Hepatitis C Virus Infection Sensitizes Human Hepatocytes to TRAIL-Induced Apoptosis in a Caspase 9-Dependent Manner

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*J Immunol* 2008; 181:4926-4935; doi: 10.4049/jimmunol.181.7.4926

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Hepatitis C virus (HCV) infection is a leading cause of liver cirrhosis and hepatocellular carcinoma (1). HCV belongs to the Flaviviridae family and has an enveloped, positive-stranded RNA genome of 9.6 kb length containing one open reading frame translated into a single polyprotein. A highly conserved, untranslated region at the 5' site serves as an internal ribosomal entry site which directs cap-independent translation. Posttranslational cleavage of the polyprotein yields in structural and nonstructural proteins including core, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B (2, 3).

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1 This study was supported by the Fritz Thyssen Stiftung (to R.F.), Germany, the Faculty of Medicine, University of Freiburg, Germany (to R.F. and A.S.-G.), and by grants of Institut National de la Santé et de la Recherche Médicale, France, the European Union (LSHM-CT-2004–503359 “VIRGIL”; to T.F.B.), Belgium, the chair of excellence program of the Agence Nationale de la Recherche (ANR-05-CECX-008; to T.F.B.), France, the Agence Nationale de la Recherche sur le SIDA et les Hépatites Virales (ANRS 06221; to T.F.B.), France, the Deutsche Forschungsgemeinschaft (Ba1417/11–2; to T.F.B.), Germany, and the Else Kröner-Fresenius Foundation, Bad Homburg, Germany (P17007/A83/06; T. F. B.). M.B.Z. was supported by the Inserm Poste Vert program in the framework of Institut National de la Santé et de la Recherche Médicale European Associated Laboratory Freiburg-Strasbourg. T.W. is supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science, from the Ministry of Health, Labour and Welfare of Japan and from the Ministry of Education, Culture, Sports, Science and Technology, and by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation.

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Received for publication December 21, 2007. Accepted for publication July 23, 2008.

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Hepatitis C Virus Infection Sensitizes Human Hepatocytes to TRAIL-Induced Apoptosis in a Caspase 9-Dependent Manner

Lin Lan,‡ Sebastian Gorke,‡ Sibylle J. Rau,§ Mirjam B. Zeisel,¶ Eberhard Hildt,¶ Kiyoshi Himmelsbach,*§ Monica Carvajal-Yepes,*§ Roman Huber,§ Takaji Wakita,§ Annette Schmitt-Graeff,** Cathy Royer,*¶ Hubert E. Blum,* Richard Fischer,§ and Thomas F. Baumert‡§¶

Apoptosis of infected cells represents a key host defense mechanism against viral infections. The impact of apoptosis on the elimination of hepatitis C virus (HCV)-infected cells is poorly understood. The TRAIL has been implicated in the death of liver cells in hepatitis-infected but not in normal liver cells. To determine the impact of TRAIL on apoptosis of virus-infected host cells, we studied TRAIL-induced apoptosis in a tissue culture model system for HCV infection. We demonstrated that HCV infection sensitizes primary human hepatocytes and Huh7.5 hepatoma cells to TRAIL induced apoptosis in a dose- and time-dependent manner. Mapping studies identified the HCV nonstructural proteins as key mediators of sensitization to TRAIL. Using a panel of inhibitors targeting different apoptosis pathways, we demonstrate that sensitization to TRAIL is caspase-9 dependent and mediated in part via the mitochondrial pathway. Sensitization of hepatocytes to TRAIL-induced apoptosis by HCV infection represents a novel antiviral host defense mechanism that may have important implications for the pathogenesis of HCV infection and may contribute to the elimination of virus-infected hepatocytes. The Journal of Immunology, 2008, 181: 4926–4935.

Abbreviations used in this paper: HCV, hepatitis C virus; PHH, primary human hepatocyte; MOI, multiplicity of infection; JFH1, Japanese fulminant hepatitis 1 isolate; HCVcc, cell culture-derived HCV; PARP, poly (ADP-ribose) polymerase; HBV, hepatitis B virus.
Bak, and other proteins, while Bcl-2 or Bcl-xL act anti-apoptotic (for review see Ref.12).

The molecular mechanisms of hepatocyte apoptosis in HCV infection are poorly understood. For both HCV structural and non-structural proteins, pro- and antiapoptotic properties have been described (13). Viral RNA has been shown to be able to induce apoptosis via activation of protein kinase R and the retinoic acid inducible gene-I-Cardif pathway (14–16). However, the significance of these interactions for productive viral infection and pathogenesis of HCV-induced liver disease remains unknown. Studies on HCV-host interactions had been hampered for a long time by the lack of an efficient tissue culture model for HCV infection. Thus, alternative model systems, such as recombinant proteins, pseudotype particles, or subgenomic replicons, had been developed for the study of defined aspects of the HCV life cycle (for review see Ref. 17). A major breakthrough for the study of HCV-host cell interaction was the recent establishment of an efficient cell culture system for HCV (18–20). This model system now allows the study of HCV-host interactions and apoptosis in the context of the complete viral life cycle in human hepatocyte-derived target cells.

To determine the impact of TRAIL on apoptosis of virus-infected host cells, we studied mechanisms of TRAIL-induced apoptosis in a tissue culture model system for HCV infection.

Materials and Methods

Cells

Primary human hepatocytes (PHH) were isolated and cultured as described (21). Human hepatoma cells Huh7.5 have been described (18, 22).

Constructs

Plasmids pJFH1, pJFH1/ΔE1E2, pJFH1/GND, pSGR-JFH1, pSGR-JFH1/GND, and pFK-Jc1 have been described (19, 23, 24) and are depicted in Fig. 1.

Electroporation of HCV RNA, cell culture-derived HCV (HCVcc) production, infection of Huh7.5 cells, and PHH

Electroporation of RNA derived from plasmids pJFH1, pJFH1/ΔE1E2, pJFH1/GND, pSGR-JFH1, pSGR-JFH1/GND, and pFK-Jc1 was performed as described (19, 23, 24). Culture supernatants from HCV JFH1, HCV Jc1, and HCV JFH1/ΔE1E2 RNA transfected cells were harvested, concentrated, and used to infect naive Huh7.5 cells (22) as well as primary human hepatocytes. Culture supernatants used to infect naive Huh7.5 cells had a TCID50 of 10^3/ml for JFH1 and 10^2–10^3/ml for Jc1.

Immunoblot analysis of viral and cellular proteins

Cell lysis, protein determination, Western blot analysis, and densitometric analysis were performed as recently described (25). Cytosolic and mitochondrial protein fractions were obtained using a ProteoExtract Subcellular Proteome Extraction Kit according to the manufacturer’s protocol (Calbiochem). Relative OD was calculated by correlation of the given protein to the human conserved N-terminal homology domain of Bcl-xL (aa 4–23), while Bcl-2 or Bcl-xL act anti-apoptotic (for review see Ref.12).

Induction of apoptosis by TRAIL

Naive and HCV RNA transfected Huh7.5 cells were seeded at a density of 2–4 × 10^3 cells/well in 6-well plates 72 h before apoptosis experiments.

Immunofluorescence staining of viral proteins in infected host cells

Fixation and immunostaining of Huh7.5 cells was performed using monoclonal anticore Abs (C1/C2) as described recently (25, 28, 29). Cells were embedded in fluorescence-mounting medium containing DAPI (4–6-diamidino-2-phenylindole; Vector Laboratories) for cell nuclei staining. Microscopy was performed using a fluorescent microscope (Leica). For detection of DNA fragmentation, a TUNEL assay was used as previously described (30). The percentage of apoptotic cells was determined by counting the number of TUNEL-positive cells. At least 300 cells were counted out of five microscopic fields at ×400 magnification for each experiment.

Statistical analysis

Data are expressed as means ± SEM (n = number of cell preparations). SEM was omitted in the figures when smaller than symbol size. Each experiment was performed at least in three independent cell preparations.

FIGURE 1. HCV constructs and viral protein expression. A, Constructs: HCV-pJFH1 resulting in the production of infectious viral particles, pJFH1/ΔE1E2 containing a deletion of the HCV envelope protein coding region preventing production of infectious viral particles, pJFH1/GND containing a mutation in NS5B protein preventing viral replication, and subgenomic replicon pSGR-JFH1 (lacking the coding region for HCV structural proteins and NS2, respectively). B, Protein expression from replication-competent constructs. Seventy-two hours following electroporation with HCV RNA transcribed from constructs depicted in A, cells were lysed and subjected to immunoblot analysis using Abs against HCV core, E2, NS3, and NS5A proteins as described in Materials and Methods.
TRAIL-induced apoptosis in human Huh7.5 hepatoma cells is caspase and Bcl-2/Bcl-xL dependent. Following incubation with TRAIL, Huh7.5 cells were lysed and protein lysates were subjected to SDS-PAGE and Western blot analysis using anti-human PARP and caspase-specific Abs. A, left panel, Time course of TRAIL-induced apoptosis with PARP-, caspase-3, caspase-8, and caspase-9 cleavage following 1–24 h incubation with TRAIL at a concentration of 50 ng/ml. Right panel, Dose-dependent induction of TRAIL-dependent apoptosis at TRAIL concentrations ranging from 1 to 200 ng/ml (incubation time 2 h). B, Mechanism of TRAIL-induced apoptosis. Huh7.5 cells were incubated with TRAIL following a 30-min preincubation with Bcl-2, Bcl-xL, and caspase inhibitors. Cells were incubated at a TRAIL concentration of 50 ng/ml (left panel), or for 2 h with different TRAIL concentrations (right panel). Inhibition of anti-apoptotic Bcl-2 and Bcl-xL using dimethyl-dihydro-dibenzoazoline dioxide and dimethoxy-dinitrosobenzyl (50 μmol/l) showed a marked induction of TRAIL-induced apoptosis. C, After 10 min preincubation time with caspase-8 and -10 inhibitors Z-IETD-FMK (20 μmol/l) and Z-AEVD-FMK (20 μmol/l), cells were incubated for 2 h at a TRAIL concentration of 50 ng/ml. The inhibition of proapoptotic caspases-8 and -10 completely abolished TRAIL induced apoptosis (lanes 2 and 4), whereas inhibition of caspase-9 by inhibitor Z-LEHDFMK (20 μmol/l) markedly reduced apoptosis (lane 3).

Results were compared using the Student’s t test; p < 0.05 was considered statistically significant.

Results

TRAIL induces apoptosis in Huh7.5 hepatoma cells, the target cell line for HCV infection

Sensitivity of human hepatoma cells to induction of apoptosis has been shown to differ among various cell lines. This is illustrated by the fact that for Huh7 cells, the parental cell line of Huh7.5, apoptosis induction as well as resistance following interaction with TRAIL have been reported (31–33). Thus, we studied mechanisms of TRAIL-induced apoptosis in Huh7.5 cells, a target cell line for infectious recombinant HCVcc. As shown in Fig. 2, TRAIL rapidly and dose-dependently induced cleavage of caspase-8, -9, and -3 (Fig. 2). The amounts of cleaved and uncleaved caspase-8, caspase-9, and PARP declined during apoptosis. This decline most likely reflects degradation of these proteins due to apoptosis-activated proteases as described in many model systems for apoptosis (34–36). Caspase activation is a marker of the susceptibility of the cell to undergo apoptosis, but not equivalent to apoptosis (for review, see Ref. 9). To confirm that Huh7.5 cells indeed underwent irreversible apoptosis following TRAIL incubation, we analyzed PARP cleavage, a marker of irreversible cell death (37). After incubating Huh7.5 cells with TRAIL at a concentration of 50 ng/ml for 2 h, cleavage of PARP was detectable (Fig. 2). These data demonstrate that TRAIL induces apoptosis in Huh7.5 hepatoma cells in a dose-dependent manner.

Next, we aimed to study the mechanism of TRAIL-induced apoptosis in hepatocyte-derived cell lines. Using defined inhibitors targeting key mediators of distinct apoptotic pathways, we studied the functional impact of Bcl-2, Bcl-xL, and caspases for TRAIL-induced cell death. Proteins belonging to the Bcl-family are important modulators of mitochondrial apoptosis (38). As shown in Fig. 2B specific inhibition of anti-apoptotic effectors Bcl-2 and Bcl-xL with dimethoxy-dihydro-dibenzoazoline dioxide and dimethoxy-dinitrosobenzyl did not result in spontaneous apoptosis in Huh7.5 cells, but enhanced TRAIL-induced apoptosis. These data indicate that TRAIL-induced apoptosis is suppressed by Bcl-2 and Bcl-xL, but not completely inhibited. Hepatocytes belong to the so-called type-II cells that need mitochondrial amplification and consecutive caspase-9 activation for apoptosis induction via death ligands (39). In Huh7.5 cells, inhibition of caspase-9 with Z-LEHD-FMK largely abolished TRAIL-induced apoptosis, as determined by cleaved PARP fragments (Fig. 2C, lane 3). Inhibition of the initiator caspases-8 and -10 with Z-IETD-FMK and Z-AEVD-FMK, completely prevented TRAIL-induced apoptosis (lanes 2 and 4). In contrast, inhibition of caspase-3 only marginally altered TRAIL-induced apoptosis (see Fig. 8). These data demonstrate that TRAIL-induced apoptosis in Huh7.5 cells, similar to hepatocytes, largely depends on mitochondrial amplification. Furthermore, our data indicate that TRAIL-induced apoptosis is blocked if downstream signaling of the receptor-activated death-inducing signaling complex via caspase-8/-10 is inhibited.

Replicating infectious HCV sensitizes Huh7.5 cells and primary human hepatocytes to TRAIL-induced apoptosis

Because many viruses including cytomegalovirus (40), influenza virus (41), and adenovirus (42) have been shown to sensitize infected cells to TRAIL-induced apoptosis, we aimed to study whether HCV infection sensitizes Huh7.5 cells to TRAIL-induced apoptosis. To address this issue, we first exposed Huh7.5 cells harboring replicating infectious HCV to TRAIL. Compared with control transfected cells, replicating infectious HCV RNA markedly and significantly enhanced TRAIL-induced apoptosis, as shown by cleaved PARP (Fig. 3A–D) and cleaved caspase-8 and -9 (Fig. 3E and F). At the single-cell level, the TUNEL assay showed a statistically significant enhancement of TRAIL-induced apoptosis in cells harboring replicating HCV RNA, but not in the control cells harboring replication-deficient control RNA (Fig. 3G and H). These results demonstrate that HCV sensitizes its host cells to TRAIL-induced apoptosis.

Next, we aimed to confirm whether sensitization to TRAIL-induced apoptosis was also present in HCV-infected cells. Thus, we incubated Huh7.5 cells with HCVcc or noninfectious control supernatants derived from cells transfected with JFH1/ΔE1E2 RNA unable to produce infectious viral particles. As shown in Fig. 4, exposure of HCV-infected Huh7.5 cells to TRAIL resulted in enhanced apoptosis compared to control cells incubated with control supernatants, as shown by cleaved PARP (Fig. 4A). Compared
to viral genome delivery by electroporation, HCV-dependent enhancement of TRAIL-induced apoptosis appeared to be lower in HCV JFH1-infected hepatoma cells (as shown by the lower level of cleaved PARP when comparing Fig. 4A to 3C). This difference was most likely due to low levels of MOI (TCID$_{50}$ of 10$^{4}$/ml for HCVcc derived from HCV-JFH1 strain) resulting in less efficient delivery of the viral genome in infection compared with transfection experiments. To address this issue, we repeated infection experiments using infectious particles of viral strain Jc1 (J6-JFH1). In contrast to the JFH1 strain, the Jc1 isolate has been shown to allow production of virus with markedly enhanced infectivity (24). Indeed, infection of Huh7.5 cells at higher MOIs with HCV virions derived from the HCV Jc1 strain (TCID$_{50}$ of 10$^{2}$–10$^{3}$/ml) resulted in similar enhancement of TRAIL-induced apoptosis as seen in transfection experiments (compare Fig. 4C with 3C).

Huh7.5 cells are derived from transformed hepatoma cells and therefore only represent a surrogate model for the natural target cell for HCV infection, the PHH. To confirm the relevance of our findings for the natural target cell of HCV infection, we reproduced key findings in HCV-infected primary human hepatocytes in contrast to Huh7.5 hepatoma cells have been shown to be resistant to TRAIL-induced apoptosis (5–7). Indeed, incubation of PHH with TRAIL did not yield in cleavage of PARP (Fig. 4D, lane 4), whereas incubation of PHH with TRAIL did not yield in cleavage of PARP (Fig. 4D, lane 4).

To confirm the relevance of our findings for the natural target cell of HCV infection, we reproduced key findings in HCV-infected primary human hepatocytes in contrast to Huh7.5 hepatoma cells have been shown to be resistant to TRAIL-induced apoptosis (5–7). Indeed, incubation of PHH with TRAIL did not yield in cleavage of PARP (Fig. 4D, lane 4), whereas incubation of PHH with TRAIL did not result in apoptosis (Fig. 4D, lane 5), HCV JFH1 infection rendered PHH sensitive to TRAIL-induced apoptosis (Fig. 4D, lane 4). Infection of PHH with HCV JFH1 was confirmed by detection of HCV NS5A protein expression in infected cells using immunoblot (Fig. 4D, lanes 2 and 3). These results confirm the relevance of HCV-dependent sensitization to TRAIL-induced apoptosis for PHH.
Interestingly, we observed that transfection of HuH7.5 cells with replication-competent full-length HCV RNA itself can result in apoptosis of HuH7.5 cells as indicated by an increase in the number of TUNEL-positive cells compared with control cells (Fig. 3).

To identify the viral factor(s) enhancing TRAIL-induced apoptosis, we determined whether HCV structural protein expression is required for HCV-TRAIL interaction. To address this question, we transfected HuH7.5 cells with a JFH1 variant (JFH1/ΔE1E2) containing a deletion in the HCV envelope coding region preventing synthesis of infectious viral particles. Because the phenotype of full-length JFH1 RNA and JFH1/ΔE1E2 was indistinguishable in regard to enhancement of TRAIL-induced apoptosis (Fig. 5), we conclude that HCV-TRAIL interaction is independent of production of infectious viral particles. To determine whether structural proteins are required for HCV-enhanced TRAIL-induced apoptosis, we compared the full-length JFH1 RNA with a JFH1 subgenomic replicon lacking the HCV core-NS2 region. Because the JFH1 subgenomic replicon exhibited a similar apoptosis phenotype as full-length JFH1 RNA (Fig. 5), we conclude that the expression of HCV structural proteins and NS2 is not required for enhancement of TRAIL-induced apoptosis and sensitization to TRAIL-induced apoptosis is mediated predominantly by the HCV nonstructural proteins.

HCV JFH1-dependent enhancement of TRAIL-induced apoptosis is caspase-9 dependent

Following mapping the viral factors required for HCV-dependent enhancement of TRAIL-induced apoptosis, we investigated the cellular factors mediating this HCV-host interaction. Several studies pointed to an involvement of TRAIL in apoptosis of virus-infected hepatocytes by an autocrine loop (43, 44). To address this question, we studied the impact of HCV replication and protein expression on the expression of TRAIL and TRAIL receptor R1 in cells harboring replicating HCV (compare lanes 1 and 5 in Fig. 4A; lanes 2 and 4 in Fig. 3, E and F; lanes 2 and 4 see in Fig. 8B).

Enhancement of TRAIL-induced apoptosis is mediated by HCV nonstructural proteins

To identify the viral factor(s) enhancing TRAIL-induced apoptosis, we determined whether HCV structural protein expression is required for HCV-TRAIL interaction. To address this question, we transfected HuH7.5 cells with a JFH1 variant (JFH1/ΔE1E2) containing a deletion in the HCV envelope coding region preventing synthesis of infectious viral particles. Because the phenotype of full-length JFH1 RNA and JFH1/ΔE1E2 was indistinguishable in regard to enhancement of TRAIL-induced apoptosis (Fig. 5), we conclude that HCV-TRAIL interaction is independent of production of infectious viral particles. To determine whether structural proteins are required for HCV-enhanced TRAIL-induced apoptosis, we compared the full-length JFH1 RNA with a JFH1 subgenomic replicon lacking the HCV core-NS2 region. Because the JFH1 subgenomic replicon exhibited a similar apoptosis phenotype as full-length JFH1 RNA (Fig. 5), we conclude that the expression of HCV structural proteins and NS2 is not required for enhancement of TRAIL-induced apoptosis and sensitization to TRAIL-induced apoptosis is mediated predominantly by the HCV nonstructural proteins.

HCV JFH1-dependent enhancement of TRAIL-induced apoptosis is caspase-9 dependent

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and Bcl-xL was suppressed in JFH1-transfected Huh7.5 cells (Fig. 7). Interestingly, expression of Bcl-2 with JFH1 appeared to reduce Bcl-2 and Bcl-xL protein expression (Fig. 7, lane 1) compared with Huh7.5 cells (Fig. 7, lane 3), suggesting an essential role for mitochondrial apoptosis in HCV infection, we inhibited the mitochondrial apoptotic pathway for TRAIL-induced apoptosis. Several forms of apoptosis do not involve caspase activation. We therefore inhibited caspases completely abolished JFH1-dependent enhancement of TRAIL-induced apoptosis. Interestingly, HCV replication alone also resulted in release of cytochrome C levels in cytosolic fractions compared with control-transfected cells incubated with TRAIL. These data suggest that cytochrome C appears to be released from the mitochondria in TRAIL-treated cells with replicating HCV and suggests that the mitochondrial pathway contributes to HCV-induced enhancement of TRAIL-induced apoptosis. Interestingly, HCV replication alone also resulted in release of cytochrome C into the cytosol (Fig. 7D) consistent with low level induction of apoptosis by JFH1 itself (see Fig. 3H).

Next, we aimed to study the impact of caspases for HCV-mediated enhancement of TRAIL-induced apoptosis. Several forms of apoptosis do not involve caspase activation. We therefore inhibited caspases with the pan-caspase inhibitor Z-VAD-FMK. Interestingly, inhibition of caspases completely abolished JFH1-dependent enhancement of TRAIL-induced apoptosis (Fig. 8A, lane 3 and Fig. 9C, lane 3). These findings indicate an essential role of caspase activation for TRAIL-induced apoptosis of Huh7.5 cells harboring replicating HCV. To determine the impact of mitochondrial apoptosis in HCV infection, we inhibited the mitochondrial-activated caspase-9. Inhibition of caspase-9 with Z-LEHD-FMK largely blocked JFH1-dependent enhancement of TRAIL-induced apoptosis (Fig. 8B, lane 5), suggesting an essential contribution of the mitochondrial apoptotic pathway for...

**FIGURE 6.** JFH1-induced apoptosis in primary human hepatocytes and Huh7.5 cells is independent of TRAIL and TRAIL-R1/-R2 expression. PHH and Huh7.5 cells were infected/transfected with virions/RNA derived from constructs depicted in Fig. 1. Seventy-two hours post transfection, Bcl-2 (A and C), Bcl-xL (B and C) expression and cytochrome C levels in cellular subfractions (D) were analyzed by Western blot analysis. Suppression of antiapoptotic Bcl-2 (A) and antiapoptotic Bcl-xL (B) protein expression in JFH1-transfected Huh7.5 cells compared with Huh7.5 cells transfected with replication-deficient HCV GND RNA was detectable. Bcl-2 and Bcl-xL protein expression was detected 72 h following infection as described above. D, Cytochrome C levels in subcellular fractions (cytosolic and organelle fractions containing mitochondria) of cells with replicating virus incubated with or without TRAIL. Cytochrome C levels in cytosolic fractions compared with control-transfected cells incubated with TRAIL. These data suggest that cytochrome C appears to be released from the mitochondria in TRAIL-treated cells with replicating HCV and suggests that the mitochondrial pathway contributes to HCV-induced enhancement of TRAIL-induced apoptosis. Interestingly, HCV replication alone also resulted in release of cytochrome C into the cytosol (Fig. 7D) consistent with low level induction of apoptosis by JFH1 itself (see Fig. 3H).

In contrast to PHH, TRAIL receptor R2 was not expressed in Huh7.5 cells (Fig. 6C). Similar to Huh7.5 cells, infection of PHH with HCV JFH1 neither increased TRAIL expression, nor enhanced protein expression of TRAIL receptors R1, respectively (Fig. 6D). These data suggest that a TRAIL autocrine loop does not play a major role for TRAIL-induced sensitization in this HCV infectious tissue culture system. The mitochondrial apoptosis pathway has been shown to play an important role in virus-induced modulations of host cell apoptosis (10). As shown in Fig. 2, we have already demonstrated that TRAIL-induced apoptosis in Huh7.5 cells is enhanced following the inhibition of Bcl-2/Bcl-xL (Fig. 2B). We therefore analyzed Bcl-2 and Bcl-xL protein expression in JFH1-transfected Huh7.5 cells and JFH1 expression. Interestingly, expression of Bcl-2 and Bcl-xL was suppressed in JFH1-transfected Huh7.5 cells (Fig. 7, A and B). These results were confirmed in PHH, where infection with JFH1 appeared to reduce Bcl-2 and Bcl-xL protein expression (Fig. 7C). The less pronounced reduction in Bcl-2/Bcl-xL protein expression observed in PHH (Fig. 7C) compared with Huh7.5 cells (Fig. 7, A and B) may be due to the lower number of cells containing replicating HCV in PHH. Incubation of Huh7.5 with a Bcl-xL mimic peptide resulted in a small but reproducible reduction (three of three experiments) of TRAIL-induced apoptosis in JFH1 replicating cells (Fig. 8D). Mitochondrial-dependent apoptosis is characterized by release of mitochondrial cytochrome C into the cytoplasm (11, 12). We therefore studied cytochrome C levels in cytosolic fractions and fractions containing the mitochondria of HCV JFH1-replicating Huh7.5 cells incubated with TRAIL. As shown in Fig. 7D, hepatoma cells with replicating virus and treated with TRAIL demonstrated an increase of cytochrome C levels in cytosolic fractions compared with control-transfected cells incubated with TRAIL. These data suggest that cytochrome C appears to be released from the mitochondria in TRAIL-treated cells with replicating HCV and suggests that the mitochondrial pathway contributes to HCV-induced enhancement of TRAIL-induced apoptosis. Interestingly, HCV replication alone also resulted in release of cytochrome C into the cytosol (Fig. 7D) consistent with low level induction of apoptosis by JFH1 itself (see Fig. 3H).

**FIGURE 7.** HCV JFH1 replication suppresses antiapoptotic Bcl-2 and Bcl-xL expression. Huh7.5 cells and PHH were transfected/infected from constructs depicted in Fig. 1. Seventy-two hours post transfection, Bcl-2 (A and C), Bcl-xL (B and C) expression and cytochrome C levels in cellular subfractions (D) were analyzed by Western blot analysis. Suppression of antiapoptotic Bcl-2 (A) and antiapoptotic Bcl-xL (B) protein expression in JFH1-transfected Huh7.5 cells compared with Huh7.5 cells transfected with replication-deficient HCV GND RNA was detectable. Bcl-2 and Bcl-xL protein expression was detected 72 h following infection as described above. D, Cytochrome C levels in subcellular fractions (cytosolic and organelle fractions containing mitochondria) of cells with replicating virus incubated with or without TRAIL. Cytochrome C levels in cytosolic fractions compared with control-transfected cells incubated with TRAIL. These data suggest that cytochrome C appears to be released from the mitochondria in TRAIL-treated cells with replicating HCV and suggests that the mitochondrial pathway contributes to HCV-induced enhancement of TRAIL-induced apoptosis. Interestingly, HCV replication alone also resulted in release of cytochrome C into the cytosol (Fig. 7D) consistent with low level induction of apoptosis by JFH1 itself (see Fig. 3H).
HCV-mediated sensitization of TRAIL-induced apoptosis. Interestingly, inhibition of caspase-3 only marginally affected TRAIL-induced apoptosis in JFH1 replicating Huh7.5 cells (Fig. 8C) indicating that caspase-3 does not play a major role in execution of TRAIL/HCV apoptosis.

TRAIL-induced apoptosis in HCV target cells results in down-regulation of viral protein expression

If HCV replication enhances TRAIL-induced apoptosis, HCV-infected cells should preferentially be eliminated. To address this question, we studied the impact of TRAIL on HCV protein expression in Huh7.5 cells and PHH harboring replicating infectious HCV. In line with this hypothesis, incubation of HCV JFH1 RNA-transfected Huh7.5 cells with TRAIL induced a significant reduction of HCV core and E2 protein expression (Fig. 9A). As shown in immunofluorescence analyses of individual cells, apoptotic Huh7.5 cells with replicating HCV exhibited a marked reduction of HCV core protein expression compared with nonapoptotic cells (Fig. 9E). These results were further confirmed by
quantification of viral protein expression using immunofluorescence analyses (data not shown). Similar to findings in Huh7.5 hepatoma cells, incubation with TRAIL appeared to result in suppression of HCV protein expression in HCV JFH1-infected PHH (Fig. 4D, lane 2, compared with lane 3). The TRAIL-dependent reduction of HCV protein expression was dependent on apoptosis induction, since preincubation of Huh7.5 cells with pan-caspase-inhibitor Z-VDAD-FMK completely restored expression of the HCV proteins core and E2, respectively (Fig. 9, C and D). These findings confirm the impact of TRAIL-induced apoptosis for HCV-host interactions and suggest that sensitization to TRAIL-induced apoptosis in cells harboring replicating HCV may contribute to control of HCV infection.

Discussion
In this study, we demonstrate that HCV infection sensitizes the host cell to TRAIL-induced apoptosis. Detailed analyses indicate that this sensitization depends on the expression of HCV nonstructural proteins NS3, NS4, and NS5. The interaction between HCV and the host cell apoptosis machinery involves caspase-9 and the mitochondrial but not the autocrine TRAIL-mediated pathway. Furthermore, we show that induction of apoptosis by TRAIL results in suppression of HCV protein expression, suggesting that this mechanism may contribute to elimination of HCV-infected hepatocytes.

Viral factors required for sensitization to TRAIL-induced apoptosis
For virtually all HCV proteins, interference with apoptosis has been reported. However, most studies assessing the effect of HCV infection on cellular apoptosis were performed in surrogate models of HCV infection, e.g., stably transfected cell lines or recombinant proteins expressed in heterologous systems. Thus, the relevance of these observations for HCV infection remained uncertain. Limitations of surrogate models of HCV infection are, among others, artificially elevated levels of expressed HCV proteins or the lack of genetic elements in subgenomic replicons (13). To overcome these limitations and to study the mechanism of apoptosis in a model system closer to HCV infection in vivo, we used the HCVcc model system allowing us to study HCV-host interactions in the context of the complete viral life cycle (18–20).

In contrast to our observations having identified the HCV nonstructural proteins as key players for sensitization of TRAIL-induced apoptosis, a recent study had demonstrated an important role of core for TRAIL-induced apoptosis (31). Isolate-specific factors (genotype 1 vs 2), different core protein levels in the infectious cell culture system (this study), and transfection of cDNA (31), as well as a dominant effect of nonstructural proteins in the infectious tissue culture system, may explain the different findings. Using a full-length replicon expressing all viral proteins, Lee et al. showed that HCV envelope glycoprotein E2 can antagonize the proapoptotic effects of HCV core protein (32). In our study, we did not observe a markedly altered induction of apoptosis in cells transfected with HCV RNA containing a deletion in the HCV envelope proteins (JFH1/ΔE1E2) compared with infectious HCV RNA (Fig. 5). Because subgenomic replicons still showed sensitization to TRAIL-induced apoptosis (Fig. 5), our findings clearly demonstrate that the HCV structural proteins may not represent the major factors that determine sensitization to TRAIL, although we cannot exclude a modulatory role of these proteins. Because subgenomic replicons lack a functional NS2 protein (Fig. 1), our data also suggest that the recently described antiapoptotic effect of NS2 in a transgenic mouse model (45) may not play a major role in the enhancement of TRAIL-induced apoptosis in HCV-infected human hepatoma cells.

Interestingly, proapoptotic effects of HCV nonstructural proteins NS3/NS4A (46, 47) and NS5A (48) have been described recently. NS3 complexed with NS4A is known to translocate to the mitochondrial membrane where direct apoptosis induction, independent from caspase-8 activation can occur (47). Conversely, NS3 has been described to cleave Cardif and thereby act antiapoptotically (49). NS5A has recently been shown to directly inhibit proapoptotic Bin1, a tumor suppressor protein with a SH3 domain, thereby facilitating apoptosis induction in hepatoma cells (48). By contrast, NS5A has sequence homologies with Bcl-2 and binds to FKBP38, thereby augmenting the antiapoptotic effect of Bcl-2 (50) and inhibiting the proapoptotic action of Bax in hepatoma cells (51). Although we cannot exclude a functional relevance of antiapoptotic properties of NSSA and NS3, our data suggest that these effects are not able to override the proapoptotic effects of the HCV nonstructural proteins mediating sensitization to TRAIL.

Mechanism of HCV-induced apoptosis: impact of host cell factors
The proapoptotic effects of both NS3/NS4A (47) and NS5A (48) have been described to converge at the level of the mitochondria and may explain the proapoptotic properties of HCV infection. In this study, we demonstrate evidence that HCV-induced enhancement of Huh7.5 cell apoptosis depends on the mitochondrial pathway, because HCV replication induced cytochrome C release into the cytosol (Fig. 7D) and inhibition of caspase-9 markedly abolished HCV-dependent TRAIL-induced apoptosis (Fig. 8). The cytochrome C release may be partly due to the HCV-induced down-regulation of Bcl-2 and Bcl-xL expression (Fig. 7, A–C). Furthermore, preincubation with a Bcl-xL mimetic peptide resulted in a small but reproducible (three of three experiments) reduction of HCV-dependent apoptosis as shown in Fig. 5D. Bcl-2 and Bcl-xL are known to be essential in inhibiting mitochondrial apoptosis (12). In line with these results, a recent study demonstrated down-regulation of antiapoptotic Bcl-2 and up-regulation of proapoptotic PUMA and Bax in freshly prepared liver sections of HCV-infected patients (5). HCV nonstructural proteins have been shown to induce ER stress (52), and Bcl-2/Bcl-xL-dependent transmission of ER-stress into a mitochondrial apoptotic signal has recently been demonstrated (38). Nevertheless, down-regulation of Bcl-2 and Bcl-xL alone seems not sufficient to explain proapoptotic properties of HCV JFH1, as inhibition of both proteins did not enhance TRAIL-induced apoptosis to the extent of the HCV-dependent apoptosis (Fig. 2), and incubation of cells with a Bcl-xL mimetic peptide was only partially modulating TRAIL-induced apoptosis in Huh7.5 cells (Fig. 8D).

Several authors have postulated an autocrine TRAIL-dependent apoptosis of hepatocytes. Mundt et al. (43) demonstrated TRAIL-dependent apoptosis in hepatocytes using an adenoviral vector expressing TRAIL, and Volkmann et al. (5) described an up-regulation of TRAIL receptors in HCV-infected human liver sections. Most recently, HCV-dependent up-regulation of TRAIL and apoptosis induction in a novel hepatoma cell line has been described (44). In the latter study, apoptosis of hepatoma cells was dependent on autologous TRAIL expression and HCV-dependent apoptosis resulted in death of all infected cells. In contrast, in PHH and Huh7.5 cells harboring replicating HCV, we observed a decreased expression of TRAIL and unchanged TRAIL receptor expression levels (Fig. 6). Moreover, immunohistochemistry of TRAIL expression in the HCV-infected liver revealed that nonparenchymal mononuclear cells, but not hepatocytes, appear to be predominant producers of TRAIL (data not shown). Taken together, these data
suggest that a TRAIL autocrine loop does not play a major role for TRAIL-induced sensitization.

Although HCV replication resulted in detectable induction of apoptosis (Fig. 3, A–H) and cytochrome C release (Fig. 7D), this induction was significantly less efficient than sensitization to TRAIL-induced apoptosis (see side-by-side experiments depicted in Fig. 3, A–H and Fig. 8). Thus, it seems unlikely that induction of endogenous TRAIL production is responsible for the identified HCV-dependent enhancement of TRAIL-induced apoptosis in Huh7.5 cells. This hypothesis is in line with several studies demonstrating that sensitivity toward TRAIL-induced apoptosis does not correlate with TRAIL receptor expression of target cells (53, 54). Whether massive HCV-induced cell death described in another hepatoma cell line (44) or moderate virus-induced apoptosis in Huh7.5 cells in the classical HCV tissue culture model cell line Huh7.5 (our study; see Fig. 3H and data not shown) more accurately reflects virus-host interactions during the natural course of HCV infection remains to be determined.

**Impact of HCV-mediated enhancement of TRAIL induced apoptosis on pathogenesis of HCV infection**

Apoptosis of virus-infected cells is a key mechanism of viral clearance in mammals (55). Moreover, several studies have pointed to a central role of TRAIL in the elimination of viruses via induction of host cell apoptosis (40–43). In line with this concept, induction of hepatocyte apoptosis has been observed in the HCV-infected liver (4, 5). Confirming these findings, our own histopathological analyses demonstrated expression of TRAIL in CD8+ T cells and CD68+ macrophages in the immediate vicinity of apoptotic hepatocytes in the HCV-infected liver (data not shown). Several studies have demonstrated TRAIL-dependent cell death by activated liver macrophages (30) and/or CD8+ T cells (40, 41, 56). Furthermore, elimination of influenza virus in mice has been shown to require TRAIL-expressing lymphocytes (41). In hepatitis B virus (HBV) infection, lymphocyte-dependent hepatocyte apoptosis has been demonstrated to depend on TRAIL (56). Taken together, these data suggest a functional impact of TRAIL-expressing mononuclear cells, including T cells and macrophages in the elimination of virus-infected hepatocytes. Interestingly, a recent study has demonstrated that another hepatotropic virus, HBV, sensitizes hepatocytes to TRAIL-induced apoptosis via the Bel-2 protein Bax (57), and in a mouse model of adenoviral hepatitis, TRAIL-mediated apoptosis was restrained by Bel-2xI (58). In line with these findings for other viruses including HBV, our results suggest that sensitization of TRAIL-induced apoptosis may play a key role in host antiviral defense mechanisms against HCV infection. This hypothesis is supported by our experimental finding that incubation of PHH and Huh7.5 cells with TRAIL resulted in a decrease of viral protein levels (Fig. 4, C and D; Fig. 9). This concept is further supported by the observation that IFN-dependent up-regulation of TRAIL on NK cells and macrophages seems crucial for elimination of viral infections (59). Furthermore, therapy of HCV-infected patients with pegylated IFN-a and ribavirin results in a rapid and sustained TRAIL elevation, suggesting a role of TRAIL in viral clearance (60).

Taken together, our results define a novel antiviral host defense mechanism which may play an important role for the control of HCV infection. HCV-induced TRAIL sensitization may have important implications for the pathogenesis of HCV infection and may contribute to the elimination of virus-infected hepatocytes.

**Acknowledgments**

We thank C. M. Rice for the gift of Huh7.5 cells, H. B. Greenberg and M. Houghton for the gift of Abs, and V. Lohmann for helpful discussions.

**Disclosures**

The authors have no financial conflict of interest.

**References**


