5′-GGA GAA GCT GGA GAC TCA GGT G-3′, SOCS1; forward, 5′-CAC TTC CGC ACA TTC CGT TCG-3′ and reverse, 5′-GAG GCC ATC TTC ACG CTA AGG-3′, IL6; forward, 5´-GGT ACA TCC TCG ACG GCA TCT-3′ and reverse, 5′-GTG CCT CTT TGC TGC TTT CAC-3′, 25OAS; forward, 5´- CCA CCT TGG AAA GTG CCG ACA ATG CAG ACA-3′ and reverse, 5′-CGA GTC TTT AAA AGC GAT TGC CAG ATG ATC -3′, MxA; forward, 5´-GCC AGC AGC TTC AGA AGG CCA TGC TGC AGC -3′ and reverse, 5′-GGG CAA GCC GGC GCC GAG CCT GCG TCA GCC -3′.

The siRNAs

The siRNAs directed against SOCS3 were designed as follows: SOCS3-HSS113312 stealth (sequence 5′- CCC AGA AGA GCC UAU UAC AUC UAC U-3 ′and 5′-AGU AGA UGU AAU AGG CUC UUC UGG G-3′, Invitrogen) was used. 10 μg in vitro-synthesized HCV-RNA and 80 pmol siRNA SOCS3-HSS113312 or MOCK or control siRNA (negative universal control Med #2, Invitrogen) were electroporated into 5 × 10 naïve Huh7.5.1 cells using the protocol described in *HCV cell culture system*. Forty-eight hours after transfection, expression levels of SOCS3 mRNA were measured by real-time PCR. The difference in IFN sensitivity between SOCS3 knock down HCV infected cells and control HCV infected cells was determined by measuring supernatants HCV core antigen 72 hours after addition of IFN.

Immunohistochemistry for HCV core

HCV-JFH1 transfected or infected Huh7.5.1 cells were cultured on 22 mm-round micro cover glasses (Matsunami, Tokyo, Japan). For detection of HCV core, cells were fixed with cold acetone for 15 min. The cells were incubated with the primary antibodies for 1 hour at 37 °C, and with Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VECTA SHIELD Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized by fluorescence microscopy (BZ-8000, KEYENCE, Osaka, Japan).

Western blot analysis

Western blotting was performed as described (Tanabe et al., 2004). Briefly, 10 μg of total cell lysate was separated by SDS-PAGE, and blotted onto a polyvinylidene fluoride (PVDF) membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and was visualized by chemiluminescence using the ECL Western Blotting Analysis System (Amersham Biosciences, Buckinghamshire, UK).

Statistical analyses

Statistical analyses were performed using Student's *t*-test; p-values of less than 0.05 were considered statistically significant.

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Appendix A. Supplementary data

Supplementary Fig. 1. Infectivity of the full-length 2b HCV RNA and 2b/JFH1 chimeric virus, JEC3F. A. Challenge of human liver-engrafted albumin-uPA/SCID mice with culture fluid from JFH1 and JEC3F cells. Cell culture fluids from the JFH1 clone and JEC3F were injected intravenously into human liver engrafted albumin-uPA/SCID mice. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined. B. Fig. 1B Challenge of human liver-engrafted albumin-uPA/SCID mice by intrahepatic injection of in vitro synthesized, full-length 2b HCV RNA. Five hundred μl of RNA solution containing 30 μg of in vitro synthesized full-length 2b HCV RNA was injected into the livers of anesthetized chimeric mice through a small abdominal incision. Serum samples were obtained from the mice every 2 weeks after injection and the HCV RNA titer was determined.

Supplementary Fig. 2. Comparisons of replication efficiency of JFH1and J6/JFH1, 2b/JFH1 chimeras after transfection into Huh7.5.1-cells. A. Structures of theJ6/JFH1 and 2b/JFH1 genomes. J6 is joined between NS2 and NS3 with JFH1. 2b-HCV is joined with JFH1within NS2 at nt. 2867. B Measurements of core protein in cell culture fluids. Ten μg of JFH1, J6/JFH1, 2b/JFH1 RNA were transfected into 5×10^6 Huh7.5.1 cells and the cells were cultured in 100 mm-diameter plates. The culture fluids from JFH1, J6/JFH1, H77/JFH1or 2b/JFH1-transfected Huh7.5.1 cells were collected separately on the days indicated and the levels of core antigen were measured. These experiments were done three times with similar results independently. *Panel B* shows representative date.

Supplementary Fig. 3. Inhibition of infection by blocking CD81. Huh 7.5.1 cells were plated into a 6 well plate at 1.4×10^5 cells per well. After 48 hours, the cells were incubated with anti-CD81 or isotypematched control antibody at the concentration indicated for 1 hour. Subsequently, cells were infected with 1 ml of JEC3F stock cell culture fluids at day 2 for 4 hours and washed with PBS. 48 hours after inoculation, anti-core immunostaining was performed with mouse anti-HCV core protein monoclonal antibody (Panels B and C). Quantification of HCV core antigen was carried out in culture fluids at 48 hours after infection (Panel A).

Supplementary Fig. 4. Comparison between 2b and JFH-1 core amino acid sequence.

Note: Supplementary materials related to this article can be found online at doi:10.1016/j.virol.2010.07.041.

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Cell culture and in vivo analyses of cytopathic hepatitis C virus mutants[☆,☆☆]

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ABSTRACT

HCV-JFH1 yields subclones that develop cytopathic plaques (Sekine-Osajima Y, et al., Virology 2008; 371:71). Here, we investigated viral amino acid substitutions in cytopathic mutant HCV-JFH1 clones and their characteristics in vitro and in vivo. The mutant viruses with individual C2441S, P2938S or R2985P signature substitutions, and with all three substitutions, showed significantly higher intracellular replication efficiencies and greater cytopathic effects than the parental JFH1 in vitro. The mutant HCV-inoculated mice showed significantly higher serum HCV RNA and higher level of expression of ER stress-related proteins in early period of infection. At 8 weeks post inoculation, these signature mutations had reverted to the wild type sequences. HCV-induced cytopathogenicity is associated with the level of intracellular viral replication and is determined by certain amino acid substitutions in HCV-NS5A and NS5B regions. The cytopathic HCV clones exhibit high replication competence in vivo but may be eliminated during the early stages of infection.

Introduction

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality (Alter, 1997). Antiviral therapeutic options against HCV have been limited to type I interferons and ribavirin and have yielded unsatisfactory responses (Fried et al., 2002). Given this situation, a precise understanding of the molecular mechanisms of interferon resistance has been a high priority of research in academia and industry.

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been overcome to some extent by the development of the HCV subgenomic replicon (Lohmann et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient and can replicate efficiently in Huh7 cells (Kato, 2001; Kato et al., 2003), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 and Huh-7.5.1 cells, allow production of higher viral titers and have a greater permissivity for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilo-bases that encode polyproteins of ~3000 amino acids. These proteins are processed post-translationally by cellular and viral proteases into at least 10 mature proteins (Sakamoto and Watanabe, 2009). The viral non-structural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been recently

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Abbreviations: HCV, hepatitis C virus; CPE, cytopathic effect; ER, endoplasmic reticulum; RdRp, RNA dependent RNA polymerase.

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reported that HCV-JFH1 transfected Huh-7.5.1 cells die when all of the cells are infected and intracellular HCV RNA reaches maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In a previous study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system and we reported that HCV-JFH1 transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity (Sekine-Osajima et al., 2008). We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5A and NS5B regions.

In this study, we investigated the mechanisms and viral nucleotide sequences involved in HCV-induced cytopathic effects using HCV-JFH1 cell culture and a newly developed cytopathic plaque-forming assay. We demonstrated that introduction of NS5A and NS5B mutations into the JFH1 clone resulted in a higher replication efficiency, although introduction of these mutations into the JFH1 subgenomic replicon has no effect on viral replication. These mutations do not affect virion entry or release of viral particles but regulate virus replication, and high levels of virus replication result in cytopathogenicity.

Results

Development of cytopathic plaques by HCV infection of Huh-7.5.1 cells

A plaque assay was performed to investigate the morphological CPE following HCV-JFH1 infection (see Materials and methods). Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1 cells. The cells were subsequently cultured in medium containing agarose. On 9 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 1A). HCV-inoculated cell cultures developed plaques as unstained areas, accompanied by rounded cells in the periphery (Fig. 1B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Those results were consistent with our previous study (Sekine-Osajima et al., 2008).

Introduction of mutations in the NS5A and NS5B regions of the JFH1 clone augmented its cytopathic effects

Among the amino acid substitutions that developed in the plaque-derived HCV-JFH1 strains, 6 of the 9 amino acid changes appeared redundantly among 5 independently isolated plaques, and clustered in the C terminal part of the NS5A and NS5B regions. To investigate the phenotype of each amino acid substitution, we constructed mutant JFH1

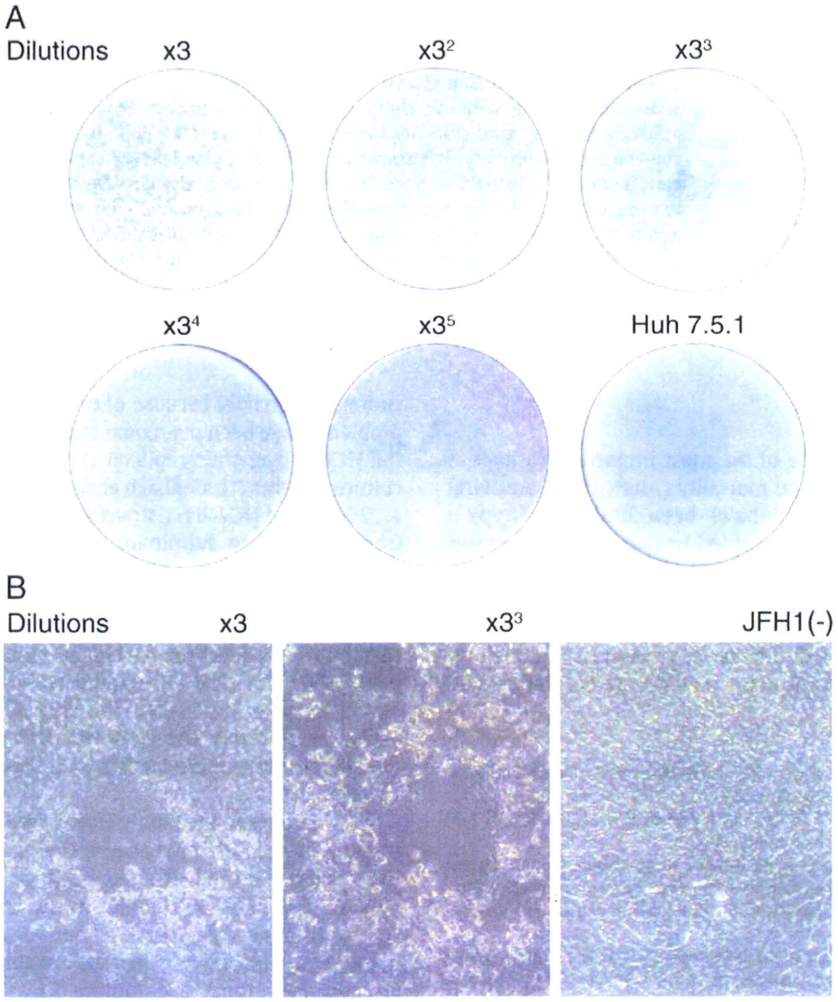

Fig. 1. The cytopathic effects of HCV-JFH1 *in vitro*. A. Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO_2 (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methyl-cellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. B. The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

clones in which we introduced separately one amino acid substitution in NS5A and five substitutions in NS5B (Fig. 2A) and transfected the mutant HCV RNAs into Huh-7.5.1 cells. To compare the electroporation efficiencies of viral RNAs, Huh-7.5.1 cells were harvested 8 h after transfection and the levels of intracellular core antigen were measured. There was no difference in the efficiencies of electroporation (Fig. 2D). The substitutions G2964D, H3004Q, and S3005N did not lead to cytopathic effects but three mutant subclones (C2441S, P2938S and R2985P) produced much more cell death compared to the wild type JFH1 (Fig. 2B). To assess the quantitative cytopathic effect seen in host cells for each of the mutants, we also performed MTS assay at 6 days post transfection. It showed that Huh-7.5.1 cells transfected with the triple mutants (C2441S, R2938S, or R2985P) induced apparently much more cytopathic effect compared to the parental JFH1 and other mutant clones, although the three mutant clones encoding the substitutions C2441S, P2938S, or R2985P did not show significant difference but

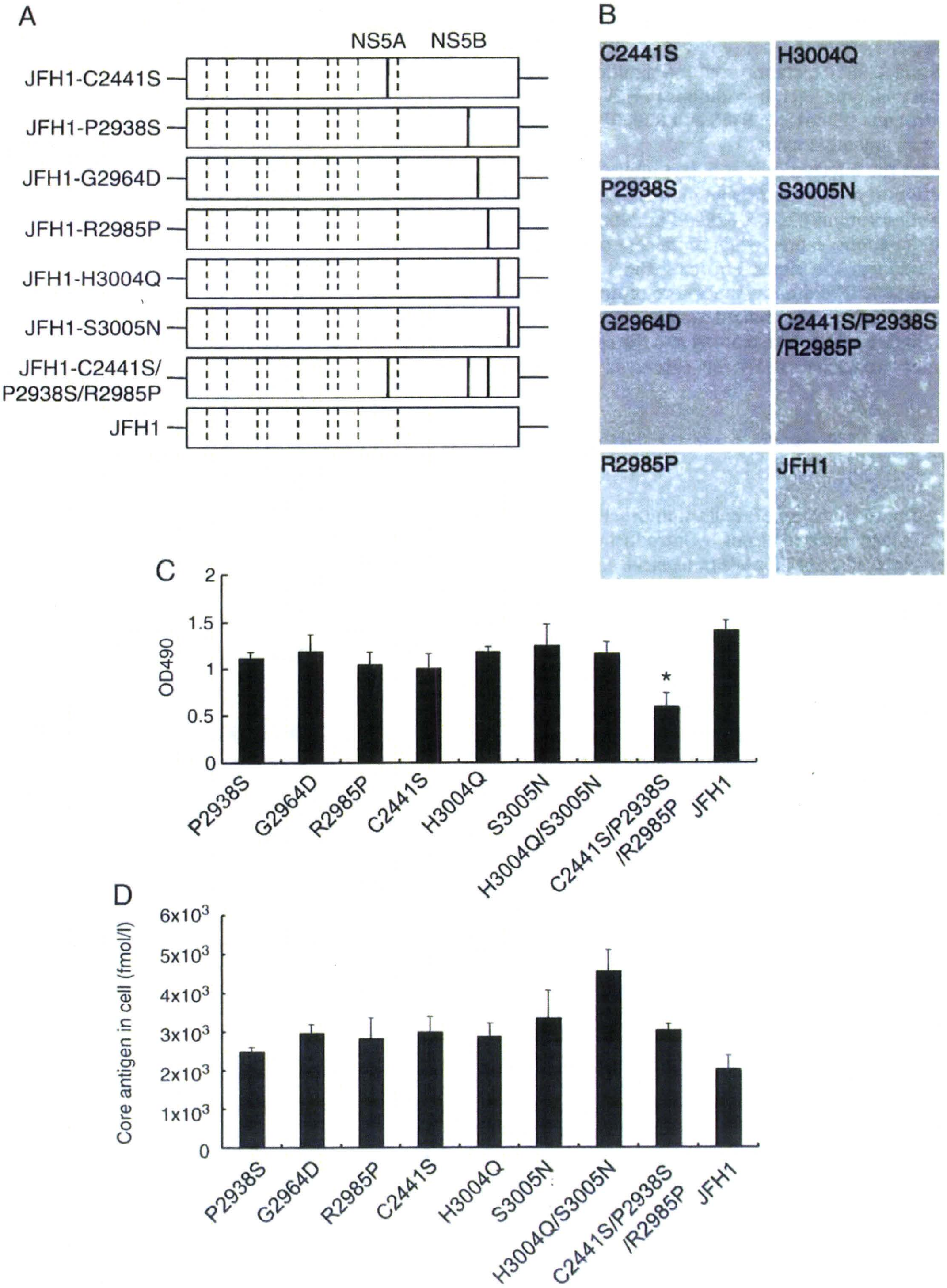

Fig. 2. Introduction of mutations into the NS5A and NS5B regions of JFH1. A. The mutations identified in the cytopathic plaque were introduced individually into the parental JFH1. Each JFH1 mutant was transfected into Huh-7.5.1 cells by electroporation. B. Huh-7.5.1 cells transfected with JFH1-mutants were observed by phase-contrast microscopy at day10 after transfection. C. MTS assay was performed to assess the quantitative cytopathic effect seen in Huh-7.5.1 cells for each of the mutants 6 days post transfection. Asterisks indicate p-values of less than 0.05 as compared with JFH1. D. Huh-7.5.1 cells were harvested at 8 h after transfection and the levels of intracellular core antigen were measured.

showed tendency to introduce more cytopathic effect than the parental JFH1 and the mutant clones encoding the substitutions G2964D, H3004Q, and S3005N (Fig. 2C).

Introduction of NS5A and NS5B mutations into the JFH1 clone led to a greater replication efficiency

To compare the expression levels of each mutant subclone, each HCV RNA was transfected and core antigen was detected subsequently in the culture medium. Similar to Fig. 2B, HCV clones with individual substitutions G2964D, H3004Q and S3005N produced significantly less core antigen or did not replicate at all. In contrast, the C2441S, P2938S and R2985P mutants produced significantly more core antigen than the wild type JFH1. In addition, an HCV clone with all 3 adaptive substitutions (C2441S, P2938S and R2985P) produced more core antigen than any other clone (Fig. 3A).

Next, we harvested the infected cells at 5 days after electroporation and performed western blotting. As shown in Fig. 3B, the three clones encoding the substitutions C2441S, P2938S, or R2985P, and the clone with all three mutations, expressed far more core protein than the parental JFH1, although the clones encoding the substitutions G2964D, H3004Q and S3005N did not express core protein. We also transferred culture media from the mutant clones onto uninfected Huh-7.5.1 cells and performed western blotting and the cells infected with the same mutant subclones as Fig. 3B expressed more core protein (Fig. 3C).

Introduction of NS5A and NS5B mutations into the JFH1 subgenomic replicon

To investigate the primary phase of replication of JFH1 mutants, we constructed JFH1 subgenomic replicons by introducing individually the six mutations in NS5A and NS5B. We transfected each replicon RNA into Huh7 cells and compared their replication levels according to the luciferase activities. Consistently with the mutant viruses, the subgenomic replicon encoding the changes C2441S, P2938S or R2985P, which produced higher amounts of core antigen, did replicate at higher levels than the other subgenomic replicons with single mutation, G2964D, H3004Q and S3005N. However, none of these mutants replicated at higher than the parental JFH1 subgenomic replicon. Furthermore, replicon with triple mutations of C2241S, P2938S and R2985P did not replicate (Fig. 4).

Introduction of NS5A and NS5B mutations into the JFH1 clone had no effect on the production of infectious virions

We sought to investigate the effects of the NS5A and NS5B mutations on virus replication and virion secretion independent of re-infection and spread of the viruses produced. Therefore, we used the S29-subclone of Huh7 cells, which cannot be infected by HCV because of a defect in CD81 expression but does support viral genomic replication and releases infectious HCV particles after transfection (Russell et al., 2008). The Huh7-S29 cells enabled us to evaluate a single cycle of infection and production of virions. Those cell lines did not show apparent cytopathic effects after transfection with HCV RNAs (data not shown). To analyze HCV particle production from cells transfected with the viral genomic RNAs transcribed in vitro, we harvested culture media and cells at 72 h post transfection and measured the core antigen levels in culture media and intracellular HCV RNA by real-time RT-PCR. The C2441S, P2938S, and R2985P mutants produced significantly greater amounts of core antigen in the culture medium than the wild type JFH1. The HCV clone carrying all three mutations produced the greatest amount of core antigen (Fig. 5A, top). Consistent with the core antigen levels in the culture media, intracellular HCV RNA levels were also higher in the cells transfected with the mutated genomes encoding separately C2441S, P2938S, and R2985P, and that with all three mutations (Fig. 5A, middle), indicating that these mutations affected virus replication. Fig. 5A bottom shows the efficiency of infectious viral particle release from each transfectant, this being expressed as the core antigen level in the culture medium adjusted by dividing by the levels of intracellular HCV RNA. There was no difference in the efficiency of release of virions by the wild type JFH1 and the genomes carrying the C2441S, P2938S or R2985P changes. These results indicated that these three mutations in NS5A and NS5B did not affect virion entry or viral particle release but did regulate virus replication, and a high level of viral replication induces cytopathogenicity. Similarly, as shown in Fig. 3B, the three clones with C2441S, P2938S or R2985P, or all three mutations expressed much higher levels of core protein than the parental JFH1, while clones with G2964D, H3004Q or S3005N mutations did not express detectable amounts of core protein (Fig. 5B).

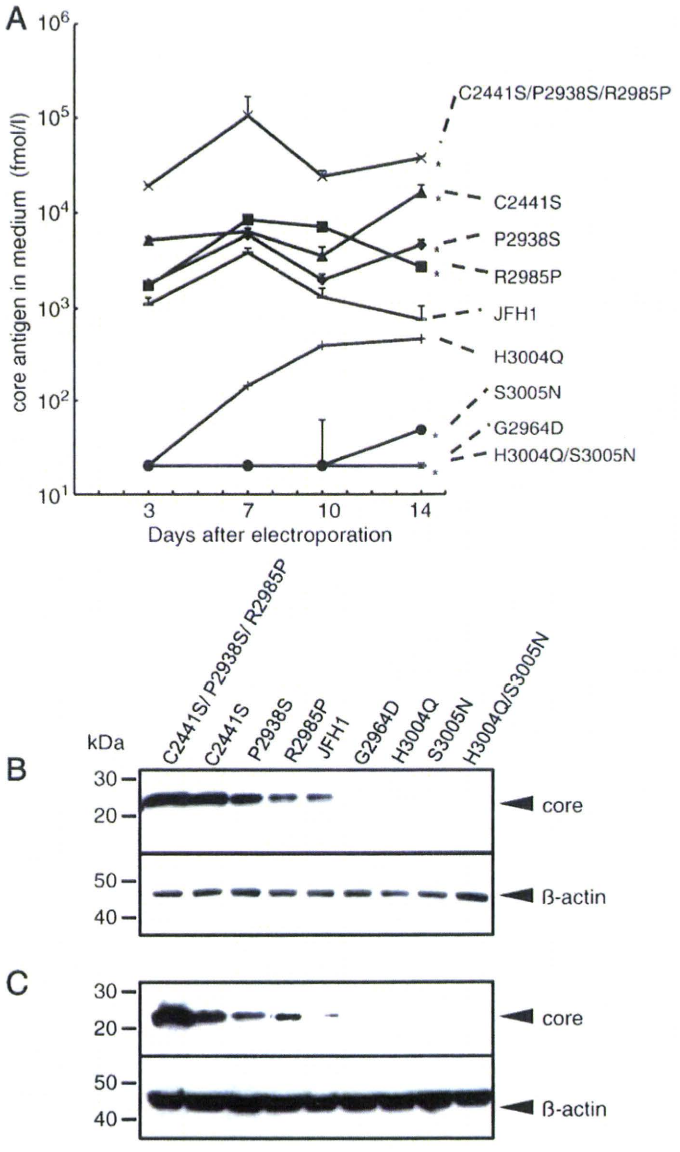

Fig. 3. Replication competences of HCV subclones with NS5A and NS5B mutations. A. Levels of core antigen in the culture medium. The culture media from transfected cells were collected on the days indicated and the levels of core antigen were measured. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Huh-7.5.1 cells transfected with JFH1 mutants were harvested at 5 days after transfection and western blotting was performed. C. The culture media from Huh-7.5.1 cells transfected with JFH1 mutants were transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 3 days after infection. Western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

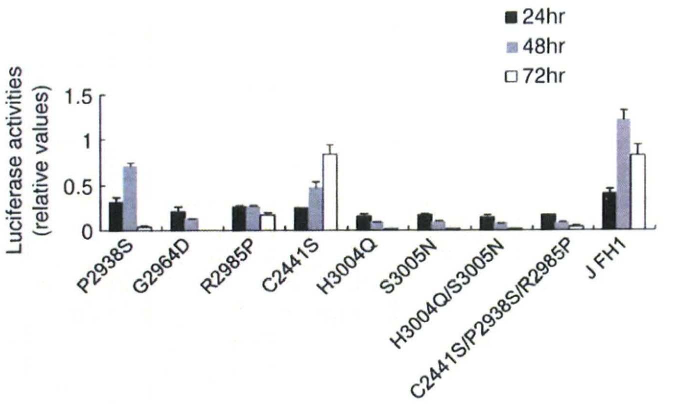

Fig. 4. Luciferase assay of the cytopathic JFH1-subgenomic replicon. Mutations were introduced into 2a-Feo subgenomic replicon and transcribed RNA for each replicon was transfected into Huh7 cells by electroporation. The cells were harvested at 24 h, 48 h and 72 h after electroporation and were used for Luciferase assay. Values are relative values to those of 8 h.

Mutations of NS5A and NS5B are associated with replication competence at earlier stages in vivo

We next used human hepatocyte chimeric mice to investigate the infectivity of the triple mutant of NS5A and NS5B. We confirmed the mouse liver chimerism greater than 70% by immunohistochemical analysis (data not shown). Culture media of the parental JFH1 and the mutant subclone with three mutations (C2441S, P2938S, and R2985P), were collected following transfection of Huh-7.5.1 cells, concentrated, and inoculated intravenously into human hepatocyte chimeric mice. We confirmed that the three mutations in NS5A and NS5B were conserved in the virus genome sequence of cell culture supernatants that were used for inoculation (data not shown). Two mice were inoculated with JFH1 and three were inoculated with the mutant virus. HCV RNA and human albumin in the sera of the mice were detected sequentially.

We repeated the same exam twice and confirmed consistency of the results. In the early phase post inoculation, the concentration of HCV RNA in serum was significantly higher in mice inoculated with the culture medium from the mutant subclone (Fig. 6A), suggesting that the mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated with virus replication in vivo. However, there was no difference in the level of HCV RNA in later period. The disparity of viral production at early time point could be influenced by the disparate numbers of infectious virus between the 2 initial inoculums. However, the sharp elevation of serum HCV RNA at day 5 after dropping at day 3 indicates that the mutants (C2441S, P2938S plus R2985P) are more replication competent at early stages in vivo. Serum levels of human albumin remained constant throughout the observed periods and showed no significant differences between wild and mutant-infected mice (Fig. 6B).

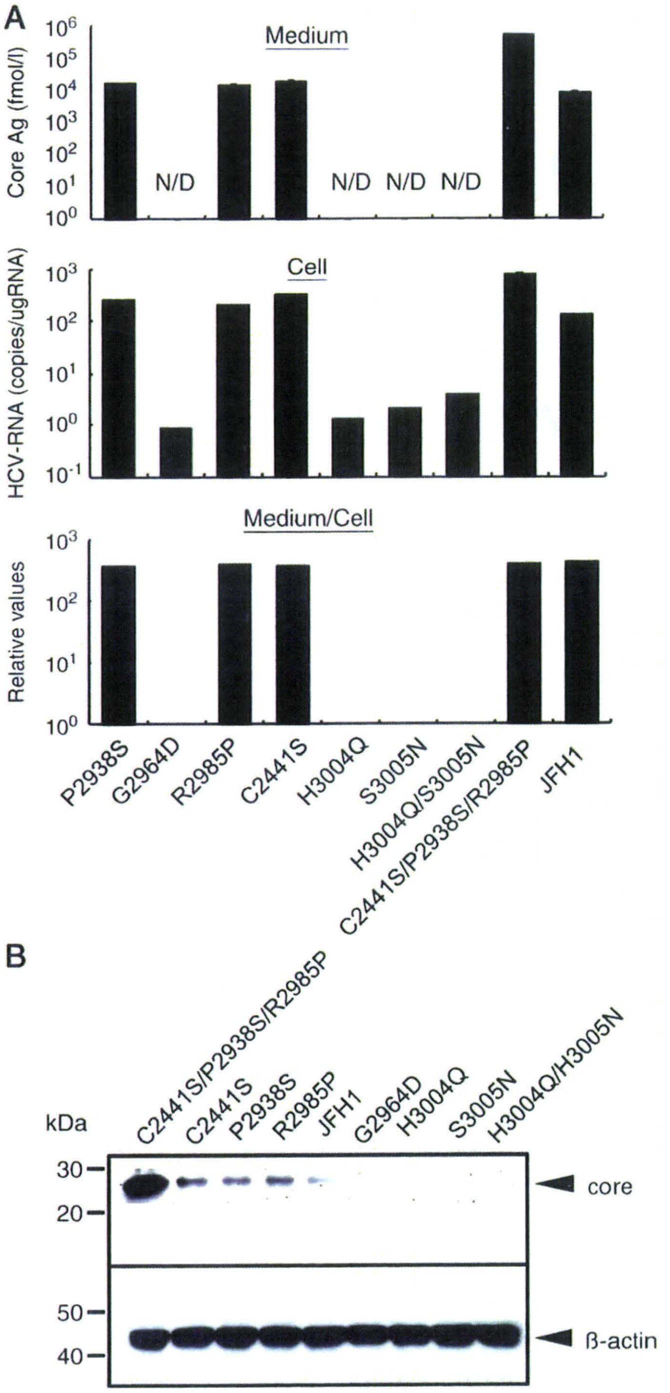

Fig. 5. Analysis of viral replication and production of viral particles using a single-cycle assay. A. Levels of core antigen in the culture media 3 days after transfection of JFH1 mutants into CD81-deficient Huh7-S29 cells (top). Levels of intracellular HCV RNA were quantified by real-time RT-PCR 3 days after transfection of JFH1 mutants into Huh7-S29 cells (middle). To determine the efficiency of infectious viral particle release from Huh7-S29 cells transfected with JFH1 mutants, the levels of core antigen in the culture media were adjusted by dividing by the levels of intracellular HCV RNA (bottom). Core Ag: Core antigen, N/D: not detectable. B. Huh7-S29 cells were harvested at 3 days after transfection of JFH1 mutants and western blotting was performed using anti-core and anti-beta-actin. kDa: kilo dalton.

We also investigated expression of ER stress-related proteins, the glucose regulated protein 78 (GRP78) and C/EBP homologous protein (CHOP), in liver of chimeric mice infected with JFH1 or the mutant in the early phase post inoculation. Human hepatocyte chimeric mice were inoculated in the same way as described above, and we verified that the level of virus titer in serum of each mouse was same as presented in Fig. 6A (data not shown). We sacrificed one each mouse that was infected with wild type or mutant JFH1 at 5 day of infection and investigated hepatic expression of GRP78 and CHOP. Liver histology showed no sign of inflammation or cytopathic cell death. However, as shown in Fig. 7, the expression level of both GRP78 and CHOP was higher in mice inoculated with the mutant viruses than the parental JFH1. There was no apparent difference in percents of hepatic chimerism between each mouse. These finding suggested that ER stress-related proteins were upregulated in the liver of HCV-infected mouse and that these responses were more strongly induced in the liver of mutant-infected mouse.

Highly adapted cytopathic mutations reverted to wild type in vivo

Finally, we analyzed the serum viral sequence at the specified time points. On days 1 and 5, the HCV genomic sequences of the mice

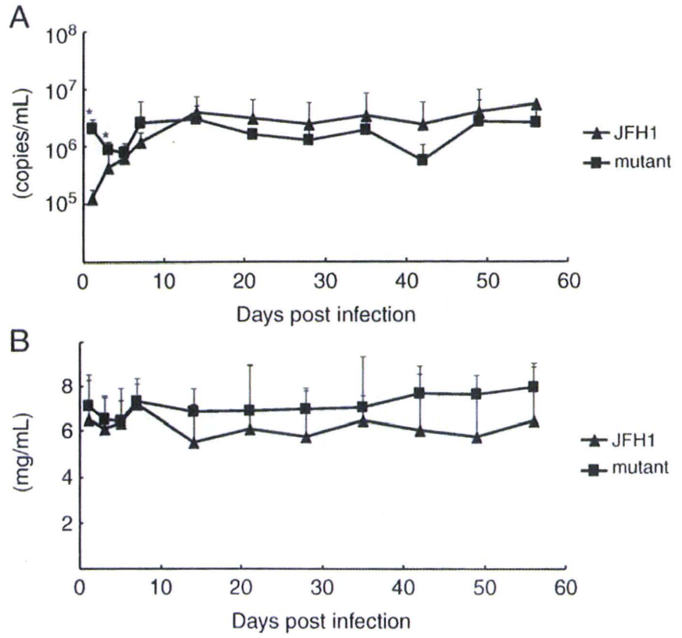

Fig. 6. In vivo analysis of cytopathic JFH1 mutants using human hepatocyte chimeric mice. A. Serial changes in HCV RNA in the sera of mice inoculated with the culture media from JFH1 mutants. The data shows the average of 2 mice for JFH1, and 3 mice for the mutant. Asterisks indicate p-values of less than 0.05 as compared with JFH1. B. Levels of human albumin in the sera of mice inoculated with the culture media from JFH1 mutants.

inoculated with the cytopathic mutant virus showed conservation of the mutations in codons 2441, 2938 and 2985. However, on days 21 and later, the mutation at codon 2985 had reverted to the wild type JFH1 sequence in all the mutant-injected mice and the mutation at codon 2938 had reverted to the wild type JFH1 sequence in two of the three mice. The C2441S mutation was more stable in the mutant-injected mice, but one mouse had lost it at day 56 (Fig. 8).

Discussion

In this study, we investigated the significance of genetic mutations in plaque-purified, cytopathic HCV-JFH1 subclones. Genetically engineered JFH1-mutants encoding C2441S, P2938S, and R2985P led to much more cell death than the wild type JFH1, and also produced significantly higher amounts of core antigen in the culture medium and inside the cells than the parental JFH1 clone. In the single-cycle production assay, which exploited a receptor-deficient Huh7 cell line, the three JFH1-mutants, JFH1-C2441S, P2938S, and R2985P produced significantly more core antigen in the culture medium and expressed equivalently higher amounts of viral genomic RNA in the cells. These data suggest that the three mutations in NS5A and NS5B (C2441S, P2938S, and R2985P) are associated directly with enhanced intracellular replication and resultant virion formation, which correlated with the extent of the cytopathic effects. Interestingly, inoculation of a cytopathogenic mutant, JFH1-C2441S/P2938S/R2985P, into human hepatocyte chimeric mice produced significantly higher plasma HCV RNA concentrations than JFH1 at ~7 days post inoculation. At a later phase of infection, however, the mutations in this mutant HCV reverted partially to the wild type sequences. Taking all things together, it is suggested that in vitro-isolated, genetically modified cytopathic HCV subclones replicate robustly in the acute phase of in vivo infection but are eliminated rapidly and substituted by in vivo adapted clones.

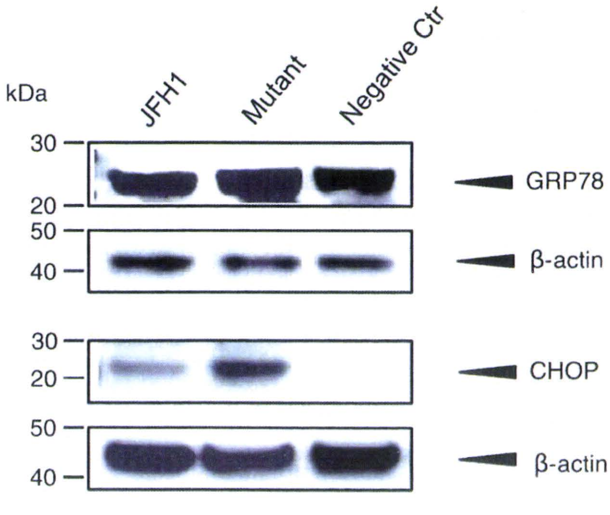

Fig. 7. Expression of ER stress-related proteins in human hepatocytes of chimeric mice infected with JFH1 or the mutant in the early phase. Western-blot analysis of the liver tissues of infected chimeric mice using anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody and anti-beta-actin. Liver samples were obtained at 5 days after inoculation. The negative control liver samples for this study was from uninfected human hepatocyte chimeric mouse.

Four of the five NS5B mutations appeared independently in several isolated subclones. This made us speculate that these amino acid substitutions may affect the enzymatic activity of RdRp. Mapping of the amino acid substitutions in the RdRp tertiary structure revealed that amino acid 2441 is located on the finger domain, and three amino acids, 2938, 2964, and 2985, are on the outer surface of the thumb domain, which corresponds to the opposite side of the nucleotide tunnel. The other substitutions, 3004 and 3005, are within the domain of the polypeptide linking the polymerase to the membrane anchor (Lesburg et al., 1999). Our preliminary study has shown that the NS5B mutations, P2938S and R2985P, did not affect cell-free enzymatic activities of the RNA polymerase. Thus, it is speculated that these mutations may affect the stability of the HCV replicase complex by altering surface affinity to other nonstructural proteins.

There are several reports on cell culture adaptive mutations in the HCV-JFH1 genome that gave more vigorous and consistent virus expression. Most studies involved prolonged cell culture of HCV-JFH1 or multiple rounds of successive passage onto naïve cells. Zhong et al. detected the E2-G451R mutation after culture for more than 60 days. The mutation led to more efficient production of infectious viral particles than wild type JFH1 (Zhong et al., 2006). Delgrange et al. conducted successive virus infections of naïve cells and identified the E2-N534K mutation that facilitated virus-CD81 attachment, and core-F172C and -P173S that increased secretion of virions (Delgrange et al., 2007). Using a similar method, Russell et al. identified E2-N417S that improved virus-cell attachment, and p7-N765D and NS2-Q1012R that increased virion production (Russell et al., 2008). Kaul et al. reported the NS5A-V2440L mutation, that was close to the C terminus and increased virion production (Kaul et al., 2007). Yi et al. used a chimeric virus of genotype 1a and JFH1 and identified the NS3-Q1251L mutation that resulted in enhanced virus production, possibly through improved interactions between NS2 and NS3 that were required for virion formation (Yi et al., 2002). Han et al. used EGFP-tagged virus and identified the mutually dependent mutations, NS3-M1290K and NS5A-T2438I, which improved virus production synergistically (Han et al., 2009).

Of note is that all of the mutations reported above promoted virion secretion or virus–cell surface interaction and none of them showed any effect on intracellular replication of viral RNA or translation of virus proteins. None of the adaptive mutations reported above overlapped with our cytopathogenic mutations. The mutations that we have identified conferred enhanced virus replication and protein expression in the early/acute stages of infection and subsequently led to massive cell death. Our data and the reports of other groups suggest that the HCV genome evolves to adapt to the host cell environment. Mutations that optimize virus secretion or virus–cell entry may be

```
                      2437    2446      2934    2943      2981    2990
           JFH1wt     DTTVCCSMSY        LGAPPLRVWK        LPEARLLDLS
           Mutant     ----S----         ----S-----        ----P-----

        [ #1 Day  1   ----S----         ----S-----        ----P-----
        |    Day 21      N/D            ----------        ----------
        |    Day 49      N/D            ----------        ----------
        |    Day 56   ----S----         ----------        ----------
        |
Mutant  | #2 Day  5   ----S-----        ----S-----        ----P-----
        |    Day 49   ----S-----        ----------        ----------
        |    Day 56   ----------        ----------        ----------
        |
        | #3 Day  1      N/D            ----S-----        ----P-----
        [    Day 56   ----S-----        ----S-----        ----------

        [ #1 Day  1   ----------        ----------        ----------
        |    Day 56   ----------        ----------        ----------
JFH1    |
        | #2 Day  1   ----------        ----------        ----------
        [    Day 56   ----------        ----------        ----------
```

Fig. 8. Nucleotide sequence analysis of virus genomes circulating in the sera of infected mice. We extracted RNA from the sera of mice inoculated with culture media from JFH1 or JFH1-mutants and analyzed the viral sequence at the specified time points. N/D is not detectable. Wt: Wild type.

required for persistent infection in vitro, while those that affect cellular viral RNA replication may possibly promote viral genetic evolution and host cell damage.

The results of in vivo experiments using human hepatocyte chimeric mice were consistent with those of virus cell culture (Figs. 5, 6 and 7). The mutant JFH1 clones showed markedly higher levels of replication than the parental JFH1 in the acute phases. However, the serum HCV titers subsequently leveled out after two weeks of infection, concomitant with reversal of some cytopathic mutations to wild type sequences. Bukh et al. reported that inoculation of the HCV-1b genome into chimpanzee liver resulted in persistent infection, although the mutation reverted rapidly to wild type (Bukh et al., 2002). In this study, the NS5A-C2441S mutation was preserved in 2 of 3 mice, while NS5B-P2938S reverted to the wild type sequences in 2 of 3 mice and NS5B-R2985P reverted to wild type sequences in all 3 mice. These results suggest that the highly adapted JFH1 genome is infectious and viable in vivo, but is not as fit in vitro.

It is not clear why the subgenomic replicons with C2441S, P2938S or R2985P mutations did not show differences in replication levels compared to the wild type JFH1 subgenomic replicon. One may speculate that this discrepancy between the results using full-length HCV genomes and replicons might be the presence or absence of the HCV structural proteins. In addition, three individual substitutions G2964D, H3004Q and S3005N did not enhance viral replication as compared with the parental JFH1 nor did express detectable amounts of core protein. It is speculated that these mutants exist in host cells through co-infection with replication-competent viral clones resulting in enhanced replication.

There is clinical evidence that suggests the pathological outcomes of hepatitis C result from the immune response of the host rather than the direct cytopathic effects of the virus (Cerny and Chisari, 1999). However, several clinical studies have shown that fulminant hepatic failure (FHF, the HCV-JFH1 strain was isolated from such a case) featured massive hepatocyte apoptosis, as characterized by caspase activation and Fas–FasL expression (Leifeld et al., 2006; Mita et al., 2005; Ryo et al., 2000). The ER stress markers, GRP78 and ATF6 are upregulated in HCV-infected liver tissue as the histological grade advances (Shuda et al., 2003). This background and our results in vitro and in vivo suggest that HCV strains with highly infectious and cytopathic gene signatures may replicate aggressively in the acute phase of infection and that certain defects in innate or adaptive immune responses against the virus could lead to severe and persistent liver damage due to cytopathic effects induced directly by HCV. Such mechanisms might explain some rare clinical features of HCV infection, such as fulminant hepatic failure and post-transplantation severe fibrosing cholestatic hepatitis (Delladetsima et al., 1999; Dixon and Crawford, 2007).

In conclusion, we identified three substitutions in cytopathic HCV-JFH1 subclones derived from plaque assay. These substitutions directly enhanced virus replication in the early phases of virus infection in vitro and in vivo. This highly enhanced replication induced ER stress-mediated apoptosis and resulted in cytopathogenicity. Further analyses of cellular effects on HCV replication may elucidate the pathogenesis of HCV infection and may define novel host factors as targets of antiviral chemotherapeutics.

Materials and methods

Cells and cell culture

Huh-7.5.1 cells (Zhong et al., 2005) (kindly provided by Dr Francis V. Chisari) and CD81 deficient Huh7-S29 cells (Russell et al., 2008) (kindly provided by Dr Rodney S. Russell and Dr Robert H. Purcell) were maintained in Dulbecco's modified minimal essential medium (DMEM, Sigma, St. Louis, MO) supplemented with 2 mmol/L L-glutamine and 10% fetal bovine serum at 37 °C under 5.0% CO_2.

Sequence analysis

The cDNA from the isolated JFH1-plaque was amplified from cytopathic virus-infected Huh-7.5.1 cells by RT-PCR and subjected to direct sequencing.

In vitro RNA synthesis and transfection

A plasmid, pJFH1full (Wakita et al., 2005), which encodes full-length HCV-JFH1 sequence, was used. In vitro RNA synthesis and transfection were conducted as previously described (Sekine-Osajima et al., 2008). Briefly, HCV RNA was synthesized from linearized pJFH1 plasmid as template and transfected into Huh-7.5.1 cells by electroporation. The transfected cells were split every 3 to 5 days. The culture media were subsequently transferred onto uninfected Huh-7.5.1 cells and Huh7-S29 cells. The levels of HCV replication and viral protein expression were detected by real-time PCR and western blotting.

Plaque assay

HCV plaque assays were performed as reported previously (Sekine-Osajima et al., 2008). Huh-7.5.1 cells were seeded in collagen-coated 60 mm-diameter plates. After overnight incubation, HCV-infected culture media were serially diluted in a final volume of 2 ml per plate and transferred onto the cell monolayer. After ~5 h of incubation, the inocula were removed and the cell monolayer was overlaid with 8 ml of culture medium containing 0.8% methylcellulose (Sigma). After 7 to 12 days culture, cytopathic plaques were visualized by staining with 0.08% crystal violet solution (Sigma). The levels of cytotoxicity were evaluated by counting the plaques and calculating the titer (plaque-forming unit/ml).

Establishment of mutant JFH1 clones

In order to introduce various mutations into the NS5A and NS5B region of JFH1, plasmid pJFH1 was digested with HindIII and the DNA fragment encompassing nt. 8231 to 9731 was subcloned into the pBluescript II SK+ phagemid vector (Stratagene, La Jolla, CA). Mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-ChangeII Site-Directed Mutagenesis Kit, Stratagene) to generate the following codon changes: P2938S, G2964D, R2985P, H3004Q and S3005N. Finally, the HindIII–HindIII fragments were subcloned back into the parental plasmid, pJFH1. A PCR fragment (nt. 7421–7839) was subcloned into the pGEM-T Easy plasmid vector (Promega, Madison, WI) and digested with RsrII and BsrGI. Finally, after introducing the codon change C2441S, the RsrII–BsrGI fragment was reinserted into the parental plasmid.

Quantification of HCV core antigen in the culture medium

The culture media from JFH1-RNA transfected Huh-7.5.1 cells and Huh7-S29 cells were collected on the days indicated, passed through a 0.45 µm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at −80 °C. The levels of core antigen in the culture media were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Western blotting

Western blotting was carried out as described previously (Itsui et al., 2009). Briefly, 10 µg of total cell lysate were separated by SDS-PAGE and blotted onto a polyvinylidene fluoride (PVDF) Western Blotting membrane. The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence using the ECL Western blotting Analysis System (Amersham Bioscience, Buckinghamshire, UK). The antibodies used were anti-core mouse monoclonal antibody (Abcam, Cambridge, MA), anti-GRP78 goat monoclonal antibody, anti-GADD153/CHOP rabbit polyclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and anti-beta-actin antibody (Sigma).

HCV subgenomic replicon constructs

The HCV subgenomic replicon plasmid, pRep-Feo, was derived from the HCV-N strain, pHCV1bneo-delS (Tanabe et al., 2004; Yokota et al., 2003). The replicon RNA was synthesized from pRep-Feo and transfected into Huh7 cells.

Luciferase reporter assay

Luciferase activity was measured using a 1420 Multilabel Counter (ARVO MX, Perkin Elmer, Waltham, MA) with a Bright-Glo Luciferase Assay System (Promega) (Tasaka et al., 2007). Assays were carried out in triplicate and the results expressed as means ± SD.

MTS assays

To evaluate cell viability, dimethylthiazol carboxymethoxy-phenyl sulfophenyl tetrazolium (MTS) assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay kit (Promega), as described previously (Sakamoto et al., 2007).

Real-time RT-PCR analysis

Total cellular RNA was isolated using an RNeasy Mini Kit (QIAGEN, Valencia, CA). Two micro-grams of total cellular RNA were used to generate cDNA from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of mRNA was quantified using TaqMan Universal PCR Master Mix (Applied Biosystems) and the ABI 7500 Real-Time PCR System (Applied Biosystems). The primers used were as follows: HCV-JFH1 sense (positions 285 to 307; 5′-GGTACTGCCTGATAGGGTGCTT-3′), HCV-JFH1 antisense (positions 349 to 375; 5′-TGGTTTTTCTTTGAGGTTTAGGATTC-3′), GAPDH sense (5′-CCTCCCGCTTCGCTCTCT-3′), and GAPDH antisense (5′-GCTGGCGACGCAAAAGA-3′).

HCV RNA inoculation into human hepatocyte chimeric mice

Housing, maintenance, and care of the mice used in this study conformed to the requirement for the humane use of animals in scientific research as defined by Animal Care and Use Committee of our institute. The culture media of Huh-7.5.1 cells transfected with parental JFH1 and JFH1 mutants were collected 10 days after transfection and passed through a 0.45 µm filter. The three mutations introduced in NS5A and NS5B were confirmed to conserve by the sequence analysis of virus genome of cell culture supernatants before inoculation. Filtrated culture medium was then pooled and concentrated using Amicon Ultra-15 (100,000 molecular weight cutoff, Millipore). 100 µl of each culture medium was injected intravenously into human hepatocyte chimeric mice (PXB mice, Phenix Bio, Hiroshima, Japan) (Mercer et al., 2001). The rate of liver chimerism of these human hepatocyte chimeric mice was confirmed more than 70% by immunohistochemical analysis. After infection, blood samples were taken serially and levels for HCV RNA and human albumin were quantified using real-time RT-PCR and an enzyme immunoassay, respectively. RNA was extracted from serum samples and subjected to direct sequence determination.

Protein extraction from human hepatocyte chimeric mice and expression of ER stress-related proteins

5 days post inoculation, mice were sacrificed and proteins were extracted from liver samples with complete Lysis-M Reagent Kit (Roche Applied Science, Indianapolis, IN). One Mini Protease Inhibitor Cocktail Tablet was dissolved into 10 ml of Lysis-M Reagent and 500 µl of this fluid was added to 50 µg of each liver sample and homogenized. The lysate was transferred to a microcentrifuge tube and centrifuged at 14,000 × g for 5 min. The supernatant containing soluble protein was transferred to a new reaction tube and 20 µg of each protein was used for western blotting to detect ER stress-related proteins.

Acknowledgments

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Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor

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A small-animal model for hepatitis C virus (HCV) infection was developed using severe combined immunodeficiency (SCID) mice encoding homozygous urokinase-type plasminogen activator (uPA) transplanted with human hepatocytes. Currently, limited information is available concerning the HCV clearance rate in the SCID mouse model and the virion production rate in engrafted hepatocytes. In this study, several cohorts of $uPA^{+/+}/SCID^{+/+}$ mice with nearly half of their livers repopulated by human hepatocytes were infected with HCV genotype 1b and used to evaluate HCV dynamics by pharmacokinetic and pharmacodynamic analyses of a specific NS3-4A protease inhibitor (telaprevir). A dose-dependent reduction in serum HCV RNA was observed. At telaprevir exposure equivalent to that in clinical studies, rapid turnover of serum HCV was also observed in this mouse model and the estimated slopes of virus decline were 0.11–0.17 $\log_{10}$ h^{-1}. During the initial phase of treatment, the $\log_{10}$ reduction level of HCV RNA was dependent on the drug concentration, which was about fourfold higher in the liver than in plasma. HCV RNA levels in the liver relative to human endogenous gene expression were correlated with serum HCV RNA levels at the end of treatment for up to 10 days. A mathematical model analysis of viral kinetics suggested that 1 g of the chimeric human liver could produce at least 10^8 virions per day, and this may be comparable to HCV production in the human liver.

INTRODUCTION

Hepatitis C virus (HCV) is a major cause for concern worldwide. More than 3 % of the world's population is chronically infected with HCV and 3–4 million people are newly infected each year (Wasley & Alter, 2000). Chronic HCV infection is relatively mild and progresses slowly; however, about 20 % of chronic hepatitis C (CHC) carriers progress to serious end-stage liver disease (Lauer & Walker, 2001; Liang *et al.*, 2000; Poynard *et al.*, 2003). The current standard treatment for HCV infection is administration of pegylated alpha interferon (PEG-IFN) in combination with ribavirin (RBV) for 48 weeks. The overall cure rates with this intervention are 40–50 % for patients with genotype 1 and more than 75 % for patients with genotypes 2 and 3 (Fried *et al.*, 2002; Manns *et al.*, 2001). Several compounds that inhibit specific stages of the virus life cycle have been clinically evaluated (Manns *et al.*, 2007; Pereira & Jacobson, 2009). Telaprevir is a novel peptidomimetic slow- and tight-binding inhibitor of HCV NS3-4A protease, which was discovered using a structure-based drug design approach (Perni *et al.*, 2006). A rapid decline in viral RNA was observed in CHC patients treated with telaprevir (Reesink *et al.*, 2006) and an increased antiviral effect of a combination of telaprevir and PEG-IFN has been reported (Forestier *et al.*, 2007). Recent clinical trials of telaprevir in combination with PEG-IFN and RBV have indicated a promising material advance in therapy for CHC patients (Hézode *et al.*, 2009; McHutchison *et al.*, 2009). First-generation HCV-specific agents have been developed despite the lack of small-animal models for HCV infection. However, early emergence of resistant variants against novel antiviral agents is a concern. Thus, the use of two or more investigation agents is strongly recommended for clinical studies in CHC patients (Sherman *et al.*, 2007). To ensure ethical and safe clinical trials, animal models continue to be necessary for the mechanistic evaluation of the ability of specific agents to inhibit the virus life cycle *in vivo* and to develop better therapeutic strategies, including combination regimens (Boonstra *et al.*, 2009). Several groups have developed a small-animal model for HCV infection using homozygous urokinase-type plasminogen activator (uPA)/severe combined immunodeficiency (SCID) ($uPA^{+/+}/SCID^{+/+}$) mice transplanted with human hepatocytes (Mercer *et al.*, 2001). These mice are susceptible to cell culture-grown HCV (HCVcc; Lindenbach *et al.*, 2006) and have been used to evaluate antiviral agents including IFN-α, BILN 2061 (an NS3-4A protease inhibitor) and HCV796 (an NS5B polymerase inhibitor) (Kneteman *et al.*, 2006, 2009; Vanwolleghem *et al.*, 2007). However, the HCV clearance rate in the SCID mouse model and the virion production rate in hepatocytes engrafted in the mouse liver are not fully understood. We also generated a mouse model with an almost humanized liver (Tateno *et al.*, 2004). Using this mouse model, we reported the infection of a genetically engineered hepatitis B virus (Tsuge *et al.*, 2005) and developed a reverse genetics system for HCV genotypes 1a, 1b and 2a after intrahepatic injection of *in vitro*-transcribed RNA as well as intravenous injection of HCVcc (Hiraga *et al.*, 2007; Kimura *et al.*, 2008). In this study, we demonstrated the rapid turnover of serum HCV RNA and the pharmacokinetics (PK) and pharmacodynamics (PD) of telaprevir treatment. We concluded after quantitative estimation and the use of a mathematical model that HCV production equivalent to that in the human liver is possible in engrafted hepatocytes in this mouse model.

RESULTS

Preliminary dose-finding study

At the beginning of this study, we attempted to determine an effective dose regimen for telaprevir in this mouse model. Nine mice were randomized and treated with telaprevir over three time periods (Table 1). The lifetime kinetics of serum HCV RNA and of human serum albumin (HSA) in blood are represented in Fig. 1. One mouse (A07) exhibited a rapid reduction in HSA in the blood, which indicated the instability of human hepatocyte grafts. As a rapid reduction in HSA levels was not observed in subsequent experiments, this mouse was excluded from the mean analysis. After 7 days of twice daily (BID) dosing in period 1, the mean $\log_{10}$ changes in HCV RNA from baseline (±SEM) after the 100 and 10 mg telaprevir kg^{-1} doses were -0.49 ± 0.094 and -0.53 ± 0.039, respectively, and no dose-dependent reduction was observed. During period 2, the dose frequency was changed from BID to three times daily (TID), and the time of serum sampling was also changed from 1 to 4 h after the last dose. After the 3-day treatment, the mean $\log_{10}$ changes of HCV RNA in 100 and 10 mg telaprevir kg^{-1} TID groups were -1.00 ± 0.166 and -0.28 ± 0.056, respectively, and the difference between the two groups was significant. To test the reproducibility of results, mice were treated with 10 or 100 mg telaprevir kg^{-1} TID for 10 days and then sacrificed 5 h after the administration of the last dose. The mean $\log_{10}$ changes in serum HCV RNA were -1.46 ± 0.265 and -0.27 ± 0.073 in the 100 and 10 mg kg^{-1} TID groups, respectively, and the difference between the means was significant.

Evaluation of HCV turnover in this mouse model

Because of the SCID nature of this mouse model, the virion clearance mechanism was of interest. Six mice with steady-state and high viral loads (9.7×10^5–1.2×10^8 copies ml^{-1}) were administered 200 mg telaprevir kg^{-1} TID for 4 days, with 5 h intervals between doses and a 14 h intermission from drug treatment each day. Because the $\log_{10}$ reduction in HCV RNA appeared to depend on the time of serum collection during the day (Fig. 2a), the mean $\log_{10}$ changes in HCV RNA were plotted against time and fitted to a linear regression model (Fig. 2b). The estimated slopes (i.e. $\log_{10}$ HCV reduction per hour) and 95 % confidence intervals (CI) on days 1, 2 and 3 were −0.165 (−0.268 to 0.0616), −0.115 (−0.131 to 0.0990) and −0.153, respectively. These regression lines also suggested that extrapolated HCV loads at the actual times of the daily first doses were 0.0530, −0.220 and −0.0948 $\log_{10}$ copies ml^{-1}, respectively. Therefore, it appeared that the viral load

Table 1. Telaprevir dose-finding experiment

Period	Duration (days)	Frequency of dose (per day)	Dose (mg kg^{-1})	No. of mice	Mean $\log_{10}$ changes ± SEM	*P* value (*t* test)
1	7	2	100	4	-0.49 ± 0.094	0.7806
			10	3*	-0.53 ± 0.039	
			0	1	−0.47	
2	3	3	100	4*	-1.00 ± 0.166	0.0064
			10	4	-0.28 ± 0.056	
3	10	3	100	3	-1.46 ± 0.265	0.0125
			10	3	-0.27 ± 0.073	

*One mouse was excluded because of instability of human hepatocyte grafts.

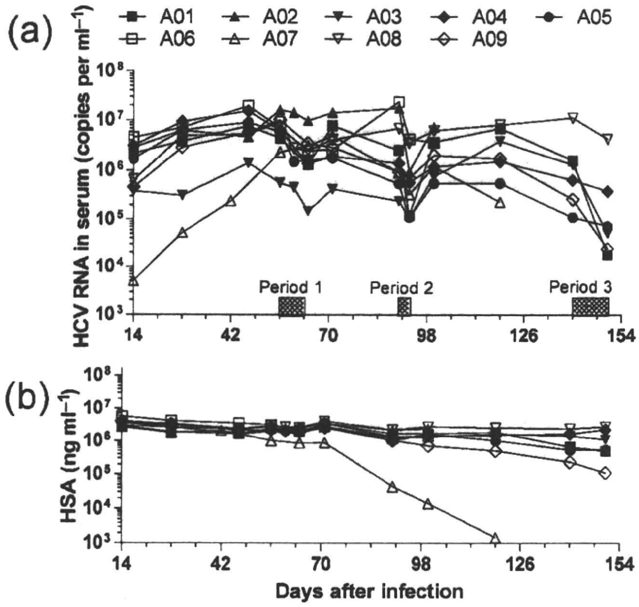

Fig. 1. Lifelong changes in serum HCV RNA and HSA in the blood of HCV-infected mice in the preliminary dose-finding experiment. Nine HCV-infected mice (A01–A09) were treated with telaprevir over three independent periods. The mice were treated with 10 mg telaprevir kg^{-1}, 100 mg telaprevir kg^{-1} or vehicle BID for 7 days (period 1), TID for 3 days (period 2) and TID for 10 days (period 3). (a) Kinetics of serum HCV RNA. (b) Kinetics of HSA level in blood. Because the HSA level indicated the stability of engrafted human hepatocytes in the mice, mouse A07 was excluded from the summary of the results in Table 1.

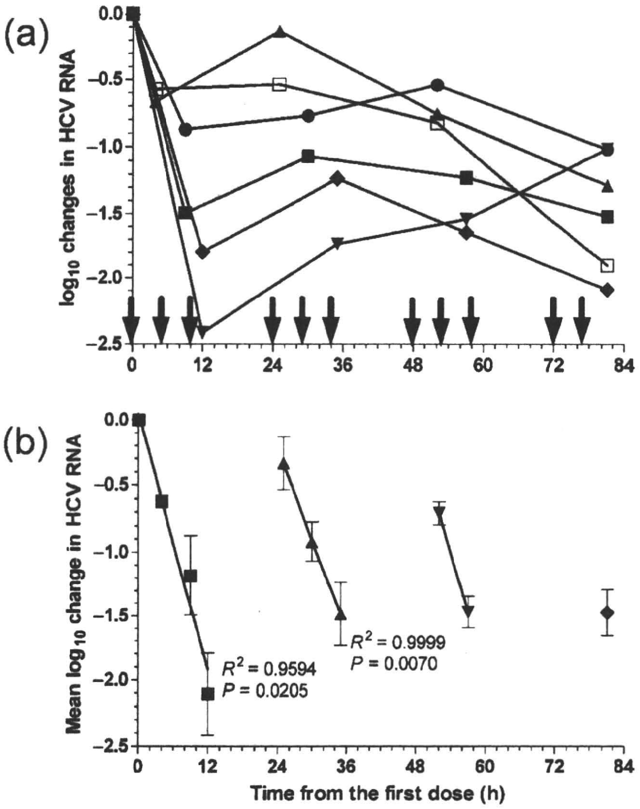

Fig. 2. Estimation of virus clearance rate. Six HCV-infected mice were treated with 200 mg telaprevir kg^{-1} TID for 4 days. Individual kinetics of log$_{10}$ reductions in serum HCV RNA (a) and of mean log$_{10}$ changes (±SEM) at each sampling time (b) are represented. Arrows indicate the times of dosing. The slopes of mean log$_{10}$ HCV RNA reduction were estimated by linear regression analysis. P and R^2 values are indicated on the figure.

reverted back towards baseline levels during the 14 h intermission from drug treatment.

PK analysis

To assess drug exposure after repeated dosing in this mouse model, mice were administered 100 or 300 mg telaprevir kg^{-1} BID for 4 days. The mice receiving 300 mg kg^{-1} BID for 4 days had a mean 2 log$_{10}$-fold HCV reduction, whereas those receiving 100 mg kg^{-1} BID had up to a 1.5 log$_{10}$-fold reduction by day 3 (Fig. 3a). Plasma telaprevir concentrations after administration of the final dose are indicated in Fig. 3(b). The estimated half-life of telaprevir in the 100 and 300 mg kg^{-1} groups was 2.4 and 3.8 h, respectively.

PK/PD analysis and the dose-dependent reduction in HCV RNA

To evaluate the correlation between telaprevir concentration and HCV reductions in this mouse model, we used another cohort of 12 HCV-infected mice with high viral loads (1.6×10^6–3.9×10^8 copies ml^{-1}). In this crossover study, mice were randomized into three groups (n=4 each), each of which underwent two periods of dosing for 5 days separated by a 1-week washout period. Serum and plasma samples were collected once daily 5 h after dosing. The mean log$_{10}$ changes in HCV RNA (±SEM) at different dose levels were calculated from the combined results of both periods (Fig. 4a). The mean log$_{10}$ reductions from baseline in the 100 and 300 mg kg^{-1} groups were approximately 1 log$_{10}$ and 1.5–2 log$_{10}$, respectively, and the difference between the two groups was statistically significant. The means calculated in each period separately are also shown in Fig. 4(b). The plasma telaprevir concentration was positively correlated with the log$_{10}$ HCV RNA reduction level in each period (Fig. 4c).

Drug concentrations and HCV levels in blood correlate with those in the liver

The correlation between telaprevir concentrations in the plasma and liver was analysed in a double logarithmic plot 5 (dose-finding cohort) or 8 h (PK and PK/PD cohorts) after the last dose (Fig. 5). The linear regression lines suggested that telaprevir concentrations in the liver were 5–

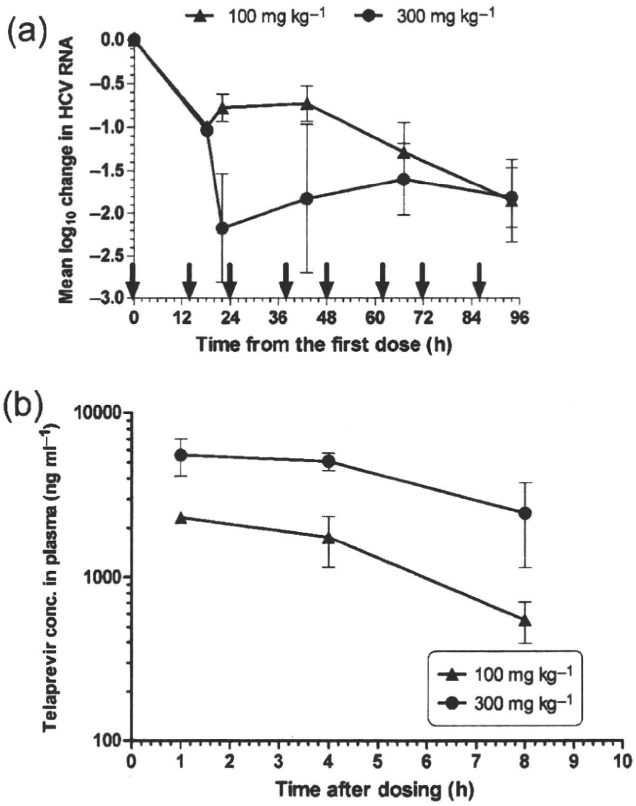

Fig. 3. PK analysis of telaprevir in the HCV-infected mouse model. Six HCV-infected mice were administered 100 ($n=3$) or 300 ($n=3$) mg telaprevir kg^{-1} BID for 4 days and serum samples were collected once daily to assess antiviral activity. After the last dose, plasma samples were collected at 1, 4 and 8 h for PK analysis. (a) Mean $\log_{10}$ changes (±SEM) in serum HCV RNA from mice treated with telaprevir. Arrows indicate the times of dosing. (b) Kinetics of telaprevir concentrations in plasma after the last dose.

10-fold higher at 5 h and approximately fourfold higher at 8 h than those in plasma. Total cellular RNA samples were extracted from two, one and four discrete small sections (approx. 50 mg) of the liver in the preliminary dose-finding, PK and PK/PD cohorts, respectively. HCV RNA levels in the total cellular RNA extract were relatively quantified by duplex real-time RT-PCR analysis using human β_2-microglobulin (hβ_2m) as an internal standard of human endogenous gene expression. Neither the threshold cycle (*Ct*) of hβ_2m ($Ct_{h\beta 2m}$) nor the *Ct* of HCV (Ct_{HCV}) correlated with total RNA from a small section of the chimeric human livers (data not shown). This result indicated that occupancy rates of human cells varied individually and/or among small sections of the chimeric human liver. Therefore, the mean difference in *Ct* ($\Delta Ct = Ct_{HCV} - Ct_{h\beta 2m}$) in each mouse was calculated and plotted against the viral load in serum (Fig. 6). After treatment with telaprevir for up to 10 days, mean ΔCt values ranged between 11 (HCV RNA content: $2^{11} = 2 \times 10^3$-fold lower than hβ_2m expression) and 17 (1×10^5-fold lower) among the HCV-infected mice and correlated linearly with $\log_{10}$ serum HCV RNA levels.

Viral dynamics model analysis

To evaluate time-dependent reductions in HCV with BID dosing, 12 HCV-infected elderly mice, which maintained high and steady-state viral loads (1.2×10^6–8.5×10^7 copies ml^{-1}) for more than 6 months, were treated with 200 mg telaprevir kg^{-1} BID for 3 days. The mice were divided into two groups, and serum samples were collected just before the second dose and 4 ($n=6$) or 8 ($n=6$) h after every two administrations. The single administration of telaprevir resulted in a mean 0.8–1.0 $\log_{10}$-fold reduction in HCV RNA in both groups. After the second dose, the pattern of viral kinetics appeared to depend on the time of serum collection, and the mean HCV RNA reduction level was higher in the 8 h group than in the 4 h group and plateaued at approximately a 2 $\log_{10}$-fold reduction in both groups after treatment for 3 days (Fig. 7). Finally, we attempted to estimate parameters of efficacy (ε) and virus clearance (c) per hour in this mouse model for comparison with estimates derived from human studies. Because the mean viral kinetics of the 8 h group was biphasic, the values in the 8 h group were used together for the mathematical model analysis. The estimated ε and c values were 0.992 (95 % CI 0.982–1.00) and 0.200 (95 % CI 0.110–0.291), respectively.

DISCUSSION

Using a mouse model with a chimeric human liver for HCV infection, we analysed the PK/PD of telaprevir treatment and investigated HCV dynamics during the initial phase of protease inhibitor treatment. All the mice in this study were expected to have more than half of their livers repopulated by human hepatocytes (Tateno *et al.*, 2004), which simulates a human drug metabolism profile (Katoh *et al.*, 2007, 2008). After the infection with HCV genotype 1b, high viral loads were maintained in the mice for more than 6 months. Recent studies have indicated the utility of a human/mouse chimera model for HCV infection to evaluate antiviral efficacy (Kneteman *et al.*, 2006, 2009) and preclinical safety (Vanwolleghem *et al.*, 2007). However, PK/PD studies and estimations of virus clearance rate have rarely been performed in this mouse model. HCV production, including intracellular replication in engrafted hepatocytes, has also not yet been elucidated. Despite the SCID nature of this mouse model, a 2 $\log_{10}$-fold HCV RNA reduction was observed within 0.5 days, as has been observed previously in CHC patients (Forestier *et al.*, 2007; Reesink *et al.*, 2006). In this mouse model, the rapid rebound in HCV load during the intermission from drug exposure indicated the rapid production and release of HCV into the circulation. This finding indicates that a virion-clearing compartment, which does not depend on T- and B-cell responses, may exist in this mouse model.

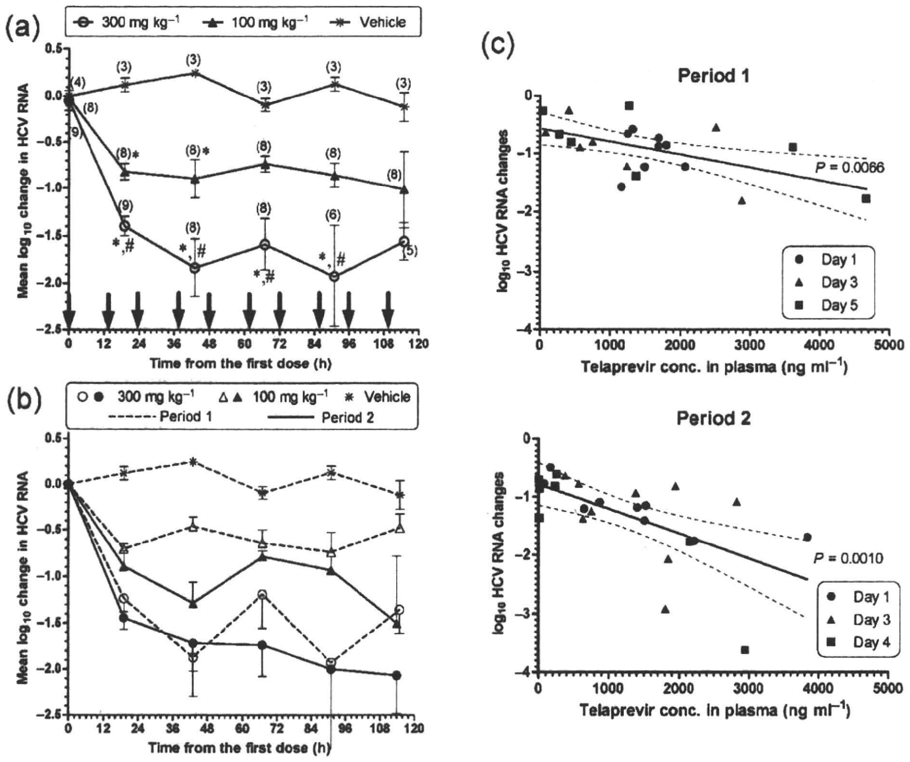

Fig. 4. PK/PD analysis and the dose-dependent reduction in HCV. Twelve HCV-infected mice were randomized into three groups (n=4 each) and then underwent two periods of telaprevir BID dosing for 5 days, separated by a 1-week washout period. Before the second period, the mice in the vehicle control group were additionally assigned to active drug groups. During the second period, mice that received the high or low doses were crossed over to the alternative treatment. Serum and plasma samples were collected once daily 5 h after dosing. Mean log$_{10}$ changes (±SEM) in serum HCV RNA were calculated from the combined results from both periods (a) and each period separately (b). Arrows indicate the times of dosing. *, $P<0.05$ versus vehicle control group; #, $P<0.05$ versus 100 mg kg^{-1} group. (c) Correlation between log$_{10}$ reduction in serum HCV and telaprevir concentrations in plasma. Linear regressions (solid lines) and 95 % CI (dashed lines) are indicated.

One possible explanation is that viral kinetics after liver transplantation in humans may play a role in HCV clearance under immunosuppressed conditions (Dahari *et al.*, 2005; Powers *et al.*, 2006; Schiano *et al.*, 2005). This observation suggests that this mouse model is capable of evaluating 'first-phase' HCV clearance after drug treatment.

In a clinical trial of telaprevir, CHC patients who exhibited a continuous decline in viral kinetics had mean plasma trough levels above 1000 ng ml^{-1}; therefore, a dose of 750 mg TID was selected for further clinical studies (Sarrazin *et al.*, 2007). When HCV-infected mice were administered 100 or 300 mg telaprevir kg^{-1}, a plasma concentration above 1000 ng ml^{-1} was maintained beyond 8 h in mice treated with 300 mg kg^{-1} but not in those treated with 100 mg kg^{-1}. This result suggests that the extrapolation of telaprevir doses from this mouse model to human studies depends on body surface area, i.e. approximately 15th of a dose in this mouse model may be equivalent to a dose in humans. In another cohort of mice treated with 100 and 300 mg telaprevir kg^{-1} BID, a dose-dependent reduction in HCV was observed and the plasma telaprevir concentration correlated significantly with the HCV reduction level. Therefore, the PK/PD results in this mouse model may be able to indicate a targeted dose range in clinical studies.

Whereas a telaprevir concentration in plasma equivalent to its dosage in clinical trials was achieved in this mouse model, the serum HCV RNA level plateaued at a decrease of approximately 2 log$_{10}$-fold within several days of treatment. A saturated reduction of approximately 2 log$_{10}$-fold after treatments with BILN 2061 and IFN was also reported in an analogous mouse model (Kneteman *et al.*, 2006; Vanwolleghem *et al.*, 2007). These observations led us to examine HCV replication in the chimeric human liver. In the relative quantification of HCV RNA against human-specific endogenous gene expression, we observed a correlation between the serum HCV RNA level and the mean ΔCt value in the liver, despite no correlation between the total RNA concentration and each Ct value of two target genes in the liver RNA extracts. This result can be interpreted to indicate that HCV replicated only in

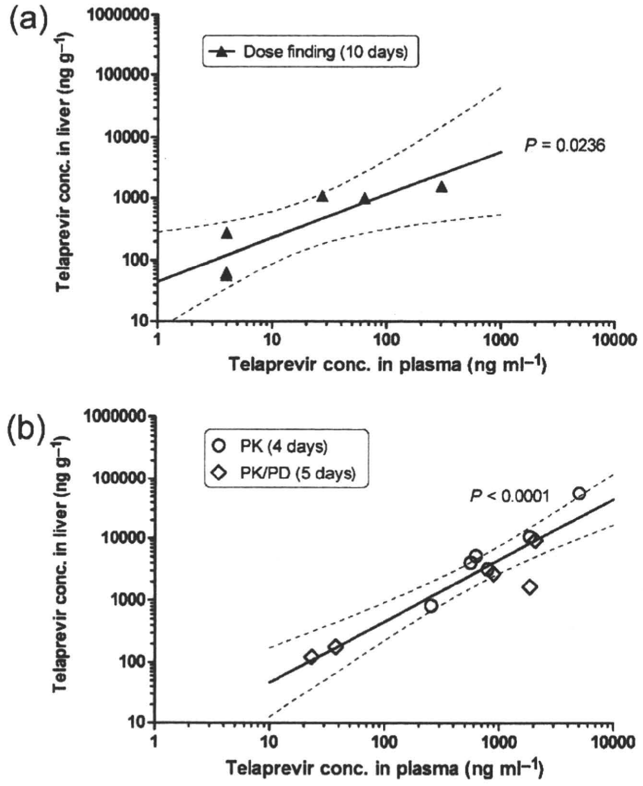

Fig. 5. Correlation between telaprevir concentrations in the liver and plasma. Telaprevir concentrations in the liver and plasma were determined at the end of the three different experiments indicated in Fig. 1 (dose-finding), Fig. 3 (PK) and Fig. 4 (PK/PD). Telaprevir concentrations in the liver were plotted against those in plasma 5 (a) or 8 (b) h after the last dose. Linear regressions (solid lines) and 95 % CI (dashed lines) are indicated.

engrafted human hepatocytes, and the observed HCV reduction in serum might reflect virus replication in the human hepatocyte grafts. Moreover, the relative content of HCV RNA was 2×10^3–1×10^5-fold lower than hβ_2m expression, whereas an HCV replicon cell line, which had approximately 1000 replicon genomes per cell (Quinkert *et al.*, 2005), contained nearly equal amounts of both genes (data not shown). HCV replication was much lower in the engrafted human hepatocytes than in an HCV replicon cell line, and HCV infected only a small portion of the engrafted human hepatocytes. It has been reported that 4–25 % of hepatocytes in a CHC patient were positive for replicative-intermediate RNA, and the mean number of viral genomes per productively infected hepatocyte ranged from 7 to 64 molecules (Chang *et al.*, 2003). Also, a more recent report suggested that the percentage of HCV antigen-positive hepatocytes in patients varied from 0 to 40 %, and the HCV content in 2000 microdissected HCV-positive cells ranged from 40 to 1800 international units using a branched DNA assay (Vona *et al.*, 2004). Therefore, we suggest that HCV replication efficiency in engrafted human hepatocytes is equivalent to that in CHC patients.

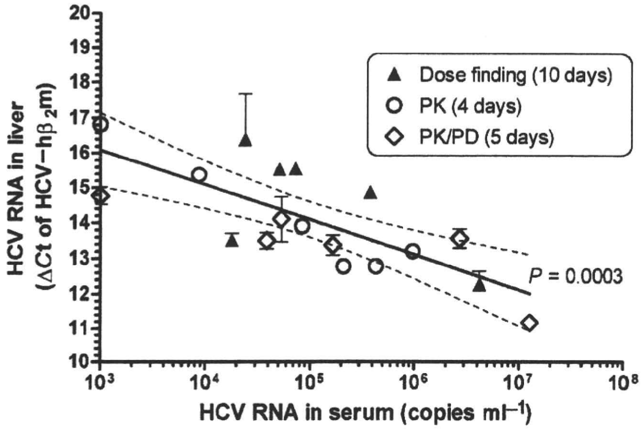

Fig. 6. Correlation between HCV content in the liver and serum. Relative quantification of HCV RNA levels in the liver was determined by the difference between threshold cycles (ΔCt) of HCV RNA and hβ_2m in a duplex real-time RT-PCR analysis. Linear regressions (solid line) and 95 % CI (dashed lines) are indicated.

The differences observed between the engrafted human hepatocytes and the HCV replicon cell line can be explained by the following assumptions: approximately 10 % of engrafted human hepatocytes are productively

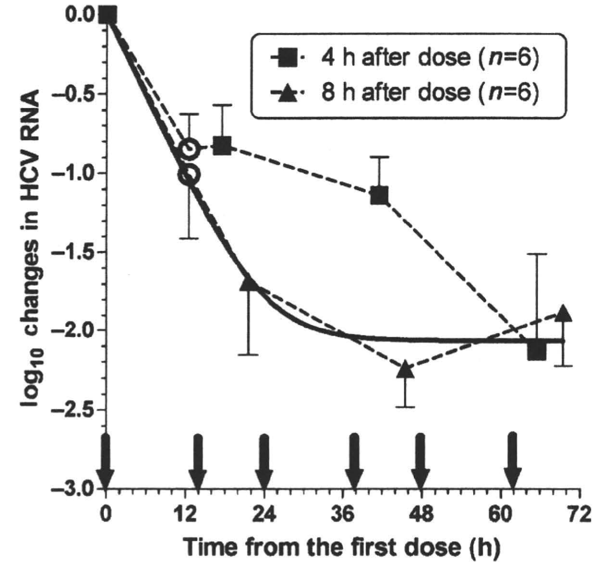

Fig. 7. Viral dynamics under BID telaprevir treatment. Mice were administered 200 mg telaprevir kg^{-1} BID at the times indicated by arrows. Serum samples were collected just before the second dose was administered and 4 (n=6) or 8 (n=6) h after every two doses were administered. Mean log$_{10}$ changes (±SEM) in serum HCV RNA are plotted. The solved equation described in Methods was fitted to the values in the 8 h group (solid line), and the estimated efficacy and virion clearance rates were 0.992 (95 % CI 0.982–1.00) and 0.200 (95 % CI 0.110–0.291), respectively.

infected and harbour approximately ten HCV genomes per cell at baseline steady state and a 2 $\log_{10}$-fold reduction is achieved with drug treatment.

Mathematical models have proven valuable in understanding the *in vivo* dynamics of HCV, and very rapid dynamic processes occur on timescales of hours to days, and slower processes occur on timescales of weeks to months (Perelson & Ribeiro, 2008). In the last experiment, we observed a biphasic decline in the HCV RNA level after BID dosing for 3 days. During the first 2 days of the treatment, a discrepancy in viral kinetics between the serum-sampling time points was noted. Similarly, fluctuations in viral kinetics during the first-phase slope were observed in patients who received IFN three times a week (Pawlotsky *et al.*, 2004). Variable efficacy rate determined by PK parameters can explain fluctuations during the first-phase slope in mathematical model analysis (Talal *et al.*, 2006). However, it is difficult to evaluate the individual temporal changes in viral and drug kinetics using a mouse model as only a limited volume of blood is available for analysis. Therefore, we assumed a constant efficacy rate (ε) and omitted a turnover rate of hepatocytes because of the short duration of treatment. The estimated clearance rate (c) in this study was 4.8 day^{-1}. Additionally, the mean slope of 0.144 $\log_{10}\ \text{h}^{-1}$ (Fig. 2b) could be transformed to $0.332\ \text{h}^{-1}=8.0\ \text{day}^{-1}$ according to the change of base of a logarithm. The estimated clearance rates in this mouse model basically agreed with estimates determined in humans infected with HCV genotype 1 and undergoing IFN-based therapies (Herrmann *et al.*, 2003; Neumann *et al.*, 1998; Pawlotsky *et al.*, 2004) or large-volume plasma apheresis (Ramratnam *et al.*, 1999). Total virion production during steady-state viral kinetics in this mouse model was calculated by multiplying c by the initial viral load (V_0) and then normalizing the extracellular fluid volume. From previous studies, it was determined that 10^{11}–10^{13} virions are produced daily in patients with high HCV loads (Neumann *et al.*, 1998; Ramratnam *et al.*, 1999). In this mouse model, the volume of extracellular fluid and weight of the liver were approximately 20 and 9 % of the body weight (data not shown), and the mean $\log_{10} V_0$ ($\pm$SEM) among the mice with mean clearance rates of 4.8 and 8.0 per day were 6.96 ± 0.26 and 7.00 ± 0.33, respectively. The results of the calculations indicated that 1 g of the chimeric human liver produced 1×10^8–2×10^8 virions per day. The typical weight of the human liver is 1–2 kg; thus, the capacity of human hepatocytes to produce HCV in this mouse model may be equivalent to that in CHC patients. In conclusion, a mouse model with a chimeric human liver can simulate HCV replication in human patients quantitatively and dynamically, and this mouse model may be suitable for preclinical evaluations of novel HCV-specific agents and other therapeutic strategies, including combination regimens.

METHODS

Generation of mice with chimeric human livers and HCV infection. The generation of $\text{uPA}^{+/+}/\text{SCID}^{+/+}$ mice and transplantation of frozen human hepatocytes was performed at PhoenixBio. Graft function was monitored on the basis of HSA levels in blood (Tsuge *et al.*, 2005). All the mice had high HSA levels, which suggested that nearly half of their livers were repopulated by human hepatocytes (Tateno *et al.*, 2004). After obtaining written informed consent, we collected sera periodically from patients who were chronically infected with HCV genotype 1b and failed to respond to PEG-IFN and RBV therapy. The mice were inoculated with the serum samples via the orbital vein after anaesthetization. The experimental protocol was approved by the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University.

Compound preparation and experimental designs. The telaprevir formulations were kindly provided by Vertex Pharmaceuticals. A telaprevir suspension was prepared as described previously (Perni *et al.*, 2006) and used in experiments 1 and 2. In the other experiments, a telaprevir suspension was prepared daily as in the tablet formulation (Forestier *et al.*, 2007; Hézode *et al.*, 2009; McHutchison *et al.*, 2009). A suspension of telaprevir was administered via oral gavage.

Experiment 1: preliminary dose-finding study. Ten out of 11 mice developed serum HCV loads greater than 10^4 copies ml^{-1}. Nine mice with high viral loads ($>10^5$ copies ml^{-1}) were randomized and administered 10 or 100 mg telaprevir kg^{-1} BID or TID over three periods. During period 1, the mice were administered 100 ($n=4$) or 10 ($n=4$) mg telaprevir kg^{-1} or vehicle ($n=1$) BID at 18 : 00 and 10 : 00 h for 7 days, and serum samples were collected before treatment and 1 h after administration in the morning on the third and/or seventh day. During period 2, the mice were administered 100 ($n=5$) or 10 ($n=4$) mg telaprevir kg^{-1} TID for 3 days, and serum samples were collected before treatment and 4 h after administration of the last dose. Three mice died between periods 2 and 3. During period 3, the mice were administered 100 ($n=3$) or 10 ($n=3$) mg telaprevir kg^{-1} TID for 10 days. The mice were sacrificed 5 h after administration of the last dose, and plasma, serum and liver samples were collected.

Experiment 2: evaluation of HCV turnover. Eleven mice were infected with HCV and eight mice survived for more than 15 weeks with steady-state and high viral loads (10^6–10^8 copies ml^{-1}). Six of the mice were administered 200 mg telaprevir kg^{-1} TID at 9 : 00, 14 : 00 and 19 : 00 h for 4 days. On day 1, serum samples were collected before dose administration, 4 h after the first and second doses were administered, and 2 h after the third dose was administered ($n=2$ each). On day 2, serum samples were collected 1 h after each of the three doses was administered ($n=2$ each). Serum samples were also collected 4 h after the first and second doses were administered on day 3 ($n=3$ each) and 4 h after the second dose was administered on day 4.

Experiment 3: PK analysis. After a washout period, six mice from experiment 2 were administered 100 or 300 mg telaprevir kg^{-1} ($n=3$ each) BID at 19 : 00 and 9 : 00 h for 4 days. Serum samples were collected before dose administration, 4 ($n=1$) or 8 ($n=2$) h after administration of the second dose, and 5 h after every two doses were administered. After the final dose was administered, plasma for PK analysis was collected at 1 and 4 h. The mice were sacrificed at 8 h, and serum, plasma and liver samples were collected.

Experiment 4: dose dependence and PK/PD analysis. Thirty-six mice were infected with HCV and 13 survived for more than 13 weeks. The median survival time of this cohort was 81 days after infection. Twelve HCV-infected mice were randomized into three groups (A–C; $n=4$ each) and underwent two periods of BID dosing for 5 days, which were separated by 1-week washout periods. During the first period, the mice in groups A, B and C were administered 300 mg telaprevir kg^{-1}, 100 mg telaprevir kg^{-1} and vehicle, respectively. Because two mice in group A and two mice in group C died before the second period, two remaining mice in group C and one back-up mouse were assigned to group A ($n=2$) and group B ($n=1$). During the second period, mice that received high or low doses were crossed over to the alternative treatment. Serum samples were collected before the first dose was administered and 5 h after every two doses were administered. Plasma samples were also collected at the same time on days 1, 3 and 5 in the first period and days 1, 3 and 4 in the second period. The mice were sacrificed 8 h after administration of the final dose, and serum, plasma and liver samples were collected.

Experiment 5: viral kinetics with BID dosing After infection of 45 mice, 12 HCV-infected mice maintained steady-state and high viral loads (1.2×10^6–8.5×10^7 copies ml^{-1}) for more than 6 months. The median survival time of this cohort was 131 days after infection. These mice were treated with 200 mg telaprevir kg^{-1} BID at 19:00 and 9:00 h for 3 days. The mice were divided into two groups and serum samples were collected just before the second dose was administered and 4 ($n=6$) or 8 ($n=6$) h after every two doses were administered.

Serum RNA extraction and HCV RNA quantification. HCV RNA was isolated from 10 µl serum under denaturing conditions using a SepaGene RV-R kit (Sanko Junyaku). The dried precipitates were dissolved in 10 µl diethylpyrocarbonate-treated water. Extracts were duplicated and assayed by quantitative real-time RT-PCR using TaqMan EZ RT-PCR core reagents (Applied Biosystems). Nucleotide positions of the probe and primer sets refer to HCV H77 strain (GenBank accession no. AF009606). The TaqMan probe 5′-6-FAM-CTGCGGAACCGGTGAGTACAC-BHQ-1-3′ (nt 148–168) was purchased from Biosearch Technologies, and the forward (5′-CGGGAGAGCCATAGTGG-3′; nt 130–146) and reverse (5′-AGTACCACAAGGCCTTTCG-3′; nt 272–290) primers were purchased from Sigma-Aldrich. The 25 µl RT-PCR mixture contained 0.2 nmol forward and reverse primers ml^{-1}, 0.3 nmol TaqMan probe ml^{-1} and 5 µl extracted RNA, and was monitored using a PRISM 7900HT sequence detection system (Applied Biosystems). The thermal profile was 2 min at 50 °C, 30 min at 60 °C for reverse transcription and 5 min at 95 °C, followed by 45 cycles of 20 s at 95 °C and 1 min at 62 °C. The HCV replicon I_{389}neo/NS3-3′/5.1 (Lohmann *et al.*, 1999) RNA was transcribed *in vitro* using a T7 RiboMax Express Large Scale RNA Production System (Promega) and purified twice using gel filtration. The concentration of this transcribed RNA was determined by absorbance at 260 nm and serially diluted 10-fold to prepare a standard curve for each assay.

Liver RNA extraction and HCV RNA quantification. A Wizard SV total RNA Isolation System (Promega) was used to obtain a DNase I-treated total RNA sample. The total RNA concentration was determined by absorbance at 260 nm. Total RNA samples were assayed by duplex real-time RT-PCR for relative quantification of HCV RNA using endogenous control gene expression of human β_2-microglobulin (hβ_2m; GenBank accession no. NM_004048), the TaqMan probe 5′-CAL Fluor Orange 560-AGTGGGATCG-AGACATGTAAGCAGCATCAT-BHQ-1-3′ (nt 401–430), and the forward and reverse primer set of 5′-TTGTCACAGCCCAA-GATAGTT-3′ (nt 379–399) and 5′-TGCGGCATCTTCAAACC-3′ (nt 434–450). To adjust the efficacy of PCR amplification of both target genes, the reaction condition was modified from the HCV single-probe assay. The temperature for extension was 60 °C, the concentration of the HCV probe was 0.24 nmol ml^{-1} and the reaction mixture contained the TaqMan probe/primer set for hβ_2m: 0.2 nmol primers ml^{-1} and 0.12 nmol TaqMan probe ml^{-1}. Because both target genes double after one cycle of PCR, a difference in Ct between HCV and hβ2m ($\Delta Ct = Ct_{HCV} - Ct_{h\beta 2m}$) theoretically indicates a relative quantity of HCV RNA per control gene expression of $2^{-\Delta\Delta Ct}$.

Determination of drug concentration. Plasma and liver samples were analysed using chiral liquid chromatography followed by tandem mass spectrometry. After reconstitution, sample extracts were separated by normal-phase chromatography on a 2×250 mm Hypersil CPS-1 column (Thermo Hypersil-Keystone) with a mobile phase of heptane : acetone : methanol (82 : 17 : 1). Analyte concentrations were determined by turbo ion spray liquid chromatography/tandem mass spectrometry in the positive-ion mode. Analysis was performed at SRL or Mitsubishi Chemical Medience.

Statistical analysis. The HCV RNA level in serum was normalized by logarithmic conversion. Statistical analysis was performed with a mixed linear model using SAS (SAS Institute). Mean differences between two groups were evaluated with Student's *t*-test. The difference compared with vehicle control at each time point was evaluated by Dunnett's multiple comparisons test. Linear and non-linear regression analyses were performed using GraphPad Prism 5 (GraphPad Software).

Viral dynamics model analysis. The basic mathematical model for the analysis of HCV infection *in vivo*, which is a system of three ordinary differential equations for uninfected cells (T), productively infected cells (I) and free virus (V), has been reviewed elsewhere (Perelson & Ribeiro, 2008). Briefly, one of the three equations ($\mathrm{d}V/\mathrm{d}t = pI - cV$), where viral particles are produced at rate p per infected cell and cleared at rate c per virion, was solved. During treatment for 2–3 days, if one assumes that the number of I is approximately constant and equal to its pre-treatment value and that the viral level was at its set-point value (V_0), then $pI = cV_0$. Using this relationship in the equation $\mathrm{d}V/\mathrm{d}t = (1-\varepsilon)pI - cV$, where ε is the effectiveness in blocking virion production, yields $\mathrm{d}V/\mathrm{d}t = (1-\varepsilon)\ cV_0 - cV$, $V(0) = V_0$ with the solution $V(t) = V_0(1-\varepsilon+\varepsilon e^{-ct})$. Because the log change of viral load at time [log $\Delta V(t)$] equals log $V(t)/V_0$, the solved equation [$\log \Delta V(t) = \log(1-\varepsilon+\varepsilon e^{-ct})$] was fitted to the values obtained in this study via non-linear least-squares regression in order to estimate ε and c.

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