HCV upregulates Bim through the ROS/JNK signalling pathway, leading to Bax-mediated apoptosis

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We previously reported that hepatitis C virus (HCV) infection induces Bax-triggered, mitochondrion-mediated apoptosis by using the HCV J6/JFH1 strain and Huh-7.5 cells. However, it was still unclear how HCV-induced Bax activation. In this study, we showed that the HCV-induced activation and mitochondrial accumulation of Bax were significantly attenuated by treatment with a general antioxidant, N-acetyl cysteine (NAC), or a specific c-Jun N-terminal kinase (JNK) inhibitor, SP600125, with the result suggesting that the reactive oxygen species (ROS)/JNK signalling pathway is upstream of Bax activation in HCV-induced apoptosis. We also demonstrated that HCV infection transcriptionally activated the gene for the pro-apoptotic protein Bim and the protein expression of three major splice variants of Bim (BimEL, BimL and BimS). The HCV-induced increase in the Bim mRNA and protein levels was significantly counteredact by treatment with NAC or SP600125, suggesting that the ROS/JNK signalling pathway is involved in Bim upregulation. Moreover, HCV infection led to a marked accumulation of Bim on the mitochondria to facilitate its interaction with Bax. On the other hand, downregulation of Bim by siRNA (small interfering RNA) significantly prevented HCV-mediated activation of Bax and caspase 3. Taken together, these observations suggest that HCV-induced ROS/JNK signalling transcriptionally activates Bim expression, which leads to Bax activation and apoptosis induction.

INTRODUCTION

Hepatitis C virus (HCV) is a major causative pathogen of chronic hepatitis, cirrhosis and hepatocellular carcinoma (Shepard et al., 2005). Apoptosis of hepatocytes has been shown to occur in patients chronically infected with HCV (Calabrese et al., 2000). Increasing evidence suggests that apoptosis of hepatocytes is involved in the pathogenesis of HCV, such as liver injury (Guicciardi & Gores, 2005; Mengshol et al., 2007). HCV-associated apoptosis involves two pathways: an immune response-associated, death receptor-mediated (extrinsic) pathway (Zhu et al., 2007) and a mitochondrion-mediated (intrinsic) pathway (Deng et al., 2008). By using an in vitro HCV infection system, we and other groups have observed that apoptosis is induced in Huh-7.5 cells in response to HCV infection (Deng et al., 2008; Mateu et al., 2008; Walters et al., 2009). Moreover, HCV-induced apoptosis was demonstrated in vivo by using the immunodeficient chimeric SCID/Alb-uPA mouse model where immune response-dependent apoptosis can be discounted (Joyce et al., 2009). These studies suggest that HCV is by nature capable of mediating apoptosis through the mitochondrion-mediated intrinsic pathway.

The mitochondrial apoptosis pathway is critically controlled through interactions between pro- and anti-apoptotic members of the Bcl-2 family. The Bcl-2 family is divided into three groups: anti-apoptotic multidomain proteins (e.g. Bcl-2, Bcl-XL, Mcl-2), pro-apoptotic multidomain proteins (e.g. Bax, Bak), and pro-apoptotic BH3-only proteins (e.g. Bim, Bid, Bad) (Tait & Green, 2010). The pro-apoptotic multidomain protein Bax is usually present in healthy cells in an inactive state, and resides in the cytosol. Upon apoptotic stimulation, Bax undergoes a conformational change exposing the N and C termini, followed by unmasking of its BH3...
domain. Then, Bax inserts its C-terminus and oligomerizes in the outer mitochondrial membrane. Consequently, disruption of the inner transmembrane potential and release of intramembrane proteins, such as cytochrome c, are triggered, which leads to the activation of caspase 9 and caspase 3 (Lalier et al., 2007; Upton et al., 2007). Evidence suggests that BH3-only protein Bim can trigger the activation of Bax either directly, by interacting with Bax, or indirectly, by antagonizing the anti-apoptotic Bcl-2 proteins, thereby allowing Bax activation to proceed (Czabotar et al., 2009; Gavathiotis et al., 2008; Strasser et al., 2011). Bim is expressed as three major isoforms (BimEL, BimL and BimS), which are generated by alternative splicing. Although these Bim isoforms all induce apoptosis, BimL is the most potent inducer of apoptosis and BimEL is thought to be the least effective at killing cells (O’Connor et al., 1998). The expression level of Bim is regulated by mRNA transcription, mRNA stability, and post-translational modifications (Gilley et al., 2003; Hübner et al., 2008; Matsui et al., 2007).

We previously reported that HCV induces mitochondrial production of reactive oxygen species (ROS) and Bax activation (Deng et al., 2008). However, the exact mechanism of HCV-induced Bax activation remained to be elucidated. Meanwhile, a possible link between ROS and Bax activation has been reported (Buccellato et al., 2004; Byun et al., 2009; D’Alessio et al., 2005; Nie et al., 2008). Increasing evidence has suggested that c-Jun NH2-terminal kinase (JNK) plays a role in Bax-mediated apoptosis by regulating the function of Bim (Lei & Davis, 2003; Okuno et al., 2004; Putcha et al., 2003). Also, we previously demonstrated that HCV-induced ROS production causes JNK activation, resulting in increased gluconeogenesis (Deng et al., 2011).

In the present study, we investigated the possible link between HCV-induced ROS/JNK signalling and Bim/Bax-mediated apoptosis using the HCV cell culture system. We report here that HCV transcriptionally upregulates Bim expression to activate Bax through the ROS/JNK signalling pathway.

RESULTS

HCV-induced ROS production is involved in Bax-mediated apoptosis

We first examined the possible link between HCV-induced ROS production and Bax activation. Consistent with our previous observations (Deng et al., 2008), we confirmed that Bax was activated in HCV-infected cells. Importantly, the HCV-induced Bax activation was abolished by treatment with the general antioxidant N-acetyl cysteine (NAC; 5 mM for 2 h) (Fig. 1a, b). Moreover, HCV-induced cleavage of the DNA repair enzyme poly(ADP-ribose) polymerase (PARP), which is an important substrate for activated caspase 3 and serves as an apoptosis marker (Oliver et al., 1999), was clearly inhibited by NAC treatment (Fig. 1c).

To further verify HCV involvement in Bax activation, we eliminated HCV by treatment with an NS5A inhibitor, daclatasvir (1 µM for 6 days) (Gao et al., 2010). The result demonstrated that, upon HCV elimination (Fig. 1d), Bax activation was abrogated (Fig. 1e, f).

To further verify that ROS induces apoptosis in Huh-7.5 cells, we examined Bax and caspase 3 activation in response to H2O2 treatment. As had been expected, Bax was markedly activated by H2O2 treatment (400 µM for 10 h), and H2O2-induced Bax activation was blocked by pretreatment with NAC (Fig. 1g, h). Also, caspase 3 activities were significantly increased in H2O2-treated cells in a dose-dependent manner (Fig. 1i). These results suggest that HCV-induced ROS production is involved in Bax-mediated apoptosis in Huh-7.5 cells.

HCV-induced JNK activation is involved in activation and mitochondrial accumulation of Bax

We previously reported that HCV induces JNK activation through increased mitochondrial ROS production (Deng et al., 2011). We therefore tested whether HCV-induced JNK activation contributes to Bax activation. We treated HCV-infected cells with the specific JNK inhibitor SP600125 (SP; 20 µM for 24 h) and examined the activation and mitochondrial accumulation of Bax. As shown in Fig. 2(a, b), treatment with SP significantly inhibited the HCV-induced Bax activation. Also, SP treatment prevented the HCV-induced mitochondrial accumulation of Bax (Fig. 2c, d). These results suggest that HCV-induced JNK activation is involved in Bax activation and mitochondrial accumulation.

HCV infection upregulates the expression of the pro-apoptotic protein Bim through the ROS/JNK signalling pathway

To clarify how HCV-induced ROS/JNK signalling is involved in Bax activation, we focused on the pro-apoptotic BH3-only protein Bim, which was reported to trigger Bax activation directly or indirectly by binding to the Bcl-2 anti-apoptotic proteins (Gavathiotis et al., 2008; Mérino et al., 2009). It is also known that Bim activity is modulated by JNK (Lei & Davis, 2003) and that transcription factors c-Jun and FoxO regulate Bim transcription by directly binding to the Bim promoter (Gilley et al., 2003; Heidari et al., 2012). Importantly, we previously found that HCV induces the sustained transcriptional activity of FoxO1 through JNK activation (Deng et al., 2011). Therefore, we sought to determine whether HCV-mediated ROS/JNK signalling affects Bim expression. For this purpose, we performed a luciferase reporter assay to examine the possible effects of HCV infection on Bim promoter activity using the plasmids pGL4.11/Bim-luc (−3.6 kb) and pGL4.11/Bim-luc (−1.2 kb), which carry 3.6 and 1.2 kb of the Bim 5’-flanking region, respectively (Fig. 3a). The result demonstrated that Bim promoter activity was...
Fig. 1. HCV-induced ROS production is involved in Bax activation and apoptosis induction. (a) Huh-7.5 cells infected with HCV and mock-infected control cells with or without NAC pretreatment (5 mM for 2 h) were subjected to indirect immunofluorescence analysis at 6 days p.i. The cells were incubated with an antibody specifically recognizing the N terminus of Bax, followed by Alexa Fluor 488-conjugated goat anti-rabbit IgG (top) and with serum from an HCV-infected patient followed by Alexa Fluor 594-conjugated goat anti-human IgG (middle). The cells were then stained with Hoechst 33342 for the nuclei (bottom). Scale bar, 10 μm. (b) The percentages of cells expressing activated Bax were determined. Data represent means ± SE of data from two independent experiments, each performed with triplicate cultures. * , P < 0.01. (c) The cleavage products of PARP in cell lysates were determined by immunoblotting at 6 days p.i. Blots were reprobed with antibodies recognizing NS3 and GAPDH. The amounts of GAPDH were measured as an internal control to verify equal amounts of sample loading. (d) The protein expression levels of HCV NS5A in lysates of HCV-infected Huh-7.5 cells and mock-infected control with or without NS5A inhibitor daclatasvir (1 μM for 6 days) were analysed by immunoblotting. Blots were reprobed
HCV induces ROS/JNK/Bim/Bax apoptotic signalling

Fig. 2. HCV-induced JNK activation is required for the activation and mitochondrial accumulation of Bax. (a) Huh-7.5 cells infected with HCV and mock-infected control cells with or without JNK inhibitor SP pretreatment (20 μM for 24 h) were subjected to indirect immunofluorescence analysis at 6 days p.i. as described in (a). Scale bar, 10 μm. (b) The percentages of cells expressing activated Bax were determined. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. *, P<0.01. (c) Cytosolic and mitochondrial fractions were prepared from HCV-infected cells and mock-infected control cells with or without SP pretreatment (20 μM for 24 h) at 6 days p.i. and analysed by immunoblotting using antibodies against Bax, NS3, Tim23 and β-tubulin. The amounts of Tim23 and β-tubulin were measured to verify equal amounts of mitochondrial and cytosolic fractions, respectively. (d) The intensities of the bands of mitochondrion-associated Bax were quantified. The intensity of the non-treated mock-infected control was arbitrarily expressed as 1.0. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. *, P<0.05.
(a) Bim promoter

Bim-luc

(-3.6 kb)

AP-1

FoxO

Bim-luc

(-1.2 kb)

FoxO

(b) Relative luciferase activity

Mock HCV

Relative luciferase activity

Bim Reporter

(-3.6 kb) (-1.2 kb)

2.5

2.0

1.5

1.0

0.5

0

(c) Relative mRNA expression

BimEL BimL BimS

Mock HCV

Days p.i.

Days p.i.

Days p.i.

2.5

2.0

1.5

1.0

0.5

0

(d) Relative mRNA expression

BimEL BimL BimS

Mock HCV

- NAC SP - NAC SP - NAC SP

- Mock - HCV - Mock - HCV - Mock - HCV

(e) HCV

4 dpi 6 dpi 8 dpi

BimEL

BimL

BimS

NS3

GAPDH

(f) Mock HCV

- NAC SP - NAC SP

BimEL

BimL

BimS

NS3

GAPDH

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Fig. 3. HCV infection upregulates the expression of the pro-apoptotic protein Bim through the ROS/JNK signalling pathway. (a) Schematic representation of human Bim promoter-driven luciferase reporter constructs pGL4.11/Bim-luc (−3.6 kb) and pGL4.11/Bim-luc (−1.2 kb). The positions of the binding sites for AP-1 and FoxO are shown. (b) pGL4.11/Bim-luc (−3.6 kb) or pGL4.11/Bim-luc (−1.2 kb) was transfected into HCV-infected Huh-7.5 cells and mock-infected control cells at 4 days p.i. At 48 h after transfection, the Bim promoter activities were measured by using a luciferase reporter assay. Luciferase activity was normalized to Renilla activity for each well to control for transfection efficiency. The value for the control cells transfected with pGL4.11/Bim-luc (−3.6 kb) was arbitrarily expressed as 1.0. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. * P < 0.01 compared with the control. (c) Quantitative RT-PCR analysis was performed to determine BimEL (left), BimL (middle) and BimS (right) mRNA expression levels in HCV-infected Huh-7.5 cells and mock-infected control cells, and the expression levels were normalized to GAPDH mRNA expression levels. The values for the control cells at 4 days p.i. were arbitrarily expressed as 1.0. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. * P < 0.01, compared with the control. (d) Quantitative RT-PCR analysis was performed to determine BimEL (left), BimL (middle) and BimS (right) mRNA expression levels in HCV-infected Huh-7.5 cells and mock-infected control cells with or without NAC (5 mM for 2 h) or SP (20 μM for 24 h) pretreatment at 6 days p.i., and expression levels were normalized to GAPDH mRNA expression levels. The values for the non-treated control cells were arbitrarily expressed as 1.0. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. * P < 0.01. (e) The protein expression levels of BimEL, BimL and BimS in lysates of HCV-infected Huh-7.5 cells and mock-infected control cells were analysed by immunoblotting. Blots were reprobed with antibodies recognizing NS3 and GAPDH. (f) The protein expression levels of BimEL, BimL, and BimS in lysates of HCV-infected cells and mock-infected control cells treated with NAC (5 mM for 2 h) or SP (20 μM for 24 h) or left untreated at 6 days p.i. were analysed by immunoblotting. Blots were reprobed with antibodies recognizing NS3 and GAPDH.

significantly potentiated by HCV infection (Fig. 3b). The HCV-induced potentiation of the Bim promoter was still observed when the region of the Bim promoter from positions −3.6 kb to −1.2 kb was deleted. It should be noted that this 1.2 kb fragment of the Bim promoter contains the FoxO-binding site (−164/−170) (Fig. 3a) (Essafi et al., 2005). These results suggest that HCV induces Bim promoter activity and that HCV-mediated FoxO1 activity is possibly involved in the activation of the Bim promoter.

Next, we examined the mRNA expression levels of three major isoforms of Bim (BimEL, BimL and BimS) by real-time quantitative reverse transcription PCR (RT-PCR) analysis. The result revealed that the mRNA expression levels of all three isoforms were significantly higher in HCV-infected cells than in control cells at 4, 6 and 8 days post-infection (days p.i.) (Fig. 3c).

We then examined the possible effects of the HCV-induced ROS/JNK signalling on Bim gene transcription by using the antioxidant NAC or the JNK inhibitor SP. The treatment of HCV-infected cells with either NAC (5 mM for 2 h) or SP (20 μM for 24 h) clearly prevented the upregulation of Bim mRNA expressions (Fig. 3d).

We further examined the protein expression levels of BimEL, BimL and BimS in HCV-infected cells by immunoblotting analysis. The results showed that the protein expression levels of these three Bim isoforms were upregulated in HCV-infected cells compared with the control cells (Fig. 3e). The HCV-induced upregulation of Bim protein was significantly negated by treatment with NAC or SP (Fig. 3f). These results suggest that the HCV-induced ROS/JNK signalling is involved in the transcriptional regulation of Bim.

HCV infection induces mitochondrial accumulation of Bim

It was previously demonstrated that BimS-induced apoptosis requires its mitochondrial localization (Weber et al., 2007). We therefore investigated whether HCV infection affects the subcellular localization of Bim. As a control, we first confirmed mitochondrial accumulation of Bax (Fig. 4a, b), which is consistent with our previous observation (Deng et al., 2008). Importantly, the amounts of all the three isoforms of Bim, BimEL, BimL and BimS, in the mitochondrial fraction markedly increased in HCV-infected cells at 4 and 6 days p.i. (Fig. 4a, c). These results suggest that HCV mediates Bim/Bax-induced apoptosis through not only upregulation of Bim expression but also its mitochondrial accumulation.

Downregulation of Bim by siRNA attenuates HCV-induced, Bax-mediated apoptosis

To investigate whether Bim protein is required for HCV-induced, Bax-mediated apoptosis, we used an siRNA approach to inhibit endogenous Bim expression, followed by examination of Bax activation and Bax-mediated apoptosis. Cells transfected with Bim siRNA for 48 h exhibited evidently reduced mRNA expression levels of the isoforms BimEL, BimL and BimS in both the HCV-infected cells and the uninfected control (Fig. 5a). Also, protein expression levels of all three Bim isoforms were attenuated by Bim siRNA treatment in both the HCV-infected cells and the...
uninfected control (Fig. 5b, upper three panels). Concomitantly, the Bim siRNA transfection significantly inhibited HCV-induced Bax activation (Fig. 5c, d), caspase 3 activation (Fig. 5e) and cleavage of PARP (Fig. 5b, fourth panel). These results suggest that HCV-induced upregulation of Bim expression is required for Bax-mediated apoptosis.

**HCV infection facilitates the interaction between Bim and Bax**

Next, we performed a co-immunoprecipitation analysis to investigate whether HCV facilitates the interaction between Bim and Bax. Cell lysates were co-immunoprecipitated using an anti-Bim rabbit monoclonal antibody. The possible Bim-associated Bax was probed by immunoblot analysis using an anti-Bax mouse monoclonal antibody. The results revealed that Bax was co-immunoprecipitated with Bim in the HCV-infected cells, but not in the uninfected control (Fig. 6a). No band was detected using control rabbit IgG for immunoprecipitation. Moreover, HCV-induced interaction between Bim and Bax was abolished by pretreatment with NAC (5 mM for 2 h) (Fig. 6b). We also observed that H₂O₂ (400 μM for 10 h) treatment induced significant interaction between Bim and Bax (Fig. 6c). These results suggest that HCV facilitates the

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**Fig. 4.** HCV infection facilitates the mitochondrial accumulation of Bim. (a) Mitochondrial and cytosolic fractions were prepared from HCV-infected HuH-7.5 cells and the mock-infected control cells at 4 and 6 days p.i. and analysed by immunoblotting using antibodies against Bim, Bax, NS3, Tim23 and β-tubulin. The amounts of Tim23 and β-tubulin were measured to verify equal amounts of mitochondrial and cytosolic fractions, respectively. The intensities of the bands of mitochondrion-associated Bax (b) and BimEL, BimL and BimS (c) in the HCV-infected cells and the mock-infected controls were quantified. The intensities of the mock-infected controls at 4 days p.i. were arbitrarily expressed as 1.0. Data represent means ± SE of data from two independent experiments, each performed with triplicate cultures. *, P<0.05; †, P<0.01, compared with the control.
Fig. 5. Bim silencing abolishes HCV-induced activation of Bax and caspase 3, and cleavage of PARP. (a) HCV-infected Huh-7.5 cells and mock-infected control cells were transfected with siRNA targeted to Bim mRNAs or control siRNA at a final concentration of 40 nM at 4 days p.i. At 48 h after transfection, quantitative RT-PCR was performed to determine BimEL (left), BimL (middle) and BimS (right) mRNA expression levels. The values for the control cells without Bim siRNA were arbitrarily expressed as 1.0. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. *, P<0.01. (b) The expressions of BimEL, BimL, BimS and the cleavage product of PARP in lysates of HCV-infected cells and mock-infected control with or without Bim siRNA (40 nM for 48 h) were analysed by immunoblotting at 6 days p.i. Blots were reprobed with antibodies recognizing NS3 and GAPDH. The relative expression levels of BimEL, BimL and BimS were quantified and are indicated below the respective lanes. (c) HCV-infected cells and mock-infected control cells with or without Bim siRNA were subjected to indirect immunofluorescence analysis at 6 days p.i. as described in the legend of Fig. 1(a). Scale bar, 10 µm. (d) The percentages of cells expressing activated Bax were determined. Data represent...
physical interaction between Bim and Bax through, at least partly, HCV-mediated Bim upregulation and mitochondrial accumulation of both Bim and Bax.

Increased Bim expression and caspase 3 activation in hepatocytes of liver tissues obtained from HCV-infected patients

In order to verify the above in vitro observations in in vivo settings, we examined whether Bim expression levels and caspase 3 activities were elevated in hepatocytes of liver tissues obtained from HCV-infected patients by immunohistochemical analysis. Normal hepatocytes obtained from patients without HCV infection barely showed staining for Bim or the activated form of caspase 3 (Fig. 7c, e) in all of four liver tissues tested. On the other hand, Bim and cleaved caspase 3 were clearly detected in hepatocytes of liver tissues obtained from one of three HCV-infected patients (Fig. 7d, f). The samples stained with normal rabbit IgG as a control showed complete negative staining (Fig. 7a, b). The positive staining for activated caspase 3 in HCV-infected liver tissues is consistent with a previous report (Bantel & Schulze-Osthoff, 2003).

DISCUSSION

The homeostasis of cell numbers in tissues is maintained by a critical balance between cell proliferation and apoptosis. Dysregulation of apoptosis/survival signals might contribute to the development of hepatocellular carcinoma (Fabregat et al., 2007). Our previous study showed that HCV infection induces Bax-mediated apoptosis (Deng et al., 2008). In the present study, we further dissected the mechanisms of HCV-induced Bax activation. Our present findings have revealed that HCV-induced ROS and activated JNK act upstream of Bax activation (Figs 1 and 2). More importantly, our results demonstrate that HCV-mediated ROS/JNK signalling transcriptionally upregulates Bim expression (Fig. 3), and that upregulated Bim translocates to mitochondria (Fig. 4) and physically interacts with Bax (Fig. 6). Our study thus suggests that Bim is a critical factor for HCV-induced, Bax-mediated apoptosis through a ROS/JNK-dependent mechanism (Fig. 8).

JNK is activated by diverse stimuli, including ROS, cytokines, endoplasmic reticulum stress, metabolic changes and infections. Upon activation, JNK signalling regulates cell death, survival, differentiation, proliferation, metabolism, insulin signalling, and tumorigenesis in the liver (Seki et al., 2012). We previously reported that the ROS/JNK signalling pathway plays a critical role in HCV-induced upregulation of hepatic gluconeogenesis (Deng et al., 2011).

In the present study, we demonstrated that ROS/JNK signalling is also required for induction of the mitochondrial intrinsic apoptotic pathway in HCV-infected cells, which is consistent with previous reports (Kamata et al., 2005; Singh et al., 2009). In related research, it was reported that activation of the JNK signalling pathway and upregulation of Bim expression are involved in the apoptosis induction by the non-nucleoside reverse transcriptase inhibitor efavirenz in primary human hepatocytes (Bumpus, 2011). Although increasing evidence suggests that activated JNK induces apoptosis through Bax activation (Kim et al., 2006; Tsuruta et al., 2004), the precise mechanism(s) by which the JNK signalling pathway regulates Bax remains unsolved in HCV-infected cells. In the present study, we focused on the role of Bim in Bax-mediated apoptosis during HCV infection. A major mechanism of functional regulation of Bim-dependent apoptosis is the regulation of Bim expression. Our present findings further support the understanding that increased Bim expression is required for Bax activation (Fig. 5). In addition, Bim function is positively and negatively regulated by phosphorylation (Ley et al., 2005). Phosphorylation of BimEL at Ser-69 by ERK1/2 promotes the proteasomal degradation of BimEL, which has been shown to promote cell survival (Luciano et al., 2003). Also, multi-site phosphorylation of BimEL (Ser-55/65/73) in mice negatively regulates BimEL stability and apoptotic activity (Hübner et al., 2008). On the other hand, phosphorylation of BimL at other sites (Thr-56, Ser-44, and/or Ser-58) by JNK causes a release of Bim from the dynein motor complexes, leading to an enhancement of the pro-apoptotic function of Bim (Lei & Davis, 2003). Therefore, we tried to examine the possible effects of HCV infection on the phosphorylation status of BimEL at Ser-69. However, we did not observe any significant effect on the BimEL phosphorylation status in our experimental system (data not shown).

By using an in vitro HCV infection system, we and other groups have observed that increased ROS production is induced in Huh-7.5 cells infected with HCV 16/JFH1 or JFH1 (Deng et al., 2008, 2011; Lin et al., 2010; Woodhouse et al., 2010). J6/JFH1 is an HCV strain that efficiently infects and replicates in Huh-7.5 cells. Recently, it was reported that lipid peroxidation in HCV-infected cells plays a role in regulating HCV replication to restrict HCV replication. It is noteworthy that JFH1 is unique among HCV strains in its resistance to lipid peroxidation, resulting in robust replication (Yamane et al., 2014). Therefore, the mechanism for the HCV-induced ROS/JNK/Bim/Bax apoptotic signalling pathway should be further explored.

means ± SE of results from two independent experiments, each performed with triplicate cultures. * P < 0.01. (e) Caspase 3 activities in HCV-infected cells and mock-infected control cells with or without Bim siRNA were determined at 6 days p.i. The caspase 3 activity of the control cells without Bim siRNA was arbitrarily expressed as 1.0. Data represent means ± SE of results from two independent experiments, each performed with triplicate cultures. * P < 0.01. (f) Relative light units.
It was recently reported that Bim directly interacts with Bax in the cytosol of mammalian cells and that the Bim–Bax complex translocates to mitochondria (Vela et al., 2013). In addition, mitochondrial localization of Bim S causes mitochondrial recruitment of Bax and apoptosis (Weber et al., 2007). Our results showed that Bim and Bax were distributed in both the cytosol and mitochondria in HCV-infected cells (Fig. 4a), and HCV triggered the interaction between Bim and Bax (Fig. 6a). Therefore, we assume that, in HCV-infected cells, Bim interacts with Bax in the cytosol, leading to the activation and the mitochondrial translocation of Bax. Alternatively, HCV-induced mitochondrial accumulation of Bim might recruit Bax to mitochondria, leading to Bax activation.

In our previous studies, we found that NS4A accumulates on mitochondria and facilitates mitochondrion-mediated apoptosis (Nomura-Takigawa et al., 2006) as do core (Lee et al., 2007) and E2 (Chiou et al., 2006). We also found that NS5A can promote mitochondrial ROS production and activate the JNK/c-Jun signalling pathway (Deng et al., 2011). However, transient expression of any single HCV genotypes and primary human hepatocytes.

Figure 6. HCV infection facilitates the interaction between Bim and Bax. (a) Lysates from HCV-infected HuH-7.5 cells and mock-infected control cells at 5 days p.i. were immunoprecipitated using an anti-Bim rabbit monoclonal antibody or normal rabbit IgG. The cell lysates and the immunoprecipitates were probed with anti-Bax or anti-Bim mouse monoclonal antibodies. Blots were reprobed with antibodies recognizing NS3 and GAPDH. (b) Lysates from HCV-infected HuH-7.5 cells and mock-infected control with or without NAC (5 mM for 2 h) pretreatment at 5 days p.i. were immunoprecipitated using an anti-Bim rabbit monoclonal antibody as described in (a). (c) Lysates from HuH-7.5 cells treated with H2O2 (400 μM for 10 h) or left untreated were immunoprecipitated using an anti-Bim rabbit monoclonal antibody as described in (a).

Figure 7. Upregulation of Bim expression and activation of caspase 3 in HCV-infected human liver tissues in vivo. Human adult liver tissues without (a, c and e) and with HCV infection (b, d and f) were stained with normal rabbit IgG (a, b) or anti-Bim rabbit monoclonal antibody (c, d), or anti-cleaved caspase 3 rabbit polyclonal antibody (e, f). Scale bar, 20 μM. Arrows indicate representative cells with positive staining.
protein tested alone, including core, NS4A and NS5A, did not significantly influence the Bim promoter activity or the expression levels of Bim protein (data not shown). Although the possibility still remains that a composite effect of multiple HCV proteins is needed to upregulate Bim expression, our result implies the possibility that HCV infection and/or replication are required for upregulation of Bim expression.

In conclusion, our present results collectively suggest that HCV-induced ROS production and JNK activation lead to increased expression and mitochondrial accumulation of Bim, facilitating Bax activation and eventually mitochondrion-mediated apoptosis.

**METHODS**

**Cells and virus.** The human hepatoma-derived cell line Huh-7.5 (Blight et al., 2002) and plasmid pFL-J6/JFH1, which encodes the entire viral genome of a chimeric strain of HCV-2a (J6/JFH1) (Lindenbach et al., 2005), were kindly provided by Dr C. M. Rice (Rockefeller University, New York, NY). We prepared the virus stock used in this study as described previously (Deng et al., 2008). A cell culture-adapted P-47 strain (Bungyoku et al., 2009; Deng et al., 2008) was used throughout the experiments. Virus infection was performed at an m.o.i. of 2.0 focus-forming units cell

**Indirect immunofluorescence.** Huh-7.5 cells seeded onto glass coverslips in a 24-well plate were infected with HCV or left uninfected. At 6 days p.i., indirect immunofluorescence was performed as described previously (Deng et al., 2008). The primary antibodies used were a conformation-specific antibody that specifically recognizes the activated form of Bax protein (NT antibody; Millipore) and HCV-infected patient’s serum that strongly reacts with HCV antigens.

**Immunohistochemistry.** Human adult liver autopsy materials and surgically resected liver tissue of patients with or without HCV infection were obtained with written informed consent. Immunohistochemical staining was performed as described previously (Inubushi et al., 2008; Kasai et al., 2009). The primary antibodies used were rabbit antibodies against Bim or cleaved caspase 3 (Cell Signaling Technology). Normal rabbit IgG (Santa Cruz Biotechnology) served as a control. The sections were then incubated with horseradish peroxidase-labelled polymer-conjugated goat anti-rabbit IgG and observed under a light microscope.

**Cell fractionation.** Cytosolic and mitochondrial fractions prepared from HCV-infected Huh-7.5 cells or the uninfected control cells were prepared by using a mitochondrial isolation kit (Pierce), as described previously (Deng et al., 2008).

**Immunoprecipitation and immunoblot analysis.** HCV-infected Huh-7.5 cells or the uninfected control cells were harvested and lysed in lysis buffer containing 50 mM Tris/HCl (pH 7.5), 144 mM NaCl, 1 mM EDTA, 1 % Triton X-100 and protease inhibitor cocktail (Roche) for 30 min on ice. After centrifugation at 4 °C at 20 400 g for 20 min, the supernatants were immunoprecipitated with the respective antibodies. Immunoprecipitation and immunoblot analyses were performed as described previously (Deng et al., 2006). The primary antibodies used were: mouse monoclonal antibodies against HCV NS3, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Millipore), Bim (H-5, Santa Cruz Biotechnology), Tim23 and Bax (BD Biosciences); rabbit polyclonal antibodies against PARP, β-tubulin (Cell Signaling Technology) and HCV NS5A (Matsui et al., 2015); and rabbit monoclonal antibody against Bim (Cell Signaling Technology). Normal rabbit IgG served as a control. Horseradish peroxidase-conjugated goat anti-mouse IgG and goat anti-rabbit IgG (Molecular Probes) were used to visualize the respective target proteins by means of an enhanced chemiluminescence detection system (ECL; GE Healthcare) and the intensity of the bands was quantified using NIH Imagej software.

**Caspase 3 activity assay.** Caspase 3 activities were measured using Caspase-Glo 3/7 (Promega), according to the manufacturer’s instructions. The resultant luminescence was measured in relative light units (RLU) using a GloMax 96 microplate luminometer (Promega).

**Real-time quantitative RT-PCR.** Total cellular RNA was isolated by using a ReliaPrep RNA Cell Miniprep System (Promega) according to the manufacturer’s instructions, and cDNA was generated by using a GoScript Reverse Transcription System (Promega). Real-time quantitative PCR was performed by using SYBR Premix Ex Taq II (TaKaRa) with SYBR Green chemistry on an ABI PRISM 7500 system.
Luciferase reporter assay. The human Bim promoter reporter constructs pGL4.11/Bim-luc (−3.6 kb) and a deletion mutant pGL4.11/Bim-luc (−1.2 kb) (Heidari et al., 2012) were kindly provided by Dr H. Harada (Virginia Commonwealth University, Richmond, VA). HCV-infected Huh-7.5 cells or the uninfected control cells prepared in a 24-well tissue culture plate were transiently transfected with the reporter constructs described above and pRL-TK-Renilla (Promega) using the X-tremeGene 9 transfection reagent (Roche). After 48 h, a luciferase assay was performed using the Dual-Luciferase reporter assay system (Promega). Firefly and Renilla luciferase activities were measured with a GloMax 96 microplate luminometer (Promega). Firefly luciferase activity was normalized to Renilla luciferase activity for each sample.

siRNA transfection. HCV-infected Huh-7.5 cells or the uninfected control cells were transfected with SignalSilence Bim siRNA I (Cell Signaling Technology) in order to silence Bim gene expression using Lipofectamine RNAiMAX (Invitrogen) for 48 h according to the manufacturers’ instructions. AllStars Negative Control siRNA (Qiagen) was used as a control.

Statistical analysis. Results are expressed as means ± SE. Statistical significance was evaluated by analysis with Student’s t-test and was defined as a P-value of < 0.05.

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