

hepatocytes, by which means HBV could evade innate immune detection and response.

Supplementary Data

Supplementary data are available at *The Journal of Infectious Diseases* online.

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In Vivo Adaptation of Hepatitis C Virus in Chimpanzees for Efficient Virus Production and Evasion of Apoptosis

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Hepatitis C virus (HCV) employs various strategies to establish persistent infection that can cause chronic liver disease. Our previous study showed that both the original patient serum from which the HCV JFH-1 strain was isolated and the cell culture-generated JFH-1 virus (JFH-1cc) established infection in chimpanzees, and that infected JFH-1 strains accumulated mutations after passage through chimpanzees. The aim of this study was to compare the *in vitro* characteristics of JFH-1 strains emerged in each chimpanzee at early and late stages of infection, as it could provide an insight into the phenomenon of viral persistence. We generated full-genome JFH-1 constructs with the mutations detected in patient serum-infected (JFH-1/S1 and S2) and JFH-1cc-infected (JFH-1/C) chimpanzees, and assessed their effect on replication, infectious virus production, and regulation of apoptosis in cell culture. The extracellular HCV core antigen secreted from JFH-1/S1-, S2-, and C-transfected HuH-7 cells was 2.5, 8.9, and 2.1 times higher than that from JFH-1 wild-type (JFH-1/wt) transfected cells, respectively. Single cycle virus production assay with a CD81-negative cell line revealed that the strain JFH-1/S2, isolated from the patient serum-infected chimpanzee at a later time point of infection, showed lower replication and higher capacity to assemble infectious virus particles. This strain also showed productive infection in human hepatocyte-transplanted mice. Furthermore, the cells harboring this strain displayed lower susceptibility to the apoptosis induced by tumor necrosis factor α or Fas ligand compared with the cells replicating JFH-1/wt. **Conclusion:** The ability of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis may be important for prolonged infection *in vivo*. Such control of viral functions by specific mutations may be a key strategy for establishing persistent infection. (HEPATOLOGY 2011;54:425-433)

Currently, approximately 200 million people are infected with hepatitis C virus (HCV) and are at continuous risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.^{1,2} Although acute HCV infection elicits innate and adaptive immune responses, the virus successfully evades clearance in approximately 75% of infected individuals.^{3,4} The mechanisms by

Abbreviations: Ag, antigen; CTL, cytotoxic T lymphocytes; FasL, Fas ligand; HCV, hepatitis C virus; JFH-1cc, cell culture-generated JFH-1 virus; JFH-1/wt, JFH-1 wild-type; MFI, mean fluorescence intensity; NK, natural killer; NS, nonstructural; PARI, poly(adenosine diphosphate ribose) polymerase; TNF- α , tumor necrosis factor α ; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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which HCV leads to persistent infection at a high frequency are not yet fully understood. Lack of appropriate animal models, except chimpanzees, has rendered such studies difficult. Human hepatocyte-transplanted mice,^{5,6} a useful small animal model to study HCV infection, are unsuitable to study the mechanisms of virus persistence because of a lack of B and T cell-mediated immunity.

HCV is a noncytopathic positive-stranded RNA virus of the *Flaviviridae* family. It primarily infects hepatocytes of humans and chimpanzees, where, thanks to error-prone RNA-dependent RNA polymerase, the infected virus accumulates a high number of mutations rapidly, thus providing opportunity for selection of viruses that have the ability to escape the immune system and establish persistent infection. Deciphering the strategies employed by HCV to establish persistence can be helpful in the development of new strategies to eradicate the virus and to stop disease progression. Until recently, the lack of an HCV strain having the ability to establish infection *in vivo* and *in vitro* was a substantial hindrance in studying the molecular mechanisms of virus persistence. This problem was solved by the identification of an HCV strain, JFH-1, that was isolated from a fulminant hepatitis patient and found to be capable of replicating and assembling infectious virus particles in chimpanzees as well as in cell culture.⁷⁻¹⁰ This clone can be used to study the molecular mechanisms by which HCV evades the host immune system and causes chronic infection.

In a previous report, we inoculated patient serum from which the JFH-1 strain was originally isolated and cell culture-generated JFH-1 virus (JFH-1cc) into two different chimpanzees.¹¹ HCV established infection in both animals within 3 days of inoculation. In the JFH-1cc-infected chimpanzee, genome sequence of predominant infecting virus at week 2 was identical to JFH-1 wild-type (JFH-1/wt [in this study, this abbreviation was used instead of JFH-1 to distinguish it from other variant strains]), and the infecting virus has four synonymous and seven nonsynonymous mutations at week 7. In the JFH-1 patient serum-infected chimpanzee, 19 synonymous and six nonsynonymous mutations were observed in predominantly circulating virus at week 2, and this number increased to 35 synonymous and 17 nonsynonymous mutations at the later stage of infection course (week 23).¹¹ From these observations, we presumed that the isolates evolved in each chimpanzee at later stages of infection might have some advantage over the viruses isolated at earlier time points for survival in infected animals. Thus, in this study, we generated JFH-1 variants con-

taining the mutations observed in these animals and assessed their effect on replication and infectious virus production in cell culture. Furthermore, we examined the effects of infection of these strains to tumor necrosis factor α (TNF- α)- or Fas ligand (FasL)-mediated apoptosis.

Materials and Methods

The complete Materials and Methods are provided in the Supporting Information.

Results

Effects of Mutations Identified in Chimpanzees. To investigate the effect of mutations on virus phenotype, we generated constructs containing the mutations observed in JFH-1 patient serum-infected chimpanzee and JFH-1cc-infected chimpanzee at various time points. The JFH-1 variants JFH-1/S1 and JFH-1/S2 contain the mutations observed in the patient serum-infected chimpanzee at week 2 and week 23, respectively, and JFH-1/C contains the mutations observed in the JFH-1cc-infected chimpanzee at week 7 (Supporting Table 1). The replication and virus production capacity of these variants in HuH-7 cells was compared with that of JFH-1/wt. After electroporation of *in vitro*-synthesized full-genome RNA of JFH-1/wt and variant strains, extracellular and intracellular HCV RNA and core antigen (Ag) were measured (Fig. 1). At day 5 posttransfection, all constructs displayed similar intracellular HCV RNA levels. However, extracellular HCV RNA level of JFH-1/C was 1.6 times higher than that of JFH-1/wt. Likewise, extracellular HCV RNA level of JFH-1/S2 was 3.4 times higher than that of JFH-1/S1 (Fig. 1A). Intracellular HCV core Ag levels of JFH-1/S2 and C were 240.9 ± 58.2 and 189.8 ± 42.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/S1 (526.1 ± 58.2 fmol/mg protein) and JFH-1/wt (511.7 ± 32.9 fmol/mg protein) at day 1, but reached comparable levels at day 5 posttransfection. On the other hand, extracellular HCV core Ag level of JFH-1/C was 2.2 times higher than that of JFH-1/wt, and that of JFH-1/S2 was 3.6 times higher than that of JFH-1/S1 at day 5 posttransfection (Fig. 1B). Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

Single Cycle Virus Production Assay. For detailed analysis of the effects of these mutations on different stages of the virus lifecycle, we used a Huh7-25 cell

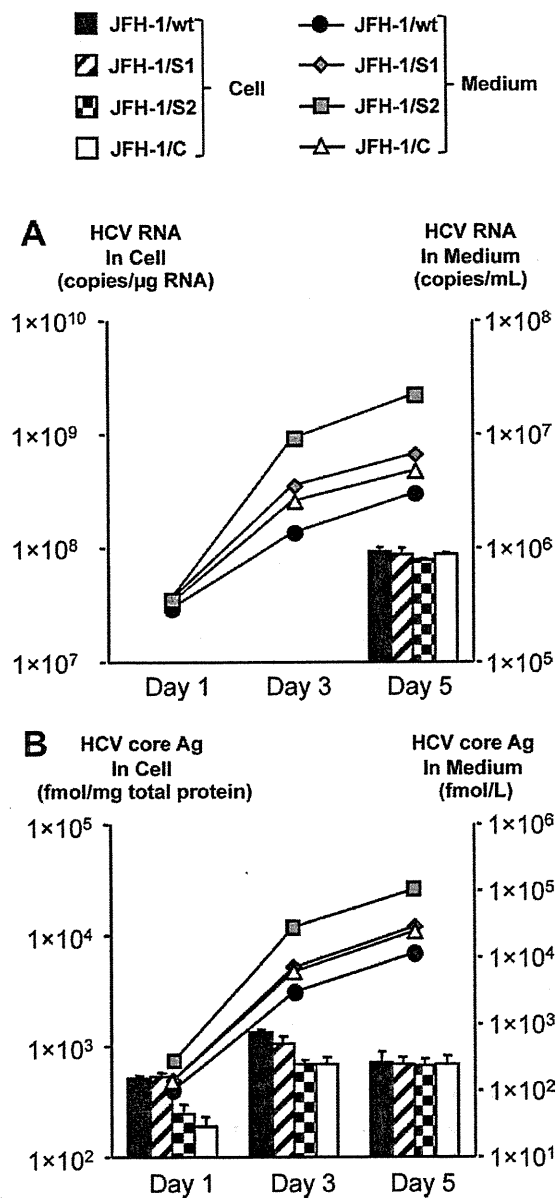


Fig. 1. Effects of *in vivo* adaptive mutations on virus production in HuH-7 cells. One million cells were transfected with 10 μ g *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean \pm SD.

line that lacks the surface expression of CD81, one of the cellular receptors for HCV entry. Three days after transfection with full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, HCV RNA levels and infectivity titer were measured, and the specific infectivity was calculated (Table 1). Intracellular HCV RNA levels of JFH-1/C and JFH-1/S2 were lower than those of JFH-1/wt and S1, suggesting lower repli-

cation efficiency of these strains. However, the intracellular infectivity titers of JFH-1/C and JFH-1/S2 were 2.03 and 11.0 times higher than those of JFH-1/wt and JFH-1/S1, respectively ($P < 0.005$). Intracellular-specific infectivities (infectivity titer/HCV RNA copy number) of JFH-1/C and JFH-1/S2 showed more pronounced difference from those of JFH-1/wt and JFH-1/S1 (3.92 times and 12.9 times higher, respectively; $P < 0.005$). The infectious virus secretion rate (extracellular infectivity titer/intracellular infectivity titer) was not significantly different between JFH-1/wt and variant strains. These data indicate that mutations identified in chimpanzees at the later time point of infection led to reduced viral replication and increased assembly of infectious virus particles without any effect on viral release in cell culture.

Subgenomic Replicon Assay. To further confirm the replication efficiencies of strains observed in chimpanzees, we generated subgenomic replicons of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C carrying the firefly luciferase reporter gene (SGR-JFH-1/Luc/wt, SGR-JFH-1/Luc/S1, SGR-JFH-1/Luc/S2, and SGR-JFH-1/Luc/C). *In vitro*-transcribed RNAs of these constructs were transfected into HuH-7 cells, and luciferase activity was measured to assess their replication capacity. The luciferase activities of SGR-JFH-1/Luc/C and SGR-JFH-1/Luc/S2 replicons were 7.30 and 7.33 times lower than those of SGR-JFH-1/Luc/wt and SGR-JFH-1/Luc/S1, respectively, at day 1 ($P < 0.00005$), suggesting attenuated replication capacities of variant replicons isolated from each animal at later time points of infection (Supporting Fig. 1A). The luciferase activity 4 hours after transfection was comparable, indicating similar levels of transfection efficiency (data not shown). Based on these data, we found that the mutations that emerged in nonstructural (NS)3-NS5B of JFH-1/S2 and JFH-1/C reduced the replication efficiency in cell culture.

Genomic Regions Responsible for Lower Replication and Higher Assembly of JFH-1/S2. To further clarify the genomic region responsible for lower replication efficiency and higher assembly rate of JFH-1/S2, we generated the chimeric constructs JFH-1/S2-wt and JFH-1/wt-S2 as described in the Supporting Materials and Methods. *In vitro*-transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 were introduced into HuH-7 cells by electroporation and intracellular and extracellular HCV RNA and core Ag were measured. At day 5 posttransfection, all constructs displayed comparable intracellular HCV RNA levels (Fig. 2). However, extracellular HCV RNA levels of JFH-1/S2 and JFH-1/S2-wt were significantly

Table 1. Infectious Virus Production and Release of JFH-1/wt and Variants in Huh7-25 Cells

Strain	Intracellular			Extracellular	Secretion Ratio (Extracellular/ Intracellular)
	HCV RNA (copies/ μ g RNA)	Infectivity Titer (ffu/well)	Specific Infectivity (ffu/copies)	Infectivity Titer (ffu/well)	
JFH-1/wt	$7.75 \times 10^8 \pm 1.04 \times 10^8$	$4.21 \times 10^2 \pm 4.32 \times 10^1$	$2.09 \times 10^{-7} \pm 7.06 \times 10^{-8}$	$1.94 \times 10^3 \pm 3.76 \times 10^1$	4.6 ± 1.3
JFH-1/S1	$7.04 \times 10^8 \pm 8.49 \times 10^7$	$4.72 \times 10^2 \pm 5.63 \times 10^1$	$2.91 \times 10^{-7} \pm 6.00 \times 10^{-8}$	$3.02 \times 10^3 \pm 2.77 \times 10^2$	5.4 ± 2.0
JFH-1/S2	$4.16 \times 10^{8**} \pm 7.47 \times 10^6$	$5.19 \times 10^{3**} \pm 8.24 \times 10^1$	$3.76 \times 10^{-6**} \pm 7.01 \times 10^{-7}$	$3.23 \times 10^{4**} \pm 3.52 \times 10^3$	6.2 ± 3.0
JFH-1/C	$3.15 \times 10^{8**} \pm 5.02 \times 10^7$	$8.59 \times 10^{2*} \pm 4.81 \times 10^1$	$8.19 \times 10^{-7*} \pm 5.68 \times 10^{-8}$	$3.68 \times 10^3 \pm 3.02 \times 10^3$	4.3 ± 1.4
JFH-1/ S2-wt	$7.07 \times 10^8 \pm 8.43 \times 10^7$	$4.40 \times 10^{3*} \pm 9.5 \times 10^1$	$2.73 \times 10^{-6*} \pm 2.35 \times 10^{-7}$	$3.0 \times 10^{4*} \pm 1.1 \times 10^3$	6.7 ± 0.7
JFH-1/ wt-S2	$4.21 \times 10^{8*} \pm 1.97 \times 10^7$	$2.7 \times 10^2 \pm 2.9 \times 10^1$	$2.02 \times 10^{-7} \pm 4.0 \times 10^{-8}$	$1.7 \times 10^3 \pm 1.3 \times 10^2$	4.5 ± 0.4

Abbreviation: ffu, focus-forming units.

* $P < 0.005$ versus JFH-1/wt.** $P < 0.005$ versus JFH-1/S1.

higher ($P < 0.0005$) than that of JFH-1/wt. On the other hand, extracellular RNA level of JFH-1/wt-S2 chimeric construct was lower than that of JFH-1/S2 and JFH-1/S2-wt and similar to that of JFH-1/wt. Likewise, extracellular core Ag levels of JFH-1/S2 and JFH-1/S2-wt were also significantly higher than that of JFH-1/wt. Intracellular HCV core Ag levels of JFH-1/S2 and JFH-1/wt-S2 on day 1 posttransfection were 240.9 ± 58.2 and 134.3 ± 17.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/wt (526.1 ± 58.2 fmol/mg protein), whereas intracellular HCV core Ag level of JFH-1/S2-wt was comparable to that of JFH-1/wt. Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

To further elucidate, we transfected Huh7-25 cells with *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 and measured HCV RNA, core Ag, and infectivity titer in the cells and culture medium. Intracellular HCV RNA levels of JFH-1/S2 and JFH-1/wt-S2 were similar and lower than those of JFH-1/wt and JFH-1/S2-wt, suggesting mutations in NS3-NS5B were responsible for lower replication efficiency of JFH-1/S2 (Table 1). Intracellular infectivity titer of JFH-1/S2 and JFH-1/S2-wt was 12.3 and 10.4 times higher, respectively, than that of JFH-1/wt ($P < 0.005$) on day 3 posttransfection. The intracellular specific infectivities of JFH-1/S2 and JFH-1/S2-wt were significantly higher than that of JFH-1/wt (18 times and 13.1 times higher, respectively; $P < 0.005$). On the other hand, intracellular specific infectivity of JFH-1/wt-S2 was comparable to that of JFH-1/wt. The infectious virus secretion rate was not significantly different among all the constructs (Table 1). These data indicate that mutations emerged in the core-NS2 region of JFH-1/S2 are responsible

for the enhanced assembly of infectious virus particles compared with JFH-1/wt.

Mapping Study for JFH-1/S2 Strain. Because our experiments with JFH-1/S2 subgenomic replicon and JFH-1/wt-S2 chimeric construct showed that mutations emerged in the NS3-NS5B region are responsible for reduced replication efficiency of JFH-1/S2, we performed mapping studies by generating various JFH-1 subgenomic replicons, each containing the mutations observed in individual nonstructural protein. Although mutations in NS4B and NS5A were associated with attenuated replication capacity of JFH-1, the most significant decrease in replication was observed with NS5B mutations (Supporting Fig. 1B).

For detailed analysis of mutations responsible for higher assembly, *in vitro*-transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, JFH-1/N397S, JFH-1/L752V, JFH-1/S2-NS2 (containing mutations G838R, A878V, and V881A), JFH-1/G838R, and JFH-1/A878V were transfected into Huh7-25 cells, and intracellular-specific infectivities were compared (Supporting Table 2). As reported previously, JFH1/G838R showed higher intracellular specific infectivity than that of JFH-1/wt, but could not reach the level of JFH-1/S2 or JFH-1/S2-wt. Among the mutants, intracellular specific infectivities of JFH1/L752V, JFH1/NS2, and JFH1/G838R were 4.02, 5.42, and 3.07 times higher than that of JFH-1/wt, but those of JFH1/N397S and JFH1/A878V were similar to that of JFH-1/wt. Thus, the combination of mutations in P7 and NS2 was found to contribute to the higher assembly of the JFH-1/S2 strain.

Human Hepatocyte-Transplanted Mouse Assay. To assess the *in vivo* infectivity of these strains, we inoculated culture medium containing 10^7 copies (HCV RNA titer measured by RTD-PCR) of JFH-1/wt, JFH-1/S1, JFH-1/S2, and C viruses into human

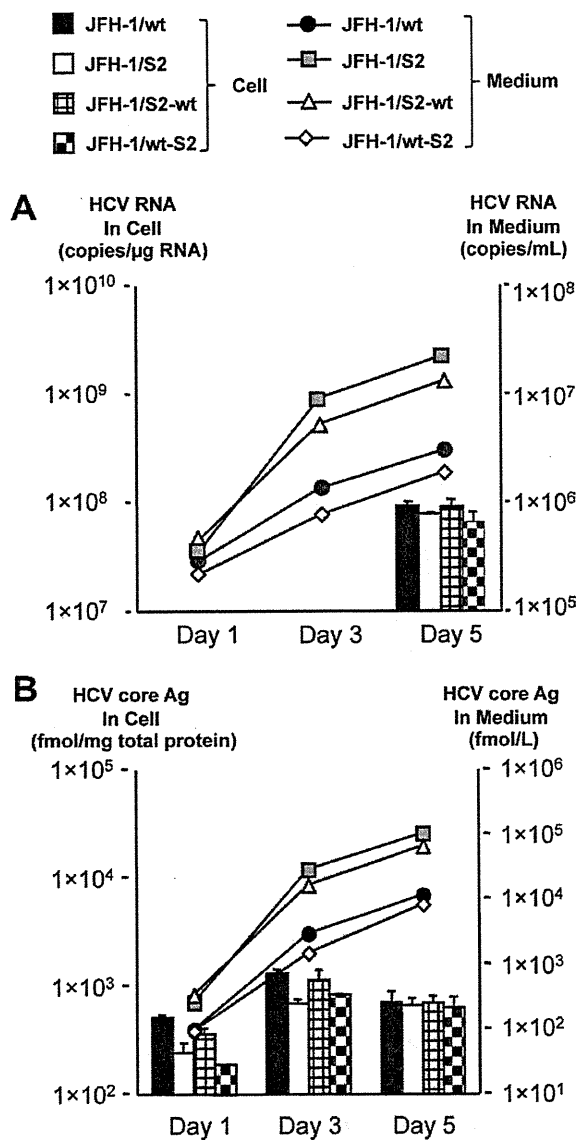


Fig. 2. Virus production of JFH-1/S2 chimeric constructs in HuH-7 cells. One million cells were transfected with 10 μ g *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean \pm SD.

hepatocyte-transplanted mice. Two mice were used for each virus. Two weeks after intravascular inoculation, all mice but one became HCV RNA-positive (Fig. 3). Two mice died 3 weeks after inoculation; one was inoculated with JFH-1/wt and had developed infection, and the other was inoculated with JFH-1/C and died without developing infection. HCV RNA levels in infected mice fluctuated, ranging from 10⁶ to 10⁹ copies/mL. We could not observe much difference of

infected HCV RNA titer among these inoculated mice. Sequence analyses of the complete open reading frames revealed that infecting JFH-1/wt virus and variant strains had no nonsynonymous mutations at the time of development of infection. From these data, we concluded that not only JFH-1/wt virus but also JFH-1/S1, JFH-1/S2, and JFH-1/C viruses were able to establish productive infection in human hepatocyte-transplanted mice.

Apoptosis Induction Assay. To investigate the survival strategy against the host defense system, we examined the susceptibility of JFH-1/wt and variant strains to TNF- α -mediated apoptosis induction. After transfection with *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, Huh-7.5.1 cells were exposed to TNF- α plus actinomycin D. Without exposure, apoptosis was observed in a limited number of HCV-positive cells (Supporting Fig. 2A). Forty-eight hours later, cells were harvested, fixed, and

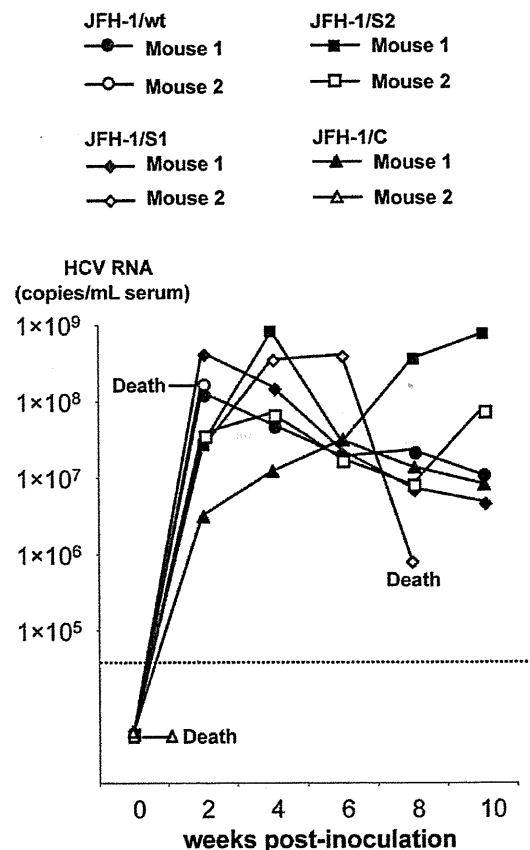


Fig. 3. *In vivo* infection study of JFH-1/wt and its variants in human hepatocyte-transplanted mice. Cell culture medium containing 1 \times 10⁷ HCV RNA copies of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C were inoculated into human hepatocyte-transplanted mice, and HCV RNA levels in mice serum were monitored.

subjected to terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and anti-HCV NS5A staining. The effects of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C transfection on apoptosis induction were determined by calculating the ratio of apoptosis between HCV-positive and HCV-negative populations and expressed as an apoptosis induction index. After treatment of JFH-1/wt-transfected cells with TNF- α , apoptosis was observed in 36.8% of the HCV-positive population and in 19.3% of the HCV-negative population, and the apoptosis induction index was 1.85 ± 0.06 (Fig. 4). The apoptosis induction indexes of JFH-1/S1-transfected and JFH-1/C-transfected cells were 1.23 ± 0.06 and 1.16 ± 0.10 , respectively, suggesting lower susceptibility to apoptosis induction compared with JFH-1/wt. On the other hand, the apoptosis induction index of JFH-1/S2 was 0.74 ± 0.17 , which was substantially lower than that of JFH-1/wt, demonstrating the more reduced apoptosis in the cells harboring this strain. Similar results were obtained by treatment with FasL plus actinomycin D (Supporting Fig. 2B). To confirm the lower susceptibility of JFH-1/S2-transfected cells, apoptosis was also detected by staining with anticlaved poly(adenosine diphosphate ribose) polymerase (PARP) antibody. The apoptosis induction indexes of JFH-1/wt and JFH-1/S2-transfected cells were 2.28 ± 0.24 and 1.15 ± 0.14 , respectively, and were consistent with TUNEL assay (Fig. 5). Although the HCV NS5A-positive rate in JFH-1/S2-transfected cells was higher than that in JFH-1/wt, the mean fluorescence intensity of the NS5A-positive population in JFH-1/S2-transfected cells was significantly lower (185.0 ± 8.7) than that in JFH-1/wt-transfected cells (395.0 ± 98.0), corresponding to the observed phenotype of the JFH-1/S2 strain in the single cycle virus production assay (i.e., lower replication efficiency and rapid spread to surrounding cells).

To clarify the genomic region responsible for lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, we examined the effect of TNF- α on the cells carrying subgenomic reporter replicons. The apoptosis induction index of SGR-JFH1/Luc/S2-transfected cells was lower than that of SGR-JFH1/Luc/wt-transfected cells (Supporting Fig. 2C); however, the difference was not as pronounced as with full-genome constructs, indicating that mutations in the NS3-NS5B region contribute to lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, but they are not sufficient to explain the difference between JFH-1/wt and JFH-1/S2. We confirmed these results by use of the chimeric

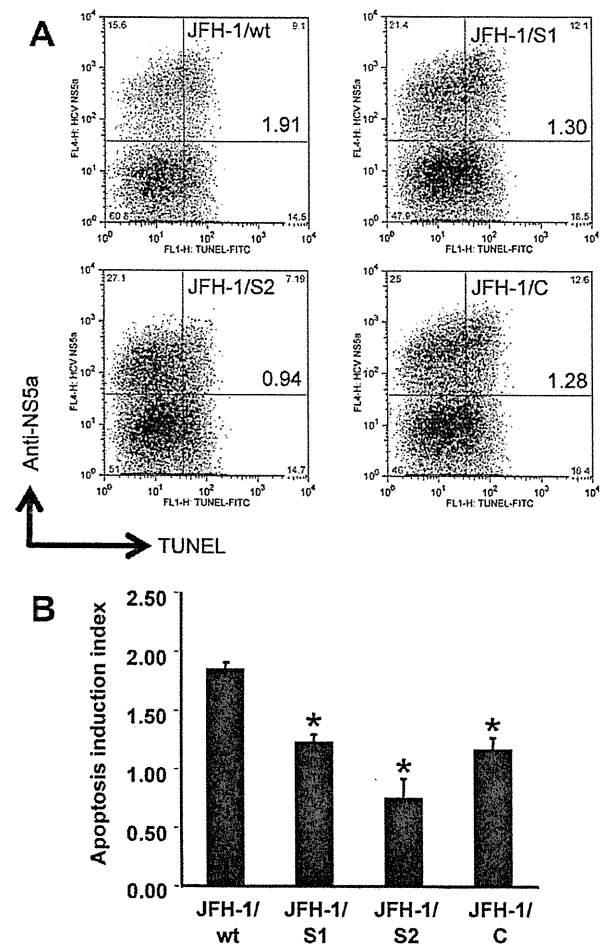


Fig. 4. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt and its variants. (A) Three million cells were transfected with 3 μ g *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. Forty-eight hours later, apoptosis was induced by exposing cells to 20 ng/mL TNF- α plus 50 ng/mL actinomycin D. Cells were harvested after 48 hours of treatment and subjected to TUNEL and anti-HCV NS5A staining. Dot plots show HCV replication and apoptosis at the single cell level. Quadrant gates were determined using unstained and a terminal deoxynucleotidyltransferase-untreated control in each culture condition. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S1-, JFH-1/S2-, and JFH-1/C-transfected cells. The mean \pm SD of three independent experiments is shown. * $P < 0.005$ versus JFH-1/wt.

constructs JFH-1/S2-wt and JFH-1/wt-S2. The apoptosis induction indexes of JFH-1/S2-wt-transfected and JFH-1/wt-S2-transfected cells were 1.42 ± 0.13 and 1.71 ± 0.08 , respectively (Fig. 5). These data indicate that both structural and nonstructural regions of JFH-1/S2 were associated with lower susceptibility to cytokine-induced apoptosis, although mutations in core-NS2 seemed to have higher contribution toward this phenotype. Together, these results indicate that the JFH-1/S2 strain, which was selected after passage in

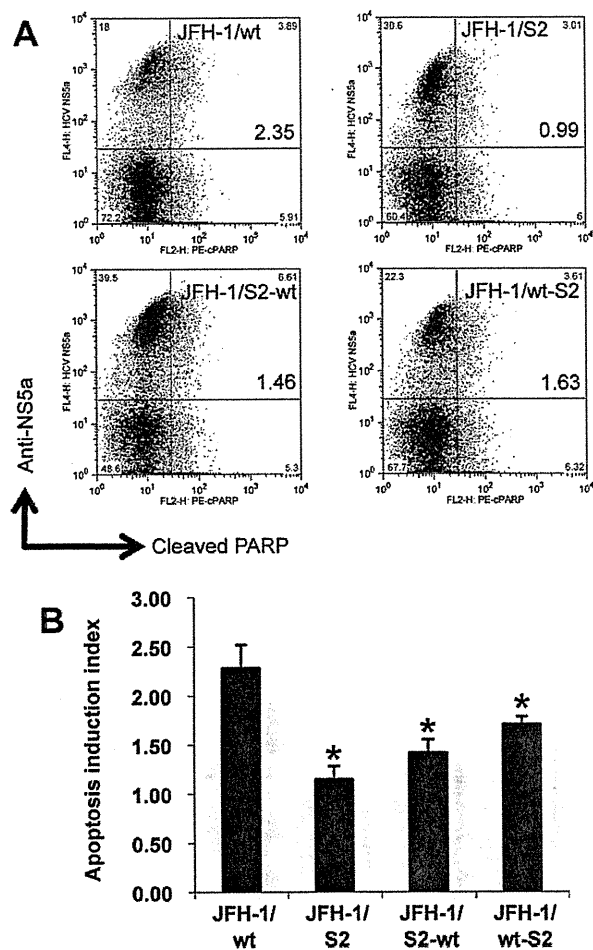


Fig. 5. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt, JFH-1/S2, and their chimeric constructs. (A) Three million cells were transfected with 3 μ g *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. Apoptosis was induced by exposing cells to 20 ng/mL TNF- α plus 50 ng/mL actinomycin D and detected by anticlaved PARP staining. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt-, JFH-1/S2-, JFH-1/S2-wt-, and JFH-1/wt-S2-transfected cells. The mean \pm SD of three independent experiments is shown. * P < 0.05 versus JFH-1/wt.

the patient serum-infected chimpanzee, acquired less susceptibility to the cytokine-induced apoptosis.

Discussion

HCV develops chronic infection in the vast majority of infected patients¹; however, the mechanisms of its persistence are still under investigation. Many viruses have evolved different strategies to cope with host immune systems, thus causing the development of persistent infection. For example, some viruses interfere with the major histocompatibility complex class I presentation of viral antigens, whereas others modulate

lymphocyte and macrophage functions, including cytokine production.¹²⁻¹⁶ In our previous study, we detected an increasing number of mutations in the HCV genome isolated from JFH-1 patient serum-infected chimpanzees. Thus, we reasoned that these detected mutations might have imparted some advantage to this virus for long-time survival. To examine this hypothesis, we compared the phenotypes of JFH-1 variant strains emerged at early and late stages of infection in JFH-1 patient serum-infected and JFH-1cc-infected chimpanzees and found that the JFH-1/S2 strain isolated from the patient serum-infected chimpanzee at a later time point of infection replicated slowly, produced more infectious viruses, and displayed reduced susceptibility to cytokine-induced apoptosis.

The JFH-1 variant strain JFH-1/C, which contains seven nonsynonymous mutations identified in the JFH-1cc-infected chimpanzee at week 7, showed comparatively slower replication kinetics and slightly enhanced infectious virus production in cell culture. The intracellular specific infectivity of this strain in Huh7-25 cells was 3.9 times higher than that of JFH-1/wt (Table 1). These characteristics might have imparted some advantage to this strain for establishing productive infection in the chimpanzee. The other JFH-1 variant strains, JFH-1/S1 and JFH-1/S2, contain 6 and 17 nonsynonymous mutations identified in the JFH-1 patient serum-infected chimpanzee at weeks 2 and 23 postinfection, respectively. Replication kinetics and infectious virus production of the JFH-1/S1 strain were comparable to that of JFH-1/wt in cultured cells (Fig. 1, Table 1). In contrast, the JFH-1/S2 strain showed lower replication efficiency. Although the intracellular HCV RNA level of this strain in Huh7-25 cells was lower than that of JFH-1/wt and JFH-1/S1, and almost the same as that of JFH-1/C (Table 1), intracellular specific infectivity was 18.0 and 12.9 times higher than that of JFH-1/wt and JFH-1/S1, respectively, suggesting a significant increase in the assembly of infectious virus particles (P < 0.005, Table 1). The enhanced capacity of this strain to assemble infectious virus particles resulted in a higher extracellular infectivity titer that contributed to the rapid spread of virus to surrounding cells. Flow cytometry analyses of cells transfected with JFH-1/wt and variant strains revealed that the percentage of the HCV NS5A-positive population in JFH-1/S2-transfected cells was higher, but the mean fluorescence intensity of the anti-NS5A signal was lower than that in JFH-1/wt-transfected cells, thus confirming higher spread and lower replication of this strain. Taken together, both JFH-1/C and JFH-1/S2 exhibited a tendency toward

decreased replication and increased infectious virus production. However, the extent of enhanced virus production was substantially lower in JFH-1/C than in JFH-1/S2, which might have led to the earlier elimination of infection in the JFH-1cc-infected chimpanzee. In other words, the potency of infectious virus production and spread seems to correspond to the duration of infection in infected animals.

The association between a lower replication efficiency and persistent infection is still unclear. It has been reported that an escape mutant with an amino acid substitution at the cytotoxic T lymphocyte (CTL) epitope in the NS3 region exhibits lower NS3/4 protease activity and replication capacity *in vitro*.^{17,18} The JFH-1/S2 strain contains the T1077A mutation in the NS3 region (Supporting Table 1), and this mutation is located close to mutations reported to be associated with immune evasion and lower replication.¹⁷ Thus, the lower replication efficiency of the JFH-1/S2 strain may be a result of an immune escape mutation at the expense of viral fitness. Meanwhile, we cannot deny the advantage of lower replication in establishing persistent infection. Lower replication may contribute to the avoidance of major histocompatibility class I-mediated antigen presentation and to escape from the host immune system. Either way, by acquiring the ability to produce more viral particles, the JFH-1/S2 strain could rapidly spread to surrounding cells, irrespective of its lower replication efficiency. Importantly, these emerged mutations did not attenuate *in vivo* infectivity, unlike cell culture adaptive mutations reported to cause attenuated infection *in vivo*.¹⁹ Upon inoculation into human hepatocyte-transplanted mice, JFH-1/S1, JFH-1/S2, and JFH-1/C strains could establish infection without any mutations, produced levels of viremia similar to JFH-1/wt, and persisted for a similar observed period of infection (Fig. 2). This observation is different from that in chimpanzees, where JFH-1/wt and JFH-1/C strains were eliminated earlier than JFH-1/S2. In contrast to chimpanzees, human hepatocyte-transplanted mice lack a CTL and natural killer (NK) cell-mediated immune system, which could be responsible for this difference.⁶ Taken together, our results suggest that along with efficient infectious virus production, the JFH-1/S2 strain might have acquired an advantage that helps it evade the CTL and NK cell-mediated immune system.

Apoptosis of virus-infected cells by the immune system is crucial as a general mechanism of clearing infections.^{20,21} The J6/JFH-1 chimeric virus has been reported to exhibit proapoptotic characteristics in cell

culture.²² However, because HCV needs to escape the host immune system in order to establish chronic infection, immune cell-mediated apoptosis may be inhibited in infected hepatocytes. In the liver, HCV-infected hepatocytes are eliminated by targeted apoptosis induced by NK cells, macrophages, and CTLs with ligand-mediated and receptor-mediated signals such as TNF- α , FasL, and TNF-related apoptosis-inducing ligand.²³⁻²⁶ Thus, we used TNF- α to mimic natural immunomediated apoptosis and found that the JFH-1/S2-replicating cells have lower susceptibility to the apoptosis induced by these cytokines. In JFH-1/S2-transfected cells, TNF- α -induced apoptosis detected by TUNEL assay was substantially lower than that of JFH-1/wt-transfected cells (Fig. 4). We confirmed it by staining with anticleaved PARP. In complete agreement with the results produced by way of TUNEL assay, the number of anticleaved PARP stained cells among JFH-1/S2-infected cells was significantly lower than that among JFH-1/wt-infected cells (Fig. 5). In our previous study, we reported that HCV-specific immune responses with T cell proliferation and interferon- γ production were maintained until the disappearance of viremia in the patient serum-infected chimpanzee.¹¹ This finding indicates that continuous selection pressure in the infected chimpanzee might have contributed to the emergence of a clone with an ability to escape the cytokine-induced apoptosis. We are not sure whether this phenotype of JFH-1/S2 is due to its lower replication efficiency and thus lower production of HCV proteins. The accumulation of viral proteins might predispose cells to the apoptosis induced by TNF- α . To answer this question, it will be necessary to investigate the genomic regions of JFH-1/S2 and cellular host factors responsible for the ability of this strain to escape the apoptosis.

By way of mapping analysis for JFH-1/S2, we could determine responsible regions; NS5B was for lower replication efficiency (Supporting Fig. 1B), and P7 and NS2 were for enhanced viral particle assembly (Supporting Table 2). For the evasion of apoptosis, we could not specify the responsible region, because both chimeric constructs, JFH-1/S2-wt and JFH-1/wt-S2, showed less susceptibility to cytokine-induced apoptosis to a certain extent. These data indicate that both structural and nonstructural regions might have contributed to the acquisition of this phenotype. Previously, a potent antiapoptotic effect of the HCV NS5A protein was described.²⁷ NS5A interacts with Bin1, which is a nucleocytoplasmic c-Myc-interacting protein with tumor suppressor and apoptotic properties, thus inhibiting Bin1-

associated apoptosis. Because JFH-1/S2 contains several mutations in the NS5A region (Supporting Table 1), one or more mutations in this protein may be associated with antiapoptotic effects.

In conclusion, we demonstrated that the JFH-1/S2 strain acquired phenotypes of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis. These phenotypes were associated with mutations that emerged 23 weeks after infection in a chimpanzee, and might have contributed to long-term infection *in vivo*. Such control of viral functions by specific mutations may be a key viral strategy to establish persistent infection.

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Elimination of hepatitis C virus by short term NS3-4A and NS5B inhibitor combination therapy in human hepatocyte chimeric mice

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See Editorial, pages 848–850

Background & Aims: The current treatment regimen for chronic hepatitis C virus (HCV) infection is peg-interferon plus ribavirin combination therapy. The majority of developing therapeutic strategies also contain peg-interferon with or without ribavirin. However, interferon is expensive and sometimes intolerable for some patients because of severe side effects.

Methods: Using human hepatocyte chimeric mice, we examined whether a short term combination therapy with the HCV NS3-4A protease inhibitor telaprevir and the RNA polymerase inhibitor MK-0608 with or without interferon eradicates the HCV from infected mice. The effect of telaprevir and MK-0608 combination therapy was examined using subgenomic HCV replicon cells.

Results: Combination therapy with the two drugs enhanced inhibition of HCV replication compared with either drug alone. In *in vivo* experiments, early emergence of drug resistance was seen in mice treated with either telaprevir or MK-0608 alone. However, emergence was prevented by the combination of these drugs. Mice treated with a triple combination therapy of telaprevir, MK-0608, and interferon became negative for HCV RNA soon after commencement of the therapy, and HCV RNA was not detected in serum of these mice 12 weeks after cessation of the

therapy. Furthermore, all mice treated with a high dose telaprevir and MK-0608 combination therapy for 4 weeks became negative for HCV RNA 1 week after the beginning of the therapy and remained negative after 18 weeks.

Conclusions: Eradication of HCV from mice with only 4 weeks of therapy without interferon points the way to future combination therapies for chronic hepatitis C patients.

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Introduction

Chronic hepatitis C virus (HCV) infection is a leading cause of cirrhosis, liver failure, and hepatocellular carcinoma [1,2]. The current standard treatment for patients chronically infected with HCV is the combination of peg-interferon (PEG-IFN) and ribavirin (RBV) [3–5]. However, this treatment results in a sustained viral response (SVR), defined as negative for HCV RNA 24 weeks after cessation of the therapy, in only about 50% of patients with genotype 1 HCV infection with high viral load [3–5]. In view of the lack of effectiveness of the current therapy, many molecules have been tested for development of novel anti-HCV therapies. Recently, a number of new selective inhibitors of HCV proteins, the so-called STAT-C (specifically targeted antiviral therapy for HCV) inhibitors, have been in development. The HCV NS3-4A protease inhibitor and the NS5B polymerase inhibitor, as well as an inhibitor of NS5A function, have been demonstrated to have potent anti-HCV effects and have proceeded to clinical trials [6].

Although the anti-viral effect of these drugs is quite potent, monotherapy using these drugs results in early emergence of drug-resistant strains [7,8]. Accordingly, these drugs are used in combination with PEG-IFN and RBV. However, because IFN-treatment is expensive and is frequently associated with serious adverse events, such as cytopenias, rash/itching, alopecia, and

Keywords: NS3-4A protease inhibitor; NS5B RNA polymerase inhibitor; Human hepatocyte chimeric mouse; Interferon.

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Abbreviations: HCV, hepatitis C virus; IFN, interferon; RBV, ribavirin; SVR, sustained virological response; STAT-C, specifically targeted antiviral therapy for HCV; uPA, urokinase-type plasminogen activator; SCID, severe combined immunodeficiency; RT-PCR, reverse transcript-polymerase chain reaction; HSA, human serum albumin.



mental disorders [3–5,9], a new treatment strategy, especially one that does not use IFN, is needed for chronic hepatitis C patients.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes that can be infected with HCV [10]. We and other groups reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs such as IFN- α and the NS3-4A protease inhibitor [11–14]. In this study, we used the NS3-4A protease inhibitor telaprevir (VX950; MP424; Mitsubishi Tanabe Pharma Co., Osaka, Japan) [15] and the NS5B RNA polymerase inhibitor MK0608 (2'-C-methyl-7-deaza-adenosine) [16] and investigated the effect of a short term combination treatment with these drugs on HCV replication both *in vitro* and *in vivo*, and showed a successful elimination of viruses in HCV-infected chimeric mice without the use of IFN. Although the dose of the drugs used in this study might be intolerable in humans, elimination of the virus without IFN by only 4 weeks of therapy sheds light on approaches to developing combination therapies using multiple STAT-C agents without IFN.

Materials and methods

Cell culture

An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally, pHc-Vibneo-delS [17]). The pRep-Feo carries a fusion gene comprising firefly luciferase (*Fluc*) and neomycin phosphotransferase, as described elsewhere [18,19]. Replicon RNA was synthesized *in vitro* by T7-RNA polymerase (Promega, Madison, WI) and transfected into Huh7 cells by electroporation. Huh7 cells were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum at 37 °C under 5% CO₂. After culturing in the presence of G418 (Wako, Osaka, Japan), cell lines stably expressing the replicons were established (Huh7/Rep-Neo).

Luciferase assay

Replicon cell lines were treated with various concentrations of either telaprevir or MK-0608 for 72 hrs, and HCV RNA replication level was quantified by internal luciferase assay. Luciferase activities were quantified using a luminometer (Lumat LB9501; Promega) and the Bright-Glo Luciferase Assay System (Promega). The 50% inhibitory concentrations (IC₅₀) were defined as the drug concentrations producing a 50% reduction in the levels of luciferase activities relative to average levels in untreated cultures.

MTT assays

Cell viability was measured under the same experimental settings using a tetrazolium (MTT)-based viability assay (BioAssay, California, USA) according to the manufacturer's directions. The 50% cytotoxic concentrations (CC₅₀) were defined as the drug concentrations producing a 50% reduction in absorbance relative to the average level in untreated cultures.

Animal treatment

Generation of the uPA^{+/+}/SCID^{+/+} mice and transplantation of human hepatocytes were performed as described recently by our group [20]. All mice were transplanted with frozen human hepatocytes obtained from the same donor. All animal protocols described in this study were performed in accordance with the guidelines of the local committee for animal experiments, and all animals received humane care. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mouse serum concentrations of human serum albumin (HSA), correlated with the repopulation index [20], were measured as previously described [21]. Eight weeks after hepatocyte transplantation, mice were intravenously injected with 100 μ l of HCV-positive human serum samples. Mouse serum samples were obtained every one or 2 weeks after HCV infection, and HSA and HCV RNA levels were measured.

Treatment with anti-HCV drugs in HCV-infected mice

Telaprevir and MK-0608 were dissolved with a specific solvent. Eight weeks after HCV infection when the mice developed stable viremia (10⁶ to 10⁹ copies/ml), mice were administered either 200 mg/kg of telaprevir or 3–50 mg/kg of MK-0608 orally twice a day for 4 weeks. The specific solvent had no anti-HCV effect in this mouse model (data not shown). To analyze the effect of the combination treatment with telaprevir and MK-0608, these drugs were mixed and given together as a cocktail. Human IFN- α -treatment was provided daily by intramuscular injection of diluted IFN solution (Otsuka Pharmaceutical Co., Ltd., Tokyo, Japan) for 4 weeks.

Human serum sample

Human serum containing a high titer of genotype 1b HCV (2.2 \times 10⁶ copies/ml) was obtained from a patient with chronic hepatitis who had provided written informed consent to participate in the study. Serum samples were divided into small aliquots and stored in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

RNA extraction and amplification

RNA extraction, nested PCR and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [12,13]. Briefly, RNA was extracted from serum samples and extracted livers using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV cDNA was performed using Light Cycler (Roche Diagnostic, Japan, Tokyo). The lower detection limit of real-time PCR is 10³ copies/ml.

Sequence analysis

The nucleotide and amino acid sequences of the NS3 and NS5B region of HCV were determined by direct sequencing following PCR amplification of cDNA after reverse transcription of HCV RNA. The primers used to amplify the NS3 region were 5'-GTGCTCCAAGCTGGCATAAC-3' and 5'-AGGACCGAGGAATCGAACAT-3' as the first (outer) primer pair and 5'-CTAGAGTGCCGTACTTCGTG-3' and 5'-ACTGATCCTGGAGGCGTAGC-3' as the second (inner) primer pair. The primers used to amplify the NS5B region were 5'-TAAGCGAGGAGGCTGGTGGAG-3' and 5'-CCTATTGGCCTGGAGTGTTT-3' as the first (outer) primer pair and 5'-GACTCAACGGTCACTGAGAG-3' and 5'-CCTATTGGCCTGGAGTGTTT-3' as the second (inner) primer pair. PCR was performed in a 25 μ l solution, consisting of a reaction buffer (12.5 μ l, 2 \times PCR buffer for FOD FX), 5 μ l 2 mM dNTPs, 0.75 μ l F primer (10 μ M), 0.75 μ l R primer (10 μ M), 1 μ l Temp DNA (10 pg–200 ng), 0.5 μ l KOD FX, 4.5 μ l D.W. RT-PCR reactions were carried out following the manufacturer's instructions (Biometra T-Personal; Montreal Biotech Inc., Kirkland, QC, Canada). Amplification conditions included an initial denaturation at 94 °C for 2 min, 35 cycles of amplification (denaturation at 94 °C for 2 min, annealing of primer at 56 °C (1st PCR) or 59 °C (2nd PCR) for 30 s; extension at 68 °C for 2 min 30 s (NS3, 1st PCR), 1 min 30 s (NS3, 2nd PCR), 2 min (NS5B, 1st PCR), or 1 min 10 s (NS5B, 2nd PCR)); and final extension at 68 °C for 5 min.

Results

Anti-viral activity of telaprevir and MK-0608 on HCV subgenomic replicon cells

The effect of telaprevir and MK-0608 on HCV replication was analyzed *in vitro* using HCV replicon cells. Huh7/Rep-Feo cells were treated with various concentrations of either telaprevir or MK-0608. Measured luciferase activity demonstrated that both drugs inhibited HCV replication in a dose-dependent manner (Fig. 1). The IC₅₀ of telaprevir and MK-0608 was 0.53 and 0.51 μ M, respectively, consistent with previous reports [7,16]. When

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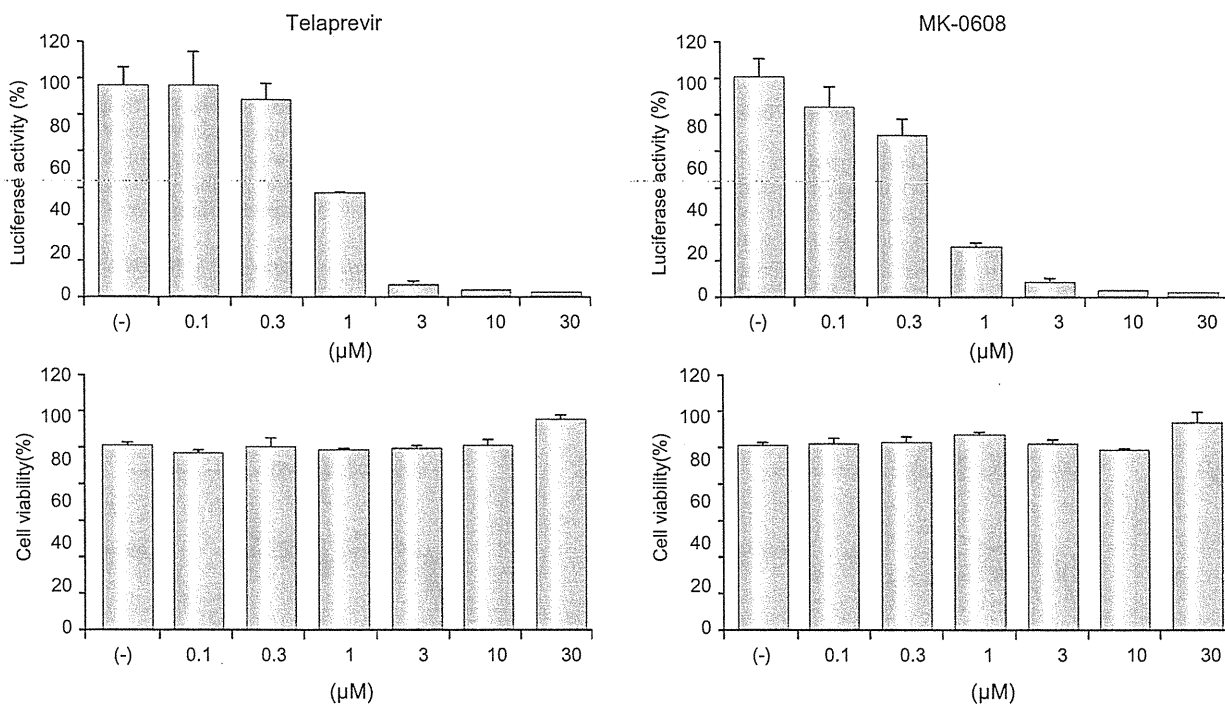


Fig. 1. *In vitro* analysis of susceptibility of HCV replicon cells to anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentrations of either telaprevir or MK-0608. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed relative to cellular viabilities (lower panel). Bars represent means \pm SD of three experiments.

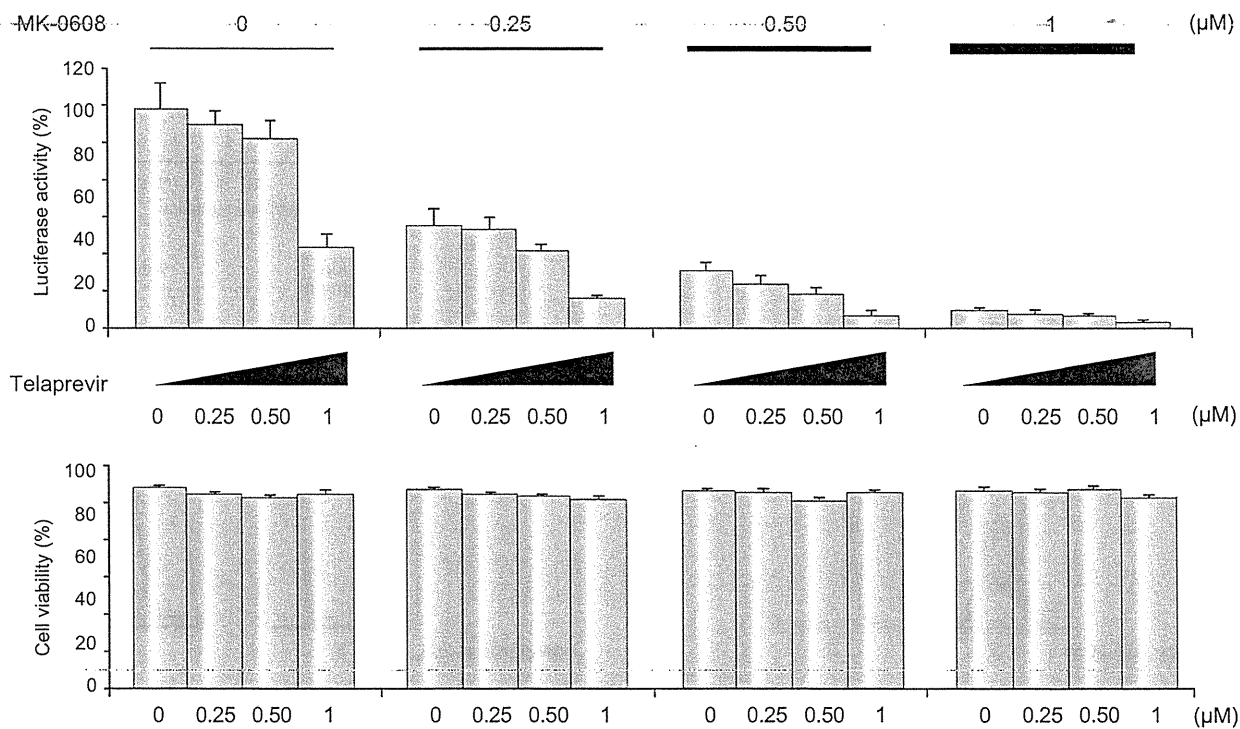


Fig. 2. *In vitro* analysis of susceptibility of HCV replicon cells to combination treatment with anti-HCV drugs. Huh7/Rep-Neo cells were treated for 72 h with the indicated concentration of MK-0608 plus telaprevir. Intracellular HCV RNA replication levels were determined as luciferase activities (upper panel), and expressed relative to cellular viabilities (lower panel). Bars are means \pm SD of 3 experiments.

telaprevir and MK-0608 were combined, the anti-HCV effect was increased without cellular damage (Fig. 2).

Effects of telaprevir and MK-0608 on HCV replication in vivo

To analyze the effect of telaprevir and MK-0608 *in vivo*, we used genotype 1b HCV-infected human hepatocyte chimeric mice. Eight HCV-infected mice were treated with either 200 mg/kg of telaprevir or 3 mg/kg of MK-0608 for 4 weeks. At the end of 1 week, treatment resulted in a 1.9 ± 0.7 log reduction of HCV RNA in telaprevir-treated mice and a 2.6 ± 0.2 log reduction in MK-0608-treated mice (Fig. 3A and C). During the treatment, the level of HSA did not decrease. Serum HCV RNA level rebounded in one of the four telaprevir-treated mice and in two

of the three MK-0608-treated mice (a MK-0608-treated mouse died after 1 week of treatment). Nucleotide and amino acid sequence analysis showed the emergence of a V36A mutation (NS3-4A protease inhibitor-resistant variant) in the NS3 region (Fig. 3B) in a telaprevir-treated mouse, and a S282T mutation (NS5B polymerase inhibitor-resistant variant) in the NS5B region (Fig. 3D) in MK-0608-treated mice, similar to clinical observations and analysis using HCV-infected chimpanzees [22,23].

Combination treatment with telaprevir and MK-0608 on HCV replication in vivo

Because mono-therapy with either telaprevir or MK0608 resulted in emergence of drug-resistant variants, we analyzed the effect of

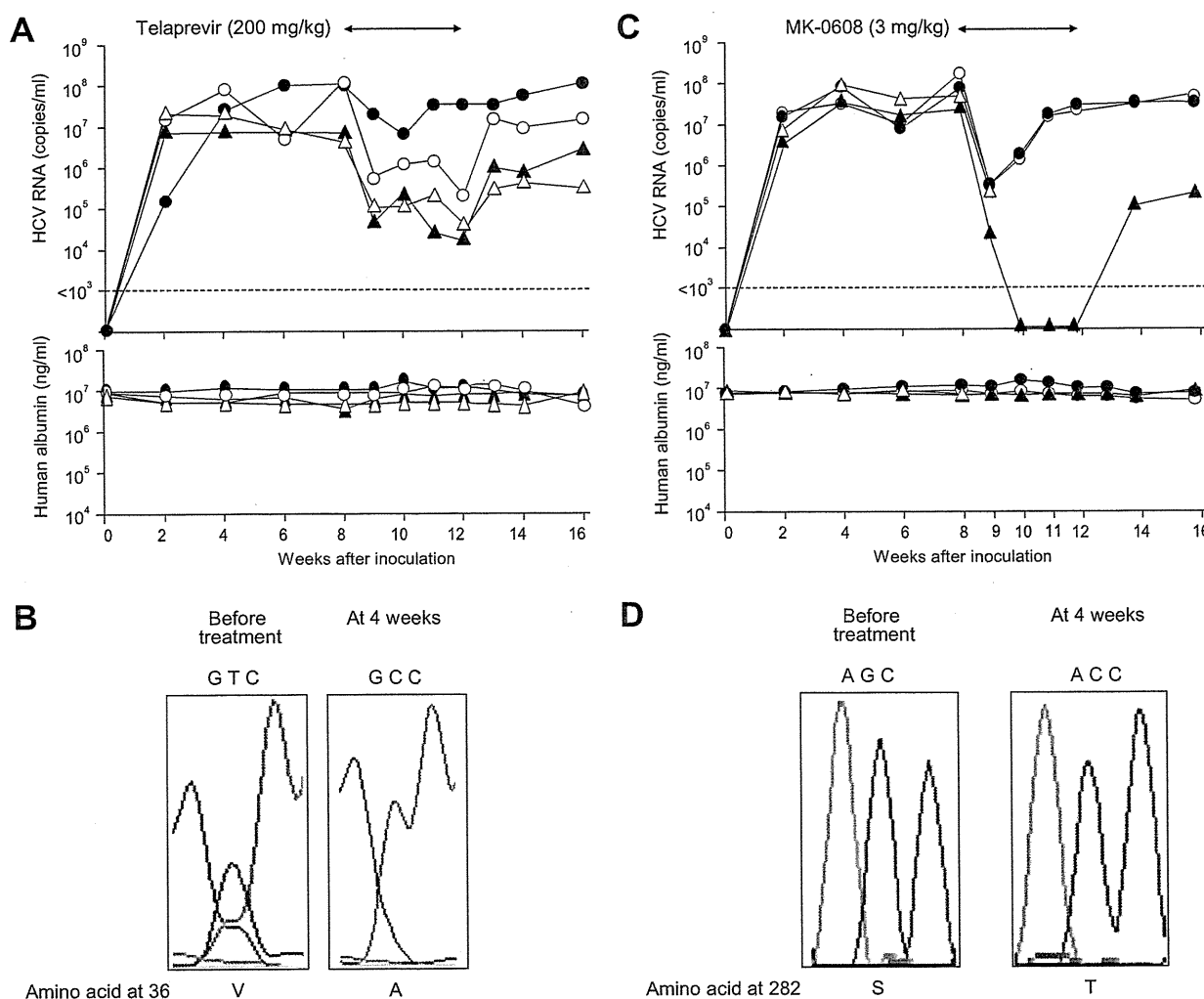


Fig. 3. Antiviral effects of either telaprevir or MK0608 monotherapy on HCV-infected mice. Mice were injected intravenously with 100 μ l of HCV-positive human serum samples. Eight weeks after HCV infection, mice were treated with either 200 mg/kg of telaprevir (A) or 3 mg/kg of MK-0608 (C) for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. The horizontal dashed line represents the detection limit (10^3 copies/ml). Note that one telaprevir-treated mouse (A, closed circle) and two MK-0608-treated mice (B, closed circle and open circle) showed a viral breakthrough during the dosing period. Nucleotide and amino acid (aa) sequence analysis of aa 36 in the HCV NS3 (B) or at aa 282 in the NS5B region (D) by direct sequencing in mice serum samples obtained before treatment and at 4 weeks.

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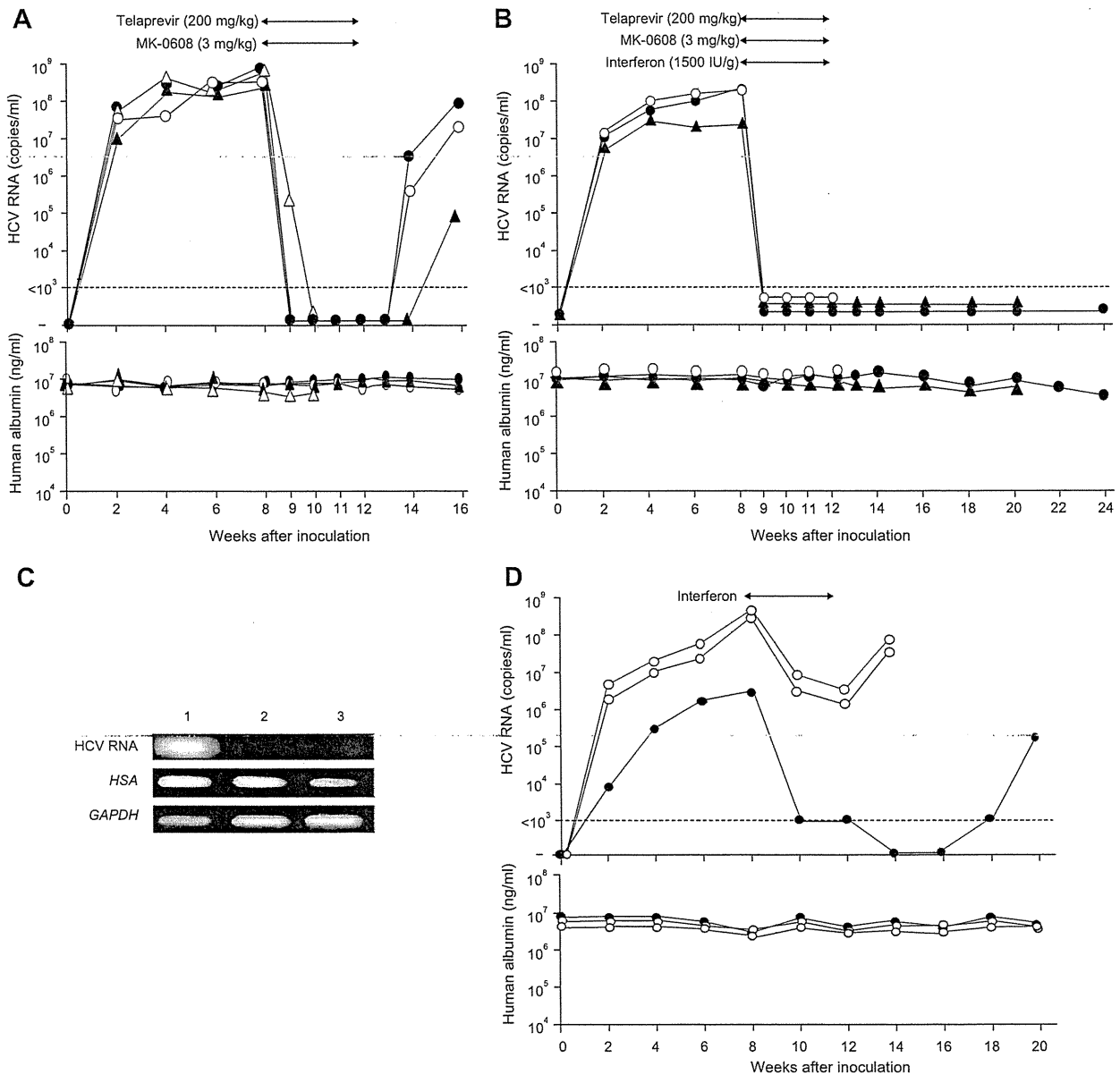


Fig. 4. Antiviral effect of combination treatment on HCV-infected mice. HCV-infected mice were treated with 200 mg/kg of telaprevir plus 3 mg/kg of MK-0608 without (A) or with (B) 1500 IU/g of human interferon-alpha for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. (C) Nested PCR of HCV RNA, human serum albumin (HSA) and GAPDH in a telaprevir, MK-0608 and interferon-alpha-treated mouse liver at 24 weeks (lane 2). Mice livers with (lane 1) or without (lane 3) HCV-infection were also analyzed. (D) HCV-infected mice were treated with either 1500 (open circles) or 7000 IU/g (closed circles) of interferon-alpha for 4 weeks.

combination treatment of these drugs with or without IFN on HCV replication *in vivo*. Four HCV-infected mice were treated with telaprevir plus MK-0608 for 4 weeks (Fig. 4A). Serum HCV RNA became negative by nested PCR with this combination treatment in all mice. One mouse died after 2 weeks of treatment. During the treatment, no emergence of resistant strains was observed in each of the remaining three mice; however, all mice became positive for HCV RNA again after cessation of the therapy. Another three mice were treated with telaprevir, MK-0608 and IFN-alpha for 4 weeks (Fig. 4B). HCV RNA became undetectable

in all three mice 1 week after the beginning of the therapy. After 4 weeks of treatment, one mouse died. In the remaining two mice, HCV RNA did not become positive after cessation of the therapy. One of the remaining two mice died at 20 weeks, and the remaining mouse was sacrificed at 24 weeks (12 weeks after the cessation of therapy). HCV was probably eliminated because no HCV RNA was detected by nested PCR in this mouse liver (Fig. 4C). As a control, HCV-infected mice were treated with 1500 IU/g/day of IFN-alpha alone for 4 weeks, resulting in a two log reduction (Fig. 4D). HCV RNA became undetectable with

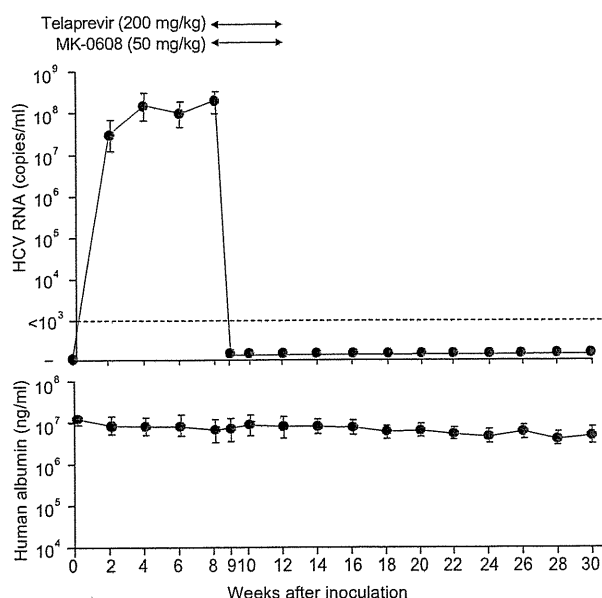


Fig. 5. High doses of MK-0608 and telaprevir combination treatment eliminates virus in HCV-infected mice. HCV-infected mice were treated with 50 mg/kg of MK-0608 and 200 mg/kg of telaprevir for 4 weeks. Mice serum samples were obtained at the indicated times, and HCV RNA titer (upper panel) and human serum albumin concentration (lower panel) were analyzed. Points represent the means \pm SD of five mice.

administration of 7000 IU/g/day of IFN- α treatment. However, the virus rebounded after cessation of the therapy.

Four-week high dose combination therapy of MK-0608 and telaprevir eliminated HCV from mice

We investigated whether combination treatment with high doses of MK-0608 and telaprevir without IFN eliminates viruses from HCV-infected mice. Five HCV-infected mice were treated with high doses of MK-0608 (50 mg/kg) and telaprevir (200 mg/kg) for 4 weeks. Serum HCV RNA titer became undetectable 1 week after commencement of the therapy and remained undetectable in all mice at 30 weeks (18 weeks after cessation of the therapy) (Fig. 5). No apparent toxicity of the drugs was observed as none of the mice showed a decrease in the level of serum HSA.

Discussion

Since we began performing treatment experiments using human hepatocyte chimeric mice with HCV, we have administered many different drugs to analyze the effects on suppression or eradication of the virus. However, until we performed the experiments described in this study, we have never observed long term absence of the virus following cessation of the therapy [12,24]. Strikingly, after only 4 weeks of triple therapy with IFN, telaprevir and MK0608, was long term absence of the virus in mouse serum after cessation of the therapy visible (Fig. 4B). Furthermore, high dose telaprevir and MK-0608 combination therapy resulted in a similar absence of the virus for 16 weeks after cessation of therapy (Fig. 5). In this study, mice were treated with 200 mg/kg of

telaprevir twice a day, and 1 week of the treatment resulted in an approximately 2 log reduction of HCV RNA (Fig. 3A), as has been observed previously in chronic hepatitis C patients treated with 450 mg of telaprevir every 8 hrs [25]. This result suggests that approximately 1/15th of a dose in this mouse model may be equivalent to a dose in humans.

During the observation period, some mice died. We do not think that this is due to the drug regimes because the chimeric mouse is weak, and approximately 50% of mice die spontaneously at week 6 after transplantation [26].

Sustained virological response, the complete elimination of the virus from the human body, is defined as testing negative for HCV RNA in serum for more than 24 weeks after cessation of the therapy. As the chimeric mouse used in this study is a weak animal, we were unable to monitor for absence of the virus beyond 24 weeks following cessation of therapy. However, negative testing for HCV RNA in mouse liver by nested PCR (Fig. 4C) 12 weeks after cessation of the therapy strongly suggests that HCV was completely eliminated from the mouse. Of course the mouse model differs from infection in humans where the virus replicates for years in the livers of infected patients. However, results of this study suggest that we will be able to eliminate the virus in humans by treating patients with regimens similar to those used in this study.

Until recently eradication of the virus with biochemical and histological improvement in chronically infected patients has long been reported only with the use of IFN or PEG-IFN [27,28]. Recently, Suzuki et al. reported for the first time eradication of the virus from chronically infected patients without IFN [29].

Elimination of the virus without IFN is desirable due to the many serious side effects of this drug [3,5–9]. However, emergence of drug resistance is a problem, as demonstrated in this study (Fig. 3) as well as in previous studies using replicon systems and HCV-infected chimpanzees [22,23]. A recent clinical study of NS3-4A and NS5B inhibitor combination therapy has reported that 13 days of this combination treatment achieved robust antiviral suppression in chronic hepatitis C patients [30]. As no study has tested the possibility of development of double drug resistant mutants, we will have to test if long term low dose treatment with any combination of STAT-C compounds might induce emergence of multi-drug resistant strains. Furthermore, as there is no report for emergence of IFN resistant strains, regimens such as combination therapy with multiple STAT-C drugs with a small or standard amount of IFN should be tested to develop the best therapy to eradicate the virus with a minimum of side effects and costs. Our further attempts to test possible combinations in mice to determine the best combination of STAT-C drugs will give us an insight into how to develop more effective therapeutic regimens in humans.

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Conflict of interest

The authors who have taken part in this study declared that they do not have anything to disclose regarding conflict of interest with respect to this manuscript.

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Acknowledgments

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ME3738 enhances the effect of interferon and inhibits hepatitis C virus replication both *in vitro* and *in vivo*

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Background & Aims: ME3738 (22 β -methoxyolean-12-ene-3 β , 24-diol), a derivative of soyasapogenol B, attenuates liver disease in several animal models of acute and chronic liver injury. ME3738 is thought to inhibit replication of hepatitis C virus (HCV) by enhancing interferon (IFN)- β production, as determined using the HCV full-length binary expression system. We examined the effect of ME3738 combined with IFN- α on HCV replication using the genotype 1b subgenomic replicon system and an *in vivo* mouse HCV model.

Methods: HCV replicon cells (ORN/3-5B/KE cells and Con1 cells) were incubated with ME3738 and/or IFN- α , and then intracellular IFN-stimulated genes (ISGs) and HCV RNA replication were analyzed by reverse-transcription-real time polymerase chain reaction and luciferase reporter assay. HCV-infected human hepatocyte chimeric mice were also treated with ME3738 and/or IFN- α for 4 weeks. Mouse serum HCV RNA titer, HCV core antigen, and ISGs expression in the liver were measured.

Results: ME3738 induced gene expression of oligoadenylate synthetase 1 and inhibited HCV replication in both HCV replicon cells. The drug enhanced the effect of IFN to significantly increase ISG expression levels, inhibit HCV replication in replicon cells, and reduce mouse serum HCV RNA and core antigen levels in mouse livers. The combination treatment was not hepatotoxic as evident histologically and did not reduce human serum albumin in mice.

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Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL, interleukin; ISG, interferon stimulated gene; MxA, myxovirus resistance protein A; OAS, oligoadenylate synthetase; PKR, double stranded RNA-dependent protein kinase; PCR, polymerase chain reaction; SCID, severe combined immunodeficiency; uPA, urokinase-type plasminogen activator; USP18, ubiquitin specific peptidase 18.

Conclusions: ME3738 inhibited HCV replication, enhancing the effect of IFN- α to increase ISG expression both *in vitro* and *in vivo*, suggesting that the combination of ME3738 and IFN might be useful therapeutically for patients with chronic hepatitis C. © 2010 European Association for the Study of the Liver. Published by Elsevier B.V. All rights reserved.

Introduction

The hepatitis C virus (HCV) infects an estimated 170 million people worldwide [1] leading to chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [2,3]. To date, the most effective therapy for viral clearance is a 48- or 72-week combination therapy of pegylated interferon (IFN)- α and ribavirin. However, successful eradication of the virus is achieved in only about 50% of treated patients [4–6]. Moreover, therapy induces significant adverse effects, such as fever, fatigue, and anemia [4], resulting in poor tolerability. More effective and less toxic treatment is, therefore, desired.

ME3738 (22 β -methoxyolean-12-ene-3 β , 24-diol), a derivative of soyasapogenol B [7], attenuates liver disease in several animal models of acute and chronic liver injury induced by concanavalin A, ethanol, lithocholate, and bile duct ligation [8–12]. ME3738 induces interleukin (IL)-6 expression, and serum amyloid A and α 1-acid glycoprotein act as downstream targets of the IL-6 signal to protect against concanavalin A-induced liver injury [8–10]. The drug also prevents the progression of hepatic fibrosis in rats with bile duct ligation through suppression of activation and collagen synthesis of hepatic stellate cells [12].

Recently, Hiasa et al. reported that ME3738 inhibited HCV replication by enhancing IFN- β production using the HCV full-length binary expression system that uses full-length genotype 1a HCV complementary DNA plasmid with a T7 promoter sequence and an adenoviral vector expressing T7 polymerase [13]. However, it is not clear if the production of IFN- β and subsequent expression of IFN-stimulated genes (ISGs) was induced by the transcribed HCV genomes through detection by innate



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immune system receptors, including RIG-I. In addition, it is also not clear whether ME3738 has anti-viral effects on genotype 1b HCV, which is the most common and most IFN-resistant genotype in Japan [14].

Recently, HCV-infected mice have been developed by inoculating HCV-infected human serum into urokinase-type plasminogen activator (uPA)-severe combined immunodeficiency (SCID) mice engrafted with human hepatocytes [15,16]. We and other groups had reported that this mouse model is useful for evaluating anti-HCV drugs such as IFN- α and anti-NS3 protease *in vivo* [17–19].

In the present study, we investigated the effects of ME3738 on HCV replication both *in vitro* and *in vivo* using the genotype 1b HCV replicon and HCV-infected human hepatocyte chimeric mice. The results demonstrate that ME3738 itself had an inhibitory effect on HCV replication, and when combined with IFN, ME3738 enhanced the anti-HCV effect of IFN by up-regulation of ISGs, such as oligoadenylate synthetase (*OAS* 1), myxovirus resistance protein A (*MxA*), and *ISG15* in HCV replicon cells. We also showed that the combination therapy increased *OAS1*, RNA-dependent protein kinase (*PKR*) and ubiquitin specific peptidase 18 (*USP18*) expression levels, and reduced virus levels effectively without liver cell damage in human hepatocyte chimeric mice.

Material and methods

Cell culture

Cells supporting replication of the genotype 1b-derived subgenomic HCV replicon, ORN/3-5B/KE cells [20] (kindly provided by N. Kato, Okayama University, Japan) and Con-1 cells [21], were cultured in Dulbecco's modified Eagle's medium (Gibco-BRL, Invitrogen Life Technology, Carlsbad, CA) supplemented with 10% fetal bovine serum, non-essential amino acids, glutamine, penicillin, and streptomycin (complete DMEM) in the presence of G418 (300 μ g/ml; Geneticin, Invitrogen, Carlsbad, CA). ORN/3-5B/KE and Con1 replicon cells (2×10^4) were seeded onto 12-well plates and incubated for 3 days with or without ME3738 (Meiji Seika Kaisha, Tokyo, Japan) [9], human IFN- α (Dainippon Sumitomo Pharma Co., Tokyo), or the combination of both drugs.

Quantitation of HCV RNA and ISG mRNAs

RNA extraction and quantitation of HCV by real-time polymerase chain reaction (PCR) were performed as described previously [19]. Briefly, RNA was extracted from mice serum, livers, or cellular lysate using SepaGene RVR (Sankojunyaku, Tokyo, Japan) and reverse transcribed with a random hexamer and a reverse transcriptase (ReverTraAce; TOYOBO, Osaka, Japan) according to the instructions provided by the manufacturer. Quantitation of HCV RNA was performed using the Real-Time PCR system (Applied Biosystems, Foster City, CA). The primers used for amplification were 5'-GAGTGTCTGTCAGCCTCCA-3' and 5'-CACTCGCAAGCACCTATCA-3'. Quantitation of ISGs (*OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*) was performed using real-time PCR Master Mix (TOYOBO) and TaqMan Gene Expression Assay primer and probe sets (PE Applied Biosystems, Foster City, CA). Thermal cycling conditions were as follows: a precycling period of 1 min at 95 °C followed by 40 cycles of denaturation at 95 °C for 15 s and annealing/extension at 60 °C for 1 min. Each ISG expression level was expressed relative to the endogenous RNA levels of the housekeeping reference gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*).

Luciferase reporter assay

After 72 h of IFN and/or ME3738 treatment, ORN/3-5B/KE cells were harvested with Renilla lysis reagent (Promega, Madison, WI) and subjected to the luciferase assay according to the manufacturer's protocol.

Western blotting

The cells were ruptured with 250 μ l lysis buffer [10 mM Tris/HCl pH 7.4, 140 mM NaCl and 0.5% (v/v) NP-40] followed by centrifugation for 2 min at 15,000g. Cell lysates were subjected to Western blotting using antibodies against NS3 (Novocastra Laboratories, UK) and β -actin (Sigma, Tokyo, Japan) as described previously [22].

WST assay

Cell viability was determined by employing tetrazolium salt, WST-8, using the WST-8 Cell Proliferation Assay Kit (Dojindo Laboratories., Kumamoto, Japan), according to the instructions provided by the manufacturer.

Human serum samples

Human serum samples containing high titers of genotype 1b HCV (2.2×10^6 copies/ml) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots were stored in liquid nitrogen until use.

Animal treatment

All animal protocols in this study were in accordance with the guidelines of the local committee for animal experiments and under approval of the Ethics Review Committee for Animal Experimentation of the Graduate School of Biomedical Sciences, Hiroshima University. We transplanted human hepatocytes into uPA^{-/-}/SCID^{+/+} mice as described previously [16]. All mice used in this study were transplanted with frozen human hepatocytes obtained from the same donor. Mice were injected intravenously with 50 μ l of HCV-positive human serum samples. Six weeks after HCV infection, mice were fed a normal chow containing 0.15% (w/w) ME3738 for 4 weeks, with or without IFN- α . IFN- α -treatment was provided daily by intramuscular injection of diluted IFN solution. Serum samples were collected every week, and human serum albumin (HSA) concentration and HCV RNA were measured. Mouse serum concentrations of HSA, which correlate with the repopulation rates, were measured as described previously [16]. Serum ME3738 concentrations were measured by liquid chromatography/mass spectrometry/mass spectrometry. After the fourth week of treatment, mice were sacrificed, and livers were either fixed with 4% buffered-paraformaldehyde for histological examination or frozen immediately in liquid nitrogen to measure HCV core antigen. To investigate the expression of ISGs in mouse livers, mice were kept for 1 week with or without 0.45% (w/w) ME3738 and then given a single injection of 1500 IU/g IFN- α . Four hours after injection, mice were sacrificed and liver samples were collected.

Quantitation of HCV core antigen in the mouse liver

Livers were homogenized in phosphate-buffered saline with 1% Triton X-100, 0.1% SDS, and 0.5% sodium deoxycholate. The homogenates were centrifuged at 20,000g for 30 min. HCV core antigen levels in the supernatant of liver homogenates were measured using enzyme immunoassay as described previously [23].

Statistical analysis

All data are expressed as mean \pm SD. Levels of HCV RNA and ISG mRNAs were compared using the Mann-Whitney *U*-test. A *p* value less than 0.05 was considered statistically significant. All statistical analyses were performed with SPSS 14.0 software (SPSS, Tokyo, Japan).

Results

Antiviral activity of ME3738 on HCV subgenomic replicon

The effect of ME3738 on HCV replication was analyzed *in vitro* using subgenomic HCV replicon cells possessing the luciferase reporter. ORN/3-5B/KE cells were treated with either IFN- α or ME3738 for 72 h. The luciferase reporter assay demonstrated that the HCV RNA replication level decreased depending on the

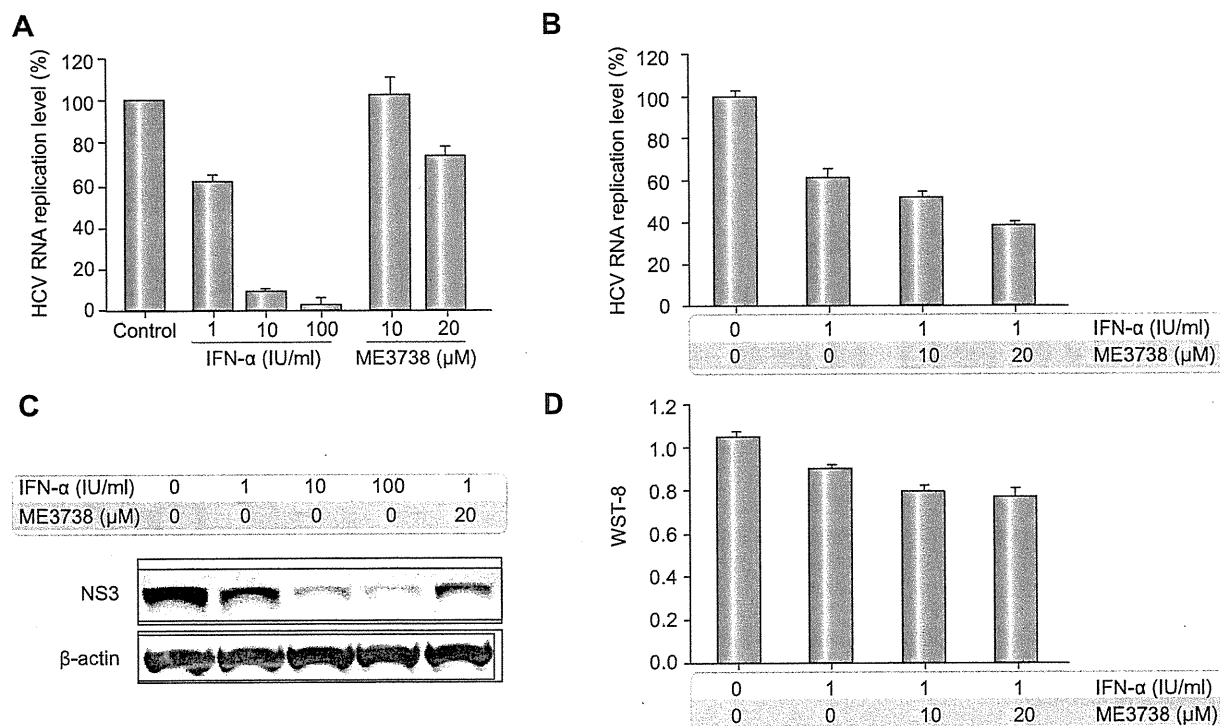


Fig. 1. Effects of ME3738 on HCV replication in the subgenomic HCV replicon, ORN/3-5B/KE cells. ORN/3-5B/KE cells were treated for 72 h with the indicated concentration of interferon (IFN)- α alone, ME3738 alone, or IFN- α plus ME3738. (A and B) Intracellular HCV RNA replication levels were determined as luciferase activity and expressed relative to cellular viability. (C) Cell lysates were analyzed by immunoblotting with antibodies to NS3 and β -actin. (D) Cellular viability was analyzed by WST assay. Data are represented as the mean \pm SD of 6 experiments. Control: cells treated with neither ME3738 nor IFN- α .

IFN-treatment dose as reported previously (Fig. 1A) [20]. Treatment with 20 μ M of ME3738 also reduced HCV RNA replication. Next, we investigated whether ME3738 enhances the effect of IFN- α . IFN- α (1 IU/ml) plus ME3738 inhibited the HCV RNA replication dose in a dependent manner with ME3738 (Fig. 1B). The level of cellular HCV NS3 protein was reduced depending on IFN- α -treatment and was reduced effectively by IFN- α /ME3738 combination treatment (Fig. 1C). The viability of cells treated with IFN- α /ME3738 combination treatment was lower than that of the control treatment and almost the same as with IFN- α treatment alone (Fig. 1D).

The effect of ME3738 was also tested in a different replicon system, Con-1 cells. ME3738 reduced HCV RNA replication dose dependently in Con-1 cells (Fig. 2A). Similar to ORN/3-5B/KE cells, IFN- α (1 IU/ml) plus ME3738 inhibited HCV RNA replication dose in a dependent manner with ME3738 (Fig. 2A), and the level of cellular HCV NS3 protein was reduced effectively by IFN- α /ME3738 combination treatment (Fig. 2B). The viability of cells treated with IFN- α /ME3738 combination treatment was lower but was not significant with IFN- α treatment alone (Fig. 2C). These results indicate that ME3738 itself has an inhibitory effect on HCV replication and enhances the effect of IFN- α .

Expression of ISGs in ME3738-treated replicon cells

We measured the levels of ISGs in drug-treated ORN/3-5B/KE cells and Con1 cells. IFN- α treatment significantly increased the expression levels of *OAS1*, *MxA*, *PKR*, *USP18* and *ISG15*, which

reached maximum levels at 24 h in ORN/3-5B/KE cells (Fig. 3A) and 8 h in Con1 cells (Fig. 3B). ME3738 treatment alone significantly increased the expression of *OAS1* in both cells. IFN- α treatment significantly increased the expression of ISGs; however, IFN- α /ME3738 combination treatment significantly induced the expressions of *OAS1*, *MxA* and *ISG15* to levels higher than IFN- α alone in both cells. These results indicate that ME3738 enhances the effect of IFN- α to increase ISG expression, and this effect may contribute to the inhibition of HCV replication.

Effect of ME3738 on HCV replication in vivo

To further analyze the effects of ME3738, we used genotype 1b HCV-infected human hepatocyte chimeric mice [17,19]. Six weeks after HCV infection, when the mice developed stable viremia (10^6 – 10^7 copies/ml, data not shown), the animals were treated with ME3738 alone, IFN- α alone, or ME3738/IFN- α for 4 weeks (Fig. 4A). Mouse serum concentrations of ME3738 increased in ME3738- and ME3738 plus IFN- α -treated mice (Table 1). ME3738 alone did not reduce the levels of HCV RNA in mice, while IFN- α -treatment reduced the HCV RNA levels, as reported previously [17]. ME3738 plus IFN- α -treatment significantly reduced HCV to levels lower than that achieved by ME3738 or IFN- α alone. We also measured the HCV core protein level in the livers of treated mice. As shown by replicon experiments, core protein levels were reduced most effectively by the ME3738/IFN- α -combination therapy (Fig. 4B). Since the level of HSA did not decrease in these treatments, it was concluded that