Hepatitis C Virus Core Protein Modulates TRAIL-Mediated Apoptosis by Enhancing Bid Cleavage and Activation of Mitochondria Apoptosis Signaling Pathway

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Hepatitis C virus (HCV) is a major human pathogen causing chronic liver disease, which leads to cirrhosis of liver and hepatocellular carcinoma. The HCV core protein, a viral nucleocapsid, has been shown to affect various intracellular events, including cell proliferation and apoptosis. However, the precise mechanisms of the effects are not fully understood. In this study, we show that HCV core protein sensitizes human hepatocellular carcinoma cell line, Huh7, conferred sensitivity to TRAIL-, but not Fas ligand-mediated apoptosis. Huh7 cells are resistant to TRAIL, despite the induction of caspase-8 after TRAIL engagement. However, HCV core protein induces TRAIL apoptosis signaling via sequential induction of caspase-8, Bid cleavage, activation of mitochondrial pathway, and effector caspase-3. HCV core protein also induces activation of caspase-9 after TRAIL engagement, and the induction of TRAIL sensitivity by HCV core protein could be reversed by caspase-9 inhibitor. Therefore, the HCV core protein-induced TRAIL-mediated apoptosis is dependent upon activation of caspase-8 downstream pathway to convey the death signal to mitochondria, leading to activation of mitochondrial signaling pathway and breaking the apoptosis resistance. These results combined indicate that the HCV core protein enhances TRAIL-, but not Fas ligand-mediated apoptotic cell death in Huh7 cells via a mechanism dependent on the activation of mitochondrial apoptosis signaling pathway. These results suggest that HCV core protein may have a role in immune-mediated liver cell injury by modulation of TRAIL-induced apoptosis. The Journal of Immunology, 2005, 174: 2160–2166.
Expression and purification of rTRAIL protein and soluble TRAIL receptors, TRAIL-R1-Fc and TRAIL-R2-Fc

The rTRAIL proteins were expressed in Escherichia coli expression system and purified with Ni column, as described (25). In brief, the coding portion of the extracellular domain of TRAIL (aa 123–314) was PCR amplified, subcloned into pRSET B vector (Invitrogen Life Technologies), and expressed in E. coli expression system. The purification of rHis-TRAIL fusion protein was performed by metal chelate column chromatography using Ni-NTA resin, according to the manufacturer’s recommendations (Qiagen). His-TRAIL was quantified by Bradford method and protein assay reagent (Bio-Rad). To generate soluble recombinant TRAIL-R1-Fc and TRAIL-R2-Fc fusion molecules, the coding sequence for the extracellular domains of human DR4/TRAIL-R1 and DR5/TRAIL-R2 was isolated by RT-PCR. The amplified products were ligated in-frame into BsuR1-HF/UC19-IgG1-Fc vector containing the human IgG1 Fc coding sequence. The fusion genes were then subcloned into pBacPAX9 vector (BD Clontech). TRAIL-R1-Fc and TRAIL-R2-Fc fusion proteins were recovered from the filtered supernatants of the recombinant virus-infected S21 cells using protein G-Sepharose beads (Pharmacia). The bound TRAIL-R1-Fc and TRAIL-R2-Fc proteins were eluted with glycine buffer (pH 3) and dialyzed into PBS.

Apoptosis assay

A sensitive ELISA that detects cytoplasmic histone-associated DNA fragments was performed, according to the manufacturer’s protocol (Cell Death Detection ELISAPLUS; Roche Mannheim Biochemicals). Human HCC cell lines were cultured in 96-well plate (10^4 cells/well) overnight, then treated with rTRAIL protein for 6 h, and harvested by centrifugation at 200 × g. The cells were lysed by incubation with lysis buffer for 30 min, followed by centrifugation at 200 × g for 10 min at room temperature. The supernatant was collected and incubated with immunoreagent for 2 h. After washing gently, the supernatant was pipetted into each well with a substrate solution and kept in the dark until development of the color was sufficient for photometric analysis. The reaction was determined in a spectrophotometer at 405 nm. In some experiments, caspase inhibitors were used. The general caspase inhibitor (Z-VAD-fmk; Bachem, Bubendorf, Switzerland), caspase-8-specific inhibitor (Z-IETD-fmk), caspase-9-specific inhibitor (Z-LEHD-fmk), or caspase-3-specific inhibitor (Z-DMGD-fmk, Calbiochem, San Diego, CA) was applied at the concentration of 20 μM to the medium 30 min before treatment.

Detection of caspase-3, caspase-8, and caspase-9 activation

The HCC cell lines were treated with rTRAIL proteins. The cell lysate of cells with or without adding rTRAIL proteins was run on SDS-PAGE and Western transferred to nitrocellulose membrane. The caspase-3, caspase-8, and caspase-9 activation was detected with anti-caspase-3 (Imgenex), anti-caspase-8, and anti-caspase-9 mAbs (Cell Signaling Technology) to identify the cleavage of procaspases on Western blot. Each lane was loaded with 20 μg of cell lysate, and the immunoblot was checked with anti-β-actin mAb to ensure the equal loading. For detecting caspase activation, anti-caspase-3, anti-caspase-8, and anti-caspase-9 mAbs with 1/500 dilution were used for blotting.

Detection of mitochondrial membrane potential (Δψm)

For detection of changes of Δψm, HCC cell lines were seeded at 10^4 cells/ml into six-well plate and grown in DMEM supplemented with 10% FCS in a 95% air-5% CO2 atmosphere at 37°C in a humidified incubator overnight. Cells were treated with 1.5 μg/ml rTRAIL protein for 12 h. Mitochondrial injury and changes in the Δψm were assessed by staining with 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolyl carbocyanine iodide (JC-1; Molecular Probes). This dye, existing as a monomer in a monomer, emitting a green fluorescence, can assume a dimeric configuration emitting red fluorescence in a reaction driven by the mitochondrial transmembrane potential (27). Thus, red fluorescence indicates intact mitochondria, whereas green fluorescence shows monomeric JC-1 that remained unprocessed due to breakdown of the mitochondrial transmembrane potential. After trypsinization by TEG buffer (0.125% trypsin, 0.05% EDTA, 0.05% glucose in PBS), the cells were resuspended in DMEM and incubated with 10 μg/ml JC-1 for 15 min at 37°C. Cells were washed with PBS twice and resuspended in PBS. Analysis was performed by FACScan, and mitochondrial function was assessed as JC-1 green (uncoupled mitochondria) or red (intact mitochondria) fluorescence (28). For confocal laser-scanning microscopy analysis of mitochondrial function, the JC-1-treated cells were excited at 488 nm, and emission was recorded simultaneously at 527 and 590 nm into independent detector.

Preparation of cytosols and analysis of cytochrome c release and Bid processing

After incubation with TRAIL, cells were washed twice with ice-cold PBS. They were suspended in 100 μl of extraction buffer (50 mM PIPES-KOH, pH 7.4, 220 mM mannitol, 68 mM sucrose, 50 mM KCl, 5 mM EGTA, 2 mM MgCl2, 1 mM DTT, and protease inhibitors) and allowed to swell on ice for 30 min. Cells were homogenized by passing the suspension through 2161The Journal of Immunology 2161

FIGURE 1. TRAIL-mediated apoptosis in human HCC cell lines. Huh7. A, Huh7 cells were incubated with rTRAIL proteins for 6 h. The cell apoptosis was measured with cell death ELISA. Values represent means ± SD of three independent experiments. B, Expression of TRAIL receptors on Huh7 cells. Huh7 cells were stained with anti-DR4/TRAIL-R1, anti-DR5/TRAIL-R2, anti-DcR1/TRAIL-R3, and anti-DcR2/TRAIL-R4 mAbs (Alexis Biochemical) and analyzed in flow cytometry (shaded histogram: isotype control). The results were representative of at least three independent experiments.
a 25-gauge needle (10 strokes). Homogenates were centrifuged at 14,000 × g for 15 min at 4°C, and supernatants were harvested and stored at −80°C. Protein content in cytosols was determined by the Bio-Rad protein assay. For analysis of cytochrome c release, 10 μg of cytosolic protein was loaded per lane. Proteins were separated on 12% SDS-PAGE and transferred to nitrocellulose sheets, which were blocked for 1 h in PBS, 0.05% Tween 20 with 5% dry milk. Blots were probed in PBS, 0.05% Tween 20, with anti-cytochrome c mAb (eBioscience) or anti-Bid polyclonal Ab (Cell Signaling Technology), and secondary Abs. The immunoblots were checked with anti-β-actin mAb to ensure the equal loading.

Results

HCV core protein enhances sensitivity to TRAIL-, but not FasL-mediated apoptosis in human HCC cell line, Huh7

TRAIL was shown to induce apoptosis in a number of different tumor cell types, but some tumor cell lines showed resistance to TRAIL-induced apoptosis. Recent studies indicate that TRAIL-induced apoptosis occurs through a caspase signaling cascade, and that resistance to TRAIL is controlled by intracellular regulators of apoptosis. To examine TRAIL-induced apoptosis in liver cells, rTRAIL proteins were used to induce apoptosis in human HCC cell line, Huh7. In the presence of TRAIL, this cell line showed only slight apoptosis even at concentration of 1.5 μg/ml, in the cell apoptosis assay by measuring cytoplasmic histone-associated DNA fragments. The results in Fig. 1B revealed that Huh7 cells were resistant to TRAIL-mediated apoptosis despite the expression of TRAIL death receptor, TRAIL-R2/DR5, but not decoy receptor (DcR), TRAIL-R4/DcR2. There were equivalent low amounts of TRAIL-R1/DR4 and TRAIL-R3/DcR1 expressed on the surface.

To investigate role of HCV core protein in regulation of TRAIL-mediated apoptosis, we further studied TRAIL-induced apoptosis in Huh7 cells transfected with HCV core (C190/Huh7) or with vector control (S2/Huh7). In the presence of TRAIL, S2/Huh7 cells showed resistance to TRAIL-mediated apoptosis, similar to the parent cell line, Huh7. However, the apoptosis indices were marked increased in HCV core-expressing C190/Huh7 cells after treatment with TRAIL in a dose-dependent manner (Fig. 2A). Furthermore, the induction of TRAIL-mediated apoptosis in C190/Huh7 cells could be specifically blocked by adding soluble TRAIL receptor, TRAIL-R1-Fc, or TRAIL-R2-Fc proteins in the culture, indicating that the cell death induced was resulted from direct interaction between TRAIL and TRAIL receptor on the cell surface (Fig. 2B). The C190/Huh7 clones with different expression levels of HCV core protein all demonstrated enhanced sensitivity to TRAIL-mediated apoptosis, and which were correlated with expression levels of HCV core protein (data not shown). We also studied FasL-induced apoptosis in S2/Huh7 cells and C190/Huh7. In contrast to apoptosis induced by TRAIL, HCV core protein did not enhance sensitivity to FasL-mediated apoptosis (Fig. 2C).
not enhance FasL-mediated apoptosis in C190/Huh7 cells (Fig. 2C). Taken together, our results indicate that HCV core protein sensitizes Huh7 cells to TRAIL-, but not FasL-mediated apoptosis.

To further investigate whether the HCV core protein-induced TRAIL sensitivity is due to up-regulation of TRAIL DRs on cell surface, we examined the expression of TRAIL receptors on cell surface before and after treatment with TRAIL. The results in Fig. 2D demonstrated that the expression of TRAIL receptors on both C190/Huh7 and S2/Huh7 cells did not appear significantly different. Moreover, the results also show that TRAIL down-modulates TRAIL-R1, but not TRAIL-R2, in C190/Huh7 cells, suggesting that the effects of enhanced TRAIL-mediated apoptosis by HCV core protein are via TRAIL-R2. These results indicate that the enhanced TRAIL sensitivity by HCV core protein is not due to up-regulation of TRAIL DRs on cell surface.

**HCV core protein induces activation of caspase-8 and its downstream pathway after TRAIL engagement**

Apoptosis can be triggered through interaction between TRAIL and its DRs, TRAIL-R1/DR4 and TRAIL-R2/DR5, on the surface of cells. This interaction results in recruitment of adaptor protein, Fas-associated death domain protein (FADD), and procaspase-8 to the cytoplasmic domain of TRAIL DR to form the death-inducing signaling complex (DISC), and initiates signaling cascade. To further delineate the intracellular signal transduction pathway modulated by HCV core protein that results in induction of TRAIL sensitivity, we investigated activation of caspase pathways after TRAIL engagement in the presence or absence of HCV core protein in Huh7 cells. The results in Fig. 3 demonstrated that the HCV core protein-induced TRAIL sensitivity was significantly inhibited by either general caspase inhibitor, Z-VAD-fmk; caspase-8-specific inhibitor, Z-IETD-fmk; or caspase-3-specific inhibitor, Z-DQMD-fmk. Furthermore, during TRAIL engagement, both caspase-3 and caspase-8 were activated in C190/Huh7 cells, resulting in cell apoptosis (Fig. 3D). In the absence of HCV core protein, TRAIL engagement induced activation of caspase-8, but not caspase-3, in S2/Huh7 cells. Nevertheless, TRAIL-induced caspase-8 activation was significantly enhanced by HCV core protein in C190/Huh7 cells (Fig. 3D). These results indicate that HCV core protein enhances TRAIL sensitivity in Huh7 cells by facilitating the generation of active caspase-8 after assembly of DISC, to further activate the caspase-8 downstream pathways, leading to beak apoptosis resistance.

**HCV core protein-induced TRAIL sensitivity is dependent on activation of mitochondria apoptosis pathway**

For detecting the caspase-processing events distal to caspase-8, we also analyzed cytochrome c released from mitochondria into cytoplasm after TRAIL engagement. The results in Fig. 4 demonstrated that TRAIL engagement induced breakdown of mitochondrial transmembrane potential in C190/Huh7, but not S2/Huh7 cells, consistent with the differences in caspase-3 activation. To further confirm the induction of mitochondria apoptosis signaling pathway by TRAIL, we analyzed cytochrome c released from mitochondria into cytoplasm after TRAIL engagement. The results in Fig. 4C demonstrated that TRAIL engagement induced cytochrome c release from mitochondria into cytosol in C190/Huh7 cells, but not S2/Huh7 cells. Moreover, results in Fig. 5 demonstrated that TRAIL engagement induces cytochrome c release from mitochondria to cytosol in C190/Huh7 cells, but not S2/Huh7 cells. These results indicate that HCV core protein-induced TRAIL sensitivity is dependent on activation of mitochondria apoptosis pathway.
protein, but not in control cells, indicates C190/Huh7 cells using signaling pathways to convey death signals from TRAIL DISC to mitochondria. For detecting the caspase-processing events distal to caspase-8, which connect caspase-8 with mitochondria, we further investigated processing of Bid after TRAIL engagement. The results in Fig. 6 demonstrated there was enhanced Bid cleavage in C190/Huh7 cells after TRAIL engagement, and this result is consistent with the enhanced activation of caspase-8 after TRAIL engagement in C190/Huh7 cells. Taken together, our results indicate that the apoptosis signal transduced from TRAIL DR is augmented by HCV core protein at the generation of active caspase-8 to cleave Bid, to further activate mitochondria signaling pathway, and breaking the apoptosis resistance.

Discussion
In this study, we presented evidences that the HCV core protein sensitizes Huh7 cells, conferred sensitivity to TRAIL-mediated apoptosis. Our studies indicate that the HCV core protein induces sensitivity to TRAIL-mediated apoptosis in Huh7 cells through a pathway involving the sequential induction of apical DISC formation, caspase-8 activation, and effector caspase-3 activity via activation of mitochondria apoptosis signaling pathway. Previous studies have shown that expression of the HCV core protein in several human and mouse cell lines enhanced TNF-induced apoptosis, probably as a result of the binding of the core protein to TNFR1 (15). One recent study has demonstrated that the HCV core protein sensitizes cells to TNF-induced apoptosis primarily by facilitating FADD recruitment to TNFR1 (13). The inhibition of JNK activation by the HCV core protein may also contribute to the increased propensity of cells for apoptosis (13). However, the mechanism of the enhanced TNF sensitivity was not clearly known, and the mechanisms regulating TRAIL apoptosis signal transduction are still not elucidated. Our results indicate the induction of TRAIL sensitivity by HCV core protein is not due to up-regulation of TRAIL DRs; instead, it is dependent upon activation of caspase-8 downstream pathway. There are two TRAIL DRs, TRAIL-R1 and TRAIL-R2, which can transduce apoptosis signals after engagement with TRAIL. The results in Fig. 2 demonstrate that the enhanced TRAIL sensitivity by HCV core protein is not due to up-regulation of TRAIL DRs on cell surface. How¬ever, the results also show that TRAIL down-modulates TRAIL-R1, but not TRAIL-R2, in C190/Huh7 cells, suggesting that the effects of enhanced TRAIL-mediated apoptosis by HCV core protein are via TRAIL-R2.

Two major pathways leading to apoptosis have been described. One pathway involves apoptosis mediated by DRs, such as CD95.
(Fas). When the FasL binds to the Fas receptor, formation of the DISC comprising the adapter molecule FADD and caspase-8 results in the active caspase-8 and process effector caspases (caspases-3, -6, and -7), thereby inducing apoptosis (extrinsic pathway). In the other pathway, various proapoptotic signals converge at the mitochondria level, provoking translocation of cytochrome c from the mitochondria to the cytoplasm. Once cytochrome c is released into cytoplasm, it binds to Apaf-1 and induces recruitment of procaspase-9. Activated caspase-9 then cleaves and activates procaspase-3 (intrinsic pathway). The role of mitochondria in TRAIL-induced apoptosis has been evaluated recently in several tumor cell lines (29, 30). Recent studies using a colon carcinoma cell line with Bax deletion (29, 30) or selected for Bax mutation (30) showed that Bax was required for TRAIL-mediated apoptosis. Thus, in these cells, the intrinsic pathway was required for TRAIL-mediated apoptosis, and Bax was essential for the mitochondrial events. Our results also suggest that the type II pathway is mainly involved in TRAIL-induced apoptosis in human hepatocytes, where mitochondria play an important role in amplifying apoptotic signals. However, the resistance to TRAIL in Huh7 cells is not likely due to Bax defect, because it could be reversed by HCV core protein. In the absence of HCV core protein, the Huh7 cells show resistance to TRAIL-induced apoptosis despite that the early events triggered by TRAIL, such as caspase-8 activation, are present (Fig. 3). However, in the presence of HCV core protein, the caspase-8 downstream signaling pathways are activated after TRAIL engagement. These results indicate that the apoptosis signal induced by TRAIL is facilitated by HCV core protein at the generation of active caspase-8 to cleave Bid, further activating mitochondrial signaling pathway, and breaking the apoptosis resistance. Our results also suggest that the apoptosis signaling from TRAIL DR is regulated by a regulator interacted with caspase-8 to prevent generation of activated caspase-8 and truncated Bid, impeding TRAIL apoptosis signal transduction. Recent studies indicated that HCV core protein could bind to LTβR and TNFR to modulate the apoptosis (10, 13). These results suggest the possibility that HCV core protein could interact with TRAIL DR to influence formation of TRAIL DISC, or induce release of the regulator to active cleavage of Bid, leading to activation of mitochondrial signaling pathway and breaking the apoptosis resistance. Discrepancies regarding the effects of the HCV core protein on the cellular apoptotic responses to DR-mediated apoptosis have been reported previously: the HCV core protein functions anti-apoptotically according to some papers (6, 10, 16, 31) and pro-apoptotically according to others (13–15). The reason for the discrepancy among these reports is still unclear. This discrepancy may be, however, explained by the possibility that it was caused by the differential regulation of death signal transduction among different DR. Recently, studies demonstrated that HCV core protein interacted with the death domain of FADD and enhanced apoptosis induced by FADD overexpression (13). However, our results demonstrated that the Huh7 cells are resistant to TRAIL-mediated apoptosis despite that the early events triggered by TRAIL, such as caspase-8 activation, are present (Fig. 3), indicating that the signal transduction from TRAIL DR is impeded downstream of caspase-8, and HCV core protein could enhance the caspase-8 downstream pathway to activate mitochondrial pathways. These results combined indicate that the HCV core protein enhances TRAIL-, but not FasL-mediated apoptotic cell death in Huh7 cells via a mechanism dependent on the activation of mitochondria apoptosis signaling pathway. These results suggest distinct intracellular signaling pathways between TRAIL- and FasL-mediated cell death in Huh7 cells.

It is interesting to note that although we demonstrate in this work that HCV core protein sensitizes Huh7 cells, conferred susceptibility to TRAIL-mediated apoptosis, the liver damage is induced subsequently by infiltrating T cells during HCV infection and the degree of apoptosis is linked to the associated inflammatory response. Therefore, the liver damage is also determined by the inflammatory response induced by HCV. Our results suggest a role for immune-mediated apoptosis of hepatocytes by infiltrating T cells. HCV infection induces T cell response and a number of inflammatory mediators, including cytokines and chemokines (32). It has been demonstrated that TRAIL expression is up-regulated in T cells activated by anti-CD3 (19, 25, 33, 34). Moreover, it was recently shown that not only does TRAIL induce apoptosis by binding to the DRs, but it also enhances T cell proliferation after TCR engagement, and augments IFN-γ secretion (25). These infiltrating T cells can then kill target cells via TRAIL/TRAIL receptor interaction. Our results, combined with recent evidence, therefore, support the possibility that HCV core protein may have a role in immune-mediated liver cell injury during HCV infection.

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References


