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Xiaohong Liang, Yugang Liu, Qiu Zhang, Lifen Gao, Lihui Han, Chunhong Ma, Lining Zhang, Youhai H. Chen and Wensheng Sun

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Hepatitis B Virus Sensitizes Hepatocytes to TRAIL-Induced Apoptosis through Bax

Xiaohong Lian,‡ Yugang Liu,‡ Qiu Zhang,‡ Lifen Gao,* Lihui Han,* Chunhong Ma,* Lining Zhang,* Youhai H. Chen,* and Wensheng Sun*‡

Hepatitis B virus (HBV) infection affects >300 million people worldwide and is a leading cause of hepatic death, cirrhosis, and hepatocellular carcinoma. While the morphological characteristics of dying hepatocytes are well documented, the molecular mechanisms leading to the death of hepatocytes during HBV infection are not well understood. TRAIL, the TNF-related apoptosis-inducing ligand, has recently been implicated in the death of hepatocytes under certain inflammatory but not normal conditions. To determine the potential roles of TRAIL in HBV-induced hepatitis, we examined the effects of HBV and its X protein (HBx) on TRAIL-induced hepatic apoptosis both in vivo and in vitro. We found that hepatitis and hepatic cell death in HBV transgenic mice were significantly inhibited by a soluble TRAIL receptor that blocks TRAIL function. We also found that HBV or HBx transfection of a hepatoma cell line significantly increased its sensitivity to TRAIL-induced apoptosis. The increase in TRAIL sensitivity were associated with a dramatic up-regulation of Bax protein expression. Knocking down Bax expression using Bax-specific small interference RNA blocked HBV-induced hepatitis and hepatocyte apoptosis. The degradation of caspases 3 and 9, but not that of Bid or caspase-8, was preferentially affected by Bax knockdown. These results establish that HBV sensitizes hepatocytes to TRAIL-induced apoptosis through Bax and that Bx-specific small interference RNA can be used to inhibit HBV-induced hepatic cell death. The Journal of Immunology, 2007, 178: 503–510.

*Institute of Immunology, School of Medicine, ‡Institute of Environmental Research, Shandong University, Jinan, People’s Republic of China; and †Institute of Pathology and Laboratory Medicine, University of Pennsylvania, Philadelphia, PA 19104

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2 X.L. and Y.L. contributed equally to this work.

3 Address correspondence and reprint requests to Dr. Youhai H. Chen, 614 BRB-II/III, Department of Pathology and Laboratory Medicine, University of Pennsylvania School of Medicine, 421 Curie Boulevard, Philadelphia, PA 19104; E-mail address: yhc@mail.med.upenn.edu or Dr. Wensheng Sun, Institute of Immunology, School of Medicine, Shandong University, Jinan, 250012 People’s Republic of China; E-mail address: wws@sdu.edu.cn

4 Abbreviations used in this paper: HBV, hepatitis B virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; Bcl-2 homology; DR, death receptor; DR5, soluble DR5; sDR5, soluble interference RNA.

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Materials and Methods

Mice

Normal BALB/c mice were obtained from the Laboratory Animal Center of Shandong University. HBV complete genome (ayw subtype) transgenic
BALB/c mice were purchased from the Transgenic Animal Central Laboratory (458 Hospital, Guang Zhou, China). The transgenic mice express high levels of HBV Ags, and their blood is positive for HBV DNA (26). All mice were housed in the Institute of Immunology Shandong University animal facilities under pathogen-free conditions. All procedures were pre-approved by the Institutional Animal Care and Use Committee.

**Plasmids**

pcDNA3-1.1HBV was generated by inserting a 3.907-kb fragment of the p3.6/E1-2HBV (provided by Prof. Y. Wang, Institute of Biochemistry and Cell Biology, Chinese Academy of Sciences, Shanghai, China) containing 110% of the HBV genome into the EcoRV site of the pcDNA3 (which contains a neomycin resistance gene under the control of the CMV promoter). pcDNA3-HBV plasmid was constructed by inserting one full copy of the HBV genome into the EcoRI restriction site of the pcDNA3. pcDNA3-HBx was generated by inserting a copy of the HBx gene into the HindIII and XbaI sites of the pcDNA3. Qiagen Plasmid Mini/Maxi kits were used to extract and purify the recombinant plasmids.

**The mouse model of human HBV-mediated hepatitis**

Five- to 6-wk-old male BALB/c mice were immunized with pcDNA3-HBV by i.m. injection. Mice injected with pcDNA3 vector served as controls. The expression of the anti-HBsAg in the serum was determined by ELISA and the presence of HBV-specific CTL in the spleen was confirmed by an in vitro CTL assay. Splenocytes from immunized mice were collected 4 wk after the immunization and were transferred into HBV transgenic BALB/c mice via tail vein (2.5 × 10⁷ cells/mouse). Forty-eight hours later, the levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in the serum were determined on a SpectraMax Plus spectrophotometer using the 3V Infinity Reagents (3V Biotech) per the manufacturer’s instructions. Liver and kidney were sectioned, stained by H&E, and examined by light microscopy.

**TRAIL blockade in vivo**

Recombinant human soluble DR5 (sDR5) was produced in Pichia Pastoris and purified as described previously (27). To block TRAIL effect in vivo, HBV transgenic mice were injected i.p. with 400 μg of purified sDR5 in 0.4 ml PBS 24 and 0 h before the splenocyte transfer.

**Liposome-mediated transfection**

Human hepatoma cell line BEL7402 was transfected with pcDNA3-1.1HBV, pcDNA3-HBx or pcDNA3 using LipofectAMINE 2000 (Invitrogen Life Technologies) per manufacturer’s instructions. Briefly, BEL7402 cells were seeded into 24-well plates with 1.5 × 10⁵ cells/well and cultured for 20 h. The medium was removed, and cells were exposed to the transfection complexes (1 μg of DNA and 2 μl of liposome mixed in 100 μl of serum-free Opti-MEM) for 6 h at 37°C. Cells were then cultured in complete medium for 24 h. The transfected cells were selected in medium containing 380 μg/ml G418 (Invitrogen Life Technologies) for ~4 wk. The G418-resistant cells were used for the experiments described here.

**TUNEL assay**

Cells were seeded into 24-well plates with 2 × 10⁵ cells/well and treated with different concentrations of recombinant soluble TRAIL (PeproTech) for 24 h. Apoptotic cells were labeled using an in situ apoptosis detection kit (Immunotech) and TUNEL. The degree of apoptosis was determined by flow cytometry.

**Detection of TRAIL receptor expression by flow cytometry**

Cells in the exponential phase of growth were trypsinized and washed twice in PBS. They were incubated with mouse anti-human DR4, DR5, DcR1, and DcR2 mAbs (Fourth Military Medical University) at 4°C for 30 min. After washing, cells were treated with FITC-conjugated goat anti-mouse IgG (Immunotech) for 30 min. The levels of the TRAIL receptors were determined by flow cytometry.

**Western blot analysis**

Cells from cultures were washed three times in PBS and incubated in the lysis buffer (50 mM Tris.Cl (pH 8.0), 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.02% sodium azide, 100 μg/ml PMSF, 1 μg/ml pepstatin, and 1 μg/ml aprotinin) for 30 min on ice. Mouse liver tissues were ground in liquid nitrogen and homogenized in the lysis buffer (8 M urea, 4% CHAPS, 10 mM Tris.Cl (pH 8.0), 0.3 mg/ml EDTA, 1% DTT, 100 μg/ml PMSF, 1 μg/ml pepstatin, and 1 μg/ml aprotinin). The homogenate was centrifuged at 16,000 × g for 45 min at 4°C. Equal amount of protein from each lysate was subjected to 12% SDS-PAGE and transferred onto a nitrocellulose membrane. After blocking for 1.5 h in 5% nonfat dried milk containing 0.1% Tween 20, the membrane was incubated at 4°C overnight in the presence of Abs to human or mouse caspase-3, caspase-8, caspase-9, Bax, B-actin (Santa Cruz Biotechnology), or Abs to human Flip or TRAIL receptors (Upstate Biotechnology). The membrane was washed and further incubated for 1 h at room temperature with HRP-conjugated secondary Abs. Following washing, immunoreactive bands were detected using the DAB kit (Maixin) or Western Lightning Chemiluminescence Reagent (Amersham Biosciences).

**Bax small interference RNA (siRNA) administration in vivo**

Fifty micrograms of pmU6-Bax451 or control pmU6 plasmids was purified and diluted in saline with a volume equivalent to 8% of the mouse body weight. Plasmid solutions were injected into HBV transgenic mice via tail vein within 8 s, according to the hydrodynamic tail vein injection method.
for naked DNA as described by Liu et al. (28). pmU6-Bax451 encodes a Bax-specific siRNA that blocks Bax expression as described in Results.

Statistical analysis
The apoptosis rates and protein ratios were analyzed by $\chi^2$ test. Enzyme and protein levels were analyzed by ANOVA.

Results
TRAIL plays an important role in HBV-induced hepatitis
Although TRAIL may not be cytotoxic to normal hepatocytes, it has been shown to induce hepatocyte apoptosis in experimental models of hepatitis (9, 10). To determine the roles of TRAIL in HBV-mediated hepatic cell death, we tested the effect of TRAIL blockade in an HBV transgenic mouse model of hepatitis. In this model, the human HBV genes are constitutively expressed in the mouse liver, causing hepatocellular carcinoma in aged animals (26). The mice do not develop spontaneous hepatitis because the immune system is tolerized against the viral Ags. However, severe acute hepatitis can be induced upon transferring activated HBV-specific CTL into these mice. The latter cells can be generated in normal syngeneic mice by immunizing them with an expression plasmid that carries the HBV genome (29). Thus, hepatitis was induced in HBV transgenic mice by injecting into them activated splenocytes from HBV immunized mice (Fig. 1a, group 4). Significant blood levels of both ALT and AST were detected in these mice but not in the controls. Histochemical analysis of liver sections 2 days after splenocyte transfer revealed acute hepatitis in this group, which was characterized by leukocyte infiltration of liver parenchyma, acidophilic, and degenerative changes as well as death of hepatocytes (Fig. 1d). By contrast, no significant hepatitis or aminotransferase release were detected in mice that were pre-treated with sDR5 (Fig. 1, a, group 5, and e). The marked inhibitory effect of sDR5 in this model is reminiscent of what was observed in other models of hepatitis in which blocking one death ligand significantly prevents the onset of the disease (30). This may be due to the interdependency of the death ligand functions in this model as recently demonstrated for TNF-$\alpha$ (30).

HBV or HBx transfection increases the sensitivity of hepatoma cells to TRAIL-induced apoptosis
Because normal hepatocytes are not sensitive to TRAIL-induced apoptosis, the observed effect of TRAIL on HBV-induced hepatitis may be due to the altered sensitivity of HBV-infected cells. To test this theory, we transfected a widely used human hepatoma cell line BEL7402 with pcDNA3 or pcDNA3-1.1HBV plasmids that carry the entire HBV genome or the HBV X gene (HBx). Transfected cells were selected in G418-containing medium and tested for HBV gene expression before being used in this study. As shown in Fig. 2, transfection of BEL7402 cells with HBV but not control pcDNA3 plasmid significantly increased their sensitivity to TRAIL-induced apoptosis. Similar results were obtained when pcDNA3-HBx was used. Specifically, following incubation with 10 ng/ml TRAIL for 24 h, 12.6 ± 2.7% control BEL7402 cells underwent apoptosis; this was...
increased to 43 ± 5.4% for BEL7402 cells transfected with HBx (p < 0.001). These results establish that HBV and HBx are able to increase the sensitivity of BEL7402 hepatocytes to TRAIL-induced apoptosis.

HBV increases Bax expression in hepatocytes

To explore the mechanisms through which HBV increases the sensitivity of hepatocytes to TRAIL-induced apoptosis, we examined a panel of apoptosis mediators/regulators in BEL7402 cells that do or do not express HBV or HBx. We found that HBV or HBx transfection did not significantly alter the levels of DR4, DR5, DcR1, DcR2, Bid, FLIP, procaspases 3, 8, and 9 (Fig. 3 and our unpublished data). Upon treatment with TRAIL, procaspases 8 and 9 are slightly degraded in BEL7402 cells leading to a reduction in the levels of these proteins as compared with control cells (Fig. 3b). Degradation of both procaspases 8 and 9 was significantly enhanced in cells transfected with HBV (Fig. 3b), which is consistent with the apoptotic results shown in Fig. 2.

Unexpectedly, Bax protein levels in both HBV and HBx transfected cells were markedly increased as compared with untransfected or pcDNA3-transfected cells (Fig. 4). Because HBx alone

**FIGURE 3.** HBV transfection does not alter the expression of TRAIL receptors, caspase-8, and caspase-9. a, BEL7402, BEL7402 transfected with pcDNA3, pcDNA3-1.1HBV, or pcDNA3-HBx were examined for TRAIL receptor expression by flow cytometry. b, BEL7402, BEL7402 transfected with pcDNA3, or pcDNA3-1.1HBV were treated with (+) or without (−) 10 ng/ml TRAIL. Procaspase-8 and procaspase-9 expressions were examined by Western blot analysis 24 h later. β-Actin was used as a loading control.

**FIGURE 4.** HBV transfection increases Bax expression in BEL7402 hepatocytes. BEL7402, BEL7402 transfected with pcDNA3, pcDNA3-1.1HBV, or pcDNA3-HBx were examined for Bax expression by Western blot analysis (a and b). The Bax/β-actin protein ratios in c were determined by densitometry.

**FIGURE 5.** HBV transgenic mice express increased levels of Bax in the liver. Livers were collected from the following groups of 6- to 7-wk-old BALB/c mice (n = 5): 1) HBV transgenic (Tg) mice injected with HBV-primed splenocytes as described in Materials and Methods 48 h earlier (group 1, HBV Tg + T cell), 2) control nontransgenic mice (group 2, control), and 3) nontreated HBV transgenic mice (group 3, HBV Tg). Bax expression in the liver was determined by Western blot (a). β-Actin was used as a loading control. The Bax/β-actin protein ratios were calculated by densitometry (b). The differences among the three groups are statistically significant (p < 0.05).

**FIGURE 6.** Bax expression in BEL7402 hepatocytes can be knocked down by a Bax-specific siRNA. BEL7402-HBx cells (BEL7402 cells stably expressing the HBx protein) were transfected with pSilencer3.1 plasmids that carry either a random sequence (pControl-Si) or Bax-specific siRNA sequences (pBax382-Si and pBax172-Si). Seventy-two hours later, Bax and β-actin expressions were determined by RT-PCR (a) and Western blot analysis (b and c). Nontransfected cells were used as controls. The Bax/β-actin protein ratios in c were determined by densitometry. The siRNA sequences used are as follows: pControl, GACTTCTAAGGCGCATG; pBax172-Si, GGTTGCCGAACTGATCAG; and pBax172-Si, GCTGAGCGAGTGCCTCAAG.
up-regulated Bax expression to a degree similar to that caused by HBV, we conclude that HBx may be the main mediator of Bax up-regulation in HBV transfected cells. HBx may up-regulate Bax expression at transcriptional, posttranscriptional, translational and posttranslational levels. By RT-PCR and real time PCR, we detected no significant differences in Bax mRNA levels between control and HBx transfected cells (data not shown). Thus, HBx may selectively up-regulate Bax protein expression without significantly altering its gene transcription.

To determine whether HBV also regulates Bax expression in our model of HBV-induced hepatitis, we examined the hepatic levels of Bax protein in HBV transgenic mice and nontransgenic mice. We found that Bax protein levels in the liver were significantly higher in nontreated HBV transgenic mice (without hepatitis) than nontransgenic mice and that hepatitis further increased the Bax level in HBV transgenic mice (Fig. 5). Because nontreated HBV transgenic mice did not develop any detectable hepatic inflammation (Fig. 1c), the increase in Bax levels may be attributed to the direct effect of the HBV gene products through Bid (31). In contrast, the difference in hepatic Bax expression between mice with and without hepatitis may be due to the inflammatory milieu in the former mice. To test this possibility, we also examined the hepatic Bax expression in mice that had developed aseptic hepatitis following injection of mitogen Con A as we described previously (10). We found that hepatic Bax protein level was increased by 2.1-fold in BALB/c mice 8 h after the onset of Con A-induced hepatitis as compared with control mice not treated with Con A ($p < 0.01$). This is consistent with our previous report that TRAIL also mediates hepatic cell death during Con A-induced hepatitis (10). Thus, both HBV gene products and inflammatory milieu may up-regulate Bax expression during HBV-induced hepatitis.

**Bax knockdown blocks the effect of HBx on TRAIL-induced apoptosis of BEL7402 hepatocytes**

Because Bax is a potent initiator of mitochondrial pathway of apoptosis, which can be activated by DRs in hepatocytes through Bid (31), its up-regulation may enhance TRAIL-induced apoptosis of HBV-transfected cells. To test this theory, we designed Bax-specific siRNAs to selectively knockdown Bax expression in HBx transfected cells. As shown in Fig. 6, one of the siRNAs, Bax382-Si, specifically and effectively diminished Bax mRNA and protein levels in BEL7402 cells. The control siRNA or Bax172-Si had no effect. To determine the effect of Bax knockdown on TRAIL-induced apoptosis of BEL7402 hepatocytes, TUNEL was performed on cells that did or did not express high levels of Bax (Fig. 7). We found that Bax knockdown significantly reduced the sensitivity of HBx transfected BEL7402 cells to TRAIL-induced apoptosis. Specifically, the rate of apoptosis in HBx transfected BEL7402 cells to TRAIL-induced apoptosis was 43 ± 5.4%; this was reduced to 12.6 ± 2.7% in Bax knockdown group, a value similar to that of control BEL7402 cells not transfected with HBx.
Bax knockdown blocks TRAIL-induced degradation of caspase-3 and caspase-9. BEL7402-HBx cells transfected with pControl-Si or pBax382-Si were treated with (+) or without (−) 10 ng/ml TRAIL for 24 h as in Fig. 7. The levels of procaspase-3, procaspase-8, procaspase-9, and Bid were determined by Western blot analysis. β-Actin was used as a loading control.

Bax knockdown blocks TRAIL-induced degradation of caspases 3 and 9 but not Bid or caspase-8

Caspases and Bid degradations are biochemical hallmarks of apoptosis. To determine the effect of Bax knockdown on caspases and Bid degradations, we examined the levels of caspases and Bid in total cell extracts by Western blot analysis. We found that TRAIL induced significant degradation (or reduction in the level) of procaspases 3, 8, and 9, as well as Bid in control BEL7402-HBx cells (Fig. 8). Remarkably, Bax knockdown using pBax382-Si selectively blocked the degradation of caspases 3 and 9 but not Bid or caspase-8 (Fig. 8). This is consistent with the theory that Bax regulates the mitochondrial but not nonmitochondrial pathway of apoptosis. Because TRAIL can activate the mitochondrial pathway of apoptosis in hepatocytes through Bid, Bax up-regulation is able to enhance TRAIL-induced apoptosis in these cells.

Bax knockdown in vivo ameliorates HBV-induced hepatitis and hepatocyte apoptosis

Results described above indicate that Bax may play a crucial role in HBV-induced hepatitis and that Bax blockade may be effective in ameliorating the disease. To test this theory, we examined the consequence of Bax knockdown in our model of HBV-induced hepatitis. As shown in Fig. 9, hydrodynamic administration of a plasmid encoding Bax siRNA significantly and specifically reduced hepatic Bax levels in mice with HBV-induced hepatitis. This was accompanied by a significant reduction of ALT and AST in the blood (Fig. 10). Histochemical analysis of the liver sections revealed less inflammation and reduced number of apoptotic cells in Bax siRNA treated group as compared with control groups (Fig. 10 and our unpublished data). Western blot analysis of hepatic extracts revealed that Bax siRNA preferentially blocked the degradation of procaspases 3 and 9 but not that of procaspase-8, an effect similar to that of Bax siRNA in vitro as shown in Fig. 8 (our unpublished data). The blockade of the caspase degradation was not complete either in vivo or in vitro, which may explain why hepatocyte apoptosis, although significantly reduced, still occurred in the presence of Bax siRNA (Figs. 7a and 10c).

Discussion

TRAIL is a promising new drug for cancer therapy because of its selective cytotoxicity to tumor cells (7, 8). However, the roles of TRAIL in physiological and pathological conditions remain poorly characterized. While TRAIL does not appear to kill hepatocytes under physiological conditions, it may do so under inflammatory conditions. HBV infection is a common inflammatory condition of the liver, which is the leading cause of cirrhosis and hepatocellular carcinoma. In this study, we show that HBV sensitizes hepatocytes to TRAIL-mediated apoptosis through up-regulating Bax. We propose that this explains why normal hepatocytes are not sensitive to TRAIL but HBV-infected cells are. Recently, Janssen et al. (24)
reported that HBV transfection of hepatoblastoma cell line Hep G2 up-regulates DR4 expression, which may help promote TRAIL-induced apoptosis of these cells. However, we did not detect any change in DR4 expression in our BEL7402 cells following HBV or HBx transfection. This discrepancy may relate to the different cell lines used in these studies and suggest that HBV may sensitize hepatocytes to TRAIL through more than one mechanism. We elected to use BEL7402 cells in the current study because the Hep G2 cells from our laboratory appear to be different from the Hep G2 cells used by Janssen et al. (24, 32) with regard to DR expression and sensitivity to TRAIL. Our Hep G2 cells express more but not less DR than Hep G2.2.15 cells that carry the HBV genes and are more sensitive to TRAIL-induced apoptosis (32). Because both Hep G2 and Hep G2.2.15 are long-term cultured cell lines, differences between them may or may not be directly caused by the HBV transgenes. The effect of spontaneous gene mutation and selection in the culture cannot be excluded. To circumvent this problem, only short-term transfected cells were used in this study. Using these cells, we discovered that HBV and HBx are potent enhancers of Bax expression.

Apoptosis can be mediated through two distinct pathways: the DR (extrinsic) pathway and the stress (mitochondria-dependent, intrinsic) pathway. The DR pathway requires FADD, which activates caspase-8/caspase-3 cascade leading to apoptosis. In contrast, stress factors, such as reactive oxygen species, induce apoptosis through mitochondria, which release cytochrome c that in turn activates the caspase cascade through Apaf-1 and caspase-9. According to the current dogma, there is little cross-talk between the two pathways at the initiating stage of apoptosis with the exception of caspase-8-Bid-mediated activation of the stress pathway in hepatocytes (31). Thus, in hepatocyte, Bcl-2-related proteins can regulate both the stress pathway and the DR pathway of apoptosis.

The Bcl-2 family includes three classes of proteins that can either promote or inhibit apoptosis. The first class is called Bcl-2-like anti-apoptotic factors. These factors all contain three to four BH1-BH3 homology (BH) domains, which are required for their antiapoptotic function. This group includes Bcl-2, Bcl-xL, Bcl-w, A1/ Bcl-1, Bax/Diva/Bcl-B, and Mcl-1. The second class is called Bax-like proapoptotic factors. These factors contain BH1-BH3 domains, which act exclusively in a proapoptotic fashion. This group consists of Bax, Bak, and Bcl/Mtd. The third class is called BH3-only proapoptotic factors. These members share only the short BH3 domain with the other Bcl-2 family members. They include Bim, Bid, Bad, Bik/Nbk, Blk, Hrk/DP5, Noxa, Puma/Bbc3, Bmf, and Bcl-G9 (33–35). Although it is well recognized that Bcl-2 family of proteins plays an essential role in the stress pathway of apoptosis, the roles of most of these proteins in hepatic cell death remain to be established. Bcl-2 is the best-characterized member of this family. It blocks apoptosis by directly or indirectly preserving the integrity of the outer mitochondrial membrane, thus preventing cytochrome c release. Bcl-2 may prevent hepatocyte apoptosis induced by DR Fas through Bid (36–38). The proapoptotic member Bax induces apoptosis by causing cytochrome c release. Mice deficient in Bax are viable, suggesting that Bax is not required for development (39). However, male Bax-/- mice are sterile due to a developmental arrest in spermatogenesis. Bax-deficient animals also have increased numbers of sympathetic and motor neurons. In vitro, sympathetic neurons from Bax-deficient mice are resistant to apoptosis induced by growth factor deprivation (39). The roles of Bax in hepatic cell death during hepatitis are not known. Our demonstration that Bax knockdown in HBx-infected hepatocytes significantly blocks TRAIL-induced apoptosis indicates that Bax is a critical regulator of hepatocyte death.

In addition to the DR-Bid connection, TRAIL may also activate the mitochondrial pathway of apoptosis through TNFR-associated factor-2 and/or receptor interacting protein, which in turn activates JNK (40–42). JNK is a member of the MAPK superfamily, which can directly activate the mitochondria-dependent apoptosis pathway through Bid (43–45). Results from several laboratories indicate that the JNK-dependent pathway of apoptosis is directly involved in DR5-induced cell death (46–48). To what degree these are true during HBV infection remains to be established.

In summary, we discovered that HBV renders hepatocytes susceptible to TRAIL-induced apoptosis through Bax. Because this may contribute to HBV-induced fulminating hepatic failure and/or progressive chronic hepatitis, inhibition of TRAIL function may be beneficial in patients. Conversely, when HBV-infected hepatocytes are not adequately removed by the immune system, bolstering TRAIL’s effect pharmacologically (e.g., via exogenous TRAIL administration) may be desirable.

Disclosures
The authors have no financial conflict of interest.

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