Research Article

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HIV infection increases HCV-induced hepatocyte apoptosis

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Background & Aims: HCV related liver disease is one of the most important complications in persons with HIV, with accelerated fibrosis progression in coinfected persons compared to those with HCV alone. We hypothesized that HCV–HIV coinfection increases HCV related hepatocyte apoptosis and that HCV and HIV influence TRAIL signaling in hepatocytes.

Methods: We analyzed the effect of HIV in JFH1-infected Huh7.5.1 cells. Apoptosis was measured by Caspase-Glo 3/7 assay and Western blotting for cleaved PARP. TRAIL, TRAIL receptor 1 (DR4), and 2 (DR5) mRNA and protein levels were assessed by real-time PCR and Western blot, respectively. We also investigated activation of caspase pathways using caspase inhibitors and assessed expression of Bid and cytochrome *C*.

Results: We found increased caspase 3/7 activity and cleaved PARP in JFH1 HCV-infected Huh7.5.1 cells in the presence of heat-inactivated HIV, compared to Huh7.5.1 cells infected with JFH1 or exposed to heat-inactivated HIV alone. Both DR4 and DR5 mRNA and protein expression were increased in JFH1-infected cells in the presence of inactivated HIV compared to Huh7.5.1 cells infected with JFH1 or exposed to heat-inactivated HIV alone. Pancaspase, caspase-8, and caspase-9 inhibition blocked apoptosis induced by HCV, inactivated HIV, and HCV plus inactivated HIV. A caspase-9 inhibitor blocked apoptosis induced by HCV, HIV, and HCV–HIV comparably to pancaspase and caspase-8 inhibitors.

HCV induced the activation of Bid cleavage and cytochrome *C* release. The addition of HIV substantially augmented this induction.

Conclusions: Our findings indicate that hepatocyte apoptosis is increased in the presence of HCV and HIV compared to HCV or HIV alone, and that this increase is mediated by DR4 and DR5 up-regulation. These results provide an additional mechanism for the accelerated liver disease progression observed in HCV–HIV co-infection.

Keywords: HCV–HIV coinfection; Apoptosis; TRAIL receptor.

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Abbreviations: JFH-1, Japanese Fulminant Hepatitis 1; TRAIL, TNF-related apoptosis-inducing ligand; PARP, poly (ADP-ribose)polymerase; TGF-β1, transforming growth factor beta1; DR4, Death receptor 4; DR5, death receptor 5.

Introduction

Hepatitis C virus (HCV) infects about 170 million people and is a leading cause of chronic liver disease worldwide [1,2]. It is a major cause of cirrhosis, a significant cause of hepatocellular carcinoma (HCC), and the leading reason for liver transplantation worldwide.

HCV and human immunodeficiency virus (HIV) frequently coexist because of their common routes of transmission, and liver disease caused by HCV has rapidly become one of the most important complications in persons with HIV [3].

Indeed, end-stage liver disease is the most frequent cause of death among HIV-infected hospitalized patients [4]. Although the cause of liver injury in HIV-infected individuals is likely multifactorial (i.e., due to coinfection and medications), a number of reports have documented histological liver abnormalities that occurred solely as a result of HIV infection [5,6]. Moreover, HIV-1 RNA [5] and p24 antigen have been isolated from livers of infected individuals [7], although direct evidence that HIV infects hepatocytes is lacking. These observations suggest that HIV may indirectly result in hepatocellular injury. The hallmark of HIV infection is depletion and exhaustion of the immune system.

Hepatocyte cell death by apoptosis is emerging as a fundamental component of virtually all acute and chronic liver diseases. The ensuing responses of cell repair, inflammation, regeneration, and fibrosis may all be triggered by apoptosis [8–11]. An increasing body of evidence from both experimental and clinical studies suggests that hepatocyte apoptosis may contribute to liver fibrogenesis. Engulfment of apoptotic bodies by hepatic stellate cells stimulates the fibrogenic activity of these cells and may be one mechanism by which hepatocyte apoptosis promotes fibrosis [12]. Furthermore, recent studies in chronic hepatitis C virus (HCV) infection demonstrate that hepatocyte apoptosis correlates with disease severity [13] and stage of fibrosis in patients with steatosis [14,15].

Death receptors 4 and 5 (DR4 and DR5), also called tumor necrosis factor-related apoptosis-inducing ligand (TRAIL)-R1 and R2, are members of the tumor necrosis factor-receptor

(TNFR) family [16,17] and receptors for TNF-related apoptosis-inducing ligand (TRAIL) [18,19]. TRAIL selectively induces apoptosis in cancer cells *in vitro* and *in vivo* with little or no toxicity toward normal cells [20–22]. DR4 and DR5 mediate TRAIL-induced apoptosis through the formation of a death inducing signaling complex (DISC) containing the death receptor, adapter proteins such as Fas-associated death domain (FADD), and initiator caspases such as pro-caspase-8 [23] or pro-caspase-10 [24,25]. Consequently, pro-caspase-8 or pro-caspase-10 is activated by autoproteolytic processing, which then cleaves and activates downstream effector caspases, such as caspase-3 (extrinsic pathway). Additionally, the Bcl-2-interacting protein Bid is also cleaved by caspase-8 [26]. Truncated-Bid causes the loss of mitochondrial membrane potential and caspase-9 cleavage, resulting in apoptosis. Thus, truncated-Bid is a mediator that transduces the pro-apoptotic signal from the extrinsic pathway to the intrinsic (mitochondrial) pathway (Fig. 1).

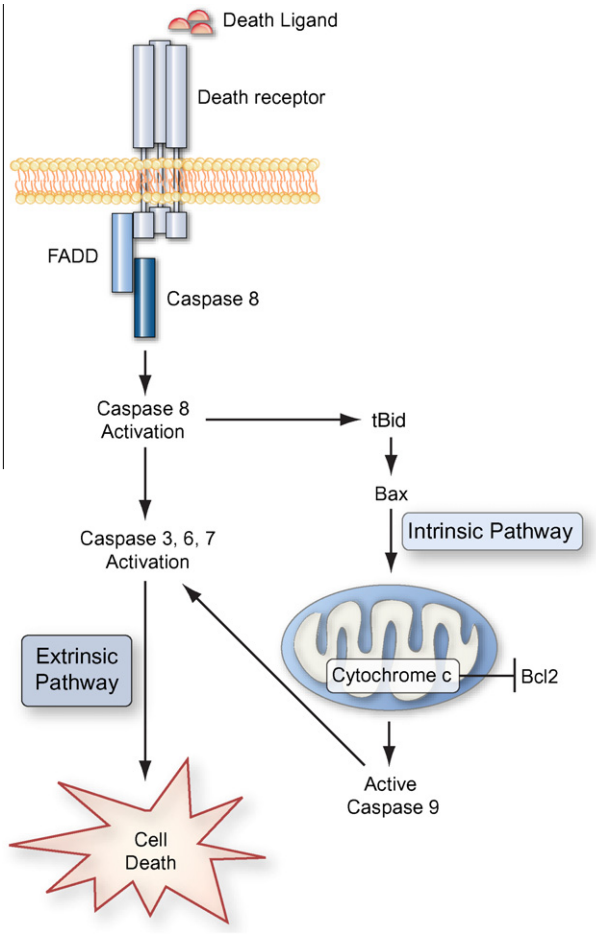

Fig. 1. Pathways of Cellular Apoptosis. Death receptor mediates death ligand-induced apoptosis through the formation of a death inducing signaling complex (DISC) containing the death receptor, adapter proteins such as the Fas-associated death domain (FADD), and initiator caspases such as pro-caspase-8 or pro-caspase-10. Consequently, pro-caspase-8 or pro-caspase-10 is activated by autoproteolytic processing, which then cleaves and activates downstream effector caspases, such as caspase-3 (extrinsic pathway). Additionally, the Bcl-2-interacting protein Bid is also cleaved by caspase-8. Truncated-Bid causes the loss of mitochondrial membrane potential and caspase-9 cleavage, resulting in apoptosis. Thus, truncated-Bid is a mediator that transduces the pro-apoptotic signal from the extrinsic pathway to the intrinsic (mitochondrial) pathway.

We hypothesized that HIV coinfection augments hepatocyte apoptosis attributable to HCV and that this represents an additional mechanism of accelerated liver disease in coinfection. We tested this hypothesis using an infectious HCV cell culture model.

Materials and methods

Cell cultures and transfection

Huh7.5.1 cells (human hepatocellular carcinoma cells) were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS). Japanese Fulminant Hepatitis 1 (JFH1) is a known tissue culture infectious strain of HCV. We transfected JFH1 RNA into Huh7.5.1 cells and collected the culture medium at 72 h post-transfection, as described previously [27]. We then inoculated naïve Huh7.5.1 cells with these culture supernatants, and confirmed JFH1 virus infection of newly inoculated cells by real-time PCR and Western blot for HCV RNA and core protein, respectively. NL4-3 is a CXCR4-tropic (T lymphocyte-tropic) HIV strain. BaL is a CCR5-tropic (macrophage-tropic) HIV strain. Heat-inactivated HIV (CXCR4 and CCR5-tropic virus) (56 °C for 30 min) was incubated with Huh7.5.1 cells or JFH1-infected Huh7.5.1 cells for 72 h at 1:4 dilution (11.25 ng/ml p24 for NL4-3; 8.75 ng/ml p24 for BaL). Naïve Huh7.5.1 cells were seeded at a density of 2×10^5 cells/well in 6-well plates 24 h before transfection. Infection of JFH1 and incubation with heat-inactivated HIV were performed simultaneously. Subsequently, cells were washed with PBS and collected 72 h later after transfection.

HIV stocks

Heat-inactivated HIV was prepared as previously described [28]. Laboratory-adapted HIV-1 strains were obtained from the NIH AIDS Research and Reference Reagent Program. A plasmid encoding the CXCR4-tropic virus NL4-3 was used to transfect HEK293T cells, and the supernatant virus was propagated using a CEM-derived T-cell line that expresses CD4, CXCR4, and CCR5 (CEM-GXR) [28]. The CCR5-tropic virus BaL [29] was also propagated using CEM-GXR cells. Viral stocks were assayed for HIV-1 p24 using the Alliance p24 Antigen ELISA Kit (PerkinElmer, Waltham, MA). Previously, we found that the increase in HCV levels mediated by heat-inactivated CCR5-tropic HIV and CXCR4-tropic HIV was abrogated by pre-incubation with neutralizing antibody to CXCR4 and CCR5 [30]. These data provided evidence for the indirect effect of HIV virus on HCV infection signaling through engagement of its co-receptors on the surface of Huh7.5.1 cells rather than through direct infection of hepatocytes.

Real-time polymerase chain reaction quantification

Total cellular RNA was harvested using the RNeasy Kit (Qiagen, Valencia, CA) according to the manufacturer's protocol. Total cDNA was synthesized by reverse transcription using the GeneAmp RNA PCR Kit (Applied Biosystems, Branchburg, New Jersey). *DR4, DR5*, *TRAIL* and *β-actin* mRNA levels were quantified by real-time PCR. Human *DR4* (GenBank#: NM_003844), Human *DR5* (GenBank# NM_003842) and Human *TRAIL* (GenBank# NM_003810) were measured using the following primers: *DR4* sense primer CTGAGCAACGCAGACTCGCTGTCCAC; *DR4* antisense primer-TCAAAGGACACGGCAGAGCCTGTGCCA; *DR5* sense primer-GGGAGCCGCTCATGAGGAAGTTGG; *DR5* antisense primer-GGCAAGTCTCTCTCCCAGCGTCTC; *TRAIL* sense primer-AATCATCAAGGAGTGGGCATTC; *TRAIL* antisense primer-ATGACCAGTTCACCATTCCTCAA. Human *β-actin* sense primer-GCACTCTT CCAGCCTTCCT; human *β-actin* antisense primer AGGTCTTTGCGGATGTCCAC. *β-Actin* was used as a control for basal RNA levels. *DR4, DR5, TRAIL,* and *β-actin* levels were quantified by real-time PCR using the Bio-Rad IQ5 (Bio-Rad Laboratories, Hercules, CA). Finnzymes SYBR green I dye (New England Biolabs, Ipswich, MA) was used for detection, as previously described [31].

Protein sample preparation

At the time of harvest, cells were washed twice with phosphate-buffered saline, and whole-cell protein samples were extracted with lysis buffer (50 mM Tris–HCl, 1 mM EDTA, 50 mM NaF, 1 mM Na_3VO_4, 1% Triton X-100, 150 mM NaCl, 10 μg/ml aprotinin, phosphatase inhibitor cocktail, and protease inhibitor cocktail) (Roche Applied Science, Indianapolis, IN).