peptides in a cotranslational and posttranslational fashion, using both viral and cellular protease activity.

A replication complex then arises from a combination of viral nonstructural proteins and cellular material. Viral NS4B, NS5A, NS5B and the NS3 helicase-NTPase domain are known to be important components of this structure, and the cellular substrate is referred to as a 'membranous web', which is a perinuclear vesiculomembranous aggregate thought to be derived from the endoplasmic reticulum. At this site, active RNA synthesis occurs.

The assembly and release of mature virions is not completely understood. Assembly likely occurs in proximity to the membranous web, and secretion may be dependant upon the ion channels formed by the p7 protein. Gastaminza et al. (2008) concluded from their studies that the virus hijacks the host machinery for assembly, maturation, degradation and secretion of VLDL, thereby explaining in part the tropism for hepatocytes.

In the liver, in situ hybridization shows up to 50% of hepatocytes to contain HCV in infection (Pal et al., 2006). Antigenic expression detected by immunohistochemistry is reported in 5% or less of hepatocytes, and in lesser numbers of biliary epithelial cells and sinusoidal lining cells (Nouri-Aria et al., 1995), although this result may be artifactually low due to antigen instability in formalin-fixed tissues. Occasional mononuclear cells also may express HCV antigens (Nouri-Aria et al., 1995). Using RT-PCR, HCV has also been detected in lymph nodes, pancreas, bone marrow, spleen, thyroid, brain and adrenal gland (Forton et al., 2004; Lerat et al., 1998). It is not known whether the virus replicates in haematopoietic cells.

Host response to HCV infection

Innate immune response to HCV

The cell immune response is important in light of the fact that the virus is largely noncytopathogenic, and mechanisms external to the infected cell are required for viral elimination. The integrated host response to HCV infection is comprised of innate and adaptive components of the immune system, with each arm modulating the kinetics of the other to some extent. In addition, there is a separate crosstalk between host and virus, as each tries to gain advantage and establish a favourable equilibrium. This initial set of interactions, in which NK cells play an important role (Khakoo et al., 2004), is crucial in determining the outcome of the infection (Gale and Foy, 2005). In approximately 20% of acutely infected patients, this process generates a strong T-cell response that leads to spontaneous resolution of infection. More often than not, however, the virus gains a marginal advantage that permits its survival at the cost of chronic hepatitis and attendant complications for the host. This discussion will focus on aspects of the innate immune response with emphasis on those features impinging upon the role of NK cells. Several recent reviews have addressed the host response to this infection with emphasis on the T-cell response (Blackburn and Wherry, 2007; Bowen and Walker, 2005; Ishii and Koziel, 2008; Li et al., 2008; Manigold and Racanelli, 2007; Neumann-Haefelin and Thimme, 2007; Neumann-Haefelin et al., 2007; Rehermann, 2007; Semmo and Klenerman, 2007).

Hepatocyte infection, IFN production and HCV countermeasures

IFN-α production indirectly activates NK cells via its effect on DCs, which provides one of many areas in which the host:virus struggle is played out. Following infection, type I IFNs are elaborated by multiple cell types, likely beginning with hepatocytes. This can be considered as the immediate early innate immune response. Li et al. (2005) showed the existence of TLR-3 as well as a retinoic acid-inducible gene (RIG) pathways in cultured hepatocytes, suggesting that these are potential in vivo mechanisms for type I IFN production by liver cells. Upon engagement of dsRNA by cell surface or intracytoplasmic membrane-bound TLR-3, binding to the adaptor protein TRIF (Toll/interleukin-1 receptor domain-containing adaptor protein inducing IFN-β) occurs, which ultimately leads to the activation of the transcription factors NFκB and IRF-3 (IFN regulatory factor-3). Similarly, intracytoplasmic RIG-1 recognition of dsRNA leads to IRF-3 activation via a separate pathway, as well as to formation of the active form of the transcription factor AP-1. These products in turn induce IFN-β gene transcription, and this protein is produced and secreted by the cell (Bode et al., 2007).

IFN-β exerts its effect in an autocrine or paracrine fashion by binding the cell surface type I IFN-α/β receptor. This engages the Jak/STAT pathway to lead to the production of the IFN-stimulated gene factor 3 (ISGF3) complex, which translocates to the nucleus and acts to enhance transcription of a family of IFN-stimulated genes. These include IRF-7, which, in conjunction with IRF-3, upregulates IFN-α transcription, thereby propagating a positive feedback mechanism to magnify the antiviral effect (Bode et al., 2007).

Reflecting the importance of this process in establishing an environment conducive to control of virus infection, HCV has evolved a number of potential strategies to combat the host offensive. Foy et al. (2003), using Huh7 hepatoma cells, demonstrated that the viral protease NS3/4 was capable of blocking phosphorylation and activity of IRF-3, resulting

in enhanced viral replication in vitro. The viral NS3/4 A protein is also able to interfere directly with both TLR-3 and RIG-1-mediated signal transduction pathways. In the former case, this involves proteolysis of the adaptor protein TRIF (Li et al., 2005) and, in the latter case, cleavage of the mitochondrial tethered adaptor protein CARDIF (caspase recruitment domain adaptor-inducing IFN-β, also known as IPS-1, MAVS or VISA) (Foy et al., 2005; Hiscott et al., 2006). Tasaka et al. (2007) also found a role for viral NS4B in the inhibition of the RIG-1 pathway. Abe et al. (2007) reported that HCV NS5A can interfere with TLR-dependent cytokine production in mouse macrophage cell lines by interacting with the death domain of the adaptor molecule MyD88. This study is of particular interest since the interaction of HCV proteins with TLR pathways represents a potentially significant and largely unexplored mechanism by which the virus may evade host immune surveillance. HCV may also interfere with the Jak/STAT signal transduction pathway, possibly by effects of the viral core protein on STAT-1, SOCS-3 (suppressor of cytokine signalling-3) and ISGF3. The controversies and implications surrounding these interactions have been recently reviewed (Bode et al., 2007). Despite these efforts by HCV, studies in the chimpanzee model show high levels of intrahepatic IFN-α production during early infection (Thimme et al., 2002). This is not associated with a concomitant decrease in HCV genomic levels, suggesting that resistance to, rather than interference with, elevated IFN-α level may be more important for viral survival.

NK cells and HCV infection

Introductory comments

Prior to a detailed discussion of NK cells in the context of HCV infection, a few general comments are in order. First, regional differences in the distribution of NK cells need to be stressed. Golden-Mason and Rosen (2006) point out that intrahepatic mononuclear cells contain a higher percentage of NK cells than is seen in the peripheral blood. Within the liver, NK cells comprise 20–30% of mononuclear cells and may account for up to half of intrahepatic lymphocytes, as compared to representing 10–15% of peripheral blood mononuclear cells. This suggests that HCV would need to have evolved specific mechanisms for long-term survival within an environment rich in NK cells (Golden-Mason and Rosen, 2006).

A number of published studies have compared the more easily obtained peripheral blood NK cell frequencies in HCV-infected patients versus uninfected patients. Such studies have led to sometimes conflicting results, raising the possibility that rapid phenotypic or functional changes of NK cells are occurring in this compartment in vivo. Examination of liver-infiltrating NK cells is technically more demanding but may provide more precise and otherwise unobtainable insights into the role of these cells in HCV infection.

A synopsis of the following discussion of NK cells and HCV infection is presented in Figure 44.1 and in Table 44.1.

A second comment relates to the issue of NK cell subpopulations. NK cells contain at least two different subpopulations according to their degree of CD56 expression, and these subpopulations differ in function as well as phenotype. There is speculation that CD56-defined subsets may play distinct roles in the pathogenesis of HCV-induced liver disease, such as in inflammation or fibrosis (Lin et al., 2004; Morishima et al., 2006). Several recent reports have demonstrated that, in chronic HCV infection, the frequency of $CD56^{dim}$ NK cells is reduced, whereas numbers of $CD56^{bright}$ NK cells are increased (Golden-Mason et al., 2008; Meier et al., 2005; Morishima et al., 2006). Future investigation may be necessary to elucidate the extent to which previously reported functional impairment of NK cells in HCV infection can be ascribed to such alterations in subset populations.

NK cell receptors and HCV infection

Effects of NK cell CD81 engagement by HCV

CD81 is a widely expressed cell surface protein that that has been mentioned previously as a cellular coreceptor for ligation of HCV to the hepatocyte. This receptor is also present on most if not all cells of the immune system. It is a member of the tetraspanin family, whose constituents share the presence of four transmembrane domains that contain small and large extracellular loops (Levy and Shoham, 2005), the latter comprising the binding site for the viral E2 protein (Drummer et al., 2002). CD81 and related proteins are thought to integrate extracellular, cytoplasmic and intramembranous components into a 'tetraspanin web' with diverse functions dependant upon context.

In the case of NK cells, the E2:CD81 interaction results in downregulation of NK cell response to activation signals from CD16, NKG2D, IL-2, IL-12, IL-15 or β1 integrin (Crotta et al., 2006; Li et al., 2004; Tseng and Klimpel, 2002). This inhibition includes a reduction of IFN-γ production, decreased release of cytolytic granules and diminished proliferative activity. Li et al. (2004) showed that NK cells cocultured with HCV replicon-containing hepatic cells secreted IFN-γ that in turn upregulated hepatic cell STAT-1 and IFN-α production, resulting in marked inhibition of HCV RNA expression. These effects could be inhibited by cross-linking CD81 by specific antibody or by antibody to IFN-γ.

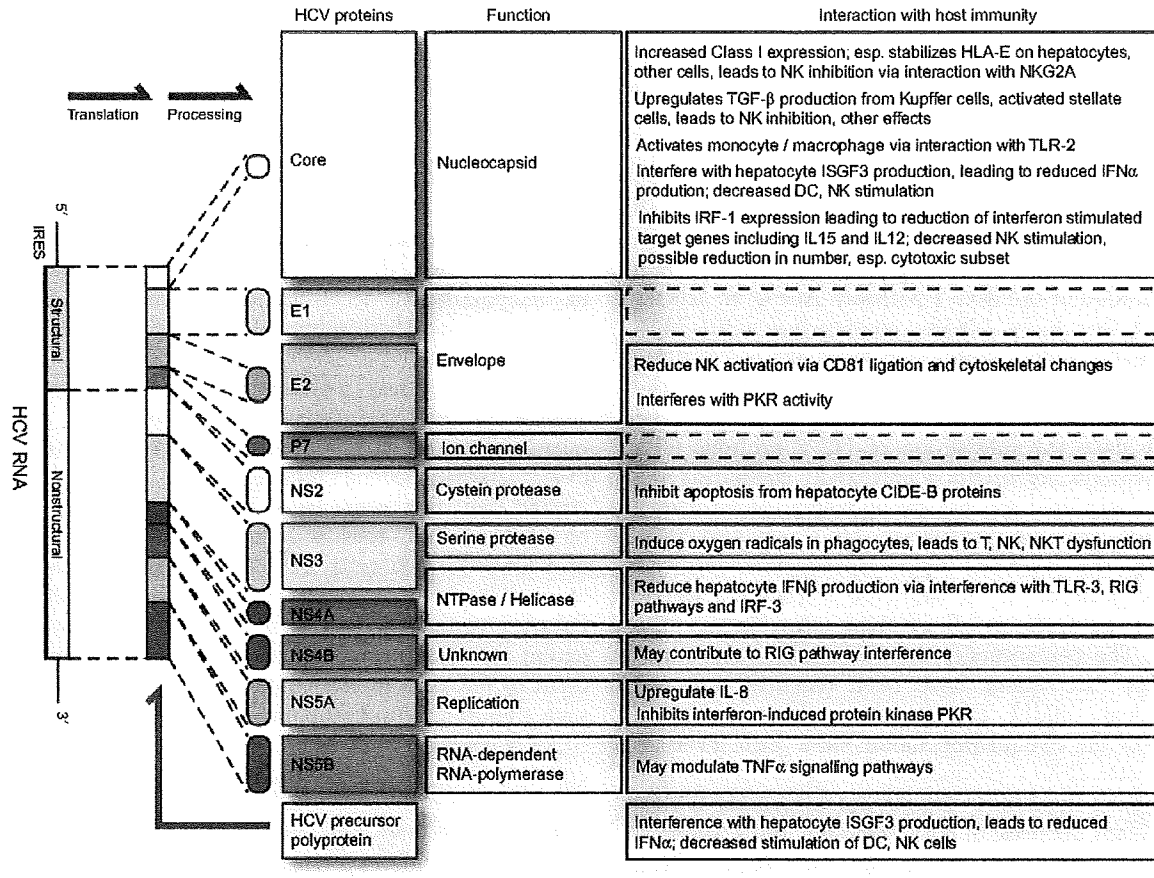

Figure 44.1 • Schematic view of HCV genomes and protein products showing associated interactions with host immunity. The HCV genome is divided into structural and nonstructural areas on the left, with the rightward direction corresponding to representations of the precursor polyprotein and individual viral proteins, respectively. Brief depictions of protein functions within the setting of the viral life cycle are followed by tabulation of probable interactions with the host immune system. The interactions are largely restricted to those involving the innate immune system. See text for additional details.

These authors concluded that NK cells, which were not directly cytotoxic to the infected hepatic cells, were potentially capable of inhibiting HCV replication via an IFN-γ-dependent pathway that was subject to viral interference via the HCV-E2:CD81 interaction. Agrati et al. (2002) suggested that CD81-associated inhibition of NK cell function might thereby contribute to a lack of viral clearance with progression to chronic infection.

Crotta et al. (2006) demonstrated that CD81 cross-linking by antibody or HCV E2 protein resulted in cytoskeletal rearrangement in NK cells as well as in T cells, as based on morphological alterations and enhanced F-actin capping. Whereas these cytoskeletal changes enhanced the response of T cells to CD3-induced TNF-α production, they decreased the CD16-mediated generation of IFN-γ and TNF-α by resting or activated NK cells. These authors were able to decrease the response of T cells and increase the response of NK cells to these stimuli by preincubation of cells with low-dose actin polymerization inhibitors. This led them to conclude that CD81 engagement induces cytoskeletal rearrangement in both NK cells and T cells, but that this process has opposite effects, leading to inhibition of NK cell responses and stimulation of T-cell responses. Since this phenomenon also extended to the inhibition of NK cell response to IL-12, they inferred that the inhibition was independent of KIR involvement. Tseng and Klimpel (2002) showed that the cross-linking of CD81 on NK cells by viral E2 inhibited both NK cell cytotoxicity and NK cell IFN-γ production, suggesting that this may be an important mechanism by which the virus can shift the balance of the early innate host immune response.

Mechanism	Effect
Weak affinity inhibitory allotype of HLA-Cw1 and KIR2DL3, potentially allowing relatively stronger stimulatory KIR interactions	Some evidence for protective antiviral effect
Strong affinity stimulatory allotype of HLA-B Bw4 and KIR3DS1	Protective in one study; increased chronic inflammation in another study
Increased inhibitory NKG2A expression in HCV NK cells	Facilitates inhibitory interaction of NK cells with HLA-E upregulated on other cells, esp. HCV-infected hepatocytes; may also lead to increased NK IL-10 and TGF-β causing defective DC interaction
Increased affinity MICA/B allotypes as ligands for activating NK NKG2D receptor	Potential role in facilitating HCV clearance
Decreased IL-15 as potential cause of decreased DC MICA/B expression	Decreased ability to stimulate NK cells; can be overcome in vitro with IL-15
Lower expression of NKp30 and NKp46 on HCV NK cells (in some studies)	Results differ among studies; possible inhibitory mediator of NK cell function in this setting
Increased NK IL-10 production upon stimulation	Potential skewing of adaptive immune response to Th2 phenotype

Interactions of NK cell receptors with HLA molecules as expressed in HCV infection

HLA class Ia levels and NK cells

Herzer et al. (2003) used recombinant adenovirus constructs to express the HCV core protein in several hepatocyte cell lines and showed upregulation of MHC class I expression on the surfaces of HepG2 cells but not on Hep3B or Huh7 cell lines. These latter cell lines lack wild-type p53, which is present in HepG2 cells, and reconstitution of these cells with wild-type p53 led to an increase in HLA class I in the setting of core protein expression. Transporter associated with surface processing-1 (TAP-1) protein, which is p53 responsive, was also upregulated. The increased expression of class I molecules led to a significant reduction of NK cell-mediated cytotoxicity as assessed in 48-hour chromium release assay, and the authors concluded that this was a likely mechanism of viral evasion against NK cell cytotoxicity in vivo.

Class Ia HLA and killer cell immunoglobulin-like receptors (KIR)

Killer cell immunoglobulin-like (KIR) receptors are clonally expressed in a stochastic fashion on NK cells. They may be stimulatory, with a short cytoplasmic tail and a charged transmembrane domain that allows association with signalling proteins, or inhibitory with a long cytoplasmic tail that contains an immunoreceptor tyrosine-based inhibitory motif (ITIM). They most often recognize class I HLA-C molecules and bind in a manner that overlaps but differs from T-cell receptor binding (Boyington et al., 2000). Different KIR bind with different affinities, and it seems likely that inhibitory KIR engage class I HLA more strongly than do stimulatory KIR (Vales-Gomez et al., 1998).This opens the possibility that different KIR haplotypes may influence the courses of various diseases, including infection with HCV. To date, epidemiologic studies have produced conflicting results.

Khakoo et al. (2004) examined the KIR and HLA-C status in 685 individuals with persistent HCV infection and 352 individuals with resolved HCV infection. Within this population, they focused on those with HLA-C allotypes containing asparagine at residue 80 (HLA-Cw group 1 alleles), which serve as ligands for the inhibitory KIRs, KIR2DL2 and KIR2DL3. Of these receptors, KIR2DL3 binds with weaker affinity. They found a protective effect in individuals who were homozygous for this HLA-C allotype and also homozygous for KIR2DL3. This protective effect was evident in those who were infected with HCV by accidental needle stick or during the course of intravenous drug abuse but not in those who were infected by blood transfusion. They hypothesized that the inhibitory effect of the relatively weakly binding KIR2DL3 could more easily be overcome by other nonvariable activating NK cell receptors. Further, they suggested that this protective effect could be overwhelmed by a massive viral inoculum, explaining the loss of protection in those who received the presumed larger viral dose during blood transfusion.

Rauch et al. (2007) performed a similar study in 142 patients with chronic HCV infection and 33 with resolved HCV infection. These individuals were part of the Swiss HIV Cohort Study and as such obtained HCV primarily as a consequence of intravenous drug abuse. These investigators were unable to find any association

between the status of KIR genotype and HLA-C ligand in their population. They felt that a lack of statistical power was unlikely but left open the possibility that the HIV positive status of their population, which differed from that of the previously cited study, may have had an effect on the results.

More recently, Romero et al. (2008) examined KIR receptor distribution and HLA class II alleles in a population of 121 intravenous drug users with chronic HCV infection and 39 others with spontaneous viral clearance. They eliminated the possibility of genetic stratification in this Puerto-Rican American cohort by analysis of autosomal short tandem repeat markers. They were able to confirm the association among homozygous KIR2DL3 status, homozygous HLA-Cw group 1 alleles and spontaneous viral clearance, and found an additional association of KIR2DL3, DRB1*1201 and spontaneous clearance. They tabulated all prior studies to date in their report and concluded that additional population studies were necessary.

A weak protective effect was also previously suggested for the combination of HLA-B Bw4 and the stimulatory KIR3DS1 (Khakoo et al., 2004). In contrast, Paladino et al. (2007) found an increased frequency of this combination in HCV-positive individuals who had progressed to cirrhosis, leading them to suggest that the presence of higher cytotoxic activity might actually be associated with progression of HCV. Lopez-Vazquez et al. (2007) in summarizing studies to date also concluded that evaluation of other large cohorts of patients with HCV infection is needed to confirm the possibility of an association between the interaction of KIR and HLA with disease progression.

HLA class Ib and NK cells

HLA-E interactions with NKG2A and NKG2C receptors HLA-E is a widely expressed member of the nonpolymorphic MHC class Ib molecules that contains a nonamer peptide binding motif that typically contains derivatives of signal peptides from other class I molecules and can present other epitopes as well (Rodgers and Cook, 2005). HLA-E can bind CD94-NKG2A (inhibitory) or CD94-NKG2C (activating) on NK cells. Nattermann et al. (2005) found that the hydrophobic peptide YLLPRRGPRL, representing an HLA-A2-restricted and known T-cell epitope derived from amino acid positions 35–44 of the HCV core protein, was able to stabilize surface HLA-E expression in an HLA-E-transfected K562 cell line. Chromium release assay showed inhibition of NK cell cytotoxicity against the HLA-E transfected HCV peptide 35- to 44-loaded K562 cells. No inhibition occurred when transfected cells were preincubated with an irrelevant peptide or when HLA-E-negative K562 target cells were preincubated with the HCV core peptide sequence. Inhibition was abolished in the presence of antibodies to either CD94 or NKG2A, implicating this receptor complex in the process.

As in vivo correlates, these investigators found increased inhibitory NKG2A expression on circulating NK cells from patients with chronic HCV infection as contrasted with those without infection (Nattermann et al., 2006). No difference was found in levels of NKG2C expression; however, reduced levels of the NK cell stimulatory receptors NKp30 and NKp46 were found in the cells of the hepatitis patients. Liver biopsy specimens from patients with chronic HCV infection demonstrated enhanced HLA-E expression in $CD68^+$ macrophages/Kupffer cells, $CD31^+$ sinusoidal endothelial cells, $CD83^+$ DCs, $CD14^+$ monocytes and hepatocytes. In the latter case, HLA-E expression was higher in hepatocytes expressing HCV core protein. These studies suggest that this T-cell epitope may also contribute to chronic viral infection by virtue of its synchronous inhibition of NK cell activity (Golden-Mason and Rosen, 2006).

Similar results were found in a study employing freshly isolated circulating NK cells from patients with chronic HCV (De Maria et al., 2007). NK cell cytotoxicity against FO1 melanoma or Daudi Burkitt lymphoma target cells were similar to those obtained from uninfected donors. However, HCV NK cells showed a significant reduction in cytolytic activity when HepG2 hepatocellular carcinoma target cells were used. These investigators also implicated the HLA-E: CD94/NKG2A ligand receptor interaction in this phenomenon and demonstrated increased expression of NKG2A on HCV NK cells compared to uninfected control cells.

Related studies (Jinushi et al., 2004) demonstrated that NKG2A ligation on NK cells is also associated with defective signals for DC maturation, discussed later.

MICA/B and NKG2D receptor MHC class I chain-related sequence (MIC) genes are thought to represent phylogenetically old members of MHC class Ib molecules (Rodgers and Cook, 2005). The two proteins in this group, MICA and MICB (MICA/B), are polymorphic, with approximately 60 and 25 known alleles, respectively; do not present peptides or associate with β-2 microglobulin; and serve as ligands for the activating NKG2D receptor on NK cells (as well as macrophages, CD8+ T cells, γδ T cells, and NKT cells) (Bauer et al., 1999; Stastny, 2006; Yokoyama and Plougastel, 2003).

Similar to the situation with KIR, the binding affinities of MICA/B to NKG2D appear to vary among different allotypes, which has been suggested as a potential influence on the threshold of NK cell activation (Steinle et al., 2001). Karacki et al. examined MICA polymorphisms in 442 individuals with chronic HCV and 228 others who cleared the virus. They found a statistically significant association with the presumed high affinity MICA*015, which occurred twice as often in patients who cleared HCV than in those with persistent disease.

The allele occurred in only 5.6% of their patient population, and no other significant associations were uncovered, leading them to conclude that MICA polymorphisms did not play a significant role in facilitating HCV clearance. However, the association was found in a small number of Black patients, as it was too rare in Whites to analyse. It would appear that further related studies in this defined patient subpopulation may provide additional information.

Studies addressing defective DC MICA upregulation leading to reduced NK cell activation (Jinushi et al., 2003a) are discussed in more detail in the upcoming section addressing NK: DC crosstalk.

Other NK cell receptors in HCV infection

Nattermann et al. (2006) found that patients with chronic HCV infection and viraemia had reduced levels of the natural cytotoxicity receptors NKp46 and NKp30 in circulating NK cells compared to healthy uninfected individuals. Further, patients who had cleared the virus following therapy with pegylated IFN-α and ribavirin exhibited levels of these receptors similar to those of uninfected controls. Although the two receptors were expressed in a proportionate fashion, there was no correlation between the level of expression and circulating viral genomic load in those patients who had viraemia. These investigators also examined receptor expression in intrahepatic cells by flow cytometry following mechanical disruption of tissue samples. At this site, they also found a lower expression of NKp30 and NKp46 in HCV patients compared to those with other liver diseases. In a redirected killing assay using antibodies against natural cytotoxicity receptors and an FcR^+ target cell line, decreased cytotoxicity was seen in HCV NK cells. These authors suggested that the combination of reduced natural cytotoxicity receptors along with increased inhibitory NKG2A expression on HCV NK cells contributed to impaired function of these cells in patients with chronic HCV infection.

In contrast, De Maria et al. (2007) reported that circulating NK cells from patients with chronic HCV infection showed increased expression of the stimulatory natural cytotoxicity receptors NKp46 and NKp30 compared to uninfected adults. This finding was unexpected, as prior studies in other conditions such as HIV infection or acute myelogenous leukaemia had shown reduced levels of these receptors, providing a rationale for positing a functional deficit of the NK cell compartment in chronic disease conditions.

HCV NK cells were not activated as shown by a lack of increased HLA-DR expression, and no correlation was found between natural cytotoxicity receptor expression density and level of viraemia. NK cell cytotoxicity was intact using FO1 melanoma target cells and could be partially inhibited by antibodies to NKp30, NKp46 or NKG2D. Cross-linking of NK CD81, which was expressed at levels comparable to healthy individuals, had no effect on cytotoxicity.

It is possible that differences in patient populations, circulating NK cell subpopulations, or methodological details may have contributed to disparate findings between these groups. Significantly, both groups of investigators concluded that NK cells play an important role in chronic viral persistence. Additional studies to further clarify this role are needed.

Interactions and crosstalk between NK cells and DC in the presence of HCV

Most studies of DCs in the setting of HCV infection have been performed in vitro or are based on chronic infection, and the relevance to the acute phase of the innate response may remain to be proven.

In response to IFN-α, or other specific inflammatory cytokines, DCs typically undergo a maturation process that includes upregulation of class MHC molecules, costimulatory molecules and production of cytokines. Myeloid DCs ($BDCA1^+$, $CD11c^+$, $CD83^+$, $CD33^+$, $HLA\text{-}DR^{bright}$, $CD14^-$) produce IL-12, IL-10, IL-18 (Kaser et al., 2004), TNFα and IFN-β. Plasmacytoid DCs ($BDCA2^+$, $BDCA4^+$, $HLA\text{-}DR^{bright}$, $CD123^{bright}$, $CD11c^-$, $CD33^-$) preferentially produce IFN-α. The cytokines IFN-α, IL-12 and IL-18 are all capable of activating NK cells.

Jinushi et al. (2003b) showed that IFN α-induced upregulation of HLA class Ib MICA/B expression on monocyte-derived DCs was able to activate resting NK cells, enhance NK cell cytotoxicity against K562 cells and increase NK cell production of IFN-γ. This was shown to require direct cell:cell contact and to be dependant upon MICA/B:NKG2D ligation. However, DCs isolated from patients with chronic HCV infection showed impaired modulation of DC MICA/B expression in response to IFN-α, as well as a decreased ability to stimulate NK cells in this circumstance. Further work by this group showed that the DC defect was related to impaired IL-15 production. Using an in vitro coculture system, this group demonstrated that the defect could be overcome with exogenous IL-15 (Jinushi et al., 2003b) but not with administration of IFN-α (Jinushi et al., 2003a). They postulated that an autocrine/paracrine loop involving IL-15 and type I IFNs subserves the ability of DCs to upregulate MICA/B and thereby activate resting NK cells, and that this pathway is blocked by an unknown mechanism in HCV infection. Perhaps related to this is the finding of Ciccaglione et al. (2007) that HCV core protein inhibits IRF-1, thereby reducing IL-15 transcription.

The actual in vivo situation is likely more complex, as Golden-Mason et al. (2004) demonstrated by showing increased IL-15 concentrations from HCV-infected

tissues, which they attributed to production by infiltrating monocytes and resident Kupffer cells. In contrast, Meier et al. (2005) found reduced levels of IL-15 in serum from patients with chronic HCV infection. Additional studies to unravel the variables likely responsible for these superficially contrasting results are needed.

Decreased IFN-α production by circulating plasmacytoid DCs has been reported in chronic HCV infection (Dolganiuc et al., 2006; Yonkers et al., 2007). Lai et al. (2007) examined these cells from fresh HCV-infected livers and showed a relative reduction in this cell type when compared to uninfected but inflamed control livers (Lai et al., 2007). In HCV infection, these cells showed higher BDCA-2 expression. These investigators found reduced numbers of IFN α-producing cells in HCV livers and suggested that this may be related to the fact that BDCA-2 ligation inhibits IFN-α/-β production in plasmacytoid DCs and increases IL-12 secretion.

A reciprocal interaction whereby NK cells from patients with chronic HCV infection may inhibit activation of DCs has also been demonstrated (Jinushi et al., 2004). In these studies, NK cells derived from uninfected donors and cocultured with liver epithelial cells were capable of inducing maturation and activation of DCs. This did not require direct contact between NK cells and DCs, as the maturation effect could be reproduced with conditioned medium from prior coculture of NK cells with Hep3B cells. In contrast, when NK cells from chronic HCV patients were used, no activation of DCs occurred. Rather, the HCV–NK cells elaborated IL-10 and TGF-β, and showed higher levels of the inhibitory receptor complex CD94/NKG2A than did normal NK cells. The investigators hypothesized that the effect was dependant upon ligation of NKG2A by hepatoma cell HLA-E. Blockade of NKG2A restored the ability of HCV–NK cells to activate DCs, concomitant with a reduction in IL-10 and TGF-β production. Further, these treated HCV–NK cells were able to stimulate DCs to produce Th1-polarized $CD4^+$ T cells (Jinushi et al., 2004).

Recently, Ebihara et al. (2008) used direct in vitro infection with the JFH1 HCV strain to address the interactions between NK cells and DCs. They were unable to directly infect monocyte-derived DCs but rather found that double-stranded viral RNA (dsRNA) was introduced into these cells via phagocytosis of apoptotic debris from the infected hepatocytes, and that this colocalized with TLR-3 within DC phagosomes. Following subsequent maturation, these cells secreted IL-6 and IFN-β and were able to activate NK cells in a manner dependant upon DC–NK cell contact. This led them to suggest that activation of NK cells via soluble factors such as type I IFN and IL-15 may only have a subsidiary role in HCV infection.

In a study of freshly separated NK cells derived from patients with chronic HCV infection, De Maria et al. (2007) showed that these cells produced increased amounts of IL-10 in addition to IFN-γ upon stimulation. They speculated that if these cells entered the liver, crosstalk with resident DCs might serve to skew the adaptive immune response to allow viral persistence.

Additional cytokine and chemokine studies relevant to the role of NK cells in HCV infection

IL-10

In the investigations of De Maria et al. (2007), challenge of HCV NK cells with FO1 melanoma cells also resulted in IFN-γ production at levels comparable to control cells, along with increased IL-10 production relative to normal NK cells. The authors suggested that the natural cytotoxicity receptors may play a role in the increased IL-10 production, with implications for Th2 skewing and viral persistence. This position finds support in the study of Knapp et al. (2003) who found that HCV patients with the promoter GG genotype at the IL-10 (−1082), which is associated with higher levels of this cytokine, were more likely to have persistent infection than those without this genotype. Kanto et al. (2004) found that myeloid and plasmacytoid DCs from patients with chronic HCV infection primed increased numbers of IL-10 producing cells relative to controls. Subsequent studies by Gelderblom et al. (2007) examined cytokine production using monocyte-derived DCs from patients with chronic HCV infection and found elevated IL-10 but not IL-12p70 secretion by these cells. Both teams also found reduced IFN-α production by DCs in chronic HCV-infected patients (Gelderblom et al., 2007; Kanto et al., 2004).

As noted earlier, HCV NK cells were capable of producing IFN-γ at normal levels in vitro. Meier et al. (2005) also found normal production of IFN-γ by HCV NK cells in response to stimulation with IL-12 plus IL-18. However, these investigators also noted a reduction of circulating NK cells in these patients and suggested that in vivo availability of IFN-γ derived from these cells may be limited.

IL-15

IL-15, discussed earlier in the context of DC–NK cell crosstalk, plays a role in the development, function and sustenance of NK cells (Becknell and Caligiuri, 2005). Golden-Mason et al. (2004) examined intrahepatic IL-15 levels using a combined approach of RT-PCR, enzyme linked immunosorbent assay and immunohistochemistry. They found a significant increase of this cytokine in HCV-infected liver samples and localized this to Kupffer cells and infiltrating monocytes. They also demonstrated that 80% of NK cells expressed the IL-2/IL-15 receptor β chain (CD 122), which led them to suggest that expression of this cytokine helped to shape the intrahepatic lymphoid population and also

likely played an additional role in the host response to HCV infection.

As noted previously, Meier et al. (2005) reported that patients with chronic HCV infection had reduced levels of circulating IL-15. They were able to promote NK cell survival in vitro with this cytokine and found that it had a preferential effect on survival of $CD56^{dim}$ cells relative to $CD56^{bright}$ cells. This corresponded to their in vivo finding of a greater proportionate decrease in the $CD56^{dim}$ versus the $CD56^{bright}$ NK cell subpopulation in peripheral blood of chronically infected patients. This translates to a shift of the ratio of cytotoxic ($CD56^{dim}$) to cytokine producing ($CD56^{bright}$) NK cell subpopulations. These investigators suggested that IL-15 might be considered as a form of adjuvant immunotherapy in these patients. The implications of NK cell subset differences in the reinterpretation of earlier studies focusing only on NK cell function were considered earlier.

IL-21

Similar in some regards to IL-15, IL-21 is a pleiotropic cytokine that is produced by activated $CD4^+$ T cells and NK T cells; enhances proliferation, activity, and survival of NK cells; and has differential effects on NK cell subsets (Skak et al., 2008; Wendt et al., 2007). To date, no studies have addressed the role of this potentially important cytokine in acute or chronic HCV infection.

TGF-β

Among other functions, transforming growth factor (TGF)-β exerts an inhibitory effect upon NK cells. This cytokine is elaborated primarily by Kupffer cells and activated stellate cells in the liver. Using HepG2 hepatoblastoma cells, Taniguchi et al. (2004) reported that HCV core protein was capable of upregulating TGF-β production directly within these cells, raising the possibility that a similar pathway may also occur in natural infection. Kimura et al. (2006) found the −509CC genotype of the TGF-β1 gene promoter to be associated with a higher clearance rate of HCV. This polymorphism is associated with lower promoter activity, concordant with the concept of TGF-β as a factor favouring viral persistence in this setting.

IL-8

IL-8 (CXCL8) is chemotactic for neutrophils but also has a variety of other effects. Khabar et al. (1997) showed that IL-8 was capable of interfering with the IFN-α pathway. The significance of this pathway for NK cell function was discussed earlier. In vitro exposure of human umbilical vein endothelial cells to HCV-like particles resulted in upregulation of IL-8 production by these cells (Balasubramanian et al., 2005). Polyak et al. (2001) found that the viral protein NS5A alone was capable of inducing IL-8 production and that this activity correlated to the results of an in vitro bioassay to detect interference with the antiviral effects of IFN-α. Thus, NS5A may interfere with the IFN-α pathway by two mechanisms: induction of IL-8 production and interference with the function of the IFN-induced double-stranded RNA-activated protein kinase (PKR) (Polyak et al., 2001).

The report by Asselah et al. (2005) provides potential insights into the timing of IL-8 changes. This group used real-time RT-PCR of 240 selected genes and examined expression levels in normal livers and livers from patients with chronic HCV infection. The latter were subdivided into varying stages of progressive fibrosis as defined using the METAVIR scoring system (Bedossa and Poynard, 1996). No difference was seen in IL-8 mRNA expression when livers with early fibrosis were compared to normal samples. However, IL-8 was significantly upregulated in livers from patients with more advanced fibrosis relative to those with only mild fibrosis, suggesting an increased role for this cytokine in more advanced disease. In contrast, livers from HCV patients with only slight fibrosis showed significant upregulation in a number of type II IFN inducible genes relative to livers from normal controls.

Other chemokines

Chemokines, or chemotactic cytokines, can evoke a number of proinflammatory effects. The production of type I IFNs consequent to HCV infection induces upregulation of the chemokine MIP-1α (macrophage inflammatory protein 1-α) from Kupffer cells (Ahmad and Alvarez, 2004) or endothelial cells, although reports vary regarding cell type (Zeremski et al., 2007). This attracts and leads to locally increased numbers of NK cells (as well as T cells, monocytes and immature DCs) mainly via the CC chemokine receptor 5 (CCR-5). IFN-γ produced by NK cells in turn stimulates hepatic sinusoidal endothelial cells to produce several additional chemokines, including MIG (monokine induced by IFN-γ, CXCL-9), IP-10 (IFN-γ-inducible protein 10, CXCL-10) and I-TAC (IFN inducible T cell α chemoattractant) (Zeremski et al., 2007). These chemokines attract activated Th1 cells that express the surface receptors CCR-5 and CXCR-3 (CXC chemokine receptor 3) (Zeremski et al., 2007), thereby providing a bridge between innate and adaptive immunity (Ahmad and Alvarez, 2004). Enrichment of intrahepatic T cells bearing these receptors has been demonstrated in patients with chronic HCV infection (Apolinario et al., 2002). IP-10 levels have been associated with the degree of lobular inflammation in these patients (Harvey et al., 2003), and this has been proposed as one of several predictive markers for both rapid and for sustained viral responses (Romero et al., 2006).

Zeremski et al. (2007) recently reviewed this topic and suggested that chemokine generation may play an important role in both viral clearance and in the propagation of chronic inflammation in this infection. In their model, early chemokine production ultimately leading to strong T cell mediated antiviral effector cells is desirable and is a likely correlate of the spontaneous resolution that occurs in 20% of acutely infected patients. In contrast, persistence of chemokine generation in the remainder of patients who generate an ineffective cell mediated response may lead to the continued and nonspecific attraction of inflammatory cells, causing continued necrosis and eventually leading to cirrhosis in the face of viral persistence.

Current therapy of HCV infection

Combined treatment with pegylated IFN-α and ribavirin remains the mainstay of therapy for patients with HCV infection. The regimen is given for 6 or 12 months. Sustained virological response is seen in approximately 55% of treated patients; 10–25% of patients have a transient response with relapse following cessation of therapy, and the remainder are nonresponders. These unsatisfactory results are further qualified by the fact that a number of patients are not eligible for or cannot tolerate therapy and are not included in these figures.

Response to therapy is manifested as a rapid decrease in circulating viral genomic levels, which is interpreted as a suppression of viral replication, followed by a slower decline thought to be related to elimination of infected cells. Treatment during the acute phase of infection appears to be more effective, leading to a reduced frequency of chronic HCV infection from the expected 80% to approximately 10%. Feld and Hoofnagle (2005) suggest that this may indicate that resistance to therapeutic IFN-α might be an acquired phenomenon that arises during the chronic phase, pointing out the need to dissect the host:viral interactions from a temporal perspective.

In one microarray study of liver biopsy samples (Chen et al., 2005), upregulation of IFN-responsive genes prior to therapy was associated with nonresponder status. This suggests (Feld and Hoofnagle, 2005) that an IFN response already in place in these patients is unable to effectively manage the infection and that additional stimulation limited to this pathway is futile. It also highlights the complexity of the host:viral immune interaction and further underscores the fact that the phenotype responsible for loss of viral control likely varies among patient subpopulations.

A major current effort is being directed towards the development of small molecule inhibitors of HCV enzymes. The major viral targets include the NS3/4 A protease and the NS5B polymerase. Several of these agents are in clinical trials and are the subject of recent reviews (De Francesco and Migliaccio, 2005; Harrison, 2007; Pawlotsky et al., 2007). Despite optimism in this area, Pawlotsky et al. (2007) point out that problems with lower antiviral efficacy in vivo compared to in vitro studies, unfavourable toxicity profiles and the development of viral resistance suggest that current therapies will remain as standard care for some time. These predictions, while presently true, are always subject to change.

Current success, however limited, with IFN-α-based regimens does indicate that stimulatory immune modulation at the level of the innate immune system may lead to either transient or prolonged viral remission. Agonists of TLR-7 and TLR-9 are currently in phase 1 clinical trials. Engagement of these receptors, normally found on plasmacytoid DCs, leads to increased IFN-α production, maturation of DCs and stimulation of NK cells.

Direct stimulation of NK cells is a potential avenue of therapy that may reduce the HCV burden in an additive or perhaps synergistic manner when combined with other therapies directed towards stimulation of adaptive immunity or against components of the viral life cycle. Based on studies of mechanisms contributing to NK cell inhibition in chronic HCV, the use of IFN-γ or IL-15 has been suggested as a possibility. Other possible approaches, such as interference with HCV E2: CD81 interaction on NK cells, stimulation of natural cytotoxicity receptors, reduction of IL-10, TGF-β, or IL-8 activity, among others, can be inferred from the earlier discussion of disordered NK cell physiology during HCV infection. Indeed, Golden-Mason and Rosen (2006) hypothesized that the NK cell is the primary target upon which HCV formulates its immune evasion strategy and that the defective crosstalk between NK cells and DCs underlies the observed T-cell defects in this disorder. However, the warning of Zeremski et al. (2007) must also be remembered: What constitutes an effective immune response in acute viral hepatitis does not necessarily imply that a similar response is desirable in later stages. A detailed understanding of the differential effects of NK cells at varying time points during the course of infection must underlie any future efforts to manipulate these powerful cells for the benefit of the host.

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Case report

Sustained virological response in a patient with chronic hepatitis C treated by monotherapy with the NS3-4A protease inhibitor telaprevir

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ABSTRACT

Here, we describe for the first time a case of sustained virological response (SVR) achieved in a patient with chronic hepatitis C (CH-C) by monotherapy with a NS3-4A protease inhibitor, telaprevir, without interferon therapy. A 59-year-old treatment-naïve Japanese man was enrolled in a phase II trial of telaprevir by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. At the start of treatment, he exhibited a low-level viremia with genotype 1b of the hepatitis C virus (HCV). After the first week of treatment with telaprevir, serum HCV RNA was undetectable, and negativity remained until the end of treatment. Moreover, he was evaluated as having a SVR after the post-treatment 24-week follow-up program. Two characteristics may explain the strong antiviral activity of telaprevir in the present case. First, although pre-treatment PCR-direct sequencing and cloning for the N-terminal in the NS3 region showed a protease inhibitor-resistant variant (I54A) in 1 of 32 independent clones, the I54A substitution has only a low-level resistance to protease inhibitors and his viral load was low. Second, when compared to a consequence sequence of 35 treatment-naïve patients with HCV genotype 1b, R130K and Q195K substitutions were unique to the present case. Although it is presently unknown whether the R130K and Q195K substitutions are related to SVR, this case suggests that long-term telaprevir monotherapy may be effective in CH-C patients with genotype 1 and a low viral load.

1. Introduction

The goals of antiviral treatment in patients with chronic hepatitis C (CH-C) are long-lasting eradication of the virus and a decrease in disease-related hepatic mortality. Standard treatment uses a combination of pegylated interferon and ribavirin (PEG-IFN-RBV), which provides a sustained virological response (SVR) rate of over 50%.[1,2] In Japan, approximately 70% of patients with CH-C are infected with genotype 1b, and those with a high titer of genotype 1b (≥ 100 KIU/mL [Amplicor; Roche Diagnostics K.K. Tokyo, Japan]) have lower rates of SVR (<50%), even on 48 weeks of PEG-IFN-RBV combination therapy.[3] Further, although treatment for CH-C is currently based on interferon (IFN), use of this agent is associated with serious adverse effects in some patients, such as mental disorders, apathy, and laboratory abnormalities.[1,2,4] Moreover, most CH-C patients in Japan over 70 years of age cannot receive IFN therapy due to either or both co-morbidities and the risk of adverse effects. For these reasons, a new treatment strategy is needed for patients with CH-C that displays high SVR rates and a favorable side-effect profile.

One recently introduced treatment strategy for CH-C is inhibition of the NS3-4A protease in the HCV polyprotein. Potential inhibitors include telaprevir (VX-950; MP-424; Mitsubishi Tanabe Pharma Co., Osaka, Japan), which has been selected as a clinical therapy candidate for the treatment of CH-C.[5] In some patients with genotype 1 and a high viral load, however, the efficacy of telaprevir monotherapy was limited, and combination therapy of telaprevir plus PEG-IFN-RBV is now standard.[6–10] On this background, we therefore report here for the first time a patient with CH-C who achieved a SVR following monotherapy with telaprevir.

2. Case report

A 59-year-old Japanese man was admitted to Toranomon Hospital, Tokyo in July 2007 following a positive result for HCV RNA at general check-up. Laboratory tests before treatment showed mild

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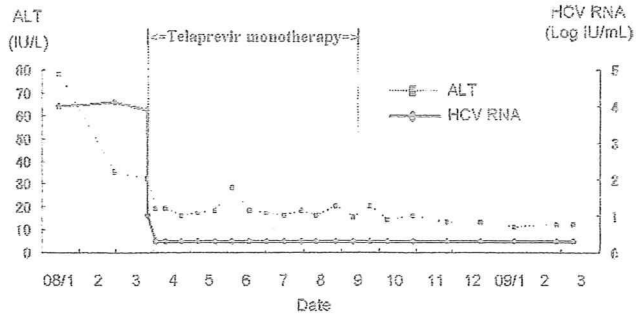

Fig. 1. Clinical course during and after 24 weeks of telaprevir monotherapy.

elevation of ALT (46 IU/L), and persistent HCV infection with genotype 1b and low-level viremia (<5 Log IU/mL [COBAS TaqMan HCV test, Roche Diagnostics K.K. Tokyo]) that continued to remain low until the start of treatment. He was diagnosed with CH-C by peritoneoscopy and liver biopsy (mild hepatitis [A1] and moderate fibrosis [F2]) at our hospital in February 2008. He had not received IFN therapy or any other antiviral drugs, and was enrolled in a phase II trial of telaprevir. Written informed consent was obtained, and the study was conducted in compliance with Good Clinical Practice and the Declaration of Helsinki. Treatment with telaprevir was started in March 2008, at which time serum HCV RNA was 3.9 Log IU/mL. Treatment was by repeat oral administration at a dose of 750 mg every 8 h for 24 weeks. Serum HCV RNA was undetectable after the first week and remained negative until the end of treatment (September 2008), and moreover remains undetectable as of March 2009. He was evaluated as having a SVR after the post-treatment 24-week follow-up program (Fig. 1).

The genome sequence for the N-terminal 609 nucleotides (203 amino acids) in the NS3 region of HCV isolates from the patient was analyzed before treatment with telaprevir. HCV RNA was extracted from 100 μL of serum and the nucleotide sequences were determined by direct sequencing and cloning. The primers used to amplify the NS3 region were NS3-F1 (5′ ACACCCCCCCGTCTGGGCACAT-3′; nucleotides 3295–3316) and NS3-AS2 (5′-GCTCTTGCCGCTGCCAGTGGGA-3′; nucleotides 4040–4019) as the first (outer) primer pair and NS3-F3 (5′-CAGGGGTGGCGGCTCCTT-3′; nucleotides 3390–3407) and NS3-AS2 as the second (inner) primer pair.[11] Thirty-five cycles of first and second amplifications were performed as follows: denaturation for 30 s at 95 °C, annealing of primers for 1 min at 63 °C, extension for 1 min at 72 °C, and final extension was performed at 72 °C for 7 min. PCR-amplified DNA was purified after agarose gel electrophoresis and amplification products of the second-round PCR were ligated with plasmid and transformed in *Escherichia coli* in a cloning kit (TA Cloning; Invitrogen, Carlsbad, CA). Dideoxynucleotide termination sequencing was performed with the BigDye Terminator v1.1 Cycle Sequencing kit (Applied Biosystems Japan, Tokyo). Sequences of 32 independent clones from the sample were determined and analyzed. The pre-treatment analyses by PCR-cloning showed a variant (T54A) resistant to protease inhibitors in 1 of the 32 clones.

We also made a consensus sequence of the NS3 region from the PCR-direct sequences of 35 treatment-naïve Japanese patients with HCV genotype 1b in our hospital (Fig. 2). Compared to the consensus sequence, there were a total of 5 identical substitution variants (V48I, P89S, S122G, R130K, Q195K) within the 32 independent clones from this patient, among which R130K and Q195K were unique to this patient.

3. Discussion

Previous studies showed that telaprevir monotherapy for HCV patients with genotype 1 and a high viral load demonstrated substantial antiviral activity, and the median maximum change was 4.77 Log IU/mL with administration of 750 mg every 8 h for 3 weeks.[6,7] In Reesink et al., HCV RNA decreased below the limit of

```
                1         10        20        30        40        50
CONSENSUS       APITAYSQQT RGLLGCIITS LTGRDKNQVE GEVQVVSTAT QSFLATCVNG
Case clone1     ---------- ---------- ---------- ---------- ---------I--
Case clone2     ---------- ---------- ---------- ---------- ---------I--
Case clone3     -----R---- ---------- ---------- ---------- ---------I--
Case clone4     ---------- ---------- ---------- ---------- ---------I--
Case clone5     ---------- ---------- ---------- ---------- ---------I--
                51                                                  100
CONSENSUS       VDWTVYHGAG SKTLAGPKGP ITQMYTNVDQ DLVGWQAPPG ARSLTPCTCG
Case clone1     ---------- ---------- ---------- ---------- ----------
Case clone2     -----F---- ---------- ---------- --------S- ----------
Case clone3     ---A------ ---------- ---------- --------S- ----------
Case clone4     ---------- ---------- ---------- --------S- -----L----
Case clone5     -----F---- ---------- ---------- --------S- ----------
                101                       130               150
CONSENSUS       SSDLYLVTRH ADVIPVRRRG DSRGSLLSPR PVSYLKGSSG GPLLCPSGHA
Case clone1     ---------- ---------- -G-------K ---------- ----------
Case clone2     ---------- ---------- -G-------K ---------- ----------
Case clone3     ---------- ---------- -G-------K ---------- ----------
Case clone4     ---------- ---------- -G-------K ---------- ----------
Case clone5     ---------- ---------- -G-------K ---------- ----------
                151                                   195  200
CONSENSUS       VGIFRAAVCT RGVAKAVDFI PVESMETTMR SPVFTDNSSP PAVPQTFQVA
Case clone1     ---------- ---------- ---------- ---------- ----K-----  15
Case clone2     ---------- ---------- ---------- ---------- ----K-----  14
Case clone3     ---------- ---------- ---------- ---------- ----K-----  1
Case clone4     ---------- ---------- ---------- ---------- ----K-----  1
Case clone5     ---------- ---------- ---------- ---------- ----K----V  1
```

Fig. 2. Evolution of the HCV NS3 gene sequence at the start of telaprevir monotherapy. Consensus sequence was made from the HCV RNA of 35 treatment-naïve Japanese patients with genotype 1b in our hospital. The number of clones within each sample of identical amino acid sequences is given on the right at the end of each sequence. Dashes indicate identical amino acid sequences.

detection (10 IU/mL) for 2 patients in the group receiving 750 mg every 8 h.[6] In some patients, however, HCV RNA levels increased between days 7 and 14, and mutations that confer resistance to telaprevir were detected. This trial of telaprevir monotherapy was therefore terminated after 2 weeks, and combination therapy of telaprevir plus PEG-IFN-RBV is now used in the USA and Europe.[8–10] Our case may therefore represent an unusual and possibly serendipitous response to long-term telaprevir monotherapy, and the efficacy of monotherapy remains unclear.

To our knowledge, this is the first report of a patient with CH-C achieving SVR by telaprevir monotherapy, without the use of IFN. Three treatment-naïve Japanese patients were enrolled in our hospital for this phase II trial of telaprevir monotherapy over 24 weeks. Before treatment, the 2 non-SVR patients had a high HCV RNA viral load (>5 Log IU/mL), while the viral load in the SVR patient remained low. Further, while HCV RNA decreased below the limit of detection (10 IU/mL) and negativity of HCV RNA remained until the end of treatment in 2 patients, HCV RNA in the other non-SVR patient reappeared after treatment cessation.

The development of drug resistance has been a challenge for treatment strategies in many viral infections. The high replication rate and the error-prone nature of viral RNA polymerases generate a viral quasi-species from which variants resistant to viral inhibitors can be selected. Recently, Kuntzen et al. reported that viral loads were high in the majority of treatment-naïve patients carrying mutations of protease and polymerase inhibitors.[12] Low viral load may therefore be an important factor for achieving SVR by telaprevir monotherapy.

It has recently been reported that CH-C patients never treated with an NS3-4A protease inhibitor may nevertheless possess variants resistant to protease inhibitors involving the HCV RNA NS3 region.[12–14] While there was a resistant variant (T54A) in this case, this mutation exhibits only low-level resistance,[7] and the number of mutant variants may have been few along with substantial suppression of HCV replication by telaprevir. This may also help to explain the effectiveness of telaprevir in this case.

Moreover, two amino acid substitutions (R130K and Q195K) were unique to this patient. We therefore checked the nucleotide sequence data in the DDBJ/EMBL/GenBank databases and found a previous report by Franco et al. on the R130K substitution (EF013801, EF013863, EF013867, EF013869).[15] Interestingly, although only a minor clone (4% of total) in that study, the viral load of the patient with the R130K substitution was also low (2364 IU/mL). To date, however, the Q195K substitution has not been reported. Their presence in this case may indicate that telaprevir has a stronger antiviral activity against HCV with these substitutions.

The NS3-4A protease targeted by protease inhibitors is required for viral polyprotein processing, an essential step in viral replication, but is also responsible for disrupting IFN responses to the infection.[16] Previous studies have shown that high concentrations of protease inhibitors may restore retinoic acid-inducible gene I (RIG-I) signaling in HCV replicon cells,[16–18] and Liang et al. also recently reported that protease inhibitors could restore interferon regulatory factor 3 (IRF-3) signaling in HCV-infected cells.[19] In our patient, telaprevir may have therefore rescued the NS3-4A-mediated blockade of IRF-3 signaling *in vivo*.

Further studies are required, such as sequencing analyses of the HCV NS3 region, and research into the rescue of IFN-β signaling through the RIG-I pathway. It is foreseeable in the future for CH-C patients to be treated by one or a combination of two or more oral drugs with high efficacy and genetic barriers to resistance and low side-effect profiles. Telaprevir may hold promise for being one of these drugs, even if only within a subset of patients, and further studies into telaprevir monotherapy or combination therapy with other oral drugs is therefore warranted. Although still an isolated response, based on our current molecular understanding of HCV infection and pharmacotherapy, this case suggests that long-term telaprevir monotherapy may be effective in other CH-C patients with genotype 1 and a low viral load.

Conflict of interest

The authors have no commercial or other associations that may pose a conflict of interest.

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Influence of Amino-Acid Polymorphism in the Core Protein on Progression of Liver Disease in Patients Infected With Hepatitis C Virus Genotype 1b

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The substitution of amino acid (aa) 70 of arginine for glutamine and/or that of aa91 of leucine for methionine in the core protein in patients infected with hepatitis C virus (HCV) genotype 1b is associated with a poor response to pegylated interferon and ribavirin. Factors influencing these substitutions were sought in 1,097 patients infected with HCV-1b who had not received antiviral treatment. HCV variants with Arg70 and Leu91 (wild-type) decreased, while those with Gln70 and/or Met91 (mutant types) increased with age ($P < 0.001$). Of the 1,097 patients, 464 (42.3%) were infected with the Gln70 variant and the remaining 633 patients with the Arg70 variant. The proportion of patients with the Gln70 variant increased with the severity of liver disease ($P < 0.001$), elevated γ-glutamyl transpeptidase (γ-GTP) levels ($P < 0.001$) and a decrease in platelet count ($P = 0.008$). In univariate analysis patients with hepatocellular carcinoma, elevated aspartate aminotransferase (AST ≥ 58 IU/L) and γ-GTP (≥61 IU/L), and decreased albumin levels (<3.9 g/dl) were more frequent in the patients with the Gln70 variant than the Arg70 variant ($P = 0.003$, 0.005, <0.001, and 0.031, respectively). In multivariate analysis HCC (odds ratio 1.829 [95% confidence interval 1.147–2.917]) and γ-GTP ≥61 IU/L (1.647 [1.268–2.139]) increased the risk for the Gln70 variant. In conclusion, the substitution of amino aa70 of Arg for Gln in patients infected with HCV-1b increases with age, and it is associated with severe liver disease accompanied by elevated AST and γ-GTP levels, as well as the development of hepatocellular carinoma. ***J. Med. Virol. 82:41–48, 2010.*** © 2009 Wiley-Liss, Inc.

KEY WORDS: cirrhosis; core protein; hepatitis C; hepatocellular carcinoma; interferon; ribavirin

INTRODUCTION

Worldwide, an estimated 170 million people are infected with hepatitis C virus (HCV) persistently [Cohen, 1999]. Decompensated cirrhosis and hepatocellular carcinoma (HCC) can develop in about 30% of patients infected with HCV [Alberti et al., 1999; Seeff, 2002]. HCV has six major genotypes and dozens of subgenotypes, and they have distinct geographic distributions and are associated with the progression of liver disease [Simmonds, 1995]. Host and virological factors can influence the severity of liver disease and the response to antiviral treatment. HCV infection in the childhood and women runs a milder course than that in adulthood and men, and the intake of alcohol accelerates the progression of liver disease [Poynard et al., 1997; Kenny-Walsh, 1999; Vogt et al., 1999; Wiese et al., 2000]. Genotypes 1 and 4 aggravate liver disease and decrease the response to antiviral treatment, in comparison with genotypes 2, 3, and 6 [Tsubota et al., 1994; Hui et al., 2003; Hadziyannis et al., 2004; Legrand-Abravanel et al., 2005; Yuen and Lai, 2006]. High levels of HCV RNA in the serum can induce severe liver disease and decrease treatment response [Tsubota et al., 1994].

In Japan, genotype 1b in a high viral load (>100 KIU/ml) accounts for >70% of HCV infection, and decreases the treatment response in patients with chronic hepatitis C [Kumada et al., 2006]. Even with pegylated interferon (PEG-IFN) combined with ribavirin, the sustained virological response for longer than 24 weeks after the withdrawal of treatment is achieved merely in

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50% of the patients with HCV-1b in high levels [Manns et al., 2001; Fried et al., 2002]. It is necessary to predict the response to PEG-IFN/ribavirin before the start of antiviral therapy, to avoid severe side-effects in the patients who will barely gain sustained virological response.

The core protein of HCV is coded for by the C gene, and consists of 191 amino acids (aa) [Rosenberg, 2001]. Although the core protein is conserved better than the other structural and non-structural proteins of HCV, polymorphisms of core protein are known, and they influence the response to antiviral treatment. In patients infected with HCV-1b, for example, the substitution of arginine at position 70 (Arg70) for glutamine (Gln70) and that of leucine at position 91 (Leu91) for methionine (Met70) decrease sustained virological response in the patients with chronic hepatitis C who are treated with PEG-IFN/ribavirin and increase the development of HCC [Akuta et al., 2007a,b,d, 2008].

In the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo, the amino-acid sequence of the core-protein was determined in 1,079 patients infected with HCV-1b who had not received antiviral treatment. The substitution of Arg70 for Gln70 and that of Leu91 or Met 91 were correlated with the age at presentation, liver function tests and the severity of liver disease. Based on the results obtained, Gln70 would contribute to the progression of chronic hepatitis C.

MATERIALS AND METHODS

Patients

During 1966–2008, 1,097 patients infected with HCV-1b visited the Department of Hepatology at the Toranomon Hospital in Metropolitan Tokyo. They were: (1) negative for hepatitis B surface antigen by radioimmunoassay (Dainabot, Tokyo, Japan) or antibody to human immunodeficiency virus type-1; (2) positive for anti-HCV by a third-generation enzyme immunoassay (Chiron Corp., Emeryville, CA) and HCV RNA by the polymerase chain reaction (PCR) (Cobas Amplicor HCV Monitor ver.2.0, Roche Diagnostics, Tokyo, Japan); (3) infected with HCV genotype 1b but not with other genotypes; (4) without previous antiviral treatment; (5) without other forms of hepatitis, including hemochromatosis, Wilson's disease, primary biliary cirrhosis, alcoholic liver disease and autoimmune liver disease; and (6) had serum samples stored at −80°C. Of the 1,097 patients, 778 (70.9%) had chronic hepatitis, 221 (20.1%) cirrhosis, and 98 (8.9%) HCC. Amino acids in the core protein at positions 70 and 91 were determined, and were correlated with liver disease and biochemical and virological markers. Informed consent was obtained from each patient in this study, and the protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki as reflected by approval by Ethic Committee of the institution.

Histopathological Diagnoses of Liver Disease

Liver biopsy was performed under laparoscopy by a modified Vim Silverman needle (Tohoku University style, Kakinuma Factory, Tokyo). The sample was fixed in 10% formalin, and was stained with hematoxylin and eosin, Masson's trichrome, silver impregnation, and periodic acid-Schiff. It contained at least six portal areas. The pathological diagnosis was made by one of the authors (H.K.) who was blinded to the clinical data. Chronic hepatitis was diagnosed based on the scoring system of Desmet et al. [1994]. Cirrhosis was diagnosed by imaging on ultrasonography (US), computed tomography (CT), or magnetic resonance imaging (MRI). HCC was diagnosed by US and/or CT. Angiography was performed when HCC was strongly suspected by US, CT, MRI, or liver biopsy. An increasing trend of tumor markers was taken into consideration for the diagnosis of HCC.

Determination of Amino-Acid Substitutions in the Core Protein

Amino acid (aa) at position 70 of Arg or Gln and aa91 of Leu or Met were determined by PCR with primers specific for each of them [Okamoto et al., 2007]. It is highly reproducible, and has a sensitivity of 94.4% in the determination of aa70 or aa91 in samples with HCV RNA titers >10 KIU/ml. The concordance of the results of this method with those of direct sequencing reached 97.1%. Amino acids at positions 70 and 91 were confirmed by direct sequencing of most samples [Akuta et al., 2005].

Statistical Analysis

Changes of Arg70/Leu91 (wild-type) and Gln70 and/or Met91 (mutant types) with age were analyzed by the Cochran–Armitage trend test (SAS version 9.1.3; SAS Institute, Inc., Cary, NC). Frequencies were compared between groups by the Kruskal–Wallis test and Fisher's exact test. Univariate and multivariate logistic regression analyses were used for the evaluation of factors independently associated with the substitution of aa70. They included the following ten variables: age, sex, liver disease, platelet count, hemoglobin, albumin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), γ-glutamyl transpeptidase (γ-GTP), and substitution of aa at position 91 in the core protein. Each variable was transformed into categorical data consisting of two simple ordinal numbers for univariate and multivariate analyses. Variables that achieved statistical significance on univariate analysis were tested by the multivariate Cox proportional hazard model to identify independent factors. Statistical comparisons were performed using SPSS ver.11.0 (SPSS, Inc., Chicago, IL). A P-value <0.05 by the two-tailed test was considered significant.

RESULTS

Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With HCV-1b

Table I lists the baseline characteristics of the 1,097 patients who were infected with HCV-1b and had not received antiviral treatment. They had the median age of 60 years and included 590 (53.8%) men. The median transaminase levels were elevated, and alpha-fetoprotein was within the normal limit (<10 μg/L). The majority of the patients (70.9%) had chronic hepatitis, while HCC had developed in 8.9% of the patients. Amino acids at positions 70 and 91 in the core protein were both the wild-type (Arg70 and Leu91) in 37.6% of them, and both mutant types (Gln70 and Met91) in 16.4%. The Gln70 variant was detected in 464 of the 1,097 (42.3%) patients.

The Prevalence of Amino-Acid Substitutions Stratified by Age and Sex

The 1,097 patients infected with HCV-1b were classified into three age groups, and the prevalence of Arg70/Leu91 (wild-type) and that of Gln70 and/or Met91 (mutant types) were compared (Fig. 1). Ag70/Leu91 decreased with age by trend analysis, from 63.6% in the patients aged ≤30 years to 36.6% in those ≥41 years ($P < 0.001$ by the Cochran–Armitage trend test). Table II lists the prevalence of the Gln70 variant in men and women stratified by the age. There were no sex differences in the prevalence of the Gln70 variant.

The Prevalence of the Gln70 Variant in Patients With Different Liver Diseases

Figure 2 compares the prevalence of the Gln70 variant among patients infected with HCV-1b who presented with different liver diseases at the baseline. The prevalence of the Gln70 variant increased with the progression of liver disease from chronic hepatitis (32.6%) to cirrhosis (43.0%) and HCC (53.1%) ($P < 0.001$ by the Kruskal–Wallis test). In patients with cirrhosis, the 126 patients with the Arg70 variant were aged with the mean of 62 years (range: 32–78 years) in comparison with the 95 patients with the Gln70 variant who were aged 59 years (25–80). In patients with HCC, the 47 patients with the Arg70 variant were aged with the mean of 66 years (range: 37–81 years) in comparison with the 51 patients with the Gln70 variant who were aged 66 years (46–78).

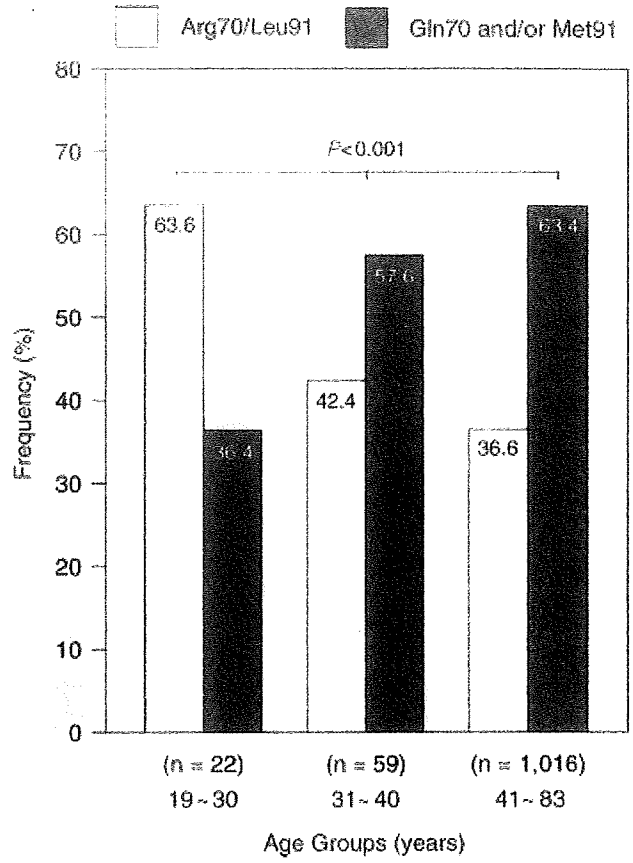

Fig. 1. The age-specific prevalence of Gln70 in treatment-naive patients infected with HCV-1b.

TABLE I. Clinical and Virological Characteristics of the 1,097 Patients Who Were Infected With Hepatitis C Virus of Genotype 1b

Age (years)	60 (19–83)
Men	590 (53.8%)
Follow-up period (years)	8 (3–28)
Hemoglobin (g/dl)	14.0 (4.5–26.8)
Platelets ($\times 10^3/mm^3$)	15.4 (2.0–34.1)
Aspartate aminotransferase (IU/L)	58 (8–617)
Alanine aminotransferase (IU/L)	69 (6–776)
Alpha-fetoprotein (μg/L)	6 (2–65,700)
Liver disease	
Chronic hepatitis	778 (70.9%)
Cirrhosis	221 (20.1%)
Hepatocellular carcinoma	98 (8.9%)
Amino acids in the core protein	
Arg70/Leu91 (double wild-type)	412 (37.6%)
Gln70/Leu91 (mutant type)	284 (25.9%)
Arg70/Met91 (mutant type)	221 (20.1%)
Gln70/Met91 (double mutant type)	180 (16.4%)

Values are the median with range in parentheses or the number with percentage in parentheses.

TABLE II. Frequency of Gln70 in the Core Protein in Patients Infected With HCV-1b Stratified by Age and Sex

Age (years)	Men	Women	Differences
19–30	23.5% (4/17)	20% (1/5)	1.0
31–40	34.1% (14/41)	38.9% (7/18)	0.773
41–50	37.2% (45/121)	40% (14/35)	0.763
51–60	39.1% (72/184)	40.1% (63/157)	0.912
61–70	36.0% (62/172)	30.1% (74/246)	0.205
70–83	45.5% (25/55)	43.5% (20/46)	0.842
Total	37.6% (222/590)	35.3% (179/507)	0.451