CB504703, 2010CB530102) and the National Natural Science Foundation of China (NSFC, Grant Nos. 81021003, 31270211).

References

[1] A.M. Gressner, The cell biology of liver fibrogenesis – an imbalance of proliferation, growth arrest and apoptosis of myofibroblasts, Cell Tissue Res. 292 (1998) 447–452.
[2] A.O. Wilensky, The pathogenesis and mechanism of cirrhosis of the liver, Am. J. Dig. Dis. 13 (1946) 367–372.
[3] R. Bataller, D.A. Brenner, Liver fibrosis, J. Clin. Invest. 115 (2005) 209–218.
[4] A. Desmouliere, Hepatic stellate cells: the only cells involved in liver fibrogenesis? A dogma challenged, Gastroenterology 132 (2007) 2059–2062.
[5] D.M. Bissell, D. Roulot, J. George, Transforming growth factor beta and the liver, Hepatology 34 (2001) 859–867.
[6] H. Zhu, L. Zeng, D. Zhu, Y. Yuan, The role of TGF-beta 1 in mice hepatic fibrosis by Schistosomiasis Japonica, J. Tongji Med. Univ. 20 (2000) 320–321 (329).
[7] X. Liu, S.T. Zhu, H. You, M. Cong, T.H. Liu, B.E. Wang, J.D. Jia, Hepatitis B virus infects hepatic stellate cells and affects their proliferation and expression of collagen type I, Chin. Med. J. (Engl.) 122 (2009) 1455–1461.
[8] W.K. Seto, C.F. Lee, C.L. Lai, P.P. Ip, D.Y. Fong, J. Fung, D.K. Wong, M.F. Yuen, A new model using routinely available clinical parameters to predict significant liver fibrosis in chronic hepatitis B, PLoS One 6 (2011) e23077.

[9] S. Clement, S. Pascarella, S. Conzelmann, C. Gonelle-Gispert, K. Guilloux, F. Negro, The hepatitis C virus core protein indirectly induces alpha-smooth muscle actin expression in hepatic stellate cells via interleukin-8, J. Hepatol. 52 (2010) 635–643.
[10] M. Coenen, H.D. Nischalke, B. Kramer, B. Langhans, A. Glassner, D. Schulte, C. Korner, T. Sauerbruch, J. Nattermann, U. Spengler, Hepatitis C virus core protein induces fibrogenic actions of hepatic stellate cells via toll-like receptor 2, Lab. Invest. 91 (2011) 1375–1382.
[11] F.H. Pace, L.E. Ferreira, A.E. Silva, M.L. Ferraz, Liver fibrosis progression in HIV/hepatitis C virus coinfected patients with normal aminotransferases levels, Rev. Soc. Bras. Med. Trop. 45 (2012) 444–447.
[12] A. Probst, T. Dang, M. Bochud, M. Egger, F. Negro, P.Y. Bochud, Role of hepatitis C virus genotype 3 in liver fibrosis progression – a systematic review and meta-analysis, J Viral Hepat. 18 (2011) 745–759.
[13] S. Martin-Vilchez, P. Sanz-Cameno, Y. Rodriguez-Munoz, P.L. Majano, F. Molina-Jimenez, M. Lopez-Cabrera, R. Moreno-Otero, E. Lara-Pezzi, The hepatitis B virus X protein induces paracrine activation of human hepatic stellate cells, Hepatology 47 (2008) 1872–1883.
[14] Y.F. Liaw, J.J. Sung, W.C. Chow, G. Farrell, C.Z. Lee, H. Yuen, T. Tanwandee, Q.M. Tao, K. Shue, O.N. Keene, J.S. Dixon, D.F. Gray, J. Sabbat, Lamivudine for patients with chronic hepatitis B and advanced liver disease, N. Engl. J. Med. 351 (2004) 1521–1531.
[15] F.M. Sanai, A. Helmy, K.I. Bzeizi, M.A. Babatin, A. Al-Qahtani, H.A. Al-Ashgar, A.S. Al-Mdani, A. Al-Akwaa, S. Almutharea, M.Q. Khan, A.S. Alghamdi, T. Farah, W. Al-Hamoudi, M. Saadeh, A.A. Abdo, Discriminant value of serum HBV DNA levels as predictors of liver fibrosis in chronic hepatitis B, J Viral Hepat. 18 (2011) 217–225.
[16] M.S. Li, T.C. Lau, S.K. Chan, C.H. Wong, P.K. Ng, J.J. Sung, H.L. Chan, S.K. Tsui, The G1613A mutation in the HBV genome affects HBeAg expression and viral replication through altered core promoter activity, PLoS One 6 (2011) e21856.
[17] H.I. Yang, H.L. Hung, M.H. Lee, J. Liu, C.L. Jen, J. Su, L.Y. Wang, S.N. Lu, S.L. You, U.H. Iloeje, C.J. Chen, Incidence and determinants of spontaneous seroclearance of hepatitis B e antigen and DNA in patients with chronic hepatitis B, Clin. Gastroenterol. Hepatol. 10 (2011) 527–534.
[18] D. Milich, T.J. Liang, Exploring the biological basis of hepatitis B e antigen in hepatitis B virus infection, Hepatology 38 (2003) 1075–1086.
[19] H. Ma, L. Wei, F. Guo, S. Zhu, Y. Sun, H. Wang, Clinical features and survival in Chinese patients with hepatitis B e antigen-negative hepatitis B virus-related cirrhosis, J. Gastroenterol. Hepatol. 23 (2008) 1250–1258.
[20] S. Vogel, R. Piantedosi, J. Frank, A. Lalazar, D.C. Rockey, S.L. Friedman, W.S. Blaner, An immortalized rat liver stellate cell line (HSC-T6): a new cell model for the study of retinoid metabolism in vitro, J. Lipid Res. 41 (2000) 882–893.
[21] L. Riccalton-Banks, R. Bhandari, J. Fry, K.M. Shakesheff, A simple method for the simultaneous isolation of stellate cells and hepatocytes from rat liver tissue, Mol. Cell. Biochem. 248 (2003) 97–102.
[22] T. Dull, R. Zufferey, M. Kelly, R.J. Mandel, M. Nguyen, D. Trono, L. Naldini, A third-generation lentivirus vector with a conditional packaging system, J. Virol. 72 (1998) 8463–8471.
[23] S. Dooley, J. Hamzavi, L. Ciuclan, P. Godoy, I. Ilkavets, S. Ehnert, E. Ueberham, R. Gebhardt, S. Kanzler, A. Geier, K. Breitkopf, H. Weng, P.R. Mertens, Hepatocyte-specific Smad7 expression attenuates TGF-beta-mediated fibrogenesis and protects against liver damage, Gastroenterology 135 (2008) 642–659.
[24] D. Eurich, M. Bahra, S. Boas-Knoop, J.F. Lock, J. Golembus, R. Neuhaus, P. Neuhaus, U.P. Neumann, Transforming growth factor beta1 polymorphisms and progression of graft fibrosis after liver transplantation for hepatitis C virus-induced liver disease, Liver Transplant 17 (2011) 279–288.
[25] S.W. Hong, K.H. Jung, H.S. Lee, H.M. Zheng, M.J. Choi, C. Lee, S.S. Hong, Suppression by fucoidan of liver fibrogenesis via the TGF-beta/Smad pathway in protecting against oxidative stress, Biosci. Biotechnol. Biochem. 75 (2011) 833–840.
[26] H. Seyhan, J. Hamzavi, E. Wiercinska, A.M. Gressner, P.R. Mertens, J. Kopp, R.E. Horch, K. Breitkopf, S. Dooley, Liver fibrogenesis due to cholestasis is associated with increased Smad7 expression and Smad3 signaling, J. Cell. Mol. Med. 10 (2006) 922–932.
[27] H. Kwon, A.S. Lok, Hepatitis B therapy, Nat. Rev. Gastroenterol. Hepatol. 8 (2011) 275–284.
[28] C.Y. Yang, T.H. Kuo, L.P. Ting, Human hepatitis B viral e antigen interacts with cellular interleukin-1 receptor accessory protein and triggers interleukin-1 response, J. Biol. Chem. 281 (2006) 34525–34536.
[29] N.M. Meindl-Beinker, S. Dooley, Transforming growth factor-beta and hepatocyte transdifferentiation in liver fibrogenesis, J. Gastroenterol. Hepatol. 23 (Suppl. 1) (2008) 122–127.
[30] S. Kanzler, A.W. Lohse, A. Keil, J. Henninger, H.P. Dienes, P. Schirmacher, S. Rose-John, K.H. zum Buschenfelde, M. Blessing, TGF-beta1 in liver fibrosis: an inducible transgenic mouse model to study liver fibrogenesis, Am. J. Physiol. 276 (1999) 1059–1068.