Hepatitis C virus-induced hepatocyte cell death and protection by inhibition of apoptosis

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Chronic hepatitis C virus (HCV) infection results in progressive liver fibrosis leading to cirrhosis and liver cancer. The mechanism for this remains unclear but hepatocyte apoptosis is thought to play a major role. Hepatocyte apoptosis in human liver tissue was determined by immunohistochemistry for cytokeratin 18 (M30 CytoDEATH) and cleaved poly(ADP-ribose) polymerase (PARP). In vitro studies were performed with replication-defective recombinant adenoviruses expressing HCV proteins (rAdHCV) to study the effects of HCV on cell death in Huh7 cells, primary mouse hepatocytes (PMoHs) and primary human hepatocytes (PHHs). Cell viability and apoptosis were studied using crystal violet assays and Western blots probed for cleaved caspase-3 and cleaved PARP, with and without treatment with the pan-caspase inhibitor Q-VD-Oph and necrostatin-1. Liver tissue of HCV-infected patients expressed elevated levels of apoptotic markers compared with HCV-negative patients. rAdHCV infection reduced cell viability compared with uninfected controls and cells infected with control virus (rAdGFP). Huh7, PMoHs and PHHs infected with rAdHCV showed significantly increased levels of apoptotic markers compared with uninfected controls and rAdGFP-infected cells. In rAdHCV-infected Huh7, treatment with Q-VD-Oph and necrostatin-1 both improved cell viability. Q-VD-Oph also reduced cleaved PARP in rAdHCV-infected Huh7 and PMoHs. Hepatocyte apoptosis is known to be increased in the livers of HCV-infected patients. HCV promoted cell death in primary and immortalized hepatocytes, and this was inhibited by Q-VD-Oph and necrostatin-1. These findings indicate that HCV-induced cell death occurs by both apoptosis and necroptosis, and provide new insights into the mechanisms of HCV-induced liver injury.

INTRODUCTION

Hepatitis C virus (HCV) infection affects ~170 million people worldwide and accounts for almost half a million deaths annually (Dore et al., 2003). Whilst there have been major advances in the understanding and treatment of HCV infection, there remain unresolved questions around the mechanisms by which HCV causes liver injury and fibrosis (Lavanchy, 2009).

Liver damage in HCV-related cirrhosis is associated with upregulation of pro-inflammatory, pro-fibrotic and pro-apoptotic genes compared with HCV-negative patients with cirrhosis (Shackel et al., 2002). Apoptosis of virus-infected hepatocytes may be an important part of the host defence mechanism against HCV as this interrupts viral replication and assists in the elimination of infected hepatocytes (Ogata et al., 2006; Teixeira et al., 2007). Virus-specific cytotoxic T-cells may also induce apoptosis of HCV-infected hepatocytes by secreting antiviral cytokines and upregulating death receptor ligands such as TNF, Fas ligand (FasL/CD95L) and TRAIL (TNF-related apoptosis-inducing ligand) (Fischer et al., 2007).

Hepatocyte cell death is thought to occur predominantly by one of three processes: apoptosis, necrosis or necroptosis. Necrosis and necroptosis result in cell membrane rupture and release of intracellular material that induces an inflammatory response. Apoptotic bodies are rapidly phagocytosed by macrophages (Lauber et al., 2004), thereby...
reducing the likelihood of activating the inflammatory cascade. Apoptosis is initiated through either the extrinsic or intrinsic pathway (Schattenberg et al., 2006). These two pathways converge on a common execution phase, which requires proteolytic activation of caspases (Fabregat et al., 2007).

Hepatocytes also undergo caspase-independent cell death known as necroptosis, which induces inflammation and thereby leads to liver damage (Brown, 2008; Vandenabeele et al., 2010). This process shares a number of the regulatory proteins involved with apoptosis and requires the interaction of receptor-interacting protein kinase (RIPK) 1 and 3 (Declercq et al., 2009). RIPK1 acts as a central switch between apoptosis, necroptosis and cell survival (Declercq et al., 2009).

The mechanism of HCV-induced hepatocyte death is not completely understood and studies based on overexpression of viral proteins have reported conflicting effects on hepatocyte cell death, showing enhancement or inhibition of apoptosis (Bantel & Schulze-Osthoff, 2003; Berg et al., 2009; Kawamura et al., 2006; Lan et al., 2008; Liang & Heller, 2004; Macdonald & Harris, 2004; Nomura-Takigawa et al., 2006; Prikhod’ko et al., 2004; Saito et al., 2006) or increased apoptosis in primary hepatocytes infected with cell-culture-derived HCV (HCVcc), but only in the presence of TRAIL (Lan et al., 2008). In this study, we evaluated hepatocyte cell death in the liver tissue of patients with chronic HCV cirrhosis compared with HCV-negative patients with and without cirrhosis. Unlike several previous reports that only studied the effects of HCV on apoptosis in immortalized cell lines or with limited studies in primary hepatocytes, we were able to correlate the findings in human liver tissue with in vitro experiments in primary human hepatocytes (PHHs) and primary mouse hepatocytes (PMoHs) to demonstrate a direct but reversible effect of HCV on hepatocyte cell death. In addition, by using replication-defective recombinant adenoviruses (rAdHCV) we were able to study the differential effects of the complete structural and/or non-structural proteins of HCV on cell death in PHHs and PMoHs.

**RESULTS**

**Hepatocyte apoptosis is upregulated in liver tissue of HCV-infected patients**

To confirm the association between HCV and apoptosis, we analysed explanted livers of 10 patients with HCV-associated cirrhosis. These were compared with liver sections from three patients with end-stage cirrhosis who successfully cleared HCV infection with antiviral treatment prior to undergoing liver transplantation, 10 patients with end-stage cirrhosis from alcoholic liver disease (HCV-negative) and nine patients without cirrhosis (healthy controls). Sections were examined for cleaved cytokeratin 18 (M30 CytoDEATH) (Fig. 1a) and cleaved poly(ADP-ribose) polymerase (PARP) (Fig. 1c). Cytokeratin 18, a cytoskeletal filament, and the intranuclear protein PARP are major substrates for caspases, and their respective cleavage products are recognized markers of activated programmed cell death.

The livers of patients with HCV-related end-stage cirrhosis showed increased staining for M30 CytoDEATH with a mean proportional area of 0.044 ± 0.004, sixfold higher than in that of patients with end-stage cirrhosis who had cleared HCV (0.007 ± 0.0008), fourfold higher than in alcoholic cirrhotic livers (0.012 ± 0.0007) and 32-fold higher than in normal livers (0.0008 ± 0.0002; P<0.0001 for all comparisons) (Fig. 1b). HCV-positive livers also demonstrated increased staining for cleaved PARP (Fig. 1d). Liver sections from patients with HCV cirrhosis had cleaved PARP levels that were 2.4-fold higher (mean proportional area 0.074 ± 0.003) than cirrhotic livers from those who had cleared HCV (0.031 ± 0.004), 2.7-fold higher than in alcoholic cirrhotic livers (0.027 ± 0.003) and 23-fold higher than normal livers (0.003 ± 0.0007; P<0.0001 for all comparisons). The increased hepatocyte cell death observed in the liver sections could be attributed directly to HCV alone as inflammatory cytokines released from HCV-activated resident liver NK-cells, Kupffer cells or T-cells could have also contributed this effect. Therefore, to understand the direct role of HCV on apoptosis, the effects of expressing HCV structural (core, E1 and E2) and/or non-structural proteins (NS3–5B) on apoptosis in Huh7 cells and primary hepatocytes in culture were investigated.

**Expression of HCV proteins in hepatocytes resulted in reduced cell viability**

The Huh7 cell line was infected with rAdHCV-CoreE1E2 and/or rAdHCV-NS3-5B at set time points to determine the specific contributions of HCV structural and non-structural proteins, respectively, on cell viability. Infection of Huh7 cells with rAdHCV-CoreE1E2 resulted in a 55% reduction in cell viability at 24 h post-infection (p.i.) compared with uninfected mock – an effect significantly greater than that seen in rAdGFP-infected control cells (4% reduction, P=0.003) (Fig. 2a). For rAdHCV-NS3-5B-infected cells, cell viability was decreased by 44% at 24 h p.i., which was also significantly greater than the effect of rAdGFP infection (P=0.004). Huh7 cells infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B combined (rAdHCV co-infection) showed a 57% reduction in cell viability at 24 h p.i. (P=0.002). The cell viability in rAdHCV co-infected cells was no greater than in mono-infected cells. This was likely to be due to the lower m.o.i. used for the individual viruses in co-infection experiments where an m.o.i. of 0.5 was used for each rAdHCV construct to maintain the final m.o.i. at 1.0 and to avoid using a higher m.o.i. (2.0) that may have resulted in excessive cell death, thereby confounding comparisons with mono-infections.

To explore the physiological relevance of the findings in cell lines, we performed the same experiments on PMoHs,
which were only co-infected with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B to concurrently express the structural and non-structural proteins in order to simulate natural infection more closely (Fig. 2b). Here, we observed a progressive decline in cell viability from 10% (P=0.003) at 24 h and 27% (P<0.0001) at 48 h, to 36% (P=0.0004) at 72 h p.i. in rAdHCV-co-infected PMoHs (Fig. 2c). These results showed that both structural and non-structural proteins of HCV were associated with loss of hepatocyte viability, both in Huh7 cells and PMoHs.

HCV proteins stimulate hepatocyte apoptosis

To determine whether the reduction in hepatocyte viability was the result of apoptosis, we examined the effects of rAdHCV infections on the expression of markers of cellular apoptosis in Huh7 cells and primary hepatocytes. For feasibility, the mono- and co-infection experiments were performed separately; however, each was done simultaneously with a rAdGFP control for comparison with the corresponding rAdHCV infections.

We observed greater levels of cleaved PARP in rAdHCV-infected Huh7 cells compared with rAdGFP at 24 h p.i. (Fig. 3a). In rAdHCV-CoreE1E2-infected cells, cleaved PARP levels were increased 1.6- (±0.64 (SEM), P=0.06), 1.8- (±0.16, P=0.03) and 2.0-fold (±0.14; P=0.003) at 24, 48 and 72 h p.i., respectively. In rAdHCV-NS3-5B-infected Huh7 cells, cleaved PARP levels were 1.2- (±0.09; P=0.20), 1.9- (±0.18; P=0.04) and 2.7-fold (±0.27; P=0.005) higher at 24, 48 and 72 h p.i., respectively, relative to rAdGFP control. With rAdHCV co-infection, cleaved PARP levels were 1.3- (±0.10, P=0.44), 1.7- (±0.17, P=0.04) and 2.4-fold (±0.19, P=0.02) greater at 24, 48 and 72 h compared with rAdGFP infection.

In addition, immunocytochemistry staining for cleaved PARP in rAdHCV-infected Huh7 cells showed a significantly greater number of hepatocytes expressing cleaved PARP per high-power field (Fig. 3b). rAdHCV-CoreE1E2 infection, rAdHCV-NS3-5B infection and co-infection with both produced 4.8-, 5.1- and 4.9-fold more cleaved PARP-expressing cells than rAdGFP-infected cells, respectively (P<0.001 for all three comparisons). These results were comparable with the immunohistochemistry studies in human liver sections.

Infection of rAdHCV in Huh7 cells did not result in changes to cleaved caspase-3 levels (results not shown), perhaps due to deranged cell death pathways inherent to the malignant Huh7 cell line. However, activation of caspase-3 was observed in rAdHCV-infected primary hepatocytes. The effect of HCV on apoptosis in primary hepatocytes was examined at 48 h p.i., as this was the time point at which changes in hepatocyte apoptosis were most reliably detected in Huh7 cells. In contrast to Huh7 cells, significant elevations in both cleaved caspase-3 and cleaved PARP were detected in rAdHCV-infected PMoHs (Fig. 4a).
Inhibition of HCV-induced hepatocyte cell death

In this study, we have demonstrated that HCV is associated directly with apoptosis in isolated hepatocytes. This was
treatment with Q-VD-OPh by 40 % (P<0.007 for all three comparisons).

Inhibition of necroptosis improved cell viability reduced by HCV, but did not reverse apoptosis induced by HCV

Given the partial effect of Q-VD-OPh in reversing HCV-induced apoptosis, we wanted to determine if infected hepatocytes might undergo programmed necrosis (necroptosis) – an alternate form of cell death. Huh7 cells co-infected with rAdHCV, in the presence or absence of TNF, were treated with necrostatin-1, a RIPK1 inhibitor that blocks necroptosis, or DMSO as a control. Remarkably, treatment with necrostatin-1 prevented cell death of rAdHCV co-infected Huh7 cells, whether or not they were treated with TNF (Fig. 6), and the level of protection was greater than that seen with the caspase inhibitor.

DISCUSSION

In this study, we have demonstrated that HCV is associated directly with apoptosis in isolated hepatocytes. This was

Inhibition of apoptosis improved cell viability reduced by HCV and reversed apoptosis induced by HCV

Treatment of Huh7 cells co-infected with rAdHCV-CoreE1E2 and rAdHCV-NS35B with the pan-caspase inhibitor Q-VD-OPh improved cell viability by 40 % (P=0.017) (Fig. 5a). A similar improvement in cell viability by blocking caspases was also seen following treatment of rAdHCV-infected PMoHs with Q-VD-OPh, indicating that HCV-induced cell death of hepatocytes is apoptotic (Fig. 5b). In vivo infected hepatocytes are likely to be subjected to inflammatory cytokines such as TNF. To test whether HCV-infected cells could be affected by such an environment, we treated rAdHCV-infected cells with TNF. Intriguingly, although TNF does not usually kill cells, rAdHCV co-infected Huh7 cells were sensitized to TNF-induced cell death and this death was blocked by Q-VD-OPh.

To confirm a role for apoptosis, we looked for cleaved PARP as a molecular marker in hepatocytes infected with rAdHCV. rAdHCV-co-infected Huh7 cells, in the presence or absence of TNF, were treated with Q-VD-OPh and compared with hepatocytes infected with rAdGFP control virus. The levels of cleaved PARP in Huh7 cells infected with rAdHCV were reduced following treatment with Q-VD-OPh (Fig. 5c). In rAdHCV-co-infected Huh7 cells, Q-VD-OPh treatment reduced cleaved PARP levels 9.4-fold (P=0.003). For rAdHCV-infected Huh7 cells sensitized with TNF, Q-VD-OPh treatment reduced cleaved PARP levels 10.6-fold (P=0.002). Similar results were seen in PMoHs infected with rAdHCV (Fig. 5d), with levels of cleaved caspase-3 reduced by 2.7-fold (P=0.001) and cleaved PARP reduced by 5.2-fold (P=0.0003) by Q-VD-OPh. Thus, treatment with Q-VD-OPh reduced, but did not completely block, HCV-associated hepatocyte cell death even following treatment with TNF.

Cleaved caspase-3 levels were increased 2.0- (±0.05), 2.1- (±0.08) and 2.4-fold (±0.15) with rAdHCV-CoreE1E2, rAdHCV-NS3-5B and rAdHCV co-infection, respectively, compared with rAdGFP-infected cells (P≤0.001), whilst in rAdHCV-CoreE1E2-infected, rAdHCV-NS3-5B-infected and co-infected PMoHs, cleaved PARP levels were raised 1.8- (±0.08), 1.8- (±0.05) and 1.8-fold (±0.04) at 48 h post-infection, respectively, compared with rAdGFP infection (P≤0.007 for all three comparisons).

In PHHs, cleaved caspase-3 levels were increased 1.5- (±0.38), 1.9- (±0.02) and 1.8-fold (±0.15) at 48 h post-infection with rAdHCV-CoreE1E2, rAdHCV-NS3-5B and co-infection, respectively, compared with rAdGFP-infected cells (P≤0.047 for all three comparisons) (Fig. 4b), whilst cleaved PARP levels were increased 1.6- (±0.12), 2.0- (±0.23) and 1.7-fold (±0.17), respectively, again significantly greater than the effect of rAdGFP infection (P≤0.01 for all three comparisons).

These data showed that HCV infection alone was sufficient to induce apoptosis in both transformed and primary hepatocytes, and no differences were seen between the structural and non-structural proteins.

Fig. 2. rAdHCV infection reduces hepatocyte viability. (a) Percentage reduction in cell viability from crystal violet assays of Huh7 cells infected with rAdGFP, rAdHCV-CoreE1E2 (CoreE1E2) and/or rAdHCV-NS3-5B (NS3-5B), at 24 h p.i., compared with mock. (b) Representative crystal violet plates showing uninfected (mock) PMoHs and the effect of rAdHCV infection. (c) Percentage reduction in cell viability from crystal violet assays of PMoHs infected with rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, at 24–72 h p.i., compared with mock. Each column represents the mean ± SEM of three experiments.

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**Fig. 3.** rAdHCV infection induces cleaved PARP in Huh7 cells. (a) Western blots of cleaved (cl.) PARP levels in Huh7 cells infected with rAdGFP, rAdHCV-CoreE1E2 and/or rAdHCV-NS3-5B. Graphs show fold increase in cleaved PARP levels relative to rAdGFP control. Each column represents the mean±SEM of three independent experiments. (b) Photomicrographs show Huh7 cells infected with rAdGFP, rAdHCV-CoreE1E2 (CoreE1E2) and/or rAdHCV-NS3-5B (NS3-5B) stained for cleaved PARP via immunocytochemistry at 72 h p.i. Representative cells stained with cleaved PARP are highlighted with arrows. Graph shows the fold increase in number of Huh7 cells staining positive for cleaved PARP per high-power field compared with rAdGFP. Each column represents the mean±SEM of three high-power fields.
observed in hepatocytes in liver sections of HCV-infected individuals, and supported by analyses in primary hepatocytes in which both the structural and non-structural proteins of HCV were able to induce hepatocyte cell death. Furthermore, the effect of HCV on hepatocyte cell death could be prevented in vitro by inhibitors of apoptosis and necroptosis.

Enhanced apoptosis has been shown to be a common feature of HCV-associated liver damage (Bantel et al., 2004; Takehara et al., 2004). In our analysis of human liver sections, we confirmed this by showing that patients with chronic HCV infection and cirrhosis have increased levels of the apoptotic markers cleaved cytokeratin 18 (M30 CytoDEATH) and cleaved PARP in their livers compared with livers from HCV-negative patients with and without cirrhosis. Although the mechanisms underlying hepatocyte cell death in an intact liver are multifactorial, the direct effect of the HCV polyproteins alone in producing hepatocyte apoptosis is likely to be of importance. Understanding the mechanism of how HCV induces hepatocyte cell death has been restricted by the limited availability of suitable in vitro and in vivo models. Nevertheless, HCV proteins have been studied widely in cell culture, and varying degrees of pro- and anti-apoptotic activities have been attributed to individual HCV proteins (Prikhod’ko et al., 2004; Saito et al., 2006). The HCV core protein has been shown to sensitize hepatocytes to TNF- and FasL-mediated apoptosis (Ruggieri et al., 1997). Core protein also induces oxidative stress, enhances mitochondrial-mediated hepatocyte apoptosis and upregulates transforming growth factor-β (Taniguchi et al., 2004), thereby promoting apoptosis and fibrogenesis. Both the E1 and E2 glycoproteins of HCV have been shown to induce hepatocyte apoptosis (Chiu et al., 2006; Ciccaglione

Fig. 4. rAdHCV infection induces cleaved (cl.) caspase-3 and cleaved PARP in primary hepatocytes. Western blots for cleaved caspase-3 and cleaved PARP in (a) PMoHs and (b) PHHs infected with rAdGFP, rAdHCV-CoreE1E2 (CoreE1E2) and/or rAdHCV-NS3-5B (NS3-5B), at 48 h p.i. Mock, Uninfected controls. Graphs show fold increase in cleaved caspase-3 and cleaved PARP levels in rAdHCV-infected primary hepatocytes relative to rAdGFP infection. Each column represents the mean ± SEM of three experiments. P values are compared with rAdGFP infection.
Fig. 5. Q-VD-Oph treatment reduces cell death in rAdHCV-infected hepatocytes. (a) Percentage reduction in cell viability from crystal violet assays of Huh7 cells infected with rAdGFP or rAdHCV-CoreE1E2 and rAdHCV-NS3-5B ± TNF, treated with Q-VD-Oph, at 24–48 h p.i., compared with mock. Co-inf., Co-infected. (b) Percentage decrease in cell viability from crystal violet assays of PMoHs co-infected with rAdHCV-CoreE1E2 and rAdHCV-NS3-5B and treated with Q-VD-Oph (QVD) compared with mock. (c) Western blots of cleaved (cl.) PARP in Huh7 cells infected with rAdGFP or rAdHCV-CoreE1E2 and rAdHCV-NS3-5B ± TNF, treated with Q-VD-Oph. Graph shows fold change in cleaved PARP levels in infected Huh7 cells treated with Q-VD-Oph relative to mock. Each column represents the mean + SEM of three experiments. (d) Western blots for cleaved caspase-3 and cleaved PARP in PMoHs infected with rAdHCV-CoreE1E2 and rAdHCV-NS3-5B treated with Q-VD-Oph at...
et al., 2003). These multifaceted effects of the individual HCV structural proteins on apoptosis support our findings of increased cell death in hepatocytes expressing the complete core–E1–E2 polyprotein.

Studies of the non-structural proteins have shown that the accumulation of NS4A on mitochondria stimulates mitochondrial-mediated apoptosis (Nomura-Takigawa et al., 2006). Similarly, the HCV NS3 protease can induce apoptosis in a caspase-8-dependent manner (Prikhod’ko et al., 2004). These findings are difficult to relate to the events that occur in natural infection as they focused predominantly on the overexpression of single viral proteins. Lan et al. (2008) reported sensitization of hepatocytes to TRAIL-induced apoptosis in an HCVcc infection model and attributed this effect to the non-structural proteins as key mediators of apoptosis. These investigators were also only able to show increased apoptosis in primary hepatocytes following sensitization of cells with TRAIL rather than a direct effect of the viral proteins or of HCVcc alone.

In contrast to these studies, we have investigated the effects of the complete structural and non-structural proteins of HCV, and demonstrated that both resulted in increased cell death in PMoHs and PHHs. By using a recombinant replication-defective adenoviral system, we were able to express the complete HCV structural and non-structural proteins separately or together in primary hepatocytes. In doing this, we were able to study any direct and differential effects of the structural compared with non-structural viral proteins. We have shown that HCV is associated directly with increased levels of hepatocyte apoptosis. Our findings are consistent with previous reports that have shown an association between hepatocyte apoptosis and liver inflammation and fibrosis in liver biopsy specimens from patients with chronic HCV (Bantel et al., 2004; Pianko et al., 2001) and in vitro models of HCV infection (Deng et al., 2008; Lan et al., 2008), and also expand on these studies by demonstrating a direct effect of HCV on isolated primary hepatocytes.

We also studied the effect of the pan-caspase inhibitor Q-VD-OPh. HCV-induced hepatocyte cell death was reduced, but not completely blocked by Q-VD-OPh, suggesting that other forms of cell death contribute to the observed effects in the setting of infection with HCV. We showed that necrostatin-1, a potent inhibitor of RIPK1, also reduced HCV-induced hepatocyte death, suggesting that necroptosis plays a role HCV-associated hepatocyte death (Declercq et al., 2009; Vandenabeele et al., 2010). The involvement of necroptotic cell death could possibly result in a greater level of liver inflammation and injury than if cell death were to occur via apoptosis alone (Vandenabeele et al., 2010).

Given the important role of TNF-induced hepatocyte apoptosis in liver injury, we investigated whether HCV-associated hepatocyte cell death could be reversed by Q-VD-OPh or necrostatin-1 in the presence of TNF. We demonstrated that HCV-infected hepatocytes were significantly sensitized to TNF-associated cell death. However,

Fig. 6. Necrostatin-1 treatment reduces cell death in rAdHCV-infected hepatocytes. Percentage reduction in cell viability from crystal violet assays of Huh7 cells infected with rAdGFP or rAdHCV-CoreE1E2 and rAdHCV-NS3-5B ± TNF, treated with necrostatin-1, at 24–48 h p.i., compared with mock. Each column represents the mean ± SEM of three experiments. Co-inf., Co-infection.
treatment of hepatocytes with either Q-VD-OPh or necrostatin-1 significantly improved cell viability and apoptosis was reduced by Q-VD-OPh.

The finding that HCV-associated hepatocyte cell death can be inhibited is significant as this provides important insights into alternative therapeutic strategies for liver disease in chronic HCV. Although direct antiviral therapies have been shown to be effective in treating chronic HCV infection, many patients with advanced liver disease fail antiviral therapy, and there may therefore be a role for the development of adjunctive therapies to prevent and reverse liver fibrosis. Caspase inhibitors have been developed for the treatment of human diseases as they reduce apoptosis and attenuate liver fibrosis in preclinical models (Canbay et al., 2004; Shiffman et al., 2010; Witek et al., 2009). TNF-mediated NFκB activation can also be prevented by SMAC mimetics, small molecules designed to inhibit inhibitor of apoptosis protein (IAP) family proteins XIAP, cIAP-1 and cIAP-2 (Petersen et al., 2007; Varfolomeev et al., 2007). Although such agents are in the relatively early stages of development, they hold promise as useful adjunctive therapies for chronic HCV infection.

In conclusion, we observed increased apoptosis within the liver tissue of HCV-infected patients compared with HCV-negative patients. In our cell culture system, we showed that infection with rAdHCV increased cell death and enhanced apoptosis in immortalized human hepatocytes (Huh7 cells), PMoHs and PHHs. We observed partial reversal of rAdHCV-induced cell death with Q-VD-OPh and improved cell viability with necrostatin-1, even in the setting of TNF-α stimulation, suggesting a novel therapeutic approach to reduce liver injury during HCV infection.

**METHODS**

**Recombinant viruses.** The HCV core–E1–E2 and NS3–NS5B genomes were amplified from pBRTM_HCV 1-3011con containing the genome of HCV H77 genotype 1a (a gift from Professor C. Rice, Rockefeller University, New York, USA) using primers listed in Table S1 (available in the online Supplementary Material). The construction of pAd-CoreE1E2 has been described previously (Chua et al., 2012). To produce recombinant adenovirus expressing the non-structural genes of HCV, the NS3–NS4B and NS4B–NS5B genomes were first amplified by PCR followed by subcloning into pShuttle (Strategene). Plasmids were then digested with XbaI/FspI and FspI/AflII, respectively, and ligated to generate the NS3–3B genome in pShuttle. The sequence was verified by DNA sequencing. Recombinant pAdTrack-CMV-HCV-NS3-5B was then generated and transformed into AdEasier-1 cells to produce pAd-HCV-NS3-5B.

The rAdHCV-CoreE1E2 and rAdHCV-NS3-5B virions were produced by transfection in 293T cells with pAd-CoreE1E2 and pAd-HCV-NS3-5B DNA followed by serial passaging in cell culture using methods described previously (Chua et al., 2012). Recombinant adenovirus expressing GFP alone (rAdGFP) was produced in the same manner to serve as a control virus. The viral titres were determined by infecting Huh7 cells with serial dilutions of viral stock and determining the percentage of GFP-expressing units by flow cytometry (FACSCanto II; Becton Dickinson). Entry of recombinant adenoviruses into hepatocytes and production of HCV RNA and proteins were confirmed by GFP expression, and Northern and Western blots (Fig. 7).

**Cells and culture.** Huh7 and 293T cells were grown in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10 % FBS and 50 μg penicillin/streptomycin ml⁻¹ (Invitrogen) at 37 °C in 5 % (v/v) CO₂.

**Preparation of PMoHs.** PMoHs were isolated from ≤12-week-old C57BL/6 mice, as described previously (Jost et al., 2009). In brief, PMoHs were extracted via ex vivo perfusion of the left liver lobe with Ca²⁺/Mg²⁺-free HEPES buffer (Invitrogen), followed by HEPES containing 500 mg collagenase IV 1 U⁻¹ (Sigma-Aldrich). Hepatocytes were separated on a 45 % Percoll density gradient (Sigma-Aldrich) and seeded at a density of 50 000 cells cm⁻² in cell culture plates coated with rat-tail collagen at 0.6 mg ml⁻¹ (Sigma-Aldrich). PMoHs were cultured overnight in complete Williams E medium (Invitrogen) containing 10 % FBS, 1 % glutamine (Sigma-Aldrich), 50 μg penicillin/streptomycin ml⁻¹ (Invitrogen) and 1:1000 gentamicin (Invitrogen). Prior to infection with recombinant adenoviruses, non-adherent cells were washed off and adherent cells incubated with complete Williams E medium supplemented with 1 % HEPES pH 7.4 (Invitrogen), 0.1 % gentamicin (Invitrogen), 1 % glutamine (Invitrogen), 1 % linoleic acid (Sigma-Aldrich), 1 % epidermal growth factor (BD Biosciences), 0.1 % ITS liquid media supplement (Sigma-Aldrich), 0.1 % insulin (Sigma-Aldrich), 0.01 % dexamethasone (Sigma-Aldrich) and 0.01 % ethanalamine (Sigma-Aldrich).

**Preparation of PHHs.** Cryopreserved PHHs were obtained from Lonza. Hepatocytes were seeded into 12-well plates coated with rat-tail type 1 collagen at 0.6 mg ml⁻¹ (Becton Dickinson) at a density of 150 000 cells cm⁻² using Hepatocyte Basal Medium (Lonza) supplemented with the contents of the Hepatocyte Culture Medium SingleQuots BulletKit (Lonza) and 2 % FBS, according to the manufacturer’s instructions. Three hours after plating, the media was replaced with fresh, pre-warmed Hepatocyte Basal Medium supplemented with Hepatocyte Culture Medium SingleQuots BulletKit without FBS and incubated at 37 °C in 5 % (v/v) CO₂.

**Hepatocyte infections.** Hepatocytes were infected with the rAdHCV viruses, rAdHCV-CoreE1E2, rAdHCV-NS3-5B or rAdGFP at m.o.i. 1.0. For co-infection with both rAdHCV-CoreE1E2 and rAdHCV-NS3-5B (rAdHCV co-infection), m.o.i. 0.5 for each rAdHCV-CoreE1E2 and rAdHCV-NS3-5B was used. PMoHs, PHHs and Huh7 cells were infected at 6, 15 and 18 h, respectively. In experiments examining inhibition of apoptosis or necroptosis, cells were treated at the time of viral infection with 50 μM Q-VD-OPh (R&D Systems) or 50 μM necrostatin-1 (Sigma-Aldrich), respectively. Controls were treated with 0.5 % DMSO. TNF (Sigma-Aldrich) at 10 ng ml⁻¹ was added where indicated to hypersensitize hepatocytes to apoptosis. All experiments were performed in triplicate.

**Western blot detection of HCV and cellular proteins.** Huh7 cells were harvested at 12, 24, 48 and 72 h p.i. in 200 μl ice-cold cell lysis buffer (Cell Signaling Technology) supplemented with 1 mM sodium molybdate, 5 mM sodium fluoride, 1 M DTT (Sigma-Aldrich) and 1 × Complete Protease Inhibitor (Roche). For PHHs and PMoHs, cells were harvested at 48 h p.i. Total cytoplasmic proteins (30 μg) were resolved by 12 % denaturing SDS-PAGE gel, transferred into Hybond-C Extra membrane (GE Healthcare) and analysed by Western blotting. HCV proteins were detected using anti-NS3 (NovoCastra), anti-NS5a (Abcam) and anti-core (Dianova) antibodies, as well as mAbs A4 (anti-E1) and H52 (anti-E2) (gifts from Professor Jean Dubuisson, Institut Pasteur, France). Induction of apoptosis was detected using anti-caspase-3 (Cell Signaling Technology) and anti-cleaved PARP antibody (Abcam), Anti-pan-actin...
was used as a loading control. Western blots were analysed with the Bio-Rad GS800 densitometer and proteins of interest were corrected for background activation induced by the rAdGFP control using Quantity One software.

**Cell viability assays.** Cell viability following mono- or co-infection with rAdHCV-CoreE1E2 and rAdHCV-NS3-5B, and following treatment with TNF, Q-VD-OPh and necrostatin-1, was determined by crystal violet staining. Huh7 cells and PMoHs (2.5 × 10^5 cells per well) were seeded in 12-well plates and infected with rAdHCV-CoreE1E2, rAdHCV-NS3-5B or rAdGFP in the presence or absence of TNF, Q-VD-OPh or necrostatin-1. At 24, 48, 72 and 96 h post-treatment, wells were washed twice with sterile PBS, and the remaining adherent viable cells on the plate were fixed and stained with 0.1% crystal violet in 1 M citric acid containing 20% methanol for 20 min at room temperature. Wells were washed thoroughly with sterile PBS to remove excess crystal violet and then air-dried. Bound dye was solubilized with 100 μl 100% DMSO for 20 min and the A_544 of the supernatants was determined using the FluoStar Optima (BMG LabTech) plate reader. Statistical analyses were performed using Prism 5.0 (GraphPad).

**Immunocytochemistry.** Sterile glass coverslips were placed in six-well plates. Wells were then seeded with Huh7 cells at a density of 3.5 × 10^5 cells per well and incubated overnight at 37 °C in 5% CO_2_. Cells were then infected with rAdHCV-CoreE1E2 and/or rAdHCV-NS3-5B, or rAdGFP as control. At 72 h p.i., cells were washed twice with sterile PBS, then fixed with ice-cold acetone for 5 min and air-dried at room temperature. Coverslips were blocked with 5% normal goat serum in PBS for 30 min and washed three times with PBS. The cells were stained overnight at 4 °C in a humidified chamber for cleaved PARP using a rabbit anti-PARP antibody (1:50; Cell Signaling Technology). Coverslips were washed three times with PBS and incubated with goat anti-rabbit polyclonal antibody (1:500; Dako) for 1 h at room temperature. Coverslips were washed three times in PBS before incubation for 30 min at room temperature with avidin–biotin Vectastain ABC (Vector). Coverslips were washed in PBS followed by the addition of 3,3′-diaminobenzidine (DAB; Sigma-Aldrich) and counterstaining with haematoxylin before mounting onto glass slides using DPX mountant (Fluka).

**Immunohistochemistry of human liver specimens.** Human liver tissue was stained for the markers of apoptosis: cleaved cytokeratin 18 (M30 CytoDEATH; Enzo Life Sciences) and cleaved PARP (Cell Signaling Technology). Immunohistochemistry was performed as described previously (Pazios et al., 2005). In brief, 4 μm sections of paraffin-embedded human liver tissue mounted on silane-coated glass slides were de-paraffinized in histolene and dehydrated in graded ethanol. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS. Non-specific proteins were blocked with Protein Block Serum-free (DakoCytomation) for 30 min at room temperature. Blocks were incubated overnight at 4 °C with either M30 CytoDEATH or cleaved PARP antibody, 1:100 in diluent as directed by the manufacturer. The following day, sections were incubated with their respective biotinylated-conjugated secondary antibody (1:200) for 1 h at room temperature, followed by incubation with the avidin–biotin Vectastain ABC system (Vector Laboratories) for 30 min. DAB (Sigma-Aldrich) was then added as a

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**Fig. 7.** Expression of HCV structural and non-structural proteins. (a) Huh7 cells infected with rAdHCV-CoreE1E2 or rAdHCV-NS3-5B were viewed under a fluorescence microscope. Infected cells expressed the reporter GFP. (b) Northern blot analysis of RNA extracted from Huh7 cells infected with rAdHCV-NS3-5B or showing HCV-specific mRNA using HCV NS3-, NS5A- and NS5B-specific [ 32P]cDNA probes. (c, d) Western blots of infected Huh7 cells, showing expression of HCV core, E1 and E2 proteins with rAdHCV-CoreE1E2 infection (c), and expression of NS3 and NS5A proteins with rAdHCV-NS3-5B infection (d).
chromogen and sections counterstained in haematoxylin. The relative staining in each group was assessed by computerized image capture quantification using MCID Analysis software (InterFocus Imaging) and the results expressed as the mean proportional area (i.e. the proportion of cells staining positive in the given area).

**Ethics statement.** This study was carried out in strict accordance with the recommendations in the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes. The PMoH protocol was approved by the Animal Ethics Committee of La Trobe University (permit 09-14 B). The study using human liver tissue was approved by the Austin Health Human Research Ethics Committee (HREC approval H2010/03979).

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**REFERENCES**


