HCV core/gC1qR interaction arrests T cell cycle progression through stabilization of the cell cycle inhibitor p27Kip1

Zhi Qiang Yao, a Audrey Eisen-Vandervelde, b Suma Ray, b and Young S. Hahn a,b, *

a Department of Pathology, Beirne Carter Center for Immunology Research, University of Virginia, Charlottesville, VA 22908, USA
b Department of Microbiology, Beirne Carter Center for Immunology Research, University of Virginia, Charlottesville, VA 22908, USA

Received 25 September 2002; returned to author for revision 30 October 2002; accepted 25 April 2003

Abstract

Hepatitis C virus (HCV) is efficient in the establishment of persistent infection. We have previously shown that HCV core protein inhibits T cell proliferation through its interaction with the complement receptor, gC1qR. Here we show that HCV core-induced inhibition of T cell proliferation involves a G0/G1 cell cycle arrest, which is reversible upon addition of anti-gC1qR antibody. Correspondingly, the expression of cyclin-dependent kinases (Cdk) 2/4 and cyclin E/D, as well as subsequent phosphorylation of retinoblastoma (pRb), is reduced in core-treated T cells in response to mitogenic stimulation. Remarkably, degradation of p27Kip1, a negative regulator of both Cdk4/cyclin D and Cdk2/cyclin E complexes, is significantly diminished in T cells treated with HCV core upon mitogenic stimulation. These data indicate that the stability of p27Kip1 by HCV core is associated with blocking activated T cells for the G1 to S phase transition and inhibiting T cell proliferation.

© 2003 Elsevier Inc. All rights reserved.

Keywords: HCV; Core protein; gC1qR; T cells; Cell cycle arrest; p27Kip1

Introduction

Hepatitis C virus (HCV) is a serious and growing threat to human health, having infected approximately 170 million people worldwide (Houghton, 1996). HCV causes a spectrum of liver diseases, ranging from an asymptomatic carrier state to end-stage diseases. Remarkably, the majority of individuals with HCV fail to resolve the infection and progress to a chronic state. Persistent HCV infection is associated with liver cirrhosis and hepatocellular carcinoma, thus becoming a leading cause of liver transplantation in the United States (Houghton, 1996). Unfortunately, no vaccine or effective treatment for HCV is currently available, and the mechanism(s) leading to HCV chronicity is not well defined.

Similar to many persistent DNA viruses, HCV, a single-stranded RNA virus, has evolved one or more mechanisms to establish and maintain viral persistent infection. Prominent features of HCV are its apparent immune evasion and quasispecies nature. Although a high rate of genetic variability may facilitate viral escape and persistence in the face of anti-viral specific immunity (Afonso et al., 1999), immune responses to HCV, both cellular and humoral, are not sufficient to resolve infection in most humans or protect against reinfection in chimpanzees (Farci et al., 1992; Alter, 1997). In addition, several studies provide compelling evidence that HCV is associated with opportunistic infections. This is well supported by increased susceptibility of chronic HCV patients to other microbial pathogens, including viruses (Liaw, 1995; Jardi et al., 2001), bacteria (Wu et al., 1995; Pellicano et al., 2000; Zucca et al., 2000; Spinzi et al., 2001), and parasites (Mohamed et al., 1998; Blanton et al., 2002). During HCV infection, anti-viral immune response is severely impaired. IgG1 restriction, low titer, and a delayed appearance of antibody response are observed (Ray and Ray, 2001). Strong and broad CTL and Th1 responses have been detected in individuals with acute hepatitis C, who subsequently clear the virus; however, these T cell responses are not sustained (Lechner et al., 2000a, 2000b). In
patients chronically infected with HCV, the frequencies of antiviral CTL are relatively low (Rehermann et al., 1996) and the proliferative response of HCV-specific CD8+ T cells was impaired (Wedemeyer et al., 2002). In addition, the production of Th1-type cytokines (i.e., IL-2 and IFN-γ) is dramatically suppressed in peripheral T cells of chronic HCV patients (Tsai et al., 1997; Lechmann et al., 1999). These observations suggest that HCV chronic infection may, at least in part, be the result of an inability to mount effective T lymphocyte responses, indicating that HCV gene product(s) might be involved in modulating or suppressing host immune responses. HCV core protein is the first protein to be synthesized during the early phases of HCV infection and core protein (genotype 1a) plays a role in impairing the host immune response to viral infection (Ray and Ray, 2001; Yao et al., 2001b).

HCV core protein is viral nucleocapsid protein with multiple functions (Ray and Ray, 2001). The presence of a putative DNA-binding motif, nuclear localization signals, and phosphorylation sites on core protein suggests its possible function as a gene regulator. These intriguing properties suggest that core protein, in concert with its cellular partners, may contribute to pathogenesis during persistent HCV infection. Recently, it has been reported that free core particles are found circulating in the bloodstream of HCV-infected patients, suggesting an immunopathological role in disease progression (Masalova et al., 1998; Maillard et al., 2001). Detection of core antigen in patient’s serum or plasma serves as a marker of HCV viremia in the serological (antibody negative) window phase (Peterson et al., 2000; Widell et al., 2002). Intriguingly, extracellular core protein is able to dampen the host immune response (Large et al., 1999; Yao et al., 2001a; Lee et al., 2001). The molecular basis of suppressing host immune response by extracellular core protein is mediated by its interaction with the complement receptor, gC1qR (Kittlesen et al., 2000). However, the precise mechanism(s) of core-induced immunosuppression remains to be identified.

An essential feature of the cellular immune response is the activation of T lymphocytes through TCR engagement by HLA-associated antigens. Ligation of TCR/CD3 provides an activation signal involving the induction of a number of genes, including the cytokine IL-2. The binding of IL-2 to its receptor is essential for TCR-stimulated cell cycle progression from G1 to S phase. Cell cycle progression is regulated by the coordinated expression and post-translational modification of cyclin-dependent kinases (Cdk) and their regulatory partners, cyclins. Specific kinase complexes, including cyclin D–Cdk4/6 and cyclin E–Cdk2, regulate the G1 to S transition by phosphorylating substrates, such as the pocket protein retinoblastoma (pRb). Additionally, two families of Cdk inhibitors (CKI), Cip/Kip and Ink4, have been identified (Sherr and Robert, 1995). The Cip/Kip members, including p21cip1, p27kip1, and p57kip2, associate with both cyclin E and D complexes, whereas the Ink4 members, including p15ink4b, p16ink4a, p18ink4c, and p19ink4d, inhibit cyclin D–Cdk4/6 complex formation (Sherr and Robert, 1995). Together, these inhibitors are critical regulators of the G1 to S transition.

During HCV infection, only a minor fraction of peripheral blood mononuclear cells (PBMC) is actually infected in vivo. Therefore, indirect mechanisms, such as contact between infected and uninfected cells or, more likely, interaction of soluble core protein, circulating in the blood of patients (Masalova et al., 1998; Maillard et al., 2001), with surface molecules on uninfected cells, may play an important role in dampening host immune response. Previously, we have demonstrated that HCV core impairs the activation of mitogen-activated protein kinase (MAPK) through an interaction with the globular C1q receptor, gC1qR (Yao et al., 2001a). This suggests that the interaction between HCV core and gC1qR may interfere with the early event of T cell activation. The importance of cell cycle regulatory proteins in T cell proliferation prompted us to examine whether these proteins might be targets for modulation by HCV core and thus contribute to the mechanism(s) of core-induced T cell unresponsiveness. We have therefore analyzed the effects of core protein on cell cycle progression, expression of essential components of G1 Cdk–cyclin complexes, and Cdk inhibitors in human PBMC in response to either anti-CD3/CD28 or Con A stimulation. We found that HCV core-induced inhibition of T lymphocyte proliferation was associated with retardation of cell cycle progression from G1 to S phase by stabilization of p27kip1, an inhibitor of cell cycle regulatory proteins.

**Results**

**HCV core inhibits cell cycle progression in human T cells in response to mitogenic stimulation**

We have previously demonstrated that HCV core inhibits T cell activation and proliferation through an interaction with gC1qR (Kittlesen et al., 2000; Yao et al., 2001a). To further determine the underlying mechanism for HCV core/gC1qR-induced inhibition of T cell function, we examined the effect of HCV core on cell cycle progression of T cells in response to mitogenic stimulation. Purified peripheral blood monocytes from healthy blood donors were treated with recombinant 6H-core or 6H-DHFR as a control and were stimulated with either Con A (Fig. 1 A, B, and C) or anti-CD3/CD28 (Fig. 1 D, E, and F). Cells were harvested at various time points after TCR stimulation and DNA content was measured by propidium iodide (PI) staining, followed by flow cytometry analysis.

As shown in Fig. 1A and D, upon 1 day of mitogenic stimulation, 2–3% of activated T cells had entered S + G2/M phase from the quiescent G0/G1 stage, and no significant difference in DNA content was observed for HCV core- or DHFR-treated cells. The degree of cell cycle progression in DHFR-treated cells was similar to...
that in mitogen-stimulated cells, suggesting that DHFR does not interfere with the cell cycle progression of T cells (data not shown). By day 3 of stimulation, DHFR-treated cells exhibited an increase in S phase progression, while an inhibition of S phase entry was observed in core-treated cells. After 5 days of stimulation, 12–13% of DHFR-treated cells were shown to be in S phase (Fig. 1C and F), while only 3–5% of those treated with core protein had progressed to S phase (Fig. 1B and E). This result suggests that HCV core arrests the cell cycle progression of activated T cells in G0/G1 and inhibits entry of these cells into S phase.

**HCV core/gC1qR interaction is directly involved in inhibition of cell cycle progression of activated T cells**

To determine whether HCV core-induced cell cycle arrest of activated T lymphocytes is mediated directly by its interaction with gC1qR, we examined the effect of anti-gC1qR antibody on abrogation of core-induced cell cycle arrest. To this end, HCV core-treated PBMC were stimulated with anti-CD3/CD28 Abs in the presence of anti-gC1qR Ab or control serum, and cell cycle progression was examined on day 5 by PI staining. As shown in Fig. 2A, 12.7% of anti-CD3/CD28-stimulated cells progressed into S/G2 phase, while only 4.6% of activated cells entered S/G2 phase in the presence of core protein. Importantly, 10.4% of core-treated cells progressed to S/G2 in the presence of anti-gC1qR Ab, suggesting that this antibody is able to partially reverse the inhibitory effect of HCV core on T cell cycle progression, while prebleed serum failed to reverse the core-induced inhibition of T cell cycle progression.

To confirm the core’s inhibitory effect on T cell cycle progression by the core/gC1qR interaction, we examined the cell cycle progression by staining PBMC with both CFDA-SE and APC-conjugated anti-CD3 Ab. CFDA-SE (CFSE) labels live cells and segregates equally between daughter cells during mitosis. Thus, the flow cytometry analysis of CFSE-labeled cells is a sensitive method to quantify the cell cycle progression. To this end, PBMC were labeled with CFSE and treated with HCV core or DHFR followed by stimulation with Con A. We also examined the cell cycle progression of core-treated cells in the presence of anti-gC1qR antibody or a control antibody. At various time points following the treatment, the cells were stained with APC-anti-CD3, and the cell cycle progression of activated T cells was analyzed by assessing the fluorescence intensity of CFSE-labeled CD3+ T cells.

As shown in Fig. 2B, upon 4 h of stimulation in both core-treated cells and untreated cells, 58% of T cells were detected in M2 (higher intensity of CFSE staining), while 42% of T cells were detected in M1 gate (lower intensity
of CFSE staining). Upon 1 day of mitogenic stimulation in untreated cells, 50% (91% at day 1 minus 42% at 4 h) of T cells had progressed from M2 to M1, which indicated the cell division, and 9% of the cells still remained in the M2 gate. In contrast, diminished cell cycle progression was observed for HCV core-treated cells upon mitogenic stimulation. Only 25% (67% at day 1 minus 42% at 4 h) of core-treated cells progressed from M2 to M1 upon mitogenic stimulation, while 30% of the cells remained in M2. In addition, the inhibition of cell cycle progression by HCV core was shown in cells following 3 or 5 days of stimulation (data not shown). Furthermore, the addition of anti-gC1qR antibody to core-treated culture restored the core-induced inhibition of cell cycle progression, confirming that gC1qR is involved in HCV core-induced cell cycle arrest.

**HCV core inhibits activation-associated Cdk/cyclin complex formation**

Cell cycle progression is driven by the sequential activation of Cdns and their regulatory partners, cyclins. As shown in Fig. 3A, activation of Cdk4/Cdk6 complexed with D-type cyclins in mid G1 phase and Cdk2–cyclin E late in G1, is particularly important for passing the G1/S restriction point. To investigate the effect of HCV core on regulation of Cdk/cyclin expression, we analyzed the expression levels of the G1/S Cdns and cyclins following stimulation with anti-CD3/CD28, in the presence of core or DHFR, by Western blot analysis. As shown in Fig. 3B, quiescent cells did not express detectable levels of Cdk2/Cdk4, while the expression of Cdk2/4 was detectable at 48 and 72 h after mitogenic stimulation.
Based on the time-dependent upregulation of Cdk2/4 expression, we next sought to examine the effect of core protein on these regulators at 48 and 72 h after TCR stimulation. As shown in Fig. 3C, HCV core inhibited the activation-dependent upregulation of Cdk2 in T cells at 48 and 72 h after TCR stimulation, compared to control DHFR-treated cells. The expression pattern of Cdk4 was found to be very similar to that of Cdk2, except that the expression level was lower than that of the former; thus, Cdk4 failed to accumulate to a detectable level at 48 h, and only a very faint band was detectable after 72 h of stimulation in the presence of core protein (Fig. 3C). Correspondingly, the levels of cyclin E and cyclin D expression, the catalytic partners of the Cdk2 and Cdk4 in lymphocytes, were similar to those of the Cdk subunits (Fig. 3C).

Since Cdk/cyclin activity is essential for passing the G1 restriction point and entering S phase, HCV core might inhibit the activation-associated accumulation of Cdk/cyclins, resulting in retardation of cell cycle progression. To examine this possibility, we analyzed Cdk2/cyclin E activity in core-treated cells by measuring kinase activity in vitro, using histone H1 as substrate. As shown in Fig. 3D, Cdk2/cyclin E activity was observed by 48 h, with an increase in substrate phosphorylation after 72 h of TCR stimulation, for cell lysates derived from DHFR-treated PBMC. However, for lysates obtained from cells treated with HCV core, a significant downregulation of Cdk2 kinase activity was observed at both 48 and 72 h after treatment (Fig. 3D), suggesting that HCV core impairs activation of Cdk/cyclin kinase activity.

Cdk/cyclin-mediated phosphorylation of pRb, which in turn activates a number of cell cycle regulatory proteins, including E2F, is critical for cell division. As seen in Fig. 4, cells stimulated with anti-CD3/CD28 in the presence of DHFR revealed a distinct doublet, suggesting that pRb is phosphorylated. In contrast, treatment of cells

---

Fig. 3. HCV core inhibits the expression of Cdk/cyclin proteins required for cell cycle progression from G1 to S phase. (A) Diagrammatic representation of the cell cycle regulatory proteins. (B) Time-dependent expression of Cdk2 and Cdk4. PBMC were stimulated with anti-CD3/CD28 for various time points (0, 24, 48, 72 h) and were analyzed for Cdk2 and Cdk4 expression by Western blot analysis. (C) Expression of Cdk2, Cdk4 cyclin E, and cyclin D. Cells were cultured in the presence of 2 μg/ml of HCV core or DHFR for 48 or 72 h followed by anti-CD3/CD28 stimulation. Cell lysates were subjected to Western blot analysis specific for Cdk2/4 and cyclin D/E. β–Actin was employed as a loading control in these experiments. The data were reproducible in two independent experiments. (D) Inhibition of Cdk2 in vitro kinase activity in core-treated PBMC. Equal amounts of cell lysates, derived from PBMC treated as above, were used to immunoprecipitate Cdk2 complexes and subsequently tested for in vitro kinase activity to phosphorylate exogenous substrate, histone H1. Phosphorylation of histone H1 was quantified and the mean OD ± SD values are shown above. The results are reproducible in two independent experiments.
with HCV core resulted in a weak single band, suggesting that core protein abolished the phosphorylation of pRb. The expression level of a control protein, β-actin, however, was not affected by core treatment. Taken together, these results demonstrate that inhibition of S-phase entry by HCV core is associated with the suppression of proteins involved in cell cycle progression, such as Cdk/cyclin complexes and pRb.

Cell cycle arrest by HCV core involves the Cdk inhibitor p27Kip1

Two classes of inhibitors have been identified that negatively regulate the kinase activity of G1-associated Cdns (Sherr and Robert, 1995). One class, Ink4 (p15Ink4b, p16Ink4a, p18Ink4c, and p19Ink4d), interferes with the formation of Cdk4/6-cyclin D complexes, while the other, Cip/Kip (p21Cip, p27Kip1, and p57Kip2), has a more generalized Cdk inhibitory activity. Among these, p27Kip1 has been implicated as a central regulator of quiescence in T cells. Downregulation of p27Kip1 in mitogen-stimulated T cells is a primary event that is obligate for the activation of G1 kinase activity and subsequent S-phase entry, whereas overexpression of p27Kip1 in T cells results in G1 arrest (Sherr and Robert, 1995).

As shown in Fig. 5A, quiescent cells express high levels of p27Kip1, correlating with the G0/G1 status of these cells. Mitogenic stimulation of PBMC resulted in a rapid decline in p27Kip1 expression over 48 h and the expression of p27Kip1 was significantly diminished after 72 h stimulation. In contrast, high levels of p27Kip1, but not β-actin, was found in core-treated cells compared to that in control cells (Fig. 5B). Expression of the closely related Cip/Kip family member, p21Cip, was rarely detectable at any time point in our system (data not shown). We also determined expression levels of Ink4 family members, p15Ink4b and p16Ink4a, which appeared to be unaffected by HCV core treatment (data not shown).
containing medium was then removed and the cells were chased for 24 h. As shown in Fig. 6B, HCV core did not affect the rate of p27Kip1 synthesis during the 6 h pulse period. After the 24 h chase, the amount of radiolabeled p27Kip1 was significantly reduced in cells treated with both medium or DHFR (relative intensity nearly three times less than that of HCV core), while it was not significantly affected in the presence of core, suggesting that the newly synthesized p27Kip1 in activated T cells was stabilized by the treatment of core protein. Taken together, these data indicate that HCV core affects the level of p27Kip1 in activated T cells through delayed degradation, rather than affecting its rate of synthesis. Furthermore, the p27Kip1 stabilization by addition of core protein into the culture on 2 days after Con A stimulation suggests that HCV core is able to inhibit the ongoing cell cycle progression after T cell activation.

Discussion

We have previously demonstrated that the HCV core/gC1qR interaction inhibits T cell proliferation by interfering with the ERK/MEK MAPK pathway (Kittlesen et al., 2000; Yao et al., 2001a). In this study, we attempted to identify the molecular events of HCV core-induced T cell unresponsiveness by determining cell cycle progression and the level of cell cycle regulatory proteins in activated T cells. Our results demonstrated that HCV core arrests T lymphocytes in G1 and blocks entry into S phase. In addition, anti-gC1qR antibody treatment could reverse the core-induced inhibition of cell cycle progression, suggesting a direct role for the HCV core/gC1qR interaction in the observed cell cycle arrest. Importantly, p27Kip1, a crucial cell cycle inhibitor, was found to be stabilized in HCV core-treated T cells. Correspondingly, we observed a deregulation of Cdk2/4–cyclinE/D complexes and pRb, both required for the G1 to S transition, in the presence of HCV core protein.

The defect in cell cycle progression induced by HCV core, described in this report, might explain the HCV core/gC1qR-mediated anti-T cell proliferative effect previously described (Kittlesen et al., 2000). It is important to point out that C1q, the natural ligand of gC1qR, also suppresses T cell proliferation by inhibiting cell cycle progression. Although the molecular mechanism of cell cycle arrest by C1q/gC1qR signaling has yet to be elucidated, upregulation of p27Kip1, as was the case for core-treated cells, might be involved. Based on our studies, we propose a model for the mechanism of HCV core-induced cell cycle arrest. As shown in Fig. 7, the HCV core/gC1qR interaction leads to inhibition of activation-associated degradation of p27Kip1, which in turn blocks Cdk/cyclin complex formation and pRb phosphorylation, thus blocking T cell cycle progression. However, the precise pathway(s) initially targeted by the core/gC1qR interaction has yet to be fully elucidated.

Efficient ligation of the TCR by high-density antigen can generate a productive T cell response characterized by cytokine secretion and clonal expansion. In contrast, suboptimal cross-linking of the TCR by antigen alone, in the absence of costimulation, is not sufficient to induce a productive immune response, but instead leads to T cell anergy (Schwardz, 1997). In contrast to primary unstimulated T
HCV core/gC1qR interaction

\[ ? \]

p27^{kip1}

\[ \downarrow \]

Cdk4/6 / Cyclin D
Cdk2 / Cyclin E

pRb phosphorylation

Inhibition of cell cycle progression

Fig. 7. Model for inhibition of cell cycle arrest of activated T cells by HCV core. The binding of HCV core on gC1qR displayed on the cell surface of T cells will trigger gene(s) involved in p27^{kip1} expression and/or stability. Thus, stable expression of p27^{kip1} in core-treated PBMC will lead to inhibit the expression of proteins responsible for cell cycle progression from G1 to S phase and arrest activated T cells at G1 phase.

cells, which can enter the cell cycle and clonally expand after antigen-specific stimulation, anergic T cells do not proliferate. Instead, they remain in a state of long-term antigen-specific unresponsiveness. These events result in increased expression of p27^{kip1}, defective Cdk/cyclin activation, inhibition of pRb phosphorylation, and blockade of G1 to S phase progression (Boussiotis et al., 2000). p27^{kip1} functions as an anergy factor and is an essential regulator responsible for inhibiting of clonal expansion of anergic T cells (Boussiotis et al., 2000). Under growth-arresting conditions, p27^{kip1} is translocated to Cdk2–cyclin E complexes, inhibiting their activity (Polyak et al., 1994; Reynolds and Massague 1997). Upon activation of T cells, especially by IL-2 mediated signaling, p27^{kip1} is phosphorylated and subsequently targeted for degradation in an ubiquitin-dependent manner (Firpo et al., 1994; Nourse et al., 1997). Interestingly, the kinases involved in this particular phosphorylation step include the Cdk2–cyclin E complex itself, for which p27^{kip1} is both an inhibitor and a substrate (Muller et al., 1997; Sheaff et al., 1997). In this report, p27^{kip1} degradation upon TCR stimulation was almost completely abolished in core-treated cells, as shown in Fig. 6B, suggesting that HCV core might drive these cells into a state of anergy. However, the molecular events required for p27^{kip1} stability, induced by the HCV core/gC1qR interaction, have yet to be defined.

Several possibilities to explain such an effect are summarized below. First, the inhibition of Cdk/cyclin complex formation by HCV core may prevent phosphorylation and degradation of p27^{kip1}, as described above (Muller et al., 1997; Sheaff et al., 1997). Thus, the core-induced inhibition of newly synthesized Cdk–cyclin complexes in mitogen-stimulated T cells may not only affect downstream targets, such as pRb, but also fail to trigger positive feedback mechanisms, which activate preexisting complexes through phosphorylation of p27^{kip1} as shown in Figs. 5 and 6B. Second, it has been shown that the activation of phosphatidylinositol 3-hydroxy kinase (PI3K) target protein kinase (B) (PKB, Akt) signaling pathway involves the activation-associated down-regulation of p27^{kip1} and T cells cycle progression (Appleman et al., 2002). Thus, HCV core may disrupt the activation of upstream kinases, such as PI3K/Akt (PKB), necessary for p27^{kip1} degradation (Avota et al., 2001; Appleman et al., 2002). We are currently examining the effect of HCV core on the alteration of PI3K/Akt activation. Third, the observed deregulation of p27^{kip1} is possibly due to impaired ERK/MEK MAPK activation by HCV core, as previously described (Yao et al., 2001a). As shown in Fig. 6B, HCV core stabilized p27^{kip1} by 24 h, suggesting that core’s effect is induced prior to this time point. Similarly, HCV core inhibits activation of ERK/MEK MAPK by 24 h (Yao et al., 2001a), suggesting that p27^{kip1} stability may occur independent of core-induced inhibition of MAPK. Thus, it is likely that these two events may happen simultaneously as the result of HCV core treatment during early T cell activation, although we do not exclude the possibility that these two pathways may overlap by cross-talk to each other. Last, following HCV core’s interaction with the gC1qR and subsequent internalization (Yao et al., unpublished data), core may form a complex with p27^{kip1}, thus stabilizing it. Similarly, HCV core has been found to associate with p21^{cip1}, also a CKI Cip/Kip family member, in vitro (Wang et al., 2000). Our attempt to establish such an interaction, by coimmunoprecipitation of HCV core with p27^{kip1}, failed to detect such an association and, thus, also excluded this possibility (data not shown). Nevertheless, the delay of p27^{kip1} degradation helps to explain our finding that Cdk/cyclin complexes and pRb fail to accumulate in the presence of HCV core, as shown in Fig. 7, thus preventing progression of T cells from G1, to S phase.

Since cell cycle progression of activated T cells is a multistep process in which many molecules must act in concert, inactivation of one pathway could partially or completely abrogate the proliferative response (Taniguchi, 1995). Thus, a number of agents involved in suppressing T cell proliferation have been reported to function through several pathways. As is observed for cells arrested by HCV core, IFN-α appears to exert a potent antiproliferative signal in several lymphoid cell lines via upregulation of the p27^{kip1}, which dephosphorylates pocket proteins and leads to G1 arrest (Erickson et al., 1999; Sangfelt et al., 1999). In addition, IL-2-induced expression of both early and late G1 Cdk complexes, such as cyclin D–Cdk4 and cyclin E–Cdk2, is also inhibited by IFN-α. Similar to the biochemical events
required for HCV core-mediated cell cycle arrest, rapamycin involves in the blockade of IL-2-mediated p27Kip1 degradation, leading to the suppression of T cell proliferation (Nourse et al., 1994). However, the molecular mechanism for the effect of other immunosuppressive agents, such as undecaploidindosin, differs from that induced by HCV core. In undecaploidindosin-treated cells, cell cycle arrest is mediated by blockade of cyclin E/A and Cdk2/4, without alteration of p27Kip1 expression (Songia et al., 1997).

While examples of virus-induced G1 arrest are not very numerous, they often include viruses known to suppress lymphocyte function. Treatment with the V3 loop of an HIV-1 clone restricts the IL-2-dependent expression of Cdk2-cyclin E activity in human T cells (Sakaida et al., 1998). Additionally, measles-virus-induced immune suppression has been found to be associated with deregulation of G1 control proteins by disruption of Akt kinase activity (Avota et al., 2001). Our data indicate that HCV core may act further upstream than these immunosuppressive drugs and viruses discussed above, as it exhibits a broader inhibitory effect on the cell cycle machinery.

HCV is characterized by a high incidence of persistent infection, suggesting the evasion of immune surveillance by this virus. Indeed, HCV core protein, one of HCV gene products, contains the immunomodulatory function (Geissler et al., 1998; Large et al., 1999; Ray and Ray, 2001). There might be a genotype difference regarding core’s effect on suppressing host immune response, as suggested by a recent study that the expression of core protein derived from HCV genotype 1b in transgenic mice didn’t elicit such an effect (Liu et al., 2002). Thus, studies on examining the immunomodulatory function of different HCV genotypes will be valuable in understanding HCV pathogenesis. In addition, the quantity of core protein circulating in the bloodstream of HCV-infected patients will be detrimental to deliver the anti-T cell proliferative signal. Several studies reported that nanomolar range of core protein is detectable in the circulating blood of HCV-infected patients (Masalova et al., 1998; Peterson et al., 2000; Maillard et al., 2001; Widell et al., 2002). It is important to point out that this concentration of core protein might be sufficient to inactivate the function of immune cells in vivo. We are currently investigating to determine the level of core protein during the acute phase of viral infection and compare circulating core level between patients who resolved infection and patients who failed to clear the virus. Nevertheless, early and sustained CD4+ and CD8+ T cell responses are crucial for controlling HCV infection (Lechner et al., 2000a, 2000b). Therefore, the HCV core-mediated inhibition of T cell cycle progression, reported here, suggests that core protein, circulating in the blood of patients upon infection, may play a pivotal role in impairing intracellular, as well as peripheral, T lymphocytes, thus facilitating the establishment of persistent HCV infection. Further elucidation of how HCV core protein modulates host immune responses to infection may provide a basis for the rational design of novel therapeutics.

Material and methods

PBMC isolation and culture

Human PBMC were isolated from peripheral blood of donors (Virginia Blood Service, Richmond, VA) by Ficoll-density centrifugation using lympholyte-H (Cedarlane Labs, Ontario, Canada). PBMC were washed twice and incubated with RPMI 1640 (Gibco BRL, Gaithersburg, MD) containing 10% (v/v) FBS (Hyclone), penicillin/streptomycin (100 μg/ml, Gibco BRL, MD), L-glutamine (2 mM), and 2-ME (5.5 × 10⁻⁵ M, Gibco BRL) at 37°C with 5% CO₂ in a humidified atmosphere for the following assays.

Antibodies and reagents

Recombinant 6H-core protein, with 6 histidine residues tagged at the N-terminal of the full-length HCV core protein (amino acid residue 2-191), was expressed and purified under native condition. A control protein, 6H-DHFR, was prepared in the same way as 6H-core protein. To activate resting human PBMC, two different T cell mitogens were employed: (1) Con A (2 μg/ml, Sigma, St. Louis, O), (2) anti-CD3 (UCHT-1, 1 μg/ml, PharrMingen, San Diego, CA) and anti-CD28 (1 μg/ml, Pharmingen). The following antibodies specific for cell cycle regulatory proteins were purchased from Pharmingen for Western blot: p15 Ink4b, p16 Ink4a, p21Cip, p27Kip1, Cdk2, Cdk4, cyclin E, cyclin D1/D2/D3, and pRb. Rabbit anti-β-actin (Sigma) was used as a loading control. Horseradish peroxidase (HRP)-anti-mouse IgG and HRP-anti-rabbit IgG conjugates (Gibco BRL, Gaithersburg, MD) served as secondary antibodies. gC1qR-specific antibodies were obtained from rabbit by injecting 6H-gC1qR fusion protein. Histone H1 from Sigma and [γ-³²P] ATP were purchased from Amersham Pharmacia Biotech (Arlington Heights, IL).

Cell cycle analysis by flow cytometry

A total of 2.5 × 10⁵ PBMC were cultured with 200 μl RPMC medium in the presence of 2 μg/ml of 6H-core or 6H-DHFR as a control protein and were stimulated with either Con A (2 μg/ml) or plate-bound anti-CD3/CD28 antibodies (1 μg/ml). At days 1, 3, and 5 after treatment, the cells were harvested in 1.5-ml Eppendorf tubes and washed two times with PBS by centrifugation at 1000g for 5 min. Cells were then resuspended in 400 μl of propidium iodide staining solution (50 μg/ml propidium iodide, 0.1% sodium citrate, 0.3% NP-40, 100 μg/ml boiled RNase in 100 mM NaAc, pH 5.0) and incubated at 4°C for 30 min. DNA histograms and statistics were determined by FACS analysis.
using CellQuest software, based on collecting 10,000 events of viable cells gated on lymphocyte populations.

To label PBMC with CFSE, 10 × 10^6 PBMC were washed two times with PBS and then incubated with 5 μM CFSE in 10 ml of prewarmed PBS at 37°C in the dark for 8 min. The incorporation of CFSE into the living cells was stopped by adding 30 ml RPMI 1640 containing 10% FBS. Following centrifugation at 1200 RPM for 5 min, CFSE-labeled PBMC were cultured in complete RPMI 1640 medium stimulated with Con A (2 μg/ml) and IL-2 (40 U/ml), in the presence or absence of core protein (2 μg/ml). Subsequently, the cells were harvested at various time points, washed with PBS, and then re-stained with APC-anti-CD3 conjugate (PharMingen) at room temperature in the dark for 1 h. The cells were washed and fixed with 1% paraformaldehyde in PBS prior to analysis of cell progression by three-color flow cytometry (BD Bioscience, San Jose CA). A total of 100,000 viable cells were collected and cell cycle progression was analyzed using CellQuest software gated on the T lymphocyte population.

To examine whether blocking the core/gC1qR interaction may abrogate the core’s inhibitory effect on cell progression, rabbit anti-gC1qR antibody (ELISA titer 1/12800) was added simultaneously to the above culture at a 1:10 dilution. Prebleed serum from the same rabbit was used as negative control. At various time points following treatment, cells were harvested for staining, followed by cytometry analysis.

**Western blot analysis**

A total of 2 × 10^6 PBMC were treated with 2 μg/ml of 6H-core or 6H-DHFR for various intervals and activated with anti-CD3/CD28 antibodies (1 μg/ml). Cell lysates were prepared by resuspending cell pellets in lysis buffer (20 mM Tris, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, and protease inhibitor cocktail. Boehringer Mannheim, GmbH, Germany) for 30 min at 4°C. Cell lysates were further disrupted and homogenized by passage through a 21-gauge needle and sonication. Cellular debris was pelleted by centrifugation and supernatants were collected and frozen at −80°C. A total of 80 μg of protein (the protein concentration was determined with BCA assay kit, Pierce, Rockford, IL) was loaded in each well and resolved by SDS-PAGE, followed by semidry transfer (Pharmacia, Piscataway, NJ) onto Hybond-P membranes (Amersham). A total of 80 μg of protein extract was subjected to immunoprecipitation with monoclonal anti-p27 Kip1 antibody (1:2000), anti-Cdk2 (1:2000), anti-pRb (1:1000), and rabbit polyclonal antibodies: anti-Cdk4 (1:2000), anti-Cdk2 (1:2000), anti-β-actin (1:500). Anti-body–antigen interaction was detected by incubation with HRP-conjugated anti-mouse or anti-rabbit IgG secondary antibodies and subsequently developed by using the enhanced chemiluminescence kit (ECL-Plus, Amersham).

**Kinase assay**

A total of 2 × 10^6 PBMC were treated with 2 μg/ml of 6H-core or 6H-DHFR for 48 and 72 h and were simultaneously stimulated with anti-CD3/CD28 antibodies (1 μg/ml). Cell lysates were prepared by resuspending cell pellets in lysis buffer as described above. Equal amounts of total protein (80 μg) from the supernatants were used for immunoprecipitation with anti-Cdk2 antibodies and protein A/G-agarose (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at 4°C with rotation. After centrifugation, the complexes were washed twice each in PBS and once in kinase buffer containing 50 mM Heps (pH 7.5), 1 mM DTT, 2.5 mM EDTA, 10 mM MgCl₂, 20 μM ATP, 10 μCi [γ-32P]ATP, and 10 μg histone H1 (Sigma) as substrate for 30 min at 30°C. The reaction was stopped by addition of 5 × SDS sample buffer, giving a final concentration of 60 mM Tris–HCl (pH 6.8), 10% glycerol, 2% SDS, and 5% β-mercaptoethanol. After denaturation, samples were subjected to SDS–PAGE and specific bands were analyzed and quantified by using a phosphorimager (Molecular Dynamics).

**p27Kip1 biosynthesis and stability assay**

After 48 h Con A stimulation, 30 × 10^6 PBMC were washed twice in methionine-free RPMI 1640 at room temperature and incubated 1 h at a concentration of 10 × 10^6 cells/ml in 6-well plates. The supernatant was aspirated and the cells were then labeled for 6 h at 37°C in medium containing 0.2 mCi of [35S]Met (Redivue Promix; Amersham) and IL-2 (100 U/ml), in the presence of 2 μg/ml 6H-core or 6H-DHFR followed by washing. Cells were chased for 24 h in medium containing IL-2 (100 U/ml) and core (2 μg/ml) or DHFR protein (2 μg/ml). Aliquots of cells (pulse and chase) were harvested and washed in ice-cold PBS. Cell lysates were prepared as described above. A total of 500 μg of labeled protein extract was subjected to immunoprecipitation with monoclonal anti-p27Kip1 bound to protein A/G-agarose (Santa Cruz Biotechnology), according to the manufacturer’s instructions. The immunoprecipitated proteins were separated by 15% SDS–PAGE. The gel was dried at 80°C for 2 h and exposed to X-ray film (Kodak) at −80°C.

**RT-PCR for Tob**

A total of 1 × 10^6 PBMC were activated with anti-CD3/CD28 antibodies (1 μg/ml) and treated with 6H-core or 6H-DHFR (2 μg/ml) for 6 h. Total RNA was isolated by the TRIZol method (Life Technologies). A total of 1 μg of RNA was DNase-treated to prevent amplification of genomic
DNA and subsequently exposed to MuLV-derived reverse transcriptase. β-Actin cDNA level from each sample was used to normalize the samples for differences in PCR efficiency. Tob mRNA quantity was determined by using endpoint dilution PCR, involving three serial 1 to 5 dilutions (1:1, 1:5, 1:25, and 1:125) of reverse transcription (RT) products for PCR amplification. Cycle conditions included an initial 2 min denaturation at 95°C and final 10-min extension at 72°C. Intervening temperatures included 30 s at 95°C, 30 s at 58°C, and 30 s at 72°C, for a total of 40 cycles. To control for genomic DNA contamination, equal amounts of cDNA from each sample were PCR amplified, without RT. The resulting PCR products were separated on a 2% BioGel (Bio 101, Carlsbad, CA). The following sets of primers were used in the PCR reaction: Tob sense: 5′-GACCCATTGAGGTTCCTACC-3′; antisense: 5′-CCATACAGAGAGTGCATTGAGG-3′; β-actin sense: 5′-CGACCGGGAATCTCGTCGTACATTAAGGA-3′; antisense: 5′-CGTCACTACTCCGGTGATCCACATTCT-3′.

Acknowledgments

We thank our colleagues for constructive criticism and comments. We also greatly appreciate the outstanding technical support to Mr. Travis Lillard and Ms. Susan Landes. This work is supported by an American Association for the Study of Liver Diseases/Schering Advanced Hepatology Fellowship (to Z.Q.Y.) and Public Health Service Grant DK066351 (to Y.S.H.).

References


