Structural proteins of *Hepatitis C virus* induce interleukin 8 production and apoptosis in human endothelial cells

Anuradha Balasubramanian,1 Neru Munshi,1 Margaret J. Koziel,2 Zongyi Hu,3 T. Jake Liang,3 Jerome E. Groopman1† and Ramesh K. Ganju†

†These authors contributed equally to this work.

Hepatitis C virus (HCV) infection is associated with inflammation of liver endothelium, which contributes to the pathogenesis of chronic hepatitis. The mechanism of this endothelitis is not understood, since the virus does not appear to infect endothelial cells productively. Here, an ‘innocent bystander’ mechanism related to HCV proteins was hypothesized and it was investigated whether the binding of HCV particles to human endothelium induced functional changes in the cells. Exposure of human umbilical vein endothelial cells (HUVECs) to HCV-like particles (HCV-LPs) resulted in increased interleukin 8 (IL8) production and induction of apoptosis. The IL8 supernatants collected after stimulation of HUVECs with HCV-LPs, BV-GUS (control baculovirus containing β-glucuronidase) and appropriate controls were used to assay the transendothelial migration of neutrophils. This assay confirmed that HCV-LP-induced IL8 was functionally active.

Using specific NF-κB inhibitors, it was also shown that HCV-LP-induced NF-κB activity mediated IL8 production in HUVECs. Apoptosis appeared to be mediated by the Fas/Fas-L pathway, as neutralizing antibodies for Fas and Fas-L significantly protected HUVECs against HCV-LP-induced apoptosis. Treatment of HUVECs with HCV-LPs also enhanced cellular Fas-L expression and augmented caspase-3 activation. This was confirmed by using a specific caspase-3 inhibitor, Z-Asp-Glu-Val-Asp-fluoromethyl ketone. As shown by blocking of specific chemokine receptors for IL8 on HUVECs, the induction of IL8 did not appear to contribute to HCV-LP-induced apoptosis. These results suggest that HCV proteins can trigger the release of inflammatory chemokines such as IL8 and cause endothelial apoptosis, thereby facilitating endothelitis.

INTRODUCTION

*Hepatitis C virus* (HCV) is a major cause of end-stage liver disease (Hoofnagle, 1997). Persistent infection can lead to chronic hepatitis complicated by cirrhosis and/or carcinoma (Saito et al., 1990; Tong et al., 1995; Okuda, 1997; Trepo et al., 1998; Ohsawa et al., 1999). HCV belongs to the family *Flaviviridae* (Choo et al., 1989; Kato et al., 1990) and its RNA genome encodes a long polyprotein. Proteolytic processing of this polyprotein produces 10 different proteins. These include three HCV structural proteins, the core protein and the two envelope glycoproteins, E1 and E2 (Miyamura & Matsuura, 1993). The core protein has been reported to modulate immune functions. It can repress the transcriptional activity of various genes including the p53 promoter in vitro (Kim et al., 1994; Ray et al., 1995, 1997) and has been implicated in Fas-mediated apoptosis both in vitro and in vivo (Ruggieri et al., 1997).

HCV has the ability to persist, despite a strong immune response. Chronic infection may result in the development of cirrhosis. A more vigorous inflammatory response is associated with a more rapid progression to cirrhosis (Poynard et al., 1997). Characteristic pathological changes associated with HCV include lymphoid nodules, steatosis, subendothelial inflammation of portal and/or terminal hepatic veins and endothelial cell damage (Lory & Zimmermann, 1997). The cause of this endothelial pathology is not yet defined. One hypothesis suggests that there is a release of chemotactic cytokines that recruit specific lymphocytes to the liver and enhance inflammatory leukocyte–endothelial cell interactions (Mackay, 1996; Adams & Lloyd, 1997; Luster, 1998; Shields et al., 1999).
We examined the possibility that HCV structural proteins might be toxic to endothelial cells independent of direct viral infection, a so-called 'innocent bystander' effect. Such a 'bystander effect' is believed to be important in the pathophysiology of human immunodeficiency virus (HIV) infection (Meyaard et al., 1992; Groux et al., 1992; Ameisen et al., 1995; Oyaizu et al., 1997). HIV and its envelope protein gp120 can induce the apoptosis of bystander CD4+ cells via binding to specific chemokine co-receptors (Banda et al., 1992). Recently, gp120 has also been shown to interact with endothelium via the CXCR4 receptor and cause endothelial cell dysfunction (Huang et al., 1999). To investigate a possible 'innocent bystander' mechanism of HCV-related endothelitis, we exposed endothelial cells to HCV particles containing structural proteins expressed in a baculovirus system. The particles contained the nucleocapsid or core protein, and the two envelope glycoproteins, E1 and E2 (Baumert et al., 1999, 1999). Exposure of human umbilical vein endothelial cells (HUVECs) to these HCV-like particles (HCV-LPs) caused the release of the inflammatory chemokine interleukin 8 (IL8) via NF-κB and triggered Fas-mediated apoptosis. Further studies revealed that these particles induced the upregulation of the Fas ligand (Fas-L) and activated caspase-3.

Our results indicate that HCV proteins can interact with endothelium and cause the release of inflammatory mediators, as well as induce programmed cell death. Such events may contribute to the pathological finding of endothelitis seen in HCV hepatitis.

**METHODS**

**Cells and cell culture.** HUVECs were grown at 37°C in 5% CO2 in endothelial cell growth medium (Clonetics) containing 2% fetal bovine serum (FBS), 12 μg bovine brain extract ml⁻¹, 10 ng human recombinant epidermal growth factor ml⁻¹, 1 μg hydrocortisone ml⁻¹ and GA-1000 (gentamicin and amphotericin B, 1 μg ml⁻¹), according to the recommendation of the supplier (Clonetics).

**Baculovirus constructs.** Recombinant HCV structural proteins (HCV-LPs) and the control baculovirus preparation were produced as described (Baumert et al., 1998). Briefly, cDNA for the HCV structural proteins (from HCV-1 strain, genotype 1b) was used to generate the recombinant baculovirus HCV particles (HCV-LPs). pFastBacHCV (which contains the coding sequences for the core, E1 and E2 proteins) was generated by PCR with the following primers: 5’-GAGACACAGCTGGGTACGAGAGAGAGGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGAGA
HCV proteins induce apoptosis in endothelial cells

Cell death detection kit (Boehringer Mannheim). Briefly, 1 × 10⁴ cells were plated in a 96-well plate and grown to 90% confluence. The cells were placed in low-serum medium (0.5% FBS) for 2 h and subsequently left untreated or treated with varying concentrations of HCV-LPs or the control BV-GUS for 24 h at 37°C. The cells were then lysed and centrifuged at 200 g to separate the cytoplasmic and nuclear fractions. Twenty microlitres of supernatant was added to a streptavidin-coated microtitre plate. Biotin-labelled anti-histone antibody was then added, followed by horseradish peroxidase-conjugated anti-DNA antibody. The increase in nucleosome degradation was calculated by comparing the values from the HCV-LP or BV-GUS cultures with that of cells in 0.5% FBS. Statistical analysis was done using Student’s t-test.

TdT-mediated dUTP nick-end labelling (TUNEL). The level of chromatin cleavage due to apoptosis was quantified using the Flourescein In situ Cell Death Detection kit (Boehringer Mannheim). Briefly, cells were plated in chamber slides (Nalge Nunc International) and then cultured in low-serum medium with or without varying concentrations of HCV-LPs or BV-GUS for 24 h. After 24 h, the medium was aspirated off and the cells washed with 1 × PBS. The air-dried cells were fixed with a 4% paraformaldehyde solution for 1 h at room temperature. Cells were then washed with PBS and incubated in permeabilization solution (0.1% Triton X-100, 0.1% sodium citrate) for 2 min on ice. Slides were rinsed twice with PBS and 50 μl of TUNEL reaction mixture was added to each sample. Samples were then incubated at 37°C in the dark for 60 min. After washing the slides again with PBS, the samples were directly analysed under a fluorescence microscope.

Western blotting. Whole-cell lysates from the HCV-LP- or BV-GUS-treated cells were prepared by lysing the cells in RIPA buffer (50 mM Tris/HCl pH 7.4, 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM PMSF, 10 μg each aprotinin, leupeptin and pepstatin ml⁻¹, 10 mM sodium vanadate, 10 mM sodium fluoride and 10 mM sodium pyrophosphate). Proteins were separated by 10% SDS-PAGE and transferred onto nitrocellulose membranes. Membranes were blocked with 5% non-fat milk for 2–3 h and then incubated with the respective primary and secondary antibodies for 2–3 h. Membranes were washed three to four times and developed by chemiluminescence (ECL system; Amersham Pharmacia Biotech).

Inhibition studies. For studies with NF-κB inhibitors, various concentrations of Bay 11-7082 and/or SN50 were added to the culture medium 1 h prior to stimulation with 1000 ng HCV-LP, control BV-GUS or LPS ml⁻¹. After 24 h of stimulation, the supernatants were used for estimation of IL8 production as described above.

Cells were treated with the Fas antagonistic ZB4 antibody or Fas-L antagonist NOX-1 antibody or their respective isotype-matched control antibodies. Antibodies were applied 2 h before the HCV-LP treatment and cell death was evaluated by sandwich ELISA.

Z-FAD-FMK, a cell-permeable general caspase inhibitor, or Z-DEVD-FMK, a specific caspase-3 inhibitor, was added to the cells at a 20 μM concentration, 2 h prior to treatment with HCV-LPs. Z-Phe-Ala-fluoromethyl ketone (Z-FA-FMK) at the same concentration was used as an inhibitor control. Cells were incubated with virus particles and the caspase inhibitors for 24 h. Apoptosis was measured by ELISA.

HUVECs were placed in low-serum medium alone or containing varying concentrations of either CXCR-1 (IL8RA)- or CXCR-2 (IL8RB)-neutralizing antibodies for 4 h. Cells were then washed and stimulated with 1000 ng HCV-LP or control BV-GUS ml⁻¹ for 24 h. After 24 h, cells were lysed as described elsewhere and subjected to Western blot analysis to check for the expression of Fas-L.

RESULTS

HCV-LP treatment of HUVECs leads to increased IL8 production

When HUVECs were exposed to HCV-LPs, increased production of the inflammatory chemokine IL8 was observed. As shown in Fig. 1(a), the IL8 level was about 2.5-fold higher in the HCV-LP-treated sample at 24 h (P < 0.023) than with either the medium control or the BV-GUS-treated HUVECs. HCV particles did not induce the production of other inflammatory cytokines such as TNF-α, IFN-γ or MCP-1 (data not shown). To determine whether IL8 induced by HCV-LPs was functionally active, the transendothelial migration of neutrophils was assessed (Fig. 1b). IL8 supernatants collected from HUVECs after stimulation with BV-GUS, HCV-LP, LPS, heat-inactivated BV-GUS or heat-inactivated HCV-LPs, along with the unstimulated control, were used for the assay. A 2.5-fold increase in the transmigration of neutrophils was observed in the presence of the HCV-LP-induced IL8 supernatant when compared with supernatant from the unstimulated control.

HCV-LPs induce NF-κB activity

NF-κB has been shown to activate various inflammatory genes. It is also widely known that IL8 gene expression is regulated by NF-κB and/or AP-1 transcription factors. HUVECs that were stimulated with HCV-LPs induced NF-κB activity. As shown in Fig. 2, when HUVECs transfected with the pNF-κB-Luc vector did not induce NF-κB activity. The reporter assay further showed that HUVECs transfected with the pTAL-Luc vector (control vector) did not induce NF-κB activity.

NF-κB mediates HCV-LP-induced IL8

To confirm that the NF-κB pathway was involved in the HCV-LP-induced production of IL8, HUVECs were stimulated with HCV-LPs in the presence of different inhibitors of NF-κB along with appropriate controls, and the supernatants were then used for IL8 estimation. HCV-LP-induced IL8 was reduced significantly (1.5-fold) in the presence of 10 μM Bay 11-7082, an inhibitor of cytokine-induced IκBα phosphorylation (Fig. 3a). A gradual decrease in the HCV-LP-induced production of IL8 was observed when increasing concentrations of the inhibitor were used. Similarly, in the presence of 10 μg SN50 ml⁻¹, a cell-permeable inhibitory peptide that blocks nuclear translocation of the NF-κB active complex, a 1.5-fold decrease in IL8 expression induced by HCV-LPs was noticed (Fig. 3b). A gradual reduction in the amount of IL8 induced

http://vir.sgmjournals.org

Downloaded from www.microbiologyresearch.org by IP: 54.70.40.11
On: Mon, 29 Jul 2019 16:13:09
by HCV-LPs was observed with increasing concentrations of SN50. In parallel, there was no obvious change in the amount of HCV-LP-induced IL8 in the presence of SN50M, a cell-permeable control peptide. These results clearly suggest that the NF-κB pathway might be involved in the induction of IL8 by HCV-LPs.

**HCV-LPs induce apoptosis in HUVECs**

HCV-LP treatment of HUVECs resulted in an approximate 2.5-fold ($P<0.0005$) increase in apoptosis over BV-GUS-treated or untreated HUVECs as measured by the histone ELISA method. As shown in Fig. 4(a), the degree of apoptosis at 24 h was found to be dose-dependent with maximal effect at a concentration of 1000 ng HCV-LPs ml$^{-1}$. The degree of apoptosis after treatment of HUVECs with the control baculovirus BV-GUS was not significantly different from the untreated HUVEC cultures over the same time periods. The induction of apoptosis measured by ELISA was confirmed using the TUNEL method. The positively stained cells, as shown in Fig. 4(b), were counted to quantitate the degree of apoptosis. About 68% of the cells were found to be TUNEL positive after HCV-LP treatment (1000 ng ml$^{-1}$) (Fig. 4b, panel v), while 32% of the cells were TUNEL positive after treatment with a similar concentration of BV-GUS (Fig. 4b, panel iv). Untreated HUVECs showed ~28% TUNEL-positive cells (Fig. 4b, panel i, control). A similar result was observed at a lower concentration (100 ng ml$^{-1}$) of HCV-LPs (Fig. 4b, panel iii) (35% TUNEL positive). HUVECs treated with 100 ng of the control baculovirus ml$^{-1}$ showed ~28% TUNEL positive staining (Fig. 4b, panel ii). These data indicate that HCV-LP treatment of HUVECs results in the induction of apoptosis.
HCV particles induce Fas-mediated apoptosis in HUVECs

To explore further the mechanism of apoptosis in HUVECs, we considered the possible involvement of known apoptotic pathways such as Fas. To this end, HUVECs were treated with 1000 ng HCV-LPs or the control BV-GUS ml⁻¹ in low-serum medium. Immunoblot analysis (Fig. 5a) showed that HCV-LP treatment of cells caused an increase in the production of Fas-L. However, there was no change in Fas receptor expression over the same period of time under similar conditions (Fig. 5b). These data indicate that HCV-LPs may increase the susceptibility of HUVECs to Fas-mediated apoptosis by upregulating the expression of Fas-L. To establish further the involvement of the Fas/Fas-L pathway in HCV-LP-mediated endothelial apoptosis, we used the Fas-antagonistic antibody ZB4 and the Fas-L-antagonistic antibody NOK-1. HUVECs were treated with varying concentrations of either antibody or the isotype-matched control antibody for 2 h. Subsequently, HCV-LPs (1000 ng ml⁻¹) were added to the cells along with the respective antibodies for 24 h. Cell death was assessed by ELISA. A dose-response study showed that the maximum inhibitory effect of these antibodies occurred at 1000 ng ml⁻¹ (data not shown). Fig. 5(c) shows that HCV-LPs were unable to induce apoptosis in HUVECs in the presence of the ZB4 or NOK-1 antibody, suggesting that the Fas/Fas-L pathway may play a key role in HCV-induced endothelial cell death.
The effect of HCV-LPs on Fas-mediated apoptosis may be due to augmentation of caspase-3 activation

To assess which downstream mediators might be operative in this HCV-LP-induced apoptosis, we examined the activation of caspase-3, a primary activator of several key apoptosis target proteins. As shown in Fig. 6(a), at 24 h the expression of the cleaved active caspase-3 fragment (17 kDa) was higher in the HCV-LP-treated cells compared with that of either the untreated or control BV-GUS-treated cells. This clearly indicates that HCV-LP treatment caused the cleavage of procaspase-3. This enhanced enzyme activation suggests that HCV-LPs may induce caspase-3 as part of the Fas downstream signalling pathway. In order to
assess further the role of caspases in this apoptotic process, we used the cell-permeable inhibitors Z-VAD-FMK (a general caspase inhibitor) or Z-DEVD-FMK (a specific caspase-3 inhibitor), or DMSO as a diluent control. HUVECs were treated with HCV-LPs (1000 ng ml\(^{-1}\)) alone or in combination with the various inhibitors for 24 h. Apoptosis was measured based on the degree of nucleosome degradation by ELISA (Fig. 6b). We found a twofold reduction (\(P<0.0005\)) in apoptosis with either the general caspase inhibitor or the specific caspase-3 inhibitor compared with cells treated only with HCV-LPs. However, no inhibition was observed when cells were treated with the inhibitor control Z-FA-FMK in the presence of HCV-LPs under similar conditions.

**IL8 production does not mediate HCV-LP-induced apoptosis in HUVECs**

We next sought to determine whether IL8 participated in apoptotic induction in HUVECs. To this end, we pretreated HUVECs with neutralizing antibodies to the IL8-specific receptors CXCR-1 (IL8RA) and CXCR-2 (IL8RB). Fig. 7(a) and (b) show that at all three concentrations (10, 100 and 1000 ng ml\(^{-1}\)) of these antibodies, HCV-LPs were still able to induce the expression of Fas-L, suggesting that IL8 production was not responsible for the induction of HUVEC apoptosis.

**DISCUSSION**

Hepatitis C infection often results in damage in different cell types within the liver. Hepatocytes are susceptible to HCV infection. Peripheral blood mononuclear cells are also thought to be productively infected by HCV (Zignego et al., 1992; Lerat et al., 1996). However, endothelial cells do not appear to be directly infected with the virus. It has previously been suggested that chemokines regulate the adherence of lymphocytes to endothelium before extravasation from
blood into tissue (Campbell et al., 2003) by activating leukocytes through changes in integrin affinity/avidity (Baltus et al., 2003). This results in firm adhesion to the vessel wall and eventual transmigration of the lymphocyte through the endothelium and into the underlying tissue to sites of infection (Johnston & Butcher, 2002). Previous studies by others suggest that in HCV infection specific chemokine–chemokine receptor interactions are involved in the recruitment of lymphocytes into the liver via either portal or sinusoidal endothelium (Shields et al., 1999; Grant et al., 2002), but little is known about the signals that position and retain lymphocytes at epithelial surfaces within the liver (Heydtmann et al., 2001). The inflammatory reaction against HCV that is believed to contribute to hepatic pathology includes polyclonal B- and T-cell responses, some of which are HCV specific (Koziel et al., 1995). Non-specific recruitment of T cells into the inflamed liver may also occur (Unutmaz et al., 1994; Nuti et al., 1998).

Since endothelial cells do not appear to be susceptible to HCV infection, we hypothesized that the binding of the HCV particles rather than infection per se might contribute to endothelial damage. Using recombinant HCV particles obtained from the cDNA of the HCV structural proteins, we analysed the effects of structural proteins on HUVECs. In support of our theory that HCV-LPs bind to endothelial cells, earlier studies have indeed reported that HCV-LPs bind to liver sinusoidal endothelial cells (LSECs), a specialized endothelial cell type with antigen-presenting cell function (Ludwig et al., 2004). Ludwig et al. (2004) further reported that this interaction of HCV-LPs with the LSECs might be through the L-SIGN antigen receptor. L-SIGN is known to be expressed in LSECs (Bashirova et al., 2001; Pohlmann et al., 2001) and it has recently been demonstrated that the C-type lectins DC-SIGN and L-SIGN/DC-SIGNR may be involved in HCV binding through their interaction with the HCV envelope glycoprotein E2 (Gardner et al., 2003; Lozach et al., 2003; Pohlmann et al., 2003). It has been suggested that L-SIGN expressed in LSECs may capture HCV from the blood and mediate the infection of adjacent hepatocytes, the main target cells of HCV.

In our study, HCV-LPs induced IL8 production but not the production of other inflammatory cytokines such as MCP-1, TNF-α or IFN-γ. In prior immunohistochemical studies of HCV infection, IL8 was shown to be expressed in infiltrating cells in the portal tract and fibrotic septa and within hepatic lobules in patients (Napoli et al., 1994; Koziel et al., 1995). Moreover, it has been reported that in HCV patients there was a correlation between the serum IL8 levels and liver fibrosis (Kaplanski et al., 1997), and that intrahepatic mRNA IL8 expression was associated with hepatic inflammation and fibrosis (Fukada et al., 1996; Shimoda et al., 1998; Masumoto et al., 1998; Mahmood et al., 2002). We have also observed enhanced IL8 production upon HCV E2 and HIV gp120 treatment in hepatocytes (Balasubramanian et al., 2003). Transmigration of neutrophils across HUVECs in the presence of the HCV-LP-induced IL8 supernatants further confirmed the functional activity of IL8. While our results also showed that IL8 expression induced by HCV-LPs was mediated by the NF-κB pathway, we do not yet know the HCV-LP-induced mechanism of NF-κB activation.

Although in chronic liver inflammation due to HCV it has been reported that E-selectin, ICAM-1 and V-CAM-1 are strongly expressed on endothelial cells from sinusoidal vessels and may play an important role in leukocyte extravasation (Volpes et al., 1992), we did not find any significant change in the basal expression of the inflammatory adhesion molecules ICAM-1, VCAM-1, E-selectin and P-selectin when HUVECs were treated with HCV-LPs (data not shown). In addition, previous studies have shown that chemokines with chemotactic activity on leukocytes may also be important in the pathogenesis of chronic hepatitis (Baggiolini et al., 1994), which is in correlation with our study showing induction of the chemokine IL8 by HCV-LPs. Our studies also revealed that HCV-LPs can induce programmed cell death in endothelial cells. Previously, the apoptosis of hepatocytes has been histologically demonstrated in patients with chronic hepatitis C (Roberts et al., 1993). To address the mechanism of this cell death, we focused on the involvement of a known apoptotic mediator, Fas. In chronic HCV infection, the immunohistochemical detection of Fas antigen in liver tissue has been demonstrated, but whether this upregulation of Fas in hepatocytes and Fas ligand in T lymphocytes is due to HCV infection or a secondary phenomenon is unknown (Hiramatsu et al., 1994). Here, we observed that treatment of HUVECs with HCV-LPs caused the upregulation of Fas-L. We further demonstrated the functional involvement of the Fas pathway by abrogating apoptosis with neutralizing antibodies for Fas and Fas-L. In addition, we recently showed that HCV E2 in conjunction with HIV gp120 induces apoptosis in hepatocytes via the Fas-mediated pathway (Munshi et al., 2003).

The binding of Fas-L to the Fas receptor is known to lead to the activation of a series of death-associated molecules including FADD (Chinnaiyan et al., 1995), an adaptor protein with a death domain. FADD in turn binds and activates procaspase-8, eventually triggering activation of other caspases such as caspase-7, -3 and -6 (Muzio et al., 1996). To characterize Fas-mediated apoptosis induced by HCV particles in more detail, we examined the effects on the activation of the caspase cascade by assessing activation of caspase-3. Caspase-3 is a downstream caspase that cleaves anti-apoptotic Bcl-2 family proteins such as Bcl-2 and Bcl-XL. This cleavage by caspase-3 destroys their anti-apoptotic functions and causes the release of their C-terminal fragments, which are pro-apoptotic (Wolf & Green, 1999). Treatment of HUVECs with HCV-LPs resulted in increased activation of caspase-3. This was confirmed using a specific caspase-3 inhibitor, Z-VAD-FMK.
With these observations in hand, we asked whether IL8 release might participate in apoptosis in HUVECs. IL8 belongs to the CXC subfamily of pro-inflammatory chemokines and is known for the induction of several cellular functions such as chemotaxis, cell adhesion and growth-modulating effects (Detmers et al., 1991; Loetscher et al., 1994; Jones et al., 1997; Rainiger et al., 1998; Wolf & Green, 1999). IL8 has been shown to mediate these effects by binding to the G protein-coupled CXCR-1 and CXCR-2 receptors (Murphy, 1997). Neutralizing antibodies against CXCR-1 and CXCR-2 had no effect on HCV-LP-induced apoptosis (data not shown), nor did these antibodies inhibit the production of Fas-L. These results indicated that IL8 production did not contribute to the induction of apoptosis.

It appears that HCV, like HIV and other viruses, may encode proteins that bind specifically to cells such as endothelium that are not susceptible to direct infection and cause damage via an ‘innocent bystander’ effect. Therapeutic interventions targeted against the interaction of endothelium and HCV structural proteins may be beneficial in limiting the pathology of HCV infection.

ACKNOWLEDGEMENTS

We thank Janet Delahanty for editing the manuscript and Daniel Kelley for preparation of the figures. This work was supported in part by National Institutes of Health grants DA15008 (J. E. G.) and AI49140 (R. K. G.).

REFERENCES


