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Molecular Mechanism of Hepatic Injury in Coinfection with Hepatitis C Virus and HIV

Anuradha Balasubramanian, Margaret Koziel, Jerome E. Groopman, and Ramesh K. Ganju
Division of Experimental Medicine and Infectious Disease, Beth Israel Deaconess Medical Center, Harvard Institutes of Medicine, Boston, Massachusetts

We have previously shown that hepatocytes exposed to hepatitis C virus (HCV) and human immunodeficiency virus (HIV) envelope proteins undergo apoptosis. In this article, we further elucidate the signaling mechanisms that mediate this effect. We found that, in human hepatocellular carcinoma (HepG2) cells, HCV E2 protein and HIV glycoprotein (gp) 120 significantly up-regulated the Fas ligand (FasL) and enhanced the formation of the Fas death-inducing signaling complex downstream of Fas receptor activation. Moreover, after stimulation with HCV E2 and HIV gp120, enhanced expression of caspases 2 and 7 and increased caspase 3 activity were observed. In addition, we showed up-regulation of the proapoptotic molecule Bid and its association with caspase 8 after treatment with these envelope proteins. We also found that HCV E2 and HIV gp120 induced a partial translocation of Bid to the mitochondria, which resulted in the release of cytochrome C and the apoptosis-inducing factor. Thus, the results of this study suggest that FasL and Bid play an important role in HCV and HIV envelope protein-induced apoptosis.

Coinfection with hepatitis C virus (HCV) is frequently found in HIV-infected persons, because the viruses share modes of transmission. It has been shown that the overall prevalence of HCV infection among HIV-infected persons is 30%–50%, with rates of coinfection as high as 90% in injection drug users and almost 100% in hemophiliac patients [1, 2]. The more-rapid progression of hepatic disease associated with HIV-HCV coinfection is poorly understood. It has been reported that coinfected patients have a higher incidence of hepatic fibrosis and cirrhosis and increased death from liver disease [3–7]. We recently observed that HCV and HIV envelope proteins induce apoptosis in hepatocytes via an “innocent bystander” mechanism, because of binding of viral proteins to the cell surface independent of direct viral infection.

Apoptosis has been observed during viral infection. Biochemical, histological, and electron microscopic analyses have revealed that liver damage via apoptosis occurs during viral hepatitis and that CD95 is involved in this apoptosis of the liver [8]. However, the mechanism of HCV and HIV envelope protein-induced apoptosis has not been clearly defined.

Two principal pathways initiate apoptosis. The intrinsic pathway emerges from the mitochondria, whereas the extrinsic pathway is activated by the ligation of death receptors [9]. Fas (CD95, APO-1) is a member of the nerve growth factor–TNF receptor superfamily, which is involved in both proliferation and apoptosis [10]. Fas has been shown to trigger apoptosis in Fas-expressing cells by binding to its natural ligand (FasL) or to specific anti-Fas agonistic antibodies [11, 12]. Mitochondria play a key role in the regulation of apoptosis. Opening of the mitochondrial permeability transition pore, which is controlled by members of the Bcl-2 family, is a decisive event in the apoptotic process. This event causes increased permeability of the outer mitochondrial membrane and the release from the intermembrane space of soluble proteins, including such apoptotic factors as cytochrome C and apoptosis-inducing factor (AIF) [13]. Although the FasL-Fas system has been studied primarily with respect to the basic
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**Figure 1.** Induction of Fas apoptosis by hepatitis C virus (HCV) E2 protein and HIV gp120. Human hepatocellular carcinoma (HepG2) cells were untreated (UN; −) or treated (+) with HCV E2 (1.5 nmol/L) and HIV gp120 (0.8 nmol/L) ("E2 + gp120") for 6, 12, and 24 h. A, Cells were labeled with Annexin V–fluorescein isothiocyanate and propidium iodide and were quantified by flow cytometry. The HepG2 cells were stimulated with hydroxy camptothecin for 24 h and were processed with the other samples as a positive control. Cell lysates were analyzed for expression of Fas ligand (FasL) with anti-FasL antibody (B) and for Fas expression with anti-Fas antibody (C). D, After 24 h of stimulation, cell lysates were immunoprecipitated (IP) with anti-Fas antibody and analyzed with anti-FasL by Western blotting (WB). Data represent 1 of 3 independent experiments done in triplicate. Results are mean ± SD. *P < .05. TCL, total cell lysate; Ab, antibody control.

**Figure 2.** Induction of Fas death–inducing signaling complex (DISC), formed during apoptosis, by hepatitis C virus (HCV) E2 protein and HIV gp120. Human hepatocellular carcinoma (HepG2) cells were untreated (−) or treated (+) with HCV E2 (1.5 nmol/L) and HIV gp120 (0.8 nmol/L) ("E2 + gp120") for 24 h. Cell lysates were immunoprecipitated (IP) with anti-Fas antibody (A) or anti-caspase 8 (B) and then were analyzed by Western blotting (WB) with anti-Fas-associated death domain (FADD) antibody. Blots in panels A and B were next immunoblotted with anti-Fas antibody or anti-caspase 8, respectively. Data represent 1 of 3 independent experiments. TCL, total cell lysate; Ab, antibody control.

**MATERIALS AND METHODS**

**Reagents.** The HCV E2 subtype 1a protein and T-trophic HIV gp120, which was derived from HIV strain IIIB, were obtained from Immuno Diagnostics and Protein Sciences, respectively. Both proteins were expressed in the baculovirus expression system and were found to be highly pure and endotoxin-free. Antibodies to Fas, FasL, Fas-associated death domain (FADD), Bid, Bak, AIF, cytochrome C, and caspases 2, 7, 8, and 9 were procured from Santa Cruz Biotechnology. 10-hydroxy camptothecin was purchased from Calbiochem. Eagle’s MEM was purchased from Invitrogen, and fetal calf serum was obtained from Sigma.

**Cell culture and stimulation of cells.** HepG2 cells were obtained from ATCC (HB-8065). Cells were cultured in ATCC medium (MEM with 2 mmol/L L-glutamine and Earle’s balanced salts adjusted to contain 1.5 g/L sodium bicarbonate, 0.1 mmol/L nonessential amino acids, 1.0 mmol/L sodium pyruvate, and 10% fetal bovine serum) supplemented with 1% penicillin (10,000 IU/mL) and streptomycin (10,000 μg/mL) at 37°C in 5% CO₂ in a water-saturated atmosphere.

Cells cultured to ∼75% confluence were starved for 3 h in the serum-free medium and then were stimulated with 1.5 nmol/L HCV E2 protein and 0.8 nmol/L HIV gp120 (i.e., 100 ng/mL each dissolved in cell culture medium). Controls received the appropriate volume of cell culture medium. After stimulation for the designated time, cells were lysed with radiolymphoprecipitation assay buffer (50 mmol/L Tris HCl [pH 7.4], 1% Nonidet P-40, and 150 mmol/L NaCl) containing 1

mechanism of its death-inducing function [14], much remains to be discovered about the unique signaling pathways that are used to select specific cells for death in disease and how different pathways are integrated to regulate the decision between death or survival of the cell.

In the present study, we analyzed the Fas pathway, which involves the formation of a death-inducing signaling complex, during stimulation of human hepatocellular carcinoma (HepG2) cells with HCV E2 protein and HIV gp120. In addition, we found that the proapoptotic molecule Bid and executioner caspases also contribute to the apoptotic effect in these cells.
Figure 3. Translocation of Bid to the mitochondria after costimulation with hepatitis C virus (HCV) E2 protein and HIV gp120. Human hepatocellular carcinoma (HepG2) cells were untreated (−) or treated (+) with HCV E2 (1.5 nmol/L) and HIV gp120 (0.8 nmol/L) (“E2 + gp120”) for 24 h. A, The cell lysates were analyzed for expression of Bid with anti-Bid antibody and for expression of Bak with anti-Bak antibody. B, Cell lysates were immunoprecipitated (IP) with anti-caspase 8 antibody and probed with anti-Bid antibody. The blot was then immunoblotted (WB; Western blotting) with anti-caspase 8 antibody. C and D, Cell lysates were fractionated by a cell fractionation kit. The purity of fractionation was determined by using anti-cyclo-oxygenase (COX) 4 antibody. C, Translocation of Bid to the mitochondria was analyzed with anti-Bid antibody. D, Release of apoptosis-inducing factor (AIF) and cytochrome C into the cytosol were analyzed by using anti-AIF antibody and anti-cytochrome C antibody, respectively. Data represent 1 of 3 independent experiments. TCL, total cell lysate; Ab, antibody control; Mito, mitochondria.

mmol/L phenylmethylsulphonyl fluoride, 10 μg/mL each aprotinin, leupeptin, and pepstatin, 10 mmol/L sodium vanadate, 10 mmol/L sodium fluoride, and 10 mmol/L sodium pyrophosphate. Total cell lysates were clarified by centrifugation at 10,000 g for 20 min. Protein concentrations were determined by use of a Bio-Rad protein assay.

**Flow cytometry.** Cells were stimulated for the designated time with HCV E2 and HIV gp120. Camptothecin (apoptotic inducer) was used as the positive control, along with appropriate negative controls. Cells were harvested with 1 mmol/L EDTA in PBS and washed thoroughly thrice with ice-cold PBS. The cells were stained with Annexin V–fluorescein isothiocyanate and propidium iodide (BD Biosciences PharMingen), according to the manufacturer’s instructions. The stained cells were then subjected to flow cytometric analysis.

**Immunoprecipitation and Western blot analysis.** Equal amounts of protein were size-fractionated on 10% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat milk and then incubated with the respective primary and secondary antibodies for 2 h each. The membranes were washed 3 times for 15 min each with Tris-buffered saline and 0.05% Tween 20 and later were developed by chemiluminescence (ECL system; Amersham Pharmacia Biotech).

For detection of proteins in the mitochondria, stimulated cells were lysed and transferred with the ApoAlert Cell Fractionation Kit (BD Biosciences Clontech), according to the manufacturer’s protocol. The cyclo-oxygenase 4 antibody provided with the kit was used to check the purity of fractionulation.

**Caspase 3 assay.** HepG2 cells were stimulated with HCV E2 and HIV gp120 for the designated time. The cells were lysed and assayed fluorometrically by use of the caspase-3 cellular activity assay kit plus (Biomol Research Laboratories), according to the manufacturer’s protocol. The specific activity was determined by measuring the fluorescence of 7-amino-4-methylcoumarin (AMC) substrate with respect to total protein.

**Statistical analysis.** Each set of experiments was repeated at least 3 times with similar results, and representative results are shown. The results presented as bar graphs are quantified as the means ± SDs. Student’s t test for paired samples was used to determine statistical significance. Differences were considered to be statistically significant at \( P \leq 0.05 \).

**RESULTS**

**Induction of Fas-mediated apoptosis by HCV E2 and HIV gp120.** Apoptosis in HCV and HIV infections has been reported elsewhere [15, 16]. However, apoptosis in coinfection with HCV and HIV has not been studied in detail. Previously, we showed, by use of TUNEL (in situ terminal deoxynucleotide transferase–mediated dUTP nick end labeling) labeling and DNA laddering, that HCV and HIV envelope proteins induced programmed cell death in HepG2 cells and primary hepatocytes [17]. Following those initial studies, we now show time-dependent apoptosis induced by HCV E2 and HIV gp120 after
staining of the cells with propidium iodide and Annexin V, as quantitated by flow cytometry (figure 1A). There was a gradual increase in apoptosis from 6 to 24 h. A slight increase in the unstimulated group was also observed under these conditions. The viability of the cells was determined by staining the cells with propidium iodide alone. In parallel, we observed an increase in FasL levels at different time intervals (figure 1B), but no significant change in Fas levels (figure 1C), indicating the possibility of a Fas-mediated apoptotic pathway. It has been reported that interaction of membrane-bound FasL with the Fas receptor induces apoptosis [10]. Here, we show a physical association between FasL and the Fas receptor after 24 h of costimulation with HCV E2 and HIV gp120 (figure 1D). This result suggests that HCV and HIV envelope proteins induce apoptosis via the Fas pathway.

**Induction of Fas death-inducing signaling complex (DISC) during apoptosis by HCV E2 and HIV gp120.** It has been shown that interaction of membrane-bound FasL with the Fas receptor induces formation of DISC [9]. Fas-DISC contains the adapter protein FADD, as well as caspases 8 and 10, which can initiate the process of apoptosis [14]. We observed associations between Fas and FADD (figure 2A) and between FADD and caspase 8 (figure 2B). However, we found only a slight increase in caspase 8 activity (data not shown).

**Translocation of Bid to the mitochondria after costimulation with HCV E2 and HIV gp120.** It has been reported that Bid is a specific proximal substrate for caspase 8 in the Fas signaling pathway [18]. We found that expression of Bid was enhanced after stimulation with HCV E2 and HIV gp120. However, no change was noticed with other proapoptotic molecules, such as Bak (figure 3A). Previous studies have shown that proapoptotic molecules, such as Bax and Bak, translocate to the mitochondria and permeabilize the mitochondrial outer membrane [19, 20]. When activated by caspase 8 through proteolytic cleavage in response to Fas signaling, Bid has been shown to translocate to the mitochondria and induce release of cytochrome C, as well as downstream caspase activation [21, 22].

We detected an association between caspase 8 and Bid protein, by use of immunoprecipitation (figure 3B), and found increased expression of Bid protein in the mitochondrial fraction (figure 3C). We also observed the release of cytochrome C and AIF into the cytosol after stimulation with HCV E2 and HIV gp120 (figure 3D). These findings imply that HCV E2 and HIV gp120 could challenge the mitochondrial integrity of the cell, via Bid, to induce apoptosis.

**Up-regulation of executioner caspases by HCV E2 and HIV gp120.** Downstream events of the Fas signaling pathway have been shown to involve activation of caspases, such as caspase 1 [23, 24], caspase 3 [25–27], caspase 4 [28], caspase 6 [29], and caspase 7 [30]. In the present study, the increased expression of caspases 2 and 7 at different time intervals was seen after stimulation with HCV E2 and HIV gp120 (figure 4A). However, no significant change was seen in the expression of caspase 9 (figure 4A) or caspase 6 (data not shown). We also observed the enhanced activity of caspase 3 at different time intervals. Caspase 3 activity was determined by the cleavage of DEVD-AMC, a fluorescent substrate (figure 4B). Therefore, it appears that HCV and HIV envelope proteins induce the expression and activity of various caspases.

**DISCUSSION**

Coinfection with HCV and HIV has been shown to result in higher rates of cirrhosis and increased death from liver disease. However, the HCV- and HIV-mediated pathogenesis of liver disease is not well known. We have previously shown that HCV and HIV envelope proteins induce the apoptosis of hepatocytes [17]. Here, we have investigated the mechanism of HCV and HIV envelope protein–induced apoptosis in hepatocytes.

In mammalian cells, 2 major apoptosis pathways have been defined: the death receptor pathway [31] and the mitochondrial pathway [32]. The most extensively characterized death receptor pathway is that of Fas-FasL–mediated apoptosis [14]. Our data show that the Fas-FasL pathway may play an important role in HCV and HIV-induced apoptosis.
role in the pathogenesis of coinfection with HCV and HIV. In vivo, activation of the Fas-mediated pathway has been shown to result in massive apoptosis of hepatocytes [33]. We have observed increased expression of FasL and its association with Fas after stimulation with HCV and HIV envelope proteins. Furthermore, increased associations of FADD with Fas and caspase 8 were also observed. This result suggests that HCV and HIV envelope proteins induce the formation of DISC, which consists of Fas, FADD, and caspase 8. DISC then initiates signaling complexes that can either process downstream caspases directly or promote a Bid-regulated mitochondrial pathway [34–36].

HCV and HIV envelope proteins also enhance the expression of Bid and its association with caspase 8. Moreover, Bid was shown to translocate to the mitochondria. These results suggest that Bid may be involved in the pathogenesis of hepatic cell death induced by the HCV and HIV envelope proteins. Previous studies have shown that Bid is important for the Fas-mediated apoptosis of hepatocytes, because these cells derive from Bid knockout mice are resistant to anti-Fas–mediated apoptosis [37]. In the present study, we have observed a slight increase in caspase 8 and caspase 3 activities. It has been reported elsewhere that hepatocytes resemble type II cells [14] and are unable to fully activate cytosolic caspases. However, the Bid-mediated mitochondrial pathway is critical for apoptosis in these cells [37, 38].

Taken together, our studies suggest that HCV E2 and HIV gp120 induce apoptosis in hepatocytes via a Fas-FasL–dependent pathway. This pathway leads to the translocation of Bid to the mitochondria and may, in turn, induce the release of cytochrome C, as well as downstream apoptotic signaling cascades.

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