CELL INJURY, REPAIR, AGING, AND APOPTOSIS

Repression of Death Receptor—Mediated Apoptosis of Hepatocytes by Hepatitis B Virus e Antigen

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Hepatitis B virus (HBV) e antigen (HBeAg) is associated with viral persistence and pathogenesis. Resistance of HBV-infected hepatocytes to apoptosis is seen as one of the primary promoters for HBV chronicity and malignancy. Fas receptor/ligand (Fas/FasL) and the tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) system plays a key role in hepatic death during HBV infection. We found that HBeAg mediates resistance of hepatocytes to FasL or TRAIL cytotoxicity in a p53-dependent manner. HBeAg further inhibited the expression of p53, total Fas, membrane-bound Fas, TNF receptor superfamily member 10a, and TNF receptor superfamily member 10b at both mRNA and protein levels. In contrast, HBeAg enhanced the expression of soluble forms of Fas through facilitation of Fas alternative mRNA splicing. In a mouse model, expression of HBeAg in mice injected with recombinant adenovirus—associated virus 8 inhibited agonistic anti-Fas antibody—induced hepatic apoptosis. Xenograft tumorigenicity assay also found that HBeAg-induced carcinogenesis was resistant to the proapoptotic effect of TRAIL and chemotherapeutic drugs. These results indicate that HBeAg may prevent hepatocytes from FasL and TRAIL-induced apoptosis by regulating the expression of the proapoptotic and antiapoptotic forms of death receptors, which may contribute to the survival and persistence of infected hepatocytes during HBV infection. (*Am J Pathol* 2019, 189: 2181–2195; <https://doi.org/10.1016/j.ajpath.2019.07.014>)

Hepatitis B virus (HBV) infection remains a major health problem worldwide because approximately 240 million individuals are chronically infected with HBV.¹ Meanwhile, the detailed mechanisms underlying chronic and acute HBV infection and deep pathophysiologic processes have not been well defined. It is well known that HBV infection may regulate the apoptotic signal to accelerate viral proliferation and hepatocellular carcinoma progression.^{2–4} Hepatic apoptosis during HBV infection is mostly mediated by death signaling from members of the tumor necrosis factor (TNF) protein family, including TNF- α , Fas ligand (FasL), and TNF-related apoptosis-inducing ligand (TRAIL).⁵ Fas, a typical glycoprotein, is abundantly distributed in the liver cell membrane and triggers apoptosis signals to the hepatocytes when Fas ligand (FasL) or agonistic anti-Fas antibody binds with it.⁶ FasL forcefully induces massive hepatic apoptosis in healthy

liver or most types of liver disease. Primary human and mice hepatocytes are sensitive to apoptosis induced by the agonistic anti-Fas antibody and active FasL.⁷ Fas is also considered a soluble molecule (sFas) beside its membrane-associated form (mFas),⁸ indicating a deeper layer of complexity in regulation of apoptosis. sFas, arising from alternatively spliced Fas mRNA, leads to proteins with deletion or disruption of the single membrane-spanning domain.⁸ Serum sFas is increased in patients with chronic and acute hepatitis B— or HBV-related hepatocellular carcinoma.^{9,10}

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The Fas/FasL apoptosis signal is strictly regulated at both receptor and intracellular apoptotic signaling events. Previous studies suggest that Fas transcription is activated by tumor suppressor gene *p53* in hepatocytes, and the cross talk between the Fas signaling and *p53* in controlling apoptosis is of clinical importance.^{11–13} The key role of *p53* in HBV-dependent control of apoptotic signal is not clear, but it has been reported that the regulation of apoptosis by HBV core protein (HBc) may be *p53* dependent and independent.^{2,14,15} Similar to FasL, TRAIL also activates the *p53*-dependent extrinsic pathway by binding to the death receptors, such as TNF receptor superfamily member 10a (DR4) and TNF receptor superfamily member 10b (DR5) at the cell surface.^{16,17}

The multifunction of the hepatitis B e antigen (HBeAg) remains unknown because it is not essential for viral replication, assembly, and infection.¹⁸ The clinical data reveal that secreted HBeAg can play an immunoregulatory role in HBV natural infection. However, intracellular HBeAg acts as a target for the specific immune response. The dual roles of the HBeAg and its ability to activate or tolerize T cells reveal the complexity of the interactions between the host and the HBeAg during viral infection.^{19,20} However, HBeAg and its precursors may function as a significant enhancer of hepatocellular carcinoma through many mechanisms, including repressing *p53* activity through interacting with NUMB.²¹ The original intention of this research is to investigate whether HBeAg is involved in controlling the Fas and TRAIL apoptosis pathway. We found that HBeAg inhibits the FasL- and TRAIL-induced apoptosis of hepatocytes *in vitro* and *in vivo* by regulating the expression of proapoptotic and antiapoptotic form of death receptors.

Materials and Methods

Ethics Statement

Cryopreserved primary human hepatocytes (PHHs) were purchased from BioreclamationIVT (Brussels, Belgium), which obtains and distributes consented human material from a network of institutional review board–approved collection sites under adherence to effective ethical and regulatory guidelines.

Cell Lines and Cell Culture

The human hepatoma cell lines HepG2 (wild-type *p53*), Huh-7 (mutant *p53*), Hep3B (deficient *p53*), and primary mouse hepatocytes (PMHs) were cultured as previously described.^{2,22} PHHs were thawed according to the manufacturer's instructions and cultured in William's Medium E (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum, 2 mmol/L L-glutamine, and 100 U/mL of penicillin with 100 mg/mL of streptomycin.

Plasmid Constructs

pcDNA3.1-HBeAg, pcDNA3.1-precure, pcDNA3.1-p22, and pcDNA3.1-*p53* were kindly provided by Dr. De-yin Guo (School of Basic Medical Sciences, Wuhan University, Wuhan, China).²¹ The promoter-driving firefly luciferase containing Fas (pGL4.10-Fas), DR4 (pGL4.10-DR4), and DR5 (pGL4.10-DR5) was constructed by ligation of the PCR-generated respective minimal promoter into the luciferase reporter plasmid pGL4.10 Vector (Promega, Madison, WI) as described previously.^{12,16,17}

The schematic diagram of the strategy used for pRep-HBV and pRep-Sal construction has been described previously.¹⁵ The *p56* plasmid, which is a pUC18 backbone that contains the full-length HBV DNA genome from strain 56 that has a high replication efficiency (3215 bp, genotype B, adw subtype; <https://www.ncbi.nlm.nih.gov/nucleotide>; accession number AF100309), was digested first with XbaI and RsrII. A 1.3-kb DNA fragment called C was recovered, which corresponds to the 249- to 1573-nt region of the HBV genome. The *p56* plasmid was then digested with SapI to release the free 56 HBV DNA, followed by self-ligation. The resulting circular DNA was digested with BamHI and XbaI, and a 2.0-kb fragment called B was recovered. Fragment B corresponds to the 1402- to 249-nt region of the HBV genome that contains the basic core promoter with Enhancer I. Next, a 0.4-kb fragment called D, which corresponds to the 1573- to 2000-nt region of the HBV genome, was amplified by PCR and digested with RsrII and SalI. The primers used included pC: 5'-CATTTCATGGCTGCTAGGC-3' and pD: 5'-ACGCGTCGACGAGATCTCGAATAGAAGGAAAG-3'. Finally, to exclude the possible influence of gene transcription upstream of the HBV DNA, a transcription blocker fragment was generated by PCR amplification, using the pSEAP-Basic plasmid (Clontech, Palo Alto, CA) as a template. The primers used were TBf: 5'-ACGCGTCGACAATAAAATATCTTTATTTTC-3' and TBb: 5'-CGCGGATCCAGAGAAATGTTCTGGCACC-3', and the 0.1-kb amplified fragment called A was digested with SalI and BamHI. Fragments A to D were ligated head-to-tail into the pREP10 vector (Invitrogen, Carlsbad, CA), which had the expression cassette, including the Rous sarcoma virus long terminal repeat and polyadenylation signal removed by SalI digestion. The recombinant plasmid, called pREP-HBV, was confirmed by restriction enzyme digest and DNA sequence analyses. The control plasmid, named pREP-SalI, was constructed by removing the expression cassette, including the Rous sarcoma virus long terminal repeat and polyadenylation signal from pREP10 by SalI digestion, followed by self-ligation.

The pRep-HBV-HBeAg(–) and pRep-HBV-HBeAg&HBc&HBx(–) triple mutation plasmids were generated by site-directed mutagenesis as described previously.²³ The pRep-HBV-HBeAg(–) contains the nonsense mutation site 28th aa of HBeAg. The pRep-HBV-HBeAg&HBc&HBx(–)

contains both the nonsense mutation site 38th aa of HBeAg and 8th aa of HBx.

Construction of Stable HBeAg-Expressing Hepatoma Cell Lines

The hepatoma cells were transfected with pHBeAg or the empty control plasmid and then selected in the presence of 400 µg/mL of hygromycin for 4 weeks. The hygromycin-resistant clones were expanded together into cell lines and screened for the extent of HBeAg protein expression by Western blot analysis.

Production of HBeAg-Expressing Recombinant Adenoviruses

The detailed procedures of construction and generation of the recombinant adenoviruses are described previously.²⁴

Generation of HBeAg-Expressing Recombinant Lentivirus

Thirty-percent confluent 293T cell monolayers in 10-cm culture dishes were cotransfected with pCDH-HBeAg-FLAG and the packaging vectors of pMDL, p-VSV-G, and pREV (Invitrogen) and cultured for 48 hours. The culture medium was collected and clarified by low-speed centrifugation. The supernatant after filtration through 0.45-µm filters was added into the culture of PHH cells grown in 6-cm culture dishes. The expression of HBeAg was confirmed by Western blot analysis.

AAV Injection

The adeno-associated virus (AAV)-8 expression system was used to achieve overexpression of wild-type HBeAg in liver. High-titer AAV-8 particles were produced and supplied by Obio Technology Corp Ltd. (Shanghai, China). A total of 2×10^{11} vg of AAV-8 in 100 µL of phosphate-buffered saline (PBS) was intravenously injected into age-matched (6 to 8 weeks old) C57BL/6 mice weighing 16 to 18 g. Mice were subsequently treated for 3 weeks.

Isolation of PMHs

PMHs were isolated from C57BL/6 male mice by a two-step collagenase perfusion technique.²² Briefly, live hepatic parenchymal cells were separated by 35% Percoll. After seeding for 4 to 6 hours, cells were refreshed with medium and cultured overnight.

Animal Experiments

All work performed with animals was in accordance with and approved by the Institutional Animal Care and Use

Committee at Fujian Medical University. The Institutional Animal Care and Use Committee protocol number for the study is M00186. Male, age-matched (6 to 8 weeks old) C57BL/6 mice (Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai, China) weighing 16 to 18 g were maintained in ventilated cages under a 12-hour light/dark cycle. Mice at 3 weeks after injection with HBeAg-expressing or empty control AAV-8 were treated intraperitoneally with the agonistic anti-Fas Jo2 antibody (554255; BD Pharmingen, San Diego, CA) diluted in 100 µL of PBS at 0.2 µg/g. The same dosage of mouse IgG (catalog number 555740; BD Biosciences, San Jose, CA) was used as a control for Jo2. Liver and blood samples were taken at 12 hours after injection of the antibody. Serum levels of alanine transaminase were determined using a standard clinical automatic analyzer (Hitachi 7020, Hitachi, Kyoto, Japan). Immediately after the blood samples were taken retro-orbitally, mice were sacrificed by cervical dislocation. The liver was excised, sectioned, and fixed overnight at 4°C in 10% formalin solution. The sections were then dehydrated, paraffin embedded, and cut at 3-mm thickness and stained with hematoxylin and eosin for histologic examination.

Tumor Xenograft Experiments and TRAIL Treatment

Tumor xenograft experiments in nude mice were performed as previously described.²⁵ One week later, mice (5 per group) were randomly assigned to one of four treatment groups: PBS, TRAIL 10 mg/kg per day i.p., doxorubicin (Adriamycin; Sigma-Aldrich) 0.1 mg/kg per day i.p., or TRAIL 10 mg/kg per day i.p. and doxorubicin 0.1 mg/kg per day i.p. Mice were treated for an additional 3 weeks. The induction of apoptosis in formalin-fixed, paraffin-embedded hepatoma xenografts was measured by the terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay (Promega).

Plasmid Transfection and RNA Interference

Transient transfections with various plasmids were performed as previously described.² siRNAs against the P53 gene *p53* siRNA (sc-29435, Santa Cruz Biotechnology, Dallas, TX) and corresponding control siRNA (sc-37007, Santa Cruz Biotechnology) were transfected into HepG2 cells in six-well plates using the X-tremeGENE siRNA Transfection Reagent (Roche Applied Science, Penzberg, Germany) according to the manufacturer's instructions. Gene silencing effect was confirmed by Western blot analysis and RT-PCR at 48 hours after transfection.

Western Blot Analysis

Western blot analysis was described previously.^{2,26} The specific antibodies used in this study included anti-FLAG (catalog number 14793, 1:1000 dilution, Cell Signaling

Technology, Beverly, MA), anti- β -tubulin (catalog number 2128, 1:1000 dilution, Cell Signaling Technology), anti-p53 (K0181-3, 1:1000 dilution, MBL, Nagoya, Japan), anti-mFas (catalog number 4233, 1:1000 dilution, Cell Signaling Technology), anti-DR4 (catalog number 42533, 1:1000 dilution, Cell Signaling Technology), anti-DR5 (catalog number 8074, 1:1000 dilution, Cell Signaling Technology), anti-Fas (ab82419, 1:1000 dilution, Abcam, Cambridge, UK), anti-HBc (sc-23947, 1:500 dilution, Santa Cruz Biotechnology), and anti-HBx (sc-57760, 1:500 dilution, Santa Cruz Biotechnology).

Treatment of Cells with Chemicals and Antibodies

In HepG2 cells or PHHs, Fas receptor stimulation was performed with 10 ng/mL of super-hFasL (ALX-522-020, Enzo Life Sciences Inc., Farmingdale, NY). DR4/DR5 receptor stimulation was performed with 1 μ g/mL of active recombinant human soluble TRAIL (catalog number 310-04, PeproTech, Rocky Hill, NJ). In PMHs, Fas receptor stimulation was performed with the agonistic monoclonal antibody anti-Fas Jo2 (catalog number 554255, BD Pharmingen) alone at 2 μ g/mL. To enhance FasL-induced apoptosis, cells were pretreated with the 60 μ g/mL of the p53 inducer bleomycin (Sigma-Aldrich) or 0.2 μ g/mL of doxorubicin (Sigma-Aldrich). To neutralize TRAIL-induced apoptosis, cells were pretreated with 10 μ g/mL of DR4 (HS101, 10 P's BVBA, Breda, the Netherlands) and/or DR5 blocking antibody (HS201, 10 P's BVBA, Breda, the Netherlands).

HBeAg Quantitative Analysis

Levels of cell medium and serum HBeAg was tested quantitatively by Architect assay (Abbott Laboratories, Chicago, IL). The Architect assay is a two-step immunoassay according to the method of chemiluminescent microparticles. Briefly, samples were mixed with paramagnetic beads presenting anti-HBeAg antibodies. After a washing step, a conjugate and reactant were added and a light signal was emitted, which was proportional to HBeAg concentration within a linear-wide range. Standardized calibration of the Architect assay was performed using the World Health Organization international standard for HBeAg (subtype adw2, genotype A). The Architect HBeAg assay is calibrated to give results in international units per milliliter.

CCK-8 Assay

Cell proliferation and viability were measured using a CCK-8 assay as previously described.²⁷

TUNEL Assay

Liver tissues were dissected and fixed in 4% paraformaldehyde overnight, dehydrated, embedded in paraffin, and then subjected to a TUNEL assay as previously reported.²⁷

Annexin V Binding Assay

Cells were treated with proapoptotic chemicals and/or antibodies. Apoptosis was detected using an fluorescein isothiocyanate annexin V apoptosis detection kit (BD Pharmingen) according to the manufacturer's instructions and analyzed by flow cytometry (FACSVerse; BD Biosciences) using FACSuite software version 1.0 (BD Biosciences).

Quantitative Real-Time PCR Analysis

Quantitative real-time PCR analysis was described previously.^{2,28}

Semiquantitative RT-PCR Analysis of mFas and sFas

Transcribed cDNA was used as a template for PCR amplification. The paired primers P1 and P2 were used for total Fas amplification and P3 and P5 for mFas/sFas amplification; primer sequences were reported previously.² PCR products were analyzed on a 2.5% agarose gel by electrophoresis and ethidium bromide staining. A digital image of the gel was obtained using a SynGene apparatus and the SynGeneGeneSnap software version 4.00.00 (SynGene, San Diego, CA). Individual band intensities were quantitated using the densitometric software Quantity One version 4.6 (Bio-Rad, Hercules, CA). sFas or mFas mRNA level was expressed as a ratio of sFas or mFas PCR product signal to that of glyceraldehyde-3-phosphate dehydrogenase.

ELISA for sFas, TNF- α , and IL-6

sFas in cell culture supernates was detected using a human sFas enzyme-linked immunosorbent assay (ELISA) kit (R&D Systems, Minneapolis, MN) according to the manufacturer's instructions. Cytokine concentrations in the mice serum were examined by ELISA (ADI-900-047 of PeproTech for TNF- α and KMC0061 of Invitrogen for IL-6). The absorbance of each well was measured at 450 nm using a microplate reader (Bio-Rad). The concentrations of the cytokines were calibrated from a dose-response curve based on reference standards.

Fas Splicing Assay

The procedures for Fas splicing assay were followed exactly as previously described.²

Caspase Enzymatic Activity Assay

Activities of caspase 3/7 were measured by using the Apo-ONETM Homogeneous Caspase-Glo 3/7 assay kit, respectively (G8091, Promega), according to the manufacturer's instructions.

Figure 1 Hepatitis B virus (HBV) e antigen (HBeAg) transfection decreases the sensitivity of hepatoma cells to Fas ligand (FasL)-induced apoptosis. **A:** Intracellular HBeAg expression in HepG2 cells verified by Western blot analysis using the FLAG tag antibody and secreted HBeAg by Architect assay. The Architect HBeAg assay is calibrated to give results in international units per milliliter. Cell viability determined by CCK-8 assay 24 hours after HepG2-pcDNA or HepG2-pHBcAg cells were treated with increasing concentrations of FasL. **B:** Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining performed on HepG2-pcDNA or HepG2-pHBcAg cells treated with FasL for 24 hours. Quantification of apoptotic cells stained with TUNEL and expressed as percentage of the number of TUNEL-positive cells to the total number of cells counted. **C:** Quantification of apoptotic cell fractions by annexin V/propidium iodide (PI) staining. Cells received the same treatments as indicated in panel B and were subjected to flow cytometric analysis. **D:** Measurements of caspase-3/7 enzymatic activity expressed as the fold change relative to that in control cells. **E:** Expression of HBeAg, HBeAg, and HBx in HepG2-pRep HBV, HepG2-pRep HBV HBeAg(-), HepG2-pRep HBV HBeAg(-), and HBeAg&HBc&HBx(-) cells was assessed by Western blot analysis. Flow cytometric analysis on the fraction of apoptotic cells 8 hours after the indicated cells were treated with FasL. **F:** HBeAg and its precursors received the same treatments as indicated in panel E and were subjected to flow cytometric analysis. Data are expressed as means $\pm$ SD. $n = 3$. * $P < 0.05$ [t -test (**A** and **D**) and χ^2 test (**B**, **C**, **E**, and **F**)]. Scale bars = 100 μ m.

Dual-Luciferase Reporter Assay

HepG2-pcDNA3.1 or HepG2-pHBeAg cells were transfected with 0.2 μ g of pGL4.10-promoter reporter plasmids and 0.2 μ g of the Renilla luciferase expression vector pRL-TK. The cells were harvested 48 hours after the transfection. A total of 20 μ g of cell lysate was used for the detection of intracellular luciferase activity by the Dual-Luciferase Reporter Assay System (Promega) following the manufacturers' recommendations. The firefly luciferase activity was normalized against the Renilla reniformis luciferase activity of the co-transfected pRL-TK to control for variations in transfection efficiency. The relative luciferase units were obtained by comparison with the pGL4.10-promoter reporters in HepG2-pcDNA3.1, which was set to 1. Luminescence was measured on a luminometer (Orion II Microplate Luminometer, Berthold Detection Systems, Pforzheim, Germany).

Statistical Analysis

The χ^2 test was used to analyze differences between the apoptosis rates. The statistical significance in the differences between mRNA and protein levels was analyzed by analysis of variance. The least significant difference method was used to further post hoc analysis. The differences between two groups were analyzed by using a *t*-test. $P < 0.05$ was considered statistically significant. All analyses were performed by IBM SPSS software version 19.0 (IBM Corp, Armonk, NY). Data are presented as means $\pm$ SD

determined from a minimum of three independent experiments each performed with triplicate cultures.

Results

HBeAg Decreases the Sensitivity of Hepatoma Cells to FasL-Induced Apoptosis

To explore the role of HBeAg in regulation of apoptosis, HepG2 (wild-type p53) sublines stably transfected with control vector or HBeAg-expressing pcDNA3.1-HBeAg were generated and their sensitivity verified to the active proapoptotic FasL. The successful expression of intracellular or secreted HBeAg was confirmed by Western blot analysis and Architect assay (Figure 1A). The CCK-8 assay was used to explore whether HBeAg expression could modulate cellular sensitivity to FasL. The FasL concentration-survival curves reveal that HepG2 cells expressing HBeAg (HepG2-pHBeAg) were less sensitive to the cytotoxic effect of FasL compared with control (Figure 1A). To explore whether the increased viability of HepG2-pHBeAg cells was due to decreased apoptosis, the frequency of apoptotic cells was tested by the TUNEL assay. A marginal but significant decrease in the rate of apoptosis after 10-ng/mL FasL treatment was detected in the HepG2-pHBeAg population compared with control (Figure 1B). Furthermore, the decreased apoptosis of HepG2 cells augmented by HBeAg was also determined by annexin V binding or caspase-3/7 activities (Figure 1, C and D). An opposite result was defined with HepG2 cells transfected with 1.2-U lengths of the HBV genome (pRep-HBV), revealing the

Figure 2 Regulation of hepatitis B virus (HBV) e antigen (HBeAg) on Fas-induced apoptosis depends on wild-type p53 status. **A** and **B**: The expression of HBeAg in Huh7 and Hep3B cells stably transfected with pHBeAg or the control vector was confirmed. Western blot analysis for HepG2 or Hep3B cells transiently transfected with p53 siRNA or p53 gene. **C**: Cell viability determined by CCK-8 assay 24 hours after Huh7-pcDNA3.1 and Huh7-pHBeAg or Hep3B-pcDNA3.1 and Hep3B-pHBeAg cells treated with increasing concentrations of Fas ligand (FasL). **D**: Annexin V staining performed on Huh7-pcDNA3.1 and Huh7-pHBeAg or Hep3B-pcDNA3.1 and Hep3B-pHBeAg cells treated with FasL for 8 hours. **E**: Annexin V staining performed on HepG2-pcDNA3.1 or HepG2-pHBeAg cells treated with FasL for 8 hours with transfection with p53 siRNA or p53 gene. Data are expressed as means $\pm$ SD. $n = 3$. $*P < 0.05$ [*t*-test (**C**) or χ^2 test (**D** and **E**)]. NC, negative control.

Figure 3 Regulation of hepatitis B virus (HBV) e antigen (HBeAg) on p53 and Fas transcription and expression. **A:** Quantitative real-time PCR analysis of p53 mRNA levels in HepG2-pcDNA3.1 and HepG2-pHBeAg cells on p53 overexpression. **B:** The effect of HBeAg transfection on p53 protein levels is shown by Western blot. **C:** Quantitative real-time PCR analysis of total Fas mRNA levels in HepG2-pcDNA3.1 and HepG2-pHBeAg cells on p53 overexpression. The primer P1 is located in exon 3/4 of the *Fas* gene and the primer P2 located in exon 4 of *Fas* gene. **D:** Quantitative real-time PCR analysis of membrane-associated Fas (mFas) mRNA levels in HepG2-pcDNA3.1 and HepG2-pHBeAg cells. The primer P3 is located in exon 3/4 of the *Fas* gene, and the primer P4 is located in exon 6 of the *Fas* gene. **E:** The effect of HBeAg transfection on mFas protein levels on p53 overexpression is shown by Western blot. **F:** Transcriptional regulation of Fas by HBeAg. HepG2-pcDNA3.1 or HepG2-pHBeAg cells were co-transfected with pGL4.10-Fas that contained the Fas promoter sequence and the Renilla luciferase expression vector pRL-TK on p53 overexpression. The level of promoter activity was evaluated 48 hours after transfection by measuring the luciferase activity normalized for variations in transfection efficiency and expressed as the fold change relative to the untreated HepG2-pcDNA3.1 cells. Data are expressed as means ± SD (**A–F**). $n = 3$. * $P < 0.05$ (analysis of variance).

enhanced sensitivity to Fas-induced apoptosis (Figure 1E). Interestingly, transfection of the HBeAg mutant pRep-HBV-HBeAg(–) into HepG2 further strengthened Fas-induced apoptosis compared with cells transfected with pRep-HBV (Figure 1E). Both HBc and HBx inhibit Fas-mediated apoptosis.^{2,28} Expectedly, transfection of HBeAg, HBc, and HBx triple mutant pRep-HBV-HBeAg&HBc&HBx(–) into HepG2 greatly increased Fas-induced apoptosis compared with cells transfected with pRep-HBV and pRep-Sall (Figure 1E). Lastly, HBeAg and its precursors (precore, p22) had the equal inhibitory effects of Fas apoptotic signal (Figure 1F). The data reveal that HBeAg may limit the sensitivity of HepG2 cells to Fas-mediated apoptosis and likely to function as an antagonist of Fas/FasL-induced hepatic apoptosis.

HBeAg Control of FasL-Induced Apoptosis Depends on Wild-Type p53 Status

The Fas receptor could be directly transactivated by p53 in hepatoma cells.^{11,29} Whether reduction of Fas-mediated

apoptosis by HBeAg was through a p53-dependent manner was tested using Huh7 and Hep3B, which represents p53 mutant³⁰ and p53 null³¹ status, respectively. The two human hepatoma cell lines were stably transfected with pcDNA3.1-HBeAg, and HBeAg expression was determined in Huh7-pHBeAg and Hep3B-pHBeAg cells (Figure 2A). Meanwhile, the effect of p53 siRNA or overexpression was verified in HepG2 and Hep3B cells (Figure 2B). Interestingly, only in the presence of wild-type p53 did FasL treatment generate effective and differential cytotoxicity to Huh7-pHBeAg or Hep3B-pHBeAg cells (Figure 2, C and D). To further confirm the key role of p53 in Fas apoptotic signal, p53 wild-type HepG2-pcDNA3.1 and HepG2-pHBeAg cells were transfected with p53 siRNA or p53 gene followed by FasL treatment then tested for annexin V binding assay. As expected, transfection with p53 siRNA fully abrogated FasL-induced apoptosis regardless of HBeAg expression, whereas p53 overexpression powerfully enhanced the apoptosis rate, with the effect being greater in control than the HepG2-pHBeAg cells (Figure 2E). Totally, the data strongly illustrate that inhibition of Fas-mediated

Figure 4 Hepatitis B virus (HBV) e antigen (HBeAg) (HBeAg) enhances soluble Fas (sFas) transcription and expression. **A:** Expression of sFas in culture medium of HepG2-pcDNA3.1 and HepG2-pHBeAg cells measured by enzyme-linked immunosorbent assay on p53 expression. **B and C:** Semiquantitative RT-PCR analysis of total Fas, membrane-associated Fas (mFas), and sFas mRNA levels in HepG2-pcDNA3.1 and HepG2-pHBeAg cells on p53 overexpression and knockdown. The PCR product using paired primers P1 and P2 represents total Fas, whereas the PCR products of paired primers P3 and P5 (located at exon 7) represent mFas and sFas varied by size. **D:** Representative agarose gel image showing the accumulation of alternatively spliced transcripts in HepG2-pcDNA3.1 and HepG2-pHBeAg cells transfected with pCMV56-Fas vector that contained human Fas genomic sequences from exons 5 to 7. The products were analyzed on agarose gels (expected size: 230 bp for exon 6 inclusion and 167 bp for exon 6 skipping). **E:** The ratio of exons 5+7 to exons 5+6+7 amplification products, equivalent to ratio of sFas to (sFas+mFas) RNA, determined densitometrically on the band intensity from three independent experiments in the pCMV56-Fas transfected HepG2-pcDNA3.1 and HepG2-pHBeAg cells. Data are expressed as means $\pm$ SD. $n = 3$. * $P < 0.05$ [analysis of variance (A–C) and t -test (E)].

hepatic apoptosis by HBeAg depends on wild-type p53 status.

HBeAg Inhibits p53 and Fas Expression

Considering that HBeAg inhibited Fas apoptotic signal, which depends on p53 expression, and that HBeAg could down-regulate p53 expression,²¹ the relative expression of p53 and Fas in control and HepG2-HBeAg cells on p53 overexpression was determined by quantitative RT-PCR and Western blot analysis. Transfection of HepG2-pcDNA3.1 and HepG2-HBeAg cells with p53 enhanced p53 expression at both mRNA (Figure 3A) and protein levels (Figure 3B), representing the effectiveness of the treatment. Meanwhile, the augmentation was less pronounced in HepG2-pHBeAg cells compared with control only at protein levels. Similar data were calculated with total Fas mRNA levels (Figure 3C) and mFas at both mRNA (Figure 3D) and protein levels (Figure 3E). Then, cells were transfected with Fas promoter–driven luciferase reporter construct pGL4.10-Fas to evaluate changes in possible HBeAg-regulated promoter inactivation. HepG2-pHBeAg cells illustrated an obvious decrease of luciferase reporter

gene activity relative to control (Figure 3F). The results suggest that HBeAg may down-regulate p53-dependent Fas expression at the transcriptional level.

HBeAg Enhances sFas Transcription and Expression

sFas arising from alternatively spliced Fas mRNA is a soluble form of Fas lacking exon 6 and has been considered as an antagonist of Fas apoptotic signal.³² The effects of HBeAg on sFas expression were subsequently evaluated in the context of p53 overexpression. In the absence of p53, the level of sFas in the culture medium of HepG2-pHBeAg cells as measured by ELISA was higher than control (Figure 4A). Transfection of cells with p53 further enhanced sFas expression, which was verified by semiquantitative PCR analysis. Although sFas mRNA levels were increased in HepG2-pHBeAg cells compared with control cells (Figure 4B) with or without p53 overexpression, knockdown of p53 significantly blocked the HBeAg-induced sFas mRNA levels (Figure 4C). The Fas splicing assay was also performed in the cells transfected with Fas splicing reporter construct pCMV56 containing the human Fas receptor sequences spanning from exon 5 to exon 7, kindly provided

Figure 5 Inhibition of tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL)-mediated apoptosis by hepatitis B virus (HBV) e antigen (HBeAg). **A:** Annexin V staining performed on Huh7-pcDNA3.1 and Huh7-pHBeAg or Hep3B-pcDNA3.1 and Hep3B-pHBeAg cells treated with TRAIL for 12 hours. **B:** Annexin V staining performed on HepG2-pcDNA3.1 or HepG2-pHBeAg cells treated with TRAIL for 12 hours with transfection with p53 siRNA or p53 gene. **C:** Annexin V staining performed on HepG2-pcDNA3.1 or HepG2-pHBeAg cells treated with TRAIL for 12 hours with pretreatment with TNF receptor superfamily member 10a (DR4) and/or TNF receptor superfamily member 10b (DR5) blocking antibody. **D:** Quantitative real-time PCR analysis of total DR4 or DR5 mRNA levels in HepG2-pcDNA3.1 and HepG2-pHBeAg cells on p53 overexpression. **E:** Representative Western blot showing the effect of HBeAg transfection on DR4 or DR5 protein levels on p53 overexpression. **F:** Transcriptional regulation of DR4 or DR5 by HBeAg. HepG2-pcDNA3.1 or HepG2-pHBeAg cells were co-transfected with pGL4.10-DR4 or DR5 and the Renilla luciferase expression vector pRL-TK on p53 overexpression. The level of promoter activity was evaluated. Data are expressed as means $\pm$ SD. $n = 3$. $*P < 0.05$ [χ^2 test (A–C) and analysis of variance (D–F)]. NC, negative control.

by Dr. J. Valca'rcel. These data revealed that relative mRNA levels of Fas exon 5/7 (representative of sFas) to Fas exons 5 to 7 in HepG2-HBeAg were obviously increased as compared with control (Figure 4, D and E). The results indicate that HBeAg promotes sFas transcription and expression via increasing transcripts of an alternatively spliced variant that specifically encodes the soluble form of the receptors.

Inhibition of TRAIL-Mediated Apoptosis by HBeAg

TRAIL is a cytokine that is produced and secreted by most normal tissue cells and causes p53-dependent apoptosis primarily in tumor or virus-infected cells by binding to certain death receptors such as DR4 and DR5.^{16,17}

Therefore, the inhibitory effect of HBeAg on p53-dependent TRAIL-mediated apoptosis was examined. Interestingly, TRAIL treatment produced effective and differential cytotoxicity to hepatoma cells expressing HBeAg only in the presence of p53 (Figure 5, A and B). To further ascertain the central role of DR4 and DR5 receptors in TRAIL-mediated apoptosis, p53 wild-type HepG2-pcDNA3.1 and HepG2-pHBeAg cells were pretreated with DR4 and/or DR5 blocking antibodies followed by exposure to TRAIL then assayed for annexin V binding assay. As expected, only pretreatment with both DR4- and DR5-blocking antibodies fully abrogated TRAIL-induced apoptotic cell death (Figure 5C), and there was no obvious difference in the apoptotic rate between HepG2-pHBeAg and control cells, which indicates combined effect of the two receptors in

Figure 6 Hepatitis B virus (HBV) e antigen (HBeAg) increases resistance to death receptor-mediated apoptosis induced by DNA damaging agents. **A–D:** Measurements of caspase-3/7 enzymatic activity expressed as the fold change relative to that in the control cells at the indicated time points after treatment. **E:** Caspase-3/7 enzymatic activity of adenovirus (Ad) green fluorescent protein (GFP) or Ad-HBeAg-infected primary mouse hepatocytes (PMHs) after Jo2 stimulation. **F:** Caspase-3/7 enzymatic activity of Ad-GFP- or Ad-HBeAg-infected primary human hepatocytes (PHHs) after Fas ligand (FasL) stimulation. **G:** Caspase-3/7 enzymatic activity of Lenti-flag or Lenti-HBeAg-infected PMHs after Jo2 stimulation. **H:** Caspase-3/7 enzymatic activity of Lenti-flag- or Lenti-HBeAg-infected PHHs after FasL stimulation. Data are expressed as means $\pm$ SD (**A–H**). $n = 3$. * $P < 0.05$ (analysis of variance).

hepatocytes. HBeAg inhibits the TRAIL apoptotic signal that is dependent on DR4 and DR5, which could be transcriptionally activated by p53.^{16,17} The inhibitory effect of HBeAg on DR4 and DR5 expression was examined. HBeAg down-regulated the expression of DR4 and DR5 at both the mRNA and protein levels on p53 overexpression

(Figure 5, D and E). Lastly, HepG2-pHBeAg cells had a significant decrease of DR4 and DR5 promoter-driven luciferase reporter gene activity relative to HepG2-pcDNA3.1 cells (Figure 5F). These data mainly illustrate that inhibition of TRAIL apoptotic signal by HBeAg is DR4 and DR5 dependent.

Figure 7 Hepatitis B virus (HBV) e antigen (HBeAg) protects C57BL/6 mice from Fas-induced hepatic apoptosis. **A:** Protein extracts collected from the liver of adeno-associated virus (AAV)-8-HBeAg and control mice were resolved on SDS/PAGE and immunoblotted with indicated antibodies. Levels of serum HBeAg was tested quantitatively by Architect assay. The Architect HBeAg assay is calibrated to give results in international units per milliliter. **B:** Macroscopic appearance of representative liver samples with hematoxylin and eosin staining and terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining 12 hours after Jo2 treatment of the different groups as indicated. **C:** The histogram summarizes the mean percentage of apoptotic cells in the livers of the indicated groups after Jo2 treatment. **D:** Caspase 8, 9, and 3/7 activity assay. Expression levels of tumor necrosis factor (TNF)- α (**E**) and IL-6 (**F**) and analyses of serum alanine aminotransferase (ALT) (**G**) showing that significantly less severity with regard to apoptosis, caspase activities, cytokine levels, and serum ALT levels is observed in AAV8-HBeAg mice. **H** and **I:** TUNEL and ALT assay shows that acute and severe cytolysis is observed in the AAV8-HBV-HBeAg(-) mice, whereas delayed and significantly less severe increase in intensity of apoptotic process are seen in AAV8-HBV mice. Return to normal values is observed within 5 days. Data are expressed as means $\pm$ SD. $n = 5$. * $P < 0.05$ [analysis of variance (**D**–**F**) and χ^2 test (**C** and **G**)]. Scale bar = 100 μ m.

Figure 8 Hepatitis B virus (HBV) e antigen (HBeAg) inhibits the antitumor effect of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in a human hepatoma xenograft model *in vivo*. **A:** *In vivo* subcutaneous tumor growth curves of HBeAg-expressing hepatoma cells and control cells. **B:** Images of all five harvested subcutaneous tumors from five nude mice of each group. **C:** Total tumor weight from each group of mice. **D:** Total tumor weight after TRAIL and/or doxorubicin treatment. **E:** The histogram summarizes the mean percentage of terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) staining apoptotic cells in the subcutaneous tumors of the indicated groups after TRAIL and/or doxorubicin treatment. Data are expressed as means $\pm$ SD. $n = 5$. * $P < 0.05$ [t -test (A and C) analysis of variance (D), and χ^2 test (E)].

HBeAg Increases Resistance to Death Receptor-Mediated Apoptosis Induced by DNA Damaging Agents

One of the hallmarks of the cancer cell phenotype is chemotherapy resistance.³³ It was therefore determined whether HBeAg could restrain the p53 apoptotic signal. To verify the correlation, two apoptosis inducers were used: bleomycin or doxorubicin, which induce p53-dependent apoptosis.¹² The results imply that the antiapoptotic effect of HBeAg was observed in bleomycin- or doxorubicin-pretreated and FasL- or TRAIL-mediated HepG2 (p53 wild-type) cells but not in Hep3B cells that express the deletion mutation of p53 or in Huh7 cells that harbors deficient mutant of p53 (Figure 6, A–D), further verifying that the antiapoptotic effect of HBeAg was p53 dependent. Because hepatoma cells may have abnormal death regulatory pathways, primary mouse or human hepatocytes were used to test whether HBeAg could also inhibit death receptor-mediated apoptosis triggered by anti-Fas Jo2 or FasL. Infection of PMHs or PHHs with an adenovirus or recombinant lentiviruses expressing HBeAg significantly suppressed Jo2- or FasL-induced apoptosis compared with the control virus-infected cells on bleomycin treatment (Figure 6, E–H). The data obviously illustrate that HBeAg can suppress the chemotherapy

agent-induced and death receptor-mediated proapoptotic signaling pathway.

HBeAg Prevents C57BL/6 Mice from Fas-Induced Hepatic Apoptosis

To determine whether the effects of HBeAg on Fas-mediated apoptosis *in vitro* could translate into responsiveness *in vivo*, anti-Fas Jo2 was injected intraperitoneally into mice to establish a Fas model. HBeAg, which had the highest ability of repressing p53 and mFas expression, was expressed in the livers of mice using a liver-targeted AAV-8. Mice that successfully expressed HBeAg had decreased p53 and Fas expression (Figure 7A). No significant morphologic changes and histologic lesions were observed between the HBeAg and AAV8-control group, indicating that HBeAg alone did not affect liver histologic findings (Figure 7B). When Jo2 was injected intraperitoneally into the AAV8-control group, severe histologic lesions of the liver were observed in mice, including morphologic changes characteristic of apoptosis. In the control mice, early onset of massive apoptosis was observed 12 hours after the injection of Jo2 (Figure 7, B and C). The AAV8-HBeAg group had a significantly decreased hepatic apoptosis. Consistent with histologic data, a remarkable reduction of caspases-3/7 activities was detected in the liver extracts of

AAV8-HBeAg group mice at 12 hours after Jo2 treatment (Figure 7D). Moreover, both reduced hepatic expression of inflammatory cytokines TNF- α and IL-6 and decreased serum alanine aminotransferase were detected in AAV8-HBeAg group mice compared with controls (Figure 7, E–G). In addition, AAV8-HBV mice with and without endogenous HBeAg expression were also used to verify the equivalent capacity of HBeAg in protecting Fas-mediated hepatocytes apoptosis from a nonlethal dose of Jo2 challenge (Figure 7, H and I). These results specifically indicate that HBeAg could prevent Fas-mediated hepatocyte cell death *in vivo*.

HBeAg Inhibits the Antitumor Effect of TRAIL in a Human Hepatoma Xenograft Model *in Vivo*

TNF- α and FasL can trigger apoptosis in solid tumors, but their clinical use has been limited by hepatotoxicity. TRAIL has a deep apoptotic effect on tumor cells but is nontoxic to human hepatocytes *in vitro* and *in vivo*.³⁴ To elucidate the effect of HBeAg expression on tumor formation *in vivo*, tumor xenograft experiments were performed in nude mice with Huh7, HepG2, and Hep3B cells stably transfected with HBeAg. Tumor xenograft experiments found that HBeAg stably expressing HepG2 cells had increased tumor growth compared with control cells as verified through tumor volume, photography, and weight measurement, and the phenotype was not observed in HBeAg stably expressing Huh7 cells or Hep3B cells (Figure 8, A–C), further verifying that the tumorigenicity of HBeAg was p53 dependent. The effects of HBeAg on TRAIL and/or chemotherapy agent-treated tumor tissues were then examined. Treatment of xenografted mice with TRAIL or doxorubicin caused stronger reduction of tumor weight in the control group than those of HBeAg stably expressing HepG2 cells (Figure 8D). The combination of TRAIL and doxorubicin was more effective than a single agent alone, which completely blocked the tumorigenicity of HBeAg, and there was no obvious difference between HepG2-pHBeAg and control cells. At last, inhibition of the antitumor effect of TRAIL and/or chemotherapy agents by HBeAg in the xenograft model was verified by the TUNEL assay (Figure 8E). Collectively, HBeAg promoted p53-dependent tumor growth and inhibited the antitumor effect of TRAIL in a human hepatoma xenograft model.

Discussion

Hepatic apoptosis, primarily mediated by extrinsic proapoptotic factors such as FasL and TRAIL, promotes the occurrence and development of several liver diseases, including nonalcoholic steatohepatitis, viral hepatitis, and hepatocarcinogenesis.³⁵ Verification of proapoptotic and antiapoptotic signal pathways relative to death receptor-mediated hepatic apoptosis would contribute

significantly to understanding of the pathophysiologic role of apoptosis in the major liver diseases. Although death receptors are vital regulators of the proapoptotic pathway in hepatocytes and have been considered the key trigger of all types of liver diseases,³⁶ the pivotal role for HBeAg in death receptor-mediated hepatic apoptosis has not been illustrated. The data suggest that HBV e protein is an effective survival factor capable of preventing hepatocytes from FasL and TRAIL-induced apoptosis through p53-dependent signaling pathway. Similar to HBc,² HBeAg also serves as a Fas alternative splicing accelerator to up-regulate sFas transcription and expression, a known antagonist of Fas apoptotic signaling.

TRAIL may induce selective apoptosis of malignant and viral infected cells. Considering that the death receptor system plays a pivotal role in the pathogenic mechanism of HBV infection^{7,37} and in the development of inflammation, fibrosis, cirrhosis, and hepatocellular carcinoma,³⁸ HBeAg expression may lead to HBV chronic infection by reinforcing the resistance of infected hepatocytes to FasL- and TRAIL-induced apoptosis. In this study, HBeAg forcefully prevented hepatocytes from FasL- and TRAIL-evoked cytotoxicity. The finding that there was no differential cytotoxicity of FasL and TRAIL between HBeAg- and control-transfected Huh7 or Hep3B cells, both of which gain p53 dysfunction, indicates that p53 may be indispensable in HBeAg-conferred apoptosis resistance, which was further verified in p53 wild-type HepG2 cells by p53 inhibition and p53 overexpression. Meanwhile, HBeAg-suppressing, chemotherapy agent-induced, and death receptor-mediated apoptosis was verified in primary human and mice hepatocytes. Furthermore, HBeAg prevented C57BL/6 mice from Fas-induced hepatic apoptosis and inhibited the antitumor effect of TRAIL in a human hepatoma xenograft model, which reflected the key role of HBeAg in apoptosis resistance and immunosuppression *in vivo*.

Figure 9 Schematic representation of hepatitis B virus (HBV) e antigen (HBeAg) regulation on p53 signaling to affect death receptor-apoptotic pathway. DR4, tumor necrosis factor receptor superfamily member 10a; DR5, tumor necrosis factor receptor superfamily member 10b; FasL, Fas ligand; mFas, membrane-associated Fas; sFas, soluble Fas; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.

HBeAg is considered a nonstructural protein, although massive HBeAg is secreted to serum and 25% of the mature protein perhaps stays in the cytoplasm,²¹ which was verified by our experiment (Figures 1F and 8A). Our research focuses on the multifunctions of the intracellular HBeAg and its precursors. HBeAg has been proposed to modulate the host immune response as a tolerogen to promote chronicity, which is a hallmark of HBV infection. Actually, HBV appears supervise the controlling of the immune response to strive for permanent and chronic infection. HBeAg or the HBV precore protein is an important viral protein hidden in HBV infection through the repression of the specific immune response. However, the mechanism for how HBeAg leads to hepatocarcinogenesis has not been illustrated. Recently, restrained p53 activity and the regulation of its downstream genes by HBeAg/HBV have been reported in the HBV-infected liver tumor tissues.^{21,39} In this work, it was illustrated that HBeAg/precore could down-regulate p53-dependent mFas, DR4, and DR5 expression and exhibit strong resistance to apoptosis induced by FasL and TRAIL *in vitro* and *in vivo*, thereby enhancing carcinogenesis.

The clinical data point out that secreted HBeAg may play an immunoregulatory role in HBV infection. Conversely, intracellular HBeAg acts as a tolerogen for the specific immune response to promote chronicity, which is a hallmark of HBV infection. Besides inhibiting p53 expression, it has been illustrated that HBeAg/precore may repress NF- κ B signal and its downstream chemokines and inflammatory cytokines.⁴⁰ HBeAg-deficient HBV variants have been described in both acute and chronic HBV infections. These variants harbor mutations in the precore region that inactivate the precore open reading frame, resulting in the deficiency of HBeAg expression. A stop codon mutation in codon 28 of the precore region occurs frequently. Chronic HBV infection has a heterogeneous clinical presentation and can be marked by fluctuations between wild-type virus and HBeAg-negative variants that may predispose patients to a more aggressive form of disease. HBeAg-deficient HBV may cause more severe inflammation response compared with the wild type, and strong inflammation activity can worsen liver diseases with a possibility of slowly developing finally into hepatocellular carcinoma, which may partly explain why HBeAg-negative mutations can also lead to hepatocellular carcinoma in certain conditions.^{18,20} Unlike the core protein, HBeAg is not indispensable for viral replication because mutations that block its expression do not interfere with the HBV package.^{18,19} However, viruses defective in HBeAg production may contribute to a more severe course of acute hepatitis B.^{41,42} Therefore, HBeAg likely possesses negative immunomodulatory roles.^{43,44} The findings that inhibition of Fas and TRAIL apoptotic signal by HBeAg provide strong evidence for the above-mentioned views.

In summary, to our knowledge, these findings are the first to illustrate that HBeAg serves to inhibit p53-dependent

FasL- and TRAIL-mediated hepatic apoptosis by down-regulating both mFas and DR4/5 expression at a transcriptional level and enhancing the expression of sFas by increasing Fas alternative splicing (Figure 9). The data indicate that HBeAg can protect hepatocytes from apoptosis induced by the Fas/FasL and TRAIL/DR system, thus leading to the survival of infected hepatocytes toward the development of HBV chronicity.

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