The HCV Core Protein Acts as a Positive Regulator of Fas-Mediated Apoptosis in a Human Lymphoblastoid T Cell Line

Chang S. Hahn,*·† Young Gyu Cho,* Beom-Sik Kang,* Isabel M. Lester,* and Young S. Hahn*·‡,1

*Beirne Carter Center for Immunology Research and †Department of Microbiology and ‡Department of Pathology, University of Virginia, Charlottesville, Virginia 22908

Hepatitis C virus (HCV) is a major human pathogen causing mild to severe liver disease worldwide and is remarkably efficient at establishing persistent infections. Previously, we have shown that the core protein has an immunomodulatory function including the suppression of T lymphocyte responses to viral infection. To investigate the underlying mechanism for the role of core protein in immune modulation, we examined the effect of core on the sensitivity of the human T cell line, Jurkat, to Fas-mediated apoptosis. The transient and stable expression of core protein in Jurkat cells increased the sensitivity of cells to Fas-mediated apoptosis when compared to control cells expressing vector DNA alone. In addition, we demonstrated that the core protein binds to the cytoplasmic domain of Fas which may enhance the downstream signaling event of Fas-mediated apoptosis. The expression of core protein did not alter the cell surface expression of Fas, indicating that the increased sensitivity of core-expressing cells to Fas ligand was not due to upregulation of Fas. Furthermore, we observed the augmentation of caspase-3 activity in core-expressing cells. These results suggest that the core protein may promote the apoptosis of immune cells during HCV infection via the Fas signaling pathway, thus facilitating HCV persistence. © 2000 Academic Press

INTRODUCTION

Fas, a member of the tumor necrosis factor receptor (TNFR) family, triggers apoptosis by transmitting a death signal through the cytoplasmic domain of Fas following engagement with Fas ligand (FasL) (Nagata, 1997; Nagata and Golstein, 1995). Fas-mediated apoptosis plays a crucial role in immune regulation through the peripheral deletion of self-reactive cells and activation-induced T cell death (Nagata and Golstein, 1995). The downstream signaling cascade involved in Fas-mediated apoptosis is well-delineated through several lines of investigations (Nagata, 1997). The binding of FasL to Fas leads to the activation of a cascade of proteases, known as caspases, that directly leads to cell death. Several components of the Fas-mediated apoptotic pathway have been identified. Upon trimerization of Fas following engagement with FasL, FADD is the first molecule recruited to the cytoplasmic domain of the Fas within Fas-bearing cells (Chinnaiyan et al., 1995). FADD in turn recruits procaspase-8 via a death effector domain interaction and activates procaspase-8, thus linking the death domains of early signaling proteins to activation of the caspase cascade (Muzio et al., 1996). Activation of these

downstream caspases leads to cleavage of substrates that include inhibitor of caspase-activated DNAse, which activates caspase-activated DNAse, poly-ADP-ribose polymerase, and numerous structural proteins including lamin, actin, and fodrin (Enari et al., 1998; Kawahara et al., 1998; Mukae et al., 1998; Sakahira et al., 1998).

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus belonging to the family Flaviviridae (Choo et al., 1989). HCV is a major cause of transfusion-associated hepatitis prior to the development of serologic tests for HCV-contaminated blood products and it remains a major agent of community-acquired hepatitis (Hollinger, 1990; Houghton et al., 1991). Characteristic features of HCV infection include the high incidence of persistent infection with a high rate of progression to chronic hepatitis (Hollinger, 1990). HCV persistent infection in the liver is strongly associated with the development of hepatocellular carcinoma (HCC) (Saito et al., 1990). There is growing evidence that HCV can replicate within peripheral blood mononuclear cells (PBMC) of chronically infected patients besides hepatocytes, suggesting a potential mechanism for the high rate of HCV persistent infection and the immunological disease induced by HCV infection (Bouffard et al., 1992; Bronowicki et al., 1998; Muller et al., 1993; Zignego et al., 1992). Some human lymphoid cell lines are susceptible to HCV infection in vitro (Ikeda et al., 1997; Mizutani et al., 1996; Shimizu et al., 1992, 1993). Furthermore, infection of extrahepatic tissues is supported by the finding that the

[1] To whom correspondence and reprint requests should be addressed at Beirne Carter Center for Immunology Research, Box 801386, University of Virginia Health Sciences Center, Charlottesville, VA 22908-1386. Fax: (804) 924-1221. E-mail: ysh5e@virginia.edu.

composition of HCV quasispecies present in liver differs from that in serum or PBMC (Cabot *et al.,* 1997; Maggi *et al.,* 1997; Navas *et al.,* 1998; Okuda *et al.,* 1999; Afonso *et al.,* 1999).

The HCV RNA genome is approximately 9.5 kb in length and encodes a single polyprotein (Houghton, 1996). This precursor protein is cleaved by the host and viral proteases to generate three structural proteins at the N-terminal end and six nonstructural proteins at the C-terminal end (Hijikata *et al.,* 1991; Houghton, 1996; Ralston *et al.,* 1993). The HCV core protein consists of 191 amino acids with an apparent molecular mass of 21 kDa. In addition to the viral capsid protein, the HCV core protein has been shown to possess a number of regulatory functions for viral and cellular gene expression (Ray *et al.,* 1995, 1997; Shih *et al.,* 1995; Srinivas *et al.,* 1996) as well as oncogenic potential (Ray *et al.,* 1996a). Recent studies indicate that it can enhance or suppress apoptosis depending on transfection methods or cell types or apoptosis-inducing agents. The stable expression of core protein enhances the apoptosis of hepatocyte cell lines, HepG2, by the Fas signaling pathway (Ruggieri *et al.,* 1997) and also increases TNF-α-induced apoptosis through the physical association between the core and the cytoplasmic domain of TNFR1 (Zhu *et al.,* 1998). In contrast, the inhibition of Fas and TNF-α mediated apoptosis of HepG2 cells was observed by transient expression of the core protein (Marusawa *et al.,* 1999). Furthermore, the core protein suppresses apoptosis in human cervical epithelial cells, HeLa, induced by cisplatin, in Chinese hamster ovary cells, CHO, by c-myc overexpression, and in human breast carcinoma cells lines, MCF7, by TNF-α (Ray *et al.,* 1996b, 1998).

In this report, we demonstrate that the transient and stable expression of HCV core protein increases the sensitivity of human T lymphocyte lines, Jurkat, to Fas-mediated apoptosis. Furthermore, we found that the HCV core protein is physically associated with the cytoplasmic domain of the Fas molecule *in vitro.* This interaction between the core and Fas leads to augmentation of the activation of caspase-3 during Fas-mediated apoptosis. These results suggest that the expression of core protein in T lymphocytes may increase the physiologic turnover of activated T lymphocytes during acute viral infection, thus facilitating HCV persistence and subsequent virus spread.

RESULTS

The HCV core protein increases the susceptibility of the human lymphoid T cell line to Fas-mediated cell death

Previously, we reported that the HCV core protein can suppress host immune responses in a murine model when delivered via infection with a recombinant vaccinia virus capable of expressing the core protein (Large *et al.,*
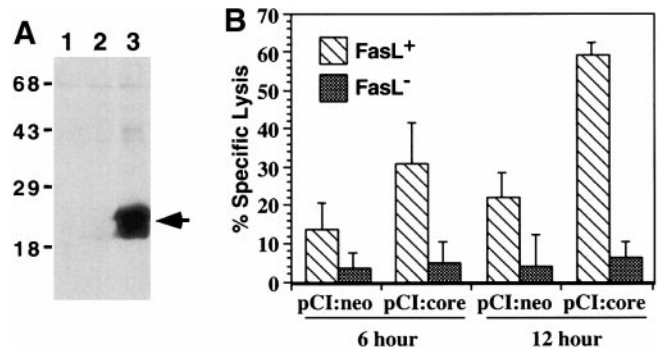

FIG. 1. Increased susceptibility of core-expressing cells to Fas-mediated cell death. (A) Expression of HCV core protein in Jurkat cells. Jurkat cells were mock transfected (lane 1) or transfected with an empty vector pCI:Neo (lane 2) or pCI:core (lane 3). Twenty-four hours after transfection, the expression of core protein was examined by Western blot analysis using HCV-core-specific antibody (anti-HCV peptide 716). (B) Increased Fas-mediated cytotoxicity in core-expressing Jurkat cells. The cells transfected with core or control plasmid DNA were labeled with ^{51}Cr and cocultured for 6 and 12 h with L:FasL$^+$ cells or L:FasL$^-$ cells. The percentage of specific lysis was measured by a standard ^{51}Cr-release assay. The results are representative of three independent experiments.

1999). The suppression phenotype includes: (1) an increased viral titer, (2) a reduced CTL activity with lower CTL precursor frequency, and (3) a reduced level of IL-2 and IFN-γ production. Based on these observations, we hypothesize that the HCV core may modulate the cell death of immune cells such as T lymphocytes. To test this possibility, we examined whether the expression of the HCV core protein influences the sensitivity of T lymphocytes to cell death induced by Fas and FasL interaction.

Jurkat cells were chosen to examine the effect of the core on Fas-mediated cell death since this cell line expresses high levels of cell surface Fas (Nagata and Golstein, 1995). Jurkat cells were transiently transfected with the HCV core plasmid (pCI:core) or an empty plasmid as a control (pCI:neo) for 24 h. pCI:neo contains the CMV promoter for heterologous gene expression and the neomycin phosphotransferase gene. The expression of core protein in transfected Jurkat cells was determined by Western blot analysis using a HCV-core-specific antibody (Fig. 1A).

Transiently transfected Jurkat cells were examined for their susceptibility to Fas-mediated cell death by coincubation with L cell transfectants expressing FasL (L:FasL$^+$) or an anti-sense FasL (L:FasL$^-$) as a control (Wang *et al.,* 1996). It has been reported that murine FasL used in our study induces Fas-mediated apoptosis of both human and murine cells (Nagata, 1997). As shown in Fig. 1B, transient expression of the core protein increased the susceptibility of Jurkat cells to Fas-mediated cell death compared to cells transfected with the control plasmid. The level of transient transfection in this exper-
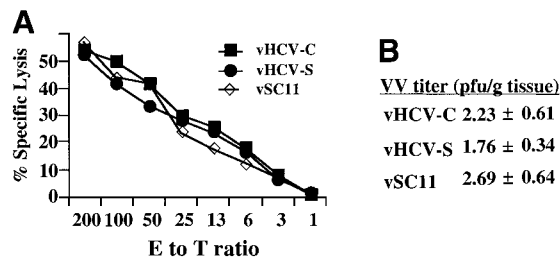
FIG. 2. Resistant phenotypes of core-induced immune suppression in *lpr* mice. (A) VV-specific CTL activity in *lpr* mice. *lpr* mice were inoculated with 5×10^7 pfu of recombinant vaccinia viruses, vHCV-C, vHCV-S, and vSC11, respectively. On day 5 of virus inoculation, primary VV-specific CTL activity was measured on P815 target cells infected with vSC11. (B) Vaccinia virus titer in liver tissue. The vaccinia virus titer was determined on BSC 40 monkey kidney cells from liver tissues harvested on day 5 after virus inoculation.

iment was 50–60% based on the expression of green fluorescent protein (GFP) in plasmid pEGFP-transfected cells under similar conditions (data not shown). This indicates that the enhanced apoptosis of cells transfected with the HCV core gene is possibly due to the effect of HCV core protein on the increased susceptibility of Fas-bearing cells to FasL.

To examine whether the core protein can increase the sensitivity of another T cell line to Fas-mediated cell death, HUT 78 cells (human T lymphoma line) were transiently transfected with pCI:core or pCI:neo. Upon treatment of these transfected T cells with FasL, cells expressing the core protein exhibited the enhanced susceptibility to Fas-mediated cell death compared to cells transfected with a control plasmid (data not shown). Taken together, these results suggest that the HCV core protein may modulate Fas-mediated cell death of T lymphocytes by altering the Fas signaling event.

It has been previously demonstrated that the HCV core protein suppresses host immune responses to viral infection including vaccinia-virus (VV)-specific CTL response and production of IFN-γ and IL-2 in mice (Large *et al.,* 1999). We hypothesize that the increased susceptibility of core-expressing T lymphocytes to Fas-mediated apoptosis as described above may account for the suppression of host immune response in mice expressing the HCV core protein. To test this possibility, we inoculated Fas-deficient *lpr* mice with recombinant VV expressing the HCV core protein (vHCV-C), structural proteins (vHCV-S), and control protein (vSC11). As shown in Fig. 2A, the mice infected with vHCV-C generated VV-specific CTL response as efficiently as mice infected with a control virus, vSC11. Consistent with the CTL activity in core-expressing mice, the level of VV titer in vHCV-C mice was as low as that in mice infected with vSC11 (Fig. 2B). This suggests that the functional expression of Fas is critical for core-induced immune suppression.

The HCV core protein is physically associated with the cytoplasmic domain of Fas *in vitro*

To understand a potential mechanism involved in the increased cell death of T lymphocytes induced by HCV core protein, we examined whether the HCV core protein can directly interact with Fas. Furthermore, the HCV core protein was previously shown to interact with a cytoplasmic domain of other TNFR family members, lymphotoxin-β receptor (LTβR), and TNFR1 (Matsumoto *et al.,* 1997; Zhu *et al.,* 1998). The potential interaction between core protein and Fas was examined *in vitro* by glutathione thiotransferase (GST) binding assay using a GST fusion protein of HCV core (GST–core) and *in vitro* translated and radiolabeled Fas. *In vitro* translated and radiolabeled full-length core protein was used as a positive control since the core protein is known to oligomerize by itself (Matsumoto *et al.,* 1996).

Figure 3A shows the result of a GST fusion binding assay. As expected, GST–core (lane 7) but not GST alone (lane 4) binds to radiolabeled core protein (21 kDa). We also found that GST–core (lane 9) but not GST alone (lane 6) interacts with the Fas protein (43 kDa). This indicates that there is a potential interaction between the HCV core protein and Fas. Surprisingly, we did not detect an interaction between the GST–core fusion protein and
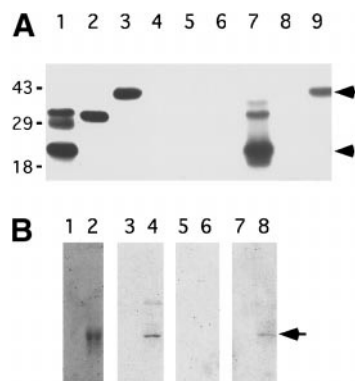

FIG. 3. The *in vitro* interaction of the HCV core protein with Fas. (A) Interaction between the GST core protein and radiolabeled Fas. The core protein (lane 1), cytoplasmic domain of TNFR1 (lane 2), and Fas (lane 3) are *in vitro* translated and radiolabeled with [^{35}S]methionine for GST binding assay. GST alone (lanes 4–6) or GST–core fusion protein (lanes 7–9) was incubated with *in vitro* translated and radiolabeled core (lanes 4, 7), TNFR1 (lanes 5, 8) or Fas (lanes 6, 9). Following incubation, GST–core fusion protein was purified using glutathione–agarose beads and separated on SDS–PAGE. Radiolabeled core or Fas binding to GST–core fusion protein was detected by autoradiography. Size markers in kilodaltons are shown to the left of the autoradiogram. (B) Far-Western analysis for the interaction of the core protein with Fas, GST (lanes 1, 3, 5, 7) or GST–core fusion protein (lanes 2, 4, 6, 8) was subjected to SDS–PAGE and transferred to nitrocellulose. The proteins bound to the membrane were renatured and incubated with either HRP-labeled anti-HCV core antibody (lanes 1, 2) or *in vitro* translated and radiolabeled HCV core (lanes 3, 4), TNFR1 (lanes 5, 6), or Fas (lanes 7, 8) and subjected to immunoblotting (lanes 1, 2) or autoradiography (lanes 3–8).
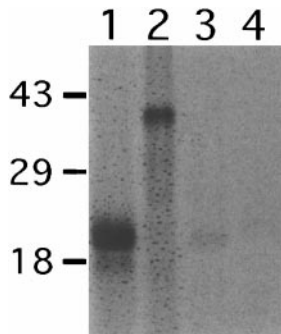
FIG. 4. Binding of core protein to the cytoplasmic domain of Fas. GST–core (lanes 1–4) was incubated with *in vitro* translated and radiolabeled core (lane 1), the full-length Fas protein (lane 2), the cytoplasmic domain of Fas (lane 3), or the extracellular region of Fas (lane 4). Following binding to glutathione resin and subsequent washing, samples were separated on SDS–PAGE and detected by autoradiography. Size markers in kilodaltons are shown to the left of the autoradiogram.

the cytoplasmic domain of TNFR1 (lane 8) as reported previously (see Discussion).

To confirm the results of these *in vitro* binding studies, we performed Far-Western analysis (Matsumoto *et al.,* 1997) as shown in Fig. 3B. GST–core and GST were separated by SDS–PAGE, transferred to a nitrocellulose membrane, and renatured. Potential interactions between core protein and Fas as well as TNFR1 were examined by incubating the membrane with radiolabeled Fas, TNFR1, or HCV core as a positive control. The presence of core protein on the membrane was confirmed by Western blot analysis using HCV-core-specific antibody (lane 2). A band corresponding to the molecular weight of the core protein (21 kDa) or GST–core (48 kDa) was detected by incubation with core protein (lane 4) or Fas (lane 8). Consistent with a GST protein binding assay, we did not detect an interaction between GST–core and TNFR1 (lane 6).

To determine whether the extracellular or cytoplasmic domain of Fas is required for the interaction with core protein, we generated plasmids capable of expressing the extracellular and cytoplasmic domains of Fas by site-directed mutagenesis. These truncated forms of Fas protein were examined for their ability to interact with the core protein by GST binding assay. As a positive control, we used radiolabeled core protein (21 kDa) and full-length Fas (43 kDa) as described above. As shown in Fig. 4, we detected a radiolabeled band corresponding to the core or full-length Fas protein. In addition, the core protein could specifically bind to the cytoplasmic domain of Fas (20 kDa) but not to the extracellular domain of Fas (22 kDa). It is notable that the intensity of the band for interaction between the core protein and the cytoplasmic domain of Fas is weaker than that of core and Fas, possibly due to the stability of the truncated form of Fas. This suggests that the HCV core protein is able to bind to the cytoplasmic domain of Fas and this interaction may

modulate Fas-mediated apoptosis by altering the Fas signaling pathway.

HCV core protein facilitates the induction of Fas-mediated apoptosis in Jurkat cells

To further examine the biological effect of the physical association between the core protein and the cytoplasmic domain of Fas on Fas-mediated apoptosis, we generated stable transfectants of Jurkat cells expressing the core protein under the control of the CMV promoter or containing an empty vector as a control. Several stable Jurkat transfectants have been established by limiting dilution in medium containing G418. When the core expression in representative clones (C4, C6, C10) of stable transfectants was examined by RT-PCR analysis, these cell lines expressed core-specific mRNA. The level of core-specific RNA in core transfectants was comparable to that in peripheral blood monocytes isolated from HCV-infected patients by RT-PCR analysis (data not shown).

To determine whether HCV core affects the differential kinetics of Fas-mediated apoptosis, we examined a time course of apoptosis induction by measuring chromosomal DNA degradation of Jurkat transfectants upon Fas/FasL interaction. The flow cytometry analysis of chromosomal DNA contents was performed to determine cellular DNA contents. The levels of Fas-mediated apoptosis was determined at various time points (40, 50, 65, 100 min) after Fas/FasL engagement. Apoptotic cells were first detected at 40 min after Fas/FasL engagement (Fig. 5A). The expression of HCV core protein in Jurkat cells enhances the kinetics for sensitivity of cells to Fas-mediated apoptosis. In contrast, less than 3% of apoptotic cells was observed when core transfectants were incubated with L:FsasL⁻ cells.

To determine whether the core/Fas interaction specifically increased the susceptibility of core-expressing cells to Fas-mediated apoptosis, we further examined the effect of core protein on Fas-independent apoptosis by treating cells with chemotherapeutic agents, etoposide and 5-fluorouacil (5-FU). These reagents have been reported to trigger the p53-dependent induction of apoptosis (Lowe *et al.,* 1993). When Jurkat and its transfectants expressing core protein were treated with various concentrations of etoposide for 24 h, the induction of apoptosis by etoposide was comparable to that of Jurkat and its transfectant expressing core protein (Fig. 5B). A similar result was obtained in cells treated with various concentrations of 5-FU (data not shown). These results suggest that core protein does not affect Fas-independent apoptosis induction such as p53 activation.

In addition, we determined whether caspase activation is required for the increased Fas-mediated apoptosis induced by core protein. As shown in Fig. 5C, Fas-mediated apoptosis in Jurkat and its transfectant was inhibited in the presence of caspase-3 and caspase-8
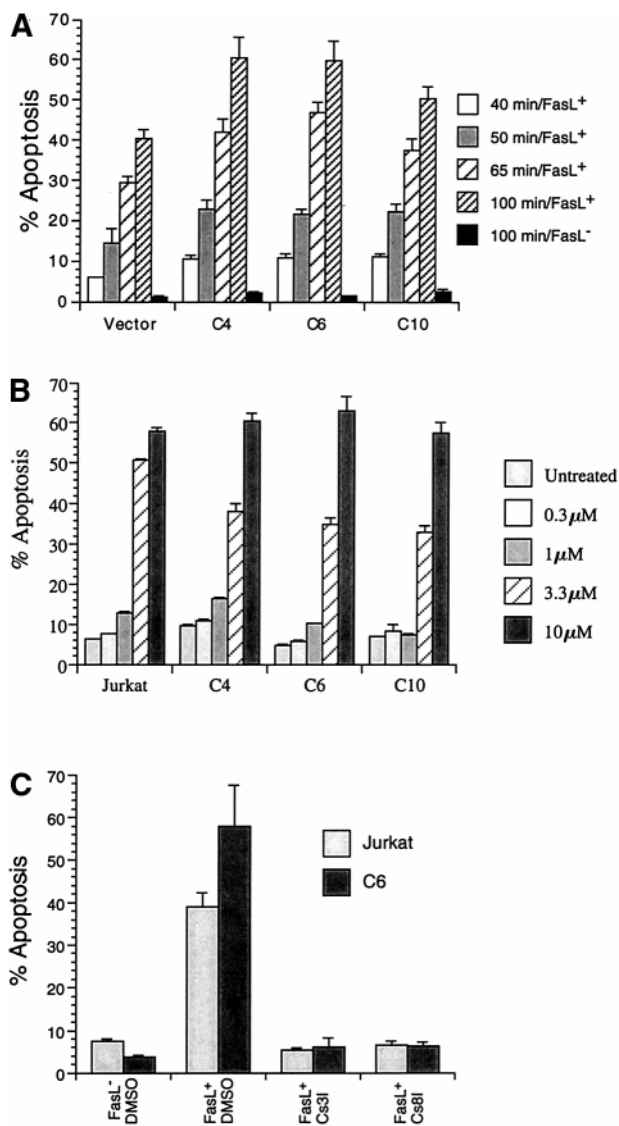
FIG. 5. Increased Fas-mediated apoptosis in stable Jurkat transfectants expressing core protein. (A) Time course of apoptosis induction upon Fas/FasL engagement. Vector transfectant and three independent stable transfectants (C4, C6, C10) expressing HCV core under the control of a CMV promoter were examined for Fas-mediated apoptosis. All experiments were performed in triplicate and at least 10^4 cells were examined for cellular DNA contents. Cells containing less than diploid DNA contents were defined as apoptotic cells. Bar graph and error bar represent mean and standard deviations, respectively. Mean value combined from three independent experiments are shown. (B) Effect of core protein on etoposide-independent apoptosis. Jurkat and its transfectants were treated with various doses of etoposide for 24 h. The degree of apoptosis was examined by the flow cytometry analysis as described above. (C) Inhibition of Fas-mediated apoptosis by inhibitors specific for caspase-3 and caspase-8 activity. Jurkat cells and its core transfectant, C6, were pretreated with vehicle (DMSO), caspase-3 inhibitor (Cs3I), or caspase-8 inhibitor (Cs8I) for 10 min. The effect of inhibitors on Fas-mediated induction of apoptosis is measured by a method as described above (Fig. 4A). At least 20,000 cells were examined for each experiment. Mean and standard deviation from four independent experiments are shown. Similar inhibition results were obtained from core transfectants, C4, and C10 (data not shown).

inhibitors. This suggests that the effect of core protein on increased Fas-mediated apoptosis upon Fas/FasL engagement depends on the activation of both caspase-3 and caspase-8.

The HCV core induces the increased Fas-mediated apoptosis through augmentation of caspase-3 activation

To investigate a potential mechanism involved in the increased Fas-mediated apoptosis induced by the core protein, we examined the downstream Fas signaling event in core transfectants, since the physical association of the core protein with the cytoplasmic domain of Fas may alter the apoptotic signaling event. First, we examined whether the interaction between the core and the cytoplasmic domain of Fas may stabilize the cell surface expression of Fas. The level of cell surface Fas expression was determined in core transfectants by flow cytometry and compared with that of control cell lines. The level of cell surface Fas expression in core transfectants (C4, C6, C10) is comparable to that in parental Jurkat or control plasmid transfected cells (data not shown). This suggests that the effect of core on increased sensitivity of Jurkat cells to Fas-mediated apoptosis may not be due to the upregulation of cell surface Fas expression. This implies that the physical association between the core protein and Fas may facilitate the activation of intracellular apoptosis signalining molecules such as caspase cascade.

To test this possibility, we examined the effect of the core protein on the activation of caspase cascade by determining the activation of caspase-3 at various time points upon Fas/FasL engagement. Caspase-3 is one of the downstream caspases activated upon Fas/FasL engagement and is responsible for activating several apoptosis target proteins, including caspase-activated DNAse (Enari et al., 1998). The activation of caspase-3 was determined by examining the cleavage of pro-caspase-3 (32 kDa) at 30, 45, and 90 min after Fas/FasL engagement. As shown in Fig. 6A, the level of pro-caspase-3 (32 kDa) in core transfectant (C4, C6, C10) is slightly lower than that in control cells (V) at 30 min after Fas/FasL engagement, suggesting the enhanced activation of caspase-3 in core transfectants. Upon 45 min post-Fas/FasL engagement, there was a dramatic decrease of procaspase-3 level in the core transfectants compared to that in control cells. All procaspase-3 was activated in both core transfectants and control cells 90 min post-Fas/FasL engagement. However, core transfectants incubated with L:FasL$^-$ showed a comparable level of procaspase-3.

Consistent with the effect of core protein on caspase-3 activation, the enzymatic activity of caspase-3 was also higher in core transfectants than in a control cell when caspase-3 activity was determined by cleavage of a
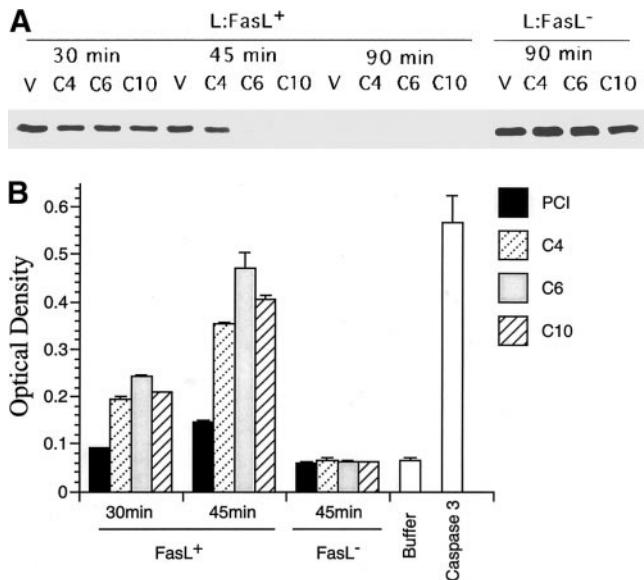
FIG. 6. Augmentation of caspase-3 activation in core transfectants upon Fas/FasL engagement. (A) Time course of caspase-3 activation upon Fas/FasL interaction. Vector transfectant or three independent clones of HCV core transfectants (C4, C6, C10) of Jurkat cells were incubated with L cells (L:FasL$^+$, L:FasL$^-$). Upon 30 min, 45 min, and 90 min after incubation, cells were collected, lysed, and analyzed for the caspase-3 activation by determining the cleavage of procaspase-3. Each lane represents total cell lysates from 2×10^5 cells. Data represent three independent experiments. (B) Caspase-3 activity in transfected Jurkat cells upon incubation with L:FasL$^+$ or L:FasL$^-$, Jurkat transfectants were coincubated with either L:FasL$^+$ or L:FasL$^-$. Upon 30 or 45 min after coincubation with L:FasL$^+$ or L:FasL$^-$, core transfectants (C4, C6, C10) of Jurkat cells or a control cell were collected, lysed, and assayed for caspase-3 activity using a colorimetric substrate of caspase-3. Ac-DEVD-pNA. Buffer alone and 10 U of purified caspase-3 were also used for assay. The enzyme assay was performed in duplicate and results are representative of two independent experiments.

colorimetric substrate of caspase-3 (Fig. 6B). In contrast, incubation of core transfectants with L:FasL$^-$ showed a basal level of caspase-3 activity. This suggests that the HCV core protein augments the activation of caspase-3 involved in the Fas downstream signaling event.

DISCUSSION

One of the most important aspects of HCV infection is its ability to establish a persistent infection in hosts. In various reports, the incidence of this persistence in infected subjects is as high as 80% (Houghton, 1996). In HCV-infected individuals, it is believed that T cell responses, including both CTL and helper T lymphocytes, are crucial in preventing viral persistence (Chang et al., 1997; Koziel, 1997). In particular, the frequency of HCV-specific CTL in the peripheral blood from chronically infected patients appears to be quite low and the detection of CTL response in these patients requires multiple peptide stimulations in vitro (Rehermann et al., 1996a,b). We have previously reported that in an experimental

murine model using infection with both VV and SIN recombinants, the expression of HCV core protein suppressed the host immune responses including CTL response and production of IFN-γ and IL-2 cytokines against viral infection (Large et al., 1999).

In this report, we examined the effect of HCV core protein on the sensitivity of Jurkat cells to Fas-mediated apoptosis and the downstream signaling event of Fas-mediated apoptosis. The transient and stable expression of the core protein in Jurkat cells increased the sensitivity of cells to Fas-mediated apoptosis compared to that in the control vector-transfected cells. This increased Fas-mediated apoptosis induced by the expression of core protein may play an important role in core-mediated immune suppression in vivo since lpr mice were resistant for core-mediated immune suppression when they were infected with VV recombinant expressing the HCV core protein, as shown in Fig. 2. This suggests that the functional expression of Fas protein may be required for core-mediated immune suppression. In addition, we found a physical association between the core protein and the cytoplasmic domain of Fas in vitro. Biochemical analysis of stable transfectants expressing the core protein revealed that the core protein augmented caspase-3 activation upon Fas/FasL engagement, possibly due to its physical association with Fas. As a consequence, the induction of apoptosis in core transfectants was more rapid compared to that in vector-transfected cells.

It is notable that the intensity of the band for interaction between the core and cytoplasmic domain of Fas is much weaker than that of the core and full-length Fas (Fig. 4). There are several possibilities for the weak intensity of the band for the interaction between the core and cytoplasmic domain of Fas. First, it might be due to a limited number of methionine residues in the cytoplasmic domain of Fas (one methionine residue), which allows weaker labeling of protein with radioactivity. Second, the instability of the cytoplasmic domain of the Fas may give rise to a weaker association with the core protein as shown in other truncated proteins, demonstrating the decreased stability of the protein (Varshavsky, 1992).

The HCV core protein is a major component of viral nucleocapsid and also multifunctions as a transcriptional regulator of various viral and cellular promoters, potentially disturbing normal cellular function. The core protein cooperates with the Ras oncogene and transforms primary rat embryo fibroblasts (Ray, 1996a), possibly by regulation of the p53 tumor suppressor promoter (Ray et al., 1997; Lu et al., 1999). Furthermore, the HCV core protein has been shown to interact with the cytoplasmic domain of LTβR and TNFR1, members of the TNFR family which play important roles in immune modulation (Matsumoto et al., 1997; Zhu et al., 1998). These observations as well as potential nuclear localization of the truncated form of the core protein (Hsieh et al., 1998;

Lo et al., 1995) implicate the role of this HCV core protein in the establishment of HCV persistence and the development of HCC.

It is of interest that the HCV core protein used in our study did not bind to the cytoplasmic domain of TNFR1 *in vitro* in contrast to studies done by Zhu and colleagues (Zhu et al., 1998). This discrepancy may be due to strain differences between the HCV Taiwan strain (genotype 1b) in experiments by Zhu et al. (Zhu et al., 1998) and the HCV Hutchinson strain (genotype 1a) (Grakoui et al., 1993) in our experiment. Although the core protein is well conserved among different HCV genotypes, there are six amino acid differences between these two strains within the putative binding region of the core protein (amino acid residues 36–119 of the core protein mapped for binding to the $LT\beta R$). Some of these changes are dramatic, such as Ser-53 to Trp, Arg-70 to Gln, Cys-91 to Leu, and Ser-106 to Asn. It is also worth noting that two substitutions at both Ser-53 and Ser-106 involve potential Ser/Thr phosphorylation sites (Shih et al., 1995).

HCV, like other hepatitis viruses, is predominantly a hepatotrophic virus. Nonetheless, several studies suggest that the infection/replication of HCV can be detected in both PBMC and bone marrow cells (Bouffard et al., 1992; Muller et al., 1993). A recent study by Bronowicki and colleagues (Bronowicki et al., 1998) strengthens the evidence for HCV replication in PBMC by taking advantage of severe combined immunodeficiency (SCID) mice that lack adaptive immune responses and thus permit xenografts of human PBMC. When PBMC isolated from HCV carriers were injected into SCID mice, the presence and replication of HCV were detected over time in PBMC of grafted mice, supporting the persistent replication of HCV in PBMC.

Why is HCV infection/replication in PBMC, and specifically lymphocytes, important for HCV pathogenesis from a biological and clinical point of view? HCV or its gene product(s) may directly affect the function of immune cells by interfering with their ability to clear HCV-infected hepatocytes. In light of our studies demonstrating that the expression of HCV core protein facilitates the induction of Fas-mediated apoptosis in Jurkat T cell lines, the HCV core protein may play an important role in the establishment of HCV persistent infection. Although Fas-mediated apoptosis is involved in downregulating normal T cell responses, it may occur with increased sensitivity or at a lower threshold in the presence of HCV core protein. Therefore, the kinetics of activation-induced apoptosis may be facilitated. If the increased Fas-mediated apoptosis induced by the core protein occurs *in vivo* during T cell activation, it might explain the high incidence of persistence during natural HCV infection in humans, presumably due to inefficient CTL activity.

Furthermore, the dysfunction in apoptosis may be linked to development of HCC in chronic HCV patients (Saito et al., 1990). Although the development of HCC in chronic HCV patients may be due to potential oncogenic properties of HCV gene products, an impairment in immune surveillance cannot be ruled out for its role in the development of HCC. If the suppression of T cell responses to viral infection occurs as a result of HCV infection in T lymphocytes, their ability of tumor surveillance would be greatly diminished. Further investigations of HCV core protein on the mechanism of T cell dysfunction would clarify many of these questions.

In summary, the HCV core protein increased Fas-mediated apoptosis of human T lymphocytes, presumably mediated by the physical interaction between the core and the cytoplasmic domain of Fas. Further analysis of the effect of the core protein on the Fas-mediated downstream signaling event suggests that the increased apoptosis of T lymphocytes is due to the upregulation of caspase-3 activation. Currently, we are in the process of examining a detailed molecular mechanism for augmentation of the downstream Fas signaling event induced by the core protein.

MATERIALS AND METHODS

DNA and plasmids

pCI·neo, pCDM8, pEGFP, and pGEX4T.3 plasmid DNA were purchased from Promega (Madison, WI), Clonetech (Palo Alto, CA), and Pharmacia Biotech (Piscataway, NJ), respectively. Plasmid pCDM8/TNFR1 capable of expressing the cytoplasmic domain of the human TNFR1 (amino acid residues 208 to 426) was a generous gift from Dr. T. H. Lee (Biotech Research Institute, Tae-Jeon, Korea) (Kim et al., 1995). Coding sequences of HCV core derived from HCV Hutchinson strain (HCV-H, genotype 1a) (Grakoui et al., 1993) were generated by juxtaposing the appropriate HCV core coding sequence of amino acid residues 1–192 in the pCI·neo plasmid followed by a translational termination codon in the plasmid. The resulting plasmid construct was named pCI·core. The open reading frame of the core protein (a region of amino acids 1 to 192) (Grakoui et al., 1993) was placed into pGEX4T.3 to generate a plasmid capable of expressing GST fusion proteins and named pGEX·core.

Human Fas cDNA was placed under the control of the phage T7 promoter in the pCDM8 plasmid. Extracellular and cytoplasmic domains of the Fas open reading frame were generated by PCR-based amplification of appropriate fragments encoding amino acid regions 1–171 and 190–335 of Fas, respectively. Oligonucleotides containing either a termination codon, 5′-AGTGCTAGC**TGG**ATC-CTTCCTCTTTG-3′, or an initiation codon, 5′-GTGAAGCT-T**ATG**GAAGTACAGAAAACATG-3′, were used for PCR amplification to generate extracellular and cytoplasmic domains of Fas, respectively. All mutagenesis products were confirmed by DNA sequencing. Resulting PCR products representing the extracellular or cytoplasmic region of Fas were inserted into plasmid pCI·neo to

produce radiolabeled protein by *in vitro* transcription and translation.

Cell lines and transfection

Jurkat and HUT 78 T cells were obtained from the American Type Culture Collection (Rockville, MD) and maintained in RPMI 1640 medium (Gibco BRL, Gaithersburg, MD) supplemented with 10% (vol/vol) fetal bovine serum (Gibco BRL) and 2 mM glutamine (Sigma Chemicals Co., St. Louis, MO). L cell transfectants, L:FasL[+], and L:FasL[−] were generous gifts from Dr. J. H. Russell (Washington University, St. Louis, MO) (Wang *et al.,* 1996). These L cell transfectants, P815, and BSC40 cells were maintained in DMEM (Gibco BRL) supplemented with 10% fetal bovine serum and 2 mM glutamine.

Jurkat or HUT78 cells (1.2×10^7 cells/0.5 ml) were transfected with 10 μg of either pCI:neo vector alone or pCI:core or pEGFP using electroporation at 250 V, 800 μF (Cell Porator, Gibco BRL). For generation of stable transfectants, Jurkat cells transfected with plasmid DNA were maintained in medium containing G148 (0.5 mg/ml) and sublines expressing the HCV core protein were established by limiting dilution (Yokoyama, 1994). The expression of HCV core protein was confirmed by RT-PCR analysis. The level of cell surface expression of Fas in Jurkat and its transfectants was examined by flow cytometry after staining cells with a murine anti-human Fas monoclonal antibody (DX2) and a secondary antibody, FITC-conjugated goat anti-mouse antibody.

^{51}Cr-release assay for Fas-mediated cytotoxicity

Transiently-transfected Jurkat cells were labeled with 150 μCi ^{51}Cr for 1 h and plated in a 96-well plate with 2×10^4 cells/well. Two groups of ^{51}Cr-labeled target cells were cocultured with either L:FasL[+] or L:FasL^{−} cells at an effector to target ratio of 1:3. One group was incubated for 6 h and another group was incubated for 12 h. After 6 and 12 h of incubation of cocultured cells, supernatants were harvested and ^{51}Cr release was determined. The percentage of specific lysis was calculated by 100 × (specific lysis-spontaneous lysis)/(total lysis-spontaneous lysis).

Vaccinia-virus-specific CTL response and virus titration in *lpr* mice

Four- to 6-week-old *lpr* mice of B10.D2 genetic background were obtained from Dr. Thomas J. Braciale (University of Virginia, Charlottesville, VA). Mice were inoculated intraperitoneally with 5×10^7 pfu of the recombinant VV, vHCV-S, vHCV-C, and vSC11. vHCV-S and vHCV-C express the HCV structural proteins (core, E1, E2) and core protein, respectively. vSC11 is a parental recombinant virus. On day 5 postinoculation, mice were sacrificed and spleens were harvested. The primary vac-cinia-virus-specific CTL response was determined by testing the ability of purified splenocytes from infected mice upon lysing the VV-infected P815 target cells. At autopsy, liver tissue was harvested to measure VV titers on BSC40 cells followed by a previously described method (Large *et al.,* 1999).

Western blot analysis

Jurkat and its transfectants were collected, washed with PBS (20 mM NaPO$_4$, pH 7.5, 150 mM NaCl), lysed in RIPA buffer (50 mM Tris.Cl, pH 7.5, 150 mM NaCl, 1 mM EGTA, 0.5% Nonidet P-40, 0.5% deoxycholate, 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride, 0.3 μm aprotinin, and 1 μM leupeptin), and separated by 12% SDS–PAGE. Proteins were transferred onto either nitrocellulose (Schleicher & Schuell, Keene, NH) or Immobilon polyvinylidene difluoride (Millipore, Bedford, MA) membranes. Membranes were blocked for more than 1 h in PBS containing 2% nonfat milk and 0.25% Tween 20 and then immunoblotted using HCV-core-specific rabbit anti-HCV peptide 716 polyclonal antibody (American Qualex, San Clemente, CA). Immunoreactive bands were detected using horseradish-peroxidase-conjugated secondary antibodies in conjunction with an enhanced chemiluminescent system (Pharmacia Biotech).

For analyzing caspase-3 activation by Western blot, Jurkat and its stable transfectants were cultured to the density of 8×10^5 cells/ml. Cells were collected and resuspended in culture medium at a concentration of 5×10^5 cells/ml. Cells (10^6) were placed in a six-well tissue culture plate containing a monolayer of L:FasL[+] or L:FasL[−]. The effector (L:FasL[+] or L:FasL[−]) to target (Jurkat and its transfectants) ratio was 1:3. To facilitate cell-to-cell contact, plates were centrifuged at 100g rcf for 5 min and placed in a 37°C incubator for specified times. Western blot analysis was performed as described above to detect caspase-3 activation using mouse monoclonal antibody against caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA).

Caspase-3 enzyme assay

To measure the caspase-3 enzymatic activity, 2×10^6 Jurkat transfectants were coincubated with 10^6 cells of either L:FasL[−] or L:FasL^{+} cells in six-well clusters for given times at 37°C. Jurkat transfectants were collected, washed with PBS, and lysed in 100 mM NaCl, 50 mM HEPES, 10 mM dithiothreitol (DTT), 1 mM EDTA, 10% glycerol, 0.1% CHAPS (pH 7.4), for 45 min on ice. Lysates were centrifuged to remove cell debris and incubated with 0.2 mM colorimetric caspase-3 substrate (Ac-DEVD-pNA) at 37°C. As a positive control, 10 U of purified human recombinant caspase-3 was used. The activity of caspase-3 was measured by reading the plate at 405 nm.

GST fusion protein binding assay

Glutathione thiotransferase and GST–core fusion protein (GST–core) were expressed in *Escherichia coli* DH5α after transforming *E. coli* with plasmids pGEX4T and pGEX:core. GST and GST–core were purified using glutathione–agarose beads (Sigma) in accordance with the supplier's recommendation. [^{35}S]Methionine-labeled HCV core (full-length), Fas, and the cytoplasmic domain of TNFR1 were generated using a coupled transcription–translation rabbit reticulocyte lysate TNT system from Promega with pCI:core, pCDM8:Fas, and pCDM8:TNFR1 plasmid DNA as templates, respectively. A total of 200 ng of GST or GST–core coupled to 20 μl of glutathione–agarose beads (50% slurry) was incubated at 4°C with [^{35}S]methionine-labeled core, Fas, and the cytoplasmic domain of TNFR1 in 600 μl of a buffer solution containing 40 mM HEPES–KOH, pH 7.5, 150 mM KCl, 0.5 mM EDTA, 5 mM MgCl$_2$, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, and 0.1% Nonidet P-40 for 1 h. To minimize potential bead loss during subsequent washes, the buffer was mixed with glutathione–agarose beads to adjust to a total bead volume of 20 μl per reaction. Following incubation, the beads were washed five times with the same buffer. The bound proteins were eluted with SDS–PAGE loading buffer and separated by SDS–PAGE and autoradiography.

"Far-Western" protein blotting assay

The GST and GST–core proteins, partially purified using glutathione–agarose resin, were separated by 12% SDS–PAGE and electrotransferred to a nitrocellulose membrane. GST–core protein has a tendency to be dissociated into GST and core protein alone. The membrane was washed with buffer A (10 mM HEPES, pH 7.5, 60 mM KCl, 1 mM EDTA, 1 mM DTT) and incubated with 5 M guanidium chloride for 10 min at 4°C and then sequentially with 2.5, 1.25, 0.625, 0.31, 0.15, and 0.08 M guanidium chloride for 5 min each to renature the proteins. The membrane was subsequently blocked with 5% nonfat milk in buffer A. Radiolabeled proteins, described in the previous section, were then incubated with the membrane in buffer A containing 5% nonfat milk. Unbound proteins were removed by washing the membrane three times with buffer A containing 1% nonfat milk and 0.05% Nonidet P-40, and the bound proteins were visualized by autoradiography.

RT-PCR analysis for core-specific gene expression

Total RNA was prepared from Jurkat cells or Jurkat transfectants with Trizol (Gibco BRL) following the manufacturer's protocol. PBMC were purified by Isopaque Ficoll gradient from HCV-infected patient's blood and RNA was isolated as described above. For RT-PCR analysis, random primed cDNA was synthesized from iso-lated total RNA using reverse transcriptase (Perkin–Elmer, Foster City, CA) according to the recommendations of the manufacturer. A portion of cDNA was used for PCR in a volume of 50 μl using 0.5 U Taq polymerase and reagents obtained from Perkin–Elmer. HCV-core-specific primers (5′ primer: 5′-AAGAAAAACCAAACG-TAACACCA-3′, region of nucleotides 365 to 387, 3′ primer: 5′-CCAGCTAGG-CCGGAGAGCCACG-3′, antisense region of nucleotides 641 to 662) were used for amplifying the cDNA encoding the HCV core protein.

Apoptosis assay by DNA content analysis

Cellular DNA contents were analyzed by flow cytometry according to a method previously described (Moorman *et al.,* 1996). In brief, Jurkat and its stable transfectants were cultured to a density of 8×10^5 cells/ml. Cells were collected and resuspended in medium at a concentration of 5×10^5 cells/ml and 5×10^5 were placed in a 24-well tissue culture plate containing L cell transfectants at a designated effector (L:FasL$^+$ or L:FasL$^-$) to target (Jurkat and its transfectants) ratio. To facilitate cell-to-cell contact, plates were centrifuged at 100g rcf for 5 min and placed in a 37°C incubator for the designated times. Jurkat cells and its stable transfectants were collected, washed with PBS, and resuspended in a staining solution containing 50 μg/ml propidium iodide, 0.3% Nonidet P-40, 100 μg/ml boiled RNase A, and 0.1% sodium citrate. Stained cells were placed at 4°C for at least 30 min to eliminate RNA and analyzed with a Becton–Dickinson FACSCalibur. DNA contents of cells were analyzed with Becton–Dickinson Cell Quest software and cells containing less than diploid DNA contents were defined as an apoptotic cell population. An average of 10^4 cells was analyzed in each sample and most assays were performed in triplicate. To examine the specificity of Fas-mediated apoptosis, Jurkat and its transfectants expressing core protein were pretreated with either caspase-3 inhibitor (100 nM Ac-DEVD-CHO in 1% DMSO Cal Biochem) or caspase-8 inhibitor (100 nM Ac-IETD-CHO in 1% DMSO, Cal Biochem) or 1% DMSO alone for 10 min at room temperature. The induction of apoptosis was measured as described above.

Apoptosis induction by etoposide and 5-FU

Jurkat and its transfectants (10^6 log phase) expressing core protein were treated with 0.1% methyl alcohol (vesicle), 0.33, 1, 3.3, and 10 μM of etoposide for 24 h. Cells were harvested by centrifugation and the induction of apoptosis was measured as described above. To examine the effect of 5-FU, 10^6 log phase Jurkat and its transfectants expressing core protein were treated with mock, 3.3, 10, 33, or 100 mM 5-FU for 48 h. Cells were harvested by centrifugation and the induction of apoptosis was measured as described above.

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