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TOPIC HIGHLIGHT

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Hepatitis C virus infection and apoptosis

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Abstract

Apoptosis is central for the control and elimination of viral infections. In chronic hepatitis C virus (HCV) infection, enhanced hepatocyte apoptosis and upregulation of the death inducing ligands CD95/Fas, TRAIL and TNFα occur. Nevertheless, HCV infection persists in the majority of patients. The impact of apoptosis in chronic HCV infection is not well understood. It may be harmful by triggering liver fibrosis, or essential in interferon (IFN) induced HCV elimination. For virtually all HCV proteins, pro- and anti-apoptotic effects have been described, especially for the core and NS5A protein. To date, it is not known which HCV protein affects apoptosis *in vivo* and whether the infectious virions act pro- or anti-apoptotic. With the availability of an infectious tissue culture system, we now can address pathophysiologically relevant issues. This review focuses on the effect of HCV infection and different HCV proteins on apoptosis and of the corresponding signaling cascades.

Key words: Hepatitis C; Spoptosis; TRAIL; CD95/Fas; TNFα; Perforin

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INTRODUCTION

Hepatitis C virus (HCV) infection persists in approximately. Eighty percent of patients and is a leading cause of liver cirrhosis and hepatocellular carcinoma[1-4]. Worldwide, about 300 million individuals are HCV infected. The only antiviral treatment available to date with PEG-INF and ribavirin does not eliminate HCV infection in a large proportion of patients, especially in HCV genotype 1 infection, and, at the same time, has multiple severe side effects. With the availability of an infectious tissue culture system, we now can address pathophysiologically relevant issues for new treatment options[1-3]. HCV belongs to the flaviviridae. It has an enveloped, positive strand RNA genome of 9.6 kb length containing one open reading frame translated into a single polyprotein. Posttranslational cleavage yields 4 structural (E1, E2, core, p7 (probably) and 6 nonstructual proteins (NS2, NS3, NS4A, NS4B, NS5A, NS5B). Six different genotypes (1 [a, b, c], 2 [a, b, c], 3 [a, b], 4a, 5a, 6a) and 52 subtypes have been described. Due to the lack of proofreading function of the RNA-dependent RNA-polymerase (NS5B), HCV has a high mutation rate and exists as genetically heterogeneous quasispecies in individual patients[5-7]. The different genotypes differ genetically from one another by at least 30%, and the different subtypes within a genotype by more than 20%. This genetic heterogeneity makes it difficult to compare apoptotic pathways obtained with different HCV genotypes. In general, apoptosis is central to viral clearance. In HCV-infected liver, however, despite enhanced hepatocyte apoptosis, viral persistence is observed.

APOPTOSIS IN HCV-INFECTED LIVER

Immune cell deficiency

The immune response to viral infections includes different components of the innate and the acquired immune system. They induce apoptosis as a host defense against viral infections. The innate immune system as the first line of defense directly activates inflammatory cells, such as macrophages (e.g., granulocytes, Kupffer cells in the liver) and natural killer (NK) cells which may directly cause death of the infected cells. On the other hand, viral RNA or proteins can bind to intracellular molecules that modulate or directly induce cell death[8]. In this immune cell-independent, virus-induced apoptosis of the host cell protein kinase R (PKR)[9,10] and the cytoplasmic RNA helicase RIG-I[11] play central roles. RIG-I activates Cardif, a cytosolic protein that localizes to the mitochondrial membrane where it acts pro-apoptotic[12,13]. PKR is also activated by interferons

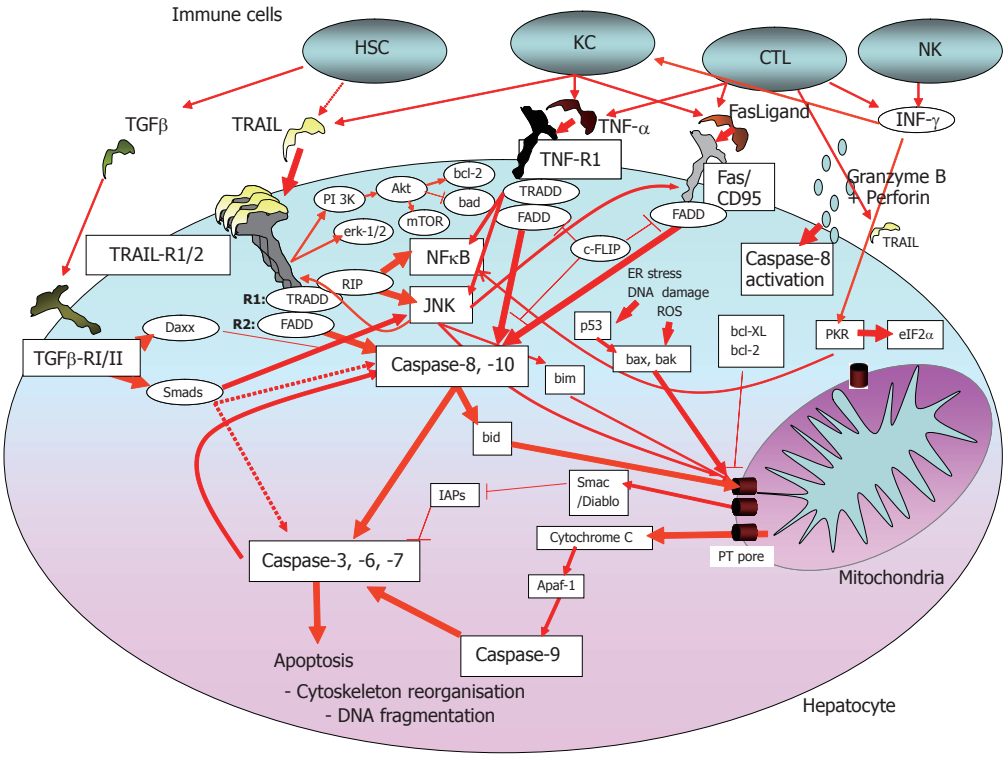

Figure 1 Signal transduction pathway of apoptosis in hepatocytes. Immune cells induce apoptosis in hepatocytes by death receptor ligands (TRAIL, TNFα, CD95Ligand, TGF-β) and granzyme B/perforin. Ligand-induced apoptosis activates caspase-8, whereas intrinsic apoptosis occurs via the mitochondrial permeability transition (PT) pore and activation of caspase-9. Caspase-9 and -8 activation converge in activation of the effector caspases-3, -6 and -7, resulting in irreversible apoptosis induction. HSC: Hepatic stellate cells; KC: Kupffer cells; CTL: Cytotoxic T-lymphocytes; NK: Natural killer cells.

(Figure 1) and acts *via* the downstream transcription factor eIF-2α[14,15]. In HCV infection, the activated innate immune system alone is obviously insufficient to eliminate the virus[16]. The acquired immune system consists of the humoral (antibody-secreting B-lymphocytes/plasma cells) and the cellular immune system (CD4+- and CD8+-T-lymphocytes). This system is essential for the clearance of most viral infections and depends on complex intercellular interactions and the recognition of viral antigens presented by specific cells (e.g., dendritic cells). CD4+-T-lymphocytes activate CD8+-T-lymphocytes, cytotoxic T lymphocytes (CTLs), macrophages and B-lymphocytes[16]. The antigen-primed CD8+-T-lymphocytes/CTLs directly kill infected cells *via* direct cell-cell-contact, and release of cytotoxic and/or antiviral cytokines (e.g., IFNγ, TNFα), whereas IFNγ and INFα are also able to eliminate the virus without killing the host cell[17,18]. In chronic HCV infection, the acquired immune system is, among others, impaired by T cell failure, dysfunction and exhaustion[19]. This failure includes CD4+- as well as CD8+-T-lymphocytes.

ENHANCED HEPATOCYTE APOPTOSIS IN HCV INFECTION *IN VIVO*

Most of the cytotoxic effects mentioned above occur *via* programmed cell death, with activation of the intracellular suicide program through specific signals. Because chronic viral infection may reflect a failure of the immune system, specific apoptosis induction may not occur. In chronic HCV infection, however, enhanced hepatocyte apoptosis has been described, independent from the HCV genotype[20]. Apoptosis varies between 0.54%[20] and 20.00% of hepatocytes[21], depending on the methods used. Typical pathomorphological features of apoptosis (e.g., nuclear fragmentation, cell shrinkage) may be seen only in a minority of hepatocytes. The close physical proximity of apoptotic hepatocytes and infiltrating lymphocytes suggests an immune cell-mediated apoptosis[20,22]. Apoptosis correlates with liver pathology[20,21] and may contribute to fibrogenesis[23]. Due to the difficulty to identify HCV infected hepatocytes, it is unknown whether apoptotic hepatocytes are indeed HCV infected. The number of HCV infected hepatocytes is in the range between 1% and 10%[24]. Therefore, we actually do not know whether apoptosis is indeed related to HCV clearance. In an animal model of cholestasis, inhibition of hepatocyte apoptosis reduced fibrogenesis[25] and excessive apoptosis lead to fulminant hepatitis[26,27]. Therefore, anti-apoptotic therapy to prevent HCV-related liver damage has been suggested[28,29]. By contrast, in a chimeric mouse-human model, pro-apoptotic gene therapy with proapoptotic Bid, engineered to contain a specific cleavage site for NS3/NS4A protease, results in a considerable decline of HCV RNA in serum[30]. The relation between PEG-IFN/ribavirin-induced viral clearance and apoptosis of infected hepatocytes is largely unknown. INFs induce apoptosis in hepatoma cells, activate pro-apoptotic PKR[10] and upregulate death receptor ligands. However, anti-apoptotic effects have also been described[7,31-33].

LIGAND-INDUCED HEPATOCYTE APOPTOSIS IN HCV INFECTION

Hepatocytes most likely represent so-called type-II cells, for which external activation of the death signaling pathway often is insufficient to induce apoptosis. Here, apoptosis requires in addition amplification by the mitochondrial pathway (intrinsic apoptosis pathway). The

latter is affected by oxidative stress, DNA damage, and viral proteins (Figure 1).

Targeted apoptosis induction *via* CTLs and macrophages largely occurs *via* the ligands and receptors of the TNFα family: TNFα/TNF-receptor 1, CD95/CD95Ligand and TRAIL/Trail receptor-1 and -2, respectively (Figure 1). Ligand binding induces the formation of a death-inducing signaling complex, resulting in the activation of caspase-8 (caspases are the proteases involved in the apoptosis signaling cascade[34]. Active caspase-8 can trigger two signaling pathways. The first pathway involves cleavage of bid, followed by mitochondria-dependent activation of caspase-9 *via* cytochrome C release and apaf-1[35] (Figure 1). Mitochondria-dependent apoptosis is amplified by pro-apoptotic bax, bad, bak and others, while molecules like bcl-2 or bcl-XL act anti-apoptotic. These proteins converge at the mitochondrial permeability transition (PT) pore that regulates release of apoptotic regulatory proteins, e.g., procaspase-9, cytochrome C, apoptosis inducing factor (AIF) or Smac/Diablo[36-38]. The second pathway involves caspase-8 activation that may bypass mitochondria resulting in the direct activation of effector caspases (caspase-3, -6, -7). Cellular inhibitors of apoptosis (IAPs, survivin, c-FLIP) are able to block caspase activation and apoptosis[39] (Figure 1).

Growth-factor activated MAP-kinases Erk-1/2 and PKB/Akt inhibit apoptosis directly (e.g., through inactivation of pro-apoptotic bad) or *via* upregulation of anti-apoptotic proteins (e.g., bcl-2). By contrast, sustained stress activation of c-jun kinase (JNK) enhances death ligand-induced apoptosis *via* bim activation and consecutive mitochondrial apoptosis or *via* enhanced death-receptor membrane trafficking[40-42]. Most death ligands, especially TNFα and TRAIL, activate NFκB, which has anti-apoptotic effects in hepatocytes by upregulation of anti-apoptotic proteins, e.g., c-FLIP and bcl-XL[43].

Death receptor ligands may be secreted by immune cells (e.g., macrophages) or may be membrane-bound. The latter form induces apoptosis more efficiently[44]. In the normal liver, INFγ-activated Kupffer cells can kill neighbouring cells *via* TRAIL and CD95Ligand[37,44]. By contrast, in injured liver, activated hepatic stellate cells release TGF-β that may induce apoptosis of hepatocytes[45,46]. While TGF-β1 expression is increased in the HCV-infected liver[22], the impact of TGF-β on hepatocyte apoptosis in HCV-infected patients remains elusive. Apart from apoptosis induction, TGF-β is a key molecule in the pathogenesis of liver fibrosis[47].

Hepatocytes undergo apoptosis in response to CD95Ligand and TNFα, whereas TRAIL presumably only induces apoptosis in infected or malignantly transformed hepatocytes/hepatoma cells, but not in normal liver cells. For all three death ligands, in chronic HCV infection, upregulation has been described[20,48-51]. Further, HCV-specific CTL clones induced CD95Ligand-, TNFα- and perforin-dependent hepatocyte apoptosis[52,53]. In HCV-infected liver, CD8+ T cells express CD95Ligand[49] and TRAIL[54] (Fischer, Blum Schmitt-Gräff *et al*, unpublished data). Interestingly, CD95Ligand-induced apoptosis did not depend on HCV infection/antigen presentation, because bystander killing of non-HCV infected hepatocytes was observed. TRAIL-induced apoptosis seems especially important in viral defense. Adenoviral-infected murine and human hepatocytes are sensitized to TRAIL-induced apoptosis, while CD95Ligand-induced cell death is not affected[50,55]. In TRAIL knock-out mice resolution of pulmonary influenza infection is TRAIL-dependent[56], and CMV infected colon epithelial cells or skin fibroblasts become sensitive to TRAIL-induced apoptosis[57]. Further, in mice infected with encephalomyocarditis virus, blocking of TRAIL resulted in higher viral titers and early death[58]. In concanamycin- and listeria-induced hepatitis, liver cell apoptosis is TRAIL-dependent[59]. PEG-INF/ribavirin therapy of patients with chronic HCV infection results in a rapid and sustained TRAIL elevation, suggesting a role of TRAIL in viral clearance[60]. Similar observations have been made for soluble CD95Ligand[61,33]. Therefore, TRAIL-induced apoptosis may play a major role in HCV defense and elimination.

Another mechanism of apoptosis involves the release of granzyme B and perforin by CTLs[62,63]. Exocytosed perforins form transmembrane channels in the target cell that allow the entry of granzyme B. Similar to death-ligand induced apoptosis, granzyme B-mediated apoptosis largely depends on caspase activation and the sensitivity of the target cell. Hepatocytes seem resistant to granzyme B mediated cell death, and CTL killing of infected hepatocytes is perforin/granzyme B- independent[29,64]. Therefore, a contribution of this apoptosis mechanism in patients with viral hepatitis is very unlikely.

MODIFIED HEPATOCYTE APOPTOSIS *IN VITRO*

Viral proteins interfere with the cellular apoptotic signaling pathway and block key cellular elements of the host cell. Until recently, the lack of an infectious HCV tissue culture system did not allow to study the impact of HCV infection on hepatocyte apoptosis. Overall, the data regarding the role of different HCV proteins are controversial and ascribe to a given viral protein pro- and anti-apoptotic effects, depending on the experimental system used. Since in most models viral proteins are overexpressed by non-viral promoters, for virtually all HCV proteins a pro-apoptotic effect has been described. Apart from the unphysiological expression of viral proteins, these models further lack the balance of intracellular viral expression of the different HCV proteins and their interactions. Especially in HCV infection, intracellular viral protein expression is very low.

Further, HCV is genetically highly variable and exists as quasispecies in a given patient. Different pro- and anti-apoptotic effects of the HCV core protein from an individual patient have been described[65], suggesting special properties of different quasispecies proteins. These protein differences may explain in part the different effects of viral proteins on apoptosis. Studies of the contribution of genotypes or quasispecies to the effects on apoptosis are largely missing. Further, experiments designed to study the impact of HCV infection on hepatocyte apoptosis must also consider the interactions between the different

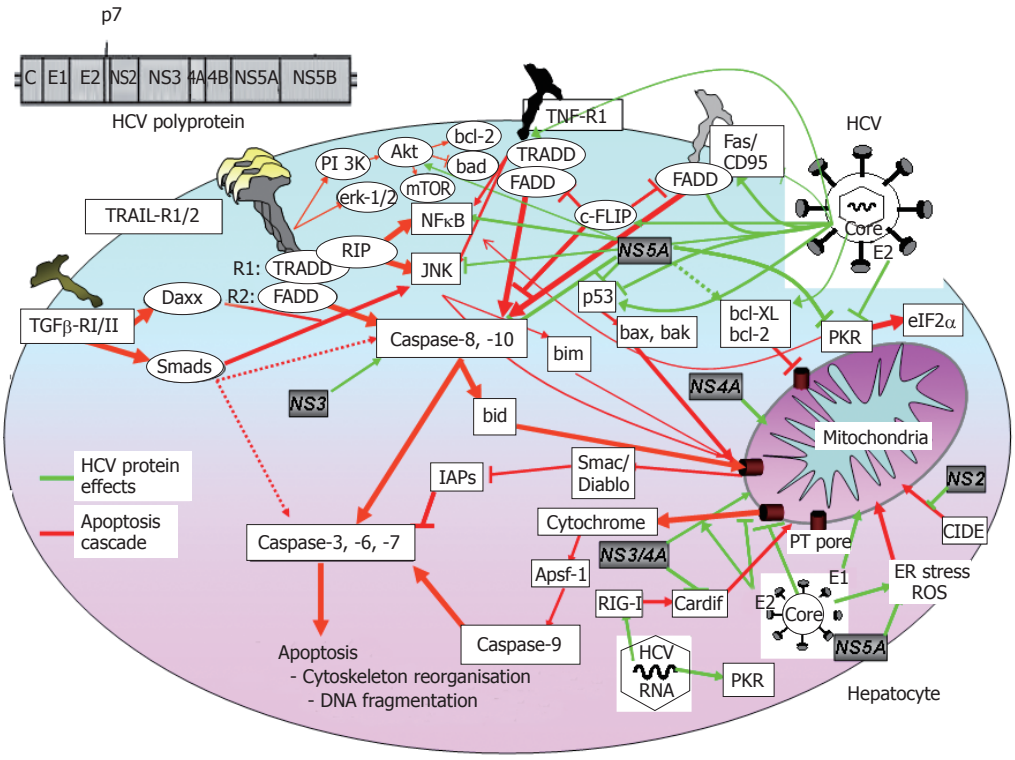

Figure 2 Interference of HCV proteins with the apoptosis cascade. Pro- and anti-apoptotic effects of HCV proteins converge at the mitochondria (e.g., NS2, NS3/4A, NS5A, E2, core), partly indirectly via *p53* (NS5A, core) and activation of PKB/Akt, c-Jun kinase JNK (core) or NFκB (NS5A). HCV interacts directly with death receptors (core), the corresponding death receptor domains (FADD) and caspase-8 (NS5A). HCV double-strand RNA-activated protein-kinase R (PKR) induced signaling *via* RIG-I (retinoic acid inducible gene-I) and Cardif is directly (E2, NS5A) and indirectly (NS3/4A) disturbed.

HCV proteins. Therefore, only models based on the complete and infectious virus may reflect to some extent the *in vivo* situation.

HCV core protein

The structural HCV core protein makes up the virion nucleocapsid[1,5,66]. The core protein has been shown to affect various cellular signaling pathways[67] and to activate different promoters, e.g., c-myc, c-fos[68-70]. It has further been shown to have pro- and anti-apoptotic effects in death ligand-mediated hepatocyte apoptosis. Core-dependent inhibition of TNF-α-[71] and CD95Ligand-induced apoptosis[72] has been described in a hepatoma cell line. In other models, overexpressed HCV core protein did not prevent CD95Ligand-induced apoptosis in hepatoma cells[73] or transgenic mice expressing HCV core protein, E1, E2 and NS2, respectively. HCV core protein inhibits CD95Ligand-mediated apoptosis by prevention of cytochrome C release from mitochondria and consecutive activation of caspase-9, -3 and -7[74]. Direct physical and pro-apoptotic interaction of the core protein with the cytoplasmatic domains of CD95, TNF-R1[75] and lymphotoxin-β[76] receptors have been reported. Further, direct binding to the downstream death domain of FADD and c-FLIP[77] has been shown to result in anti-apoptotic effects. Recently, inhibition of the TGF-β-pathway by direct interaction of the core protein with the DNA-binding domain of Smad3, important apoptosis mediators of TGF-β-receptor-I/II, has been demonstrated[65].

Several studies demonstrated binding of the HCV core protein to p53, either inhibiting or activating p53[69,78-80] with consecutive anti- or pro-apoptotic effects. In some studies apoptosis was inhibited in hepatoma through core-dependent phosphorylation and activation of STAT3 that induces the anti-apoptotic bcl-XL[81,82]. Other studies showed core-induced apoptosis through mitochondrial cytochrome C release and indirect activation of bax[83,84]. TRAIL-induced apoptosis in hepatoma cells seems enhanced by core-dependent bid-cleavage[83]. Mitochondrial functions are altered by core-induced oxidative stress, making cells more susceptible to apoptosis[85]. Machida *et al*[86] showed HCV-dependent production of reactive oxygen species (ROS), lowering of the mitochondrial transmembrane potential and consecutive caspase-independent cell death.

Taken together, it remains unclear whether HCV core protein inhibits or induces death receptor-mediated apoptosis of hepatocytes (Figure 2).

HCV envelope proteins E1 and E2

HCV proteins E1 and E2 are envelope proteins, that mediate viral binding and entry[7,87]. In a transgenic mouse model expressing HCV proteins, CD95Ligand-mediated hepatocyte apoptosis is inhibited by E1, E2, NS2 and core, respectively. The activation of mitochondrial apoptosis (intrinsic pathway) is involved, because release of cytochrome C and caspase-9, but not caspase-8 activation are inhibited. To date, the contribution of the individual HCV proteins was not investigated[74]. In E1-expressing hepatoma cells, apoptosis depends on the presence of the C-terminal transmembrane domain of E1, presumably altering membrane permeability of E1[88,89].

Inhibition of TRAIL-induced apoptosis in hepatoma cells by E2, presumably through inhibition of mitochondrial cytochrome C release has been demonstrated[90], while E1 had no effect and core did not counteract the anti-apoptotic effect of E2. Comparable results were obtained in core-E1-E2 transfected hepatoma cells or transgenic mice. In both models, core-E1-E2 induced less apoptosis than core-transfected

cells/transgenic mice and controls, respectively[91]. By contrast, E2 induces mitochondria-related and caspase-dependent apoptosis in the same hepatoma cell line[92]. These controversial data may reflect the use of different promoters that overexpress E2, while at the same time, the HCV genotype or the individual sequence of E2 have not been considered. Therefore, it still remains unclear whether HCV E1 has apoptosis-modulating activity *in vivo*, and whether HCV E2 acts anti- or pro-apoptotic (Figure 2).

HCV nonstructural proteins

The non-structural HCV proteins NS2 and NS3 are the two viral proteases required for posttranslational cleavage of non-structural proteins. NS2 is a transmembrane protein localized in the endoplasmatic reticulum (ER) that directly binds and inhibits CIDE-B-induced apoptosis (cell death-inducing DFF45 (DNA-fragmentation-factor)-like effector[93]). CIDE-B-induced apoptosis is assumed to occur *via* the mitochondrial pathway[94,95]. Its role in hepatocyte apoptosis and viral hepatitis remain to be determined, however.

NS3 has a helicase and NTPase activity that are involved in RNA replication[7]. Importantly, NS3 prevents viral RNA-induced pro-apoptotic RIG-I effects by specific cleavage of downstream Cardif, a protein that translocates to the mitochondrial membrane when activated[13]. The precise role of Cardif in hepatocyte apoptosis and viral hepatitis is unknown, however. In contrast, NS3 induces caspase-8 dependent apoptosis in hepatocytes[96] and in dendritic cells[97]; the underlying mechanism remains unknown.

HCV NS4A is a cofactor that binds to NS3. NS4A alone and complexed with NS3 is localized in mitochondria and induces the release of cytochrome C and caspase-8 independent apoptosis[98]. NS4B is an integral ER membrane protein that may play a role in anchoring the replication complex[6,7]. A role in the apoptotic signaling pathway has not yet been described.

The function of NS5A is not yet well defined. NS5A interferes with the response to IFN and seems to play an important role in viral replication[5,7]. NS5A has sequence homologies with bcl-2 and binds to FKBP38, thereby augmenting the anti-apoptotic effect of bcl-2[99] and inhibiting the pro-apoptotic action of bax in hepatoma cells[100]. Anti-apoptotic effects of NS5A are further mediated by cytoplasmatic sequestering of p53[101], activation of PI3-kinase-Akt/PKB survival pathway[102], activation of STAT3 with enhanced expression of bcl-XL and p21[103] and activation of NFκB[104]. By contrast, the direct inhibition of pro-apoptotic bin1, a tumor suppressor protein with a SH3 domain, has been described in hepatoma cells[105], and a direct NS5A-induced apoptosis has also been shown[97,106]. NS5B is the viral RNA-dependent RNA polymerase[5-7]. There are no studies demonstrating a role of NS5B in apoptotisis of hepatocytes/hepatoma cells, while a pro-apoptotic effect of NS5B has been demonstrated in dendritic cells[97].

In conclusion, similar to HCV structural proteins, the effect of non-structural proteins on hepatocyte apoptosis *in vivo* remains unclear.

CONCLUSION

The role of apoptosis in HCV infection is not well defined. Kinetics and extent of hepatocyte apoptosis as well as the pro- and anti-apoptotic mechanisms involved remain unclear. It remains further unclear whether enhanced hepatocyte apoptosis in HCV infection is related to viral clearance, and whether it has a therapeutic benefit.

Most experimental models have fundamental shortcomings and there are no data from primary hepatocytes, tissue cultures or animal models. The majority of the data were obtained with different tumor cell lines that may in themselves be inhomogeneous. Different HCV genotypes and quasispecies may induce different effects, and most studies employ nonphysiologically overexpressed viral proteins. In HCV infected patients, by comparison, only very low quantities of HCV proteins are detectable, and the balanced expression of these proteins may be essential. Therefore, the results obtained to date have to be interpreted with great cautious. The now available infectious tissue culture systems[1-3] as well as future *in vivo* model systems may give answers to these questions, may better reflect the *in vivo* situation and may help to clarify the interference of HCV with apoptotic pathways and its role in the pathogenesis of HCV infection and clearance.

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