文献
1) Asahina Y, Tsuchiya K, Tamaki N, et al. Effect of aging on risk for hepatocellular carcinoma in chronic hepatitis C virus infection. Hepatology 2010；52：518-27.
2) Enomoto N, Sakuma I, Asahina Y, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996；334：77-81.
3) Asahina Y, Izumi N, Hirayama I, et al. Potential relevance of cytoplasmic viral sensors and related regulators involving innate immunity in antiviral response. Gastroenterology 2008；134：1396-405.
4) Tanaka Y, Nishida N, Sugiyama M, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009；41：1105-9.
5) Ge D, Fellay J, Thompson AJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009；461：399-401.
6) Thomas DL, Thio CL, Martin MP, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009；461：798-801.

Impact of Viral Amino Acid Substitutions and Host Interleukin-28B Polymorphism on Replication and Susceptibility to Interferon of Hepatitis C Virus

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Amino acid (aa) substitutions of core 70 and 91 and in the NS5A (nonstructural protein 5A) interferon sensitivity determining region (ISDR) as well as genetic polymorphisms in the host interleukin-28B (IL28B) locus affect the outcome of interferon (IFN)-based therapies for patients with chronic hepatitis C. The combination of these factors and the quasispecies nature of the virus complicate understanding of the underlying mechanism. Using infectious hepatitis C virus (HCV) genotype 1b clone HCV-KT9, we introduced substitutions at both core aa70 (Arg to Gln) and aa91 (Leu to Met). We also introduced four and nine ISDR aa substitutions into core mutant HCV-KT9. Using human hepatocyte chimeric mice with different IL28B genotypes, we examined the infectivity, replication ability, and susceptibility to IFN of these clones. Although aa substitutions in the ISDR significantly impaired infectivity and replication ability of the virus, core aa70 and 91 substitutions did not. The effect of IFN treatment was similar in core wild-type and mutant viruses. Interestingly, virus titer was significantly higher in mice with the favorable IL28B allele (rs8099917 TT and rs12979860 CC) in the transplanted hepatocytes than in mice with hepatocytes from rs8099917 TG and rs12979860 TT donors ($P < 0.001$). However, the effect of IFN was significantly greater, and intrahepatic expression levels of IFN-stimulated genes were significantly higher in mice with the favorable IL28B allele. *Conclusion*: Our data suggest that HCV replication levels and response to IFN are affected by human hepatocyte IL28B single-nucleotide polymorphism genotype and mutations in the ISDR. The mechanism underlying the clinically observed association of wild-type core protein in eradication-favorable host cells should be investigated further. (HEPATOLOGY 2011;54:764-771)

Chronic hepatitis C virus (HCV) infection is the leading cause of cirrhosis, liver failure, and hepatocellular carcinoma.[1,2] Interferon (IFN) is an essential component of therapy for patients with chronic HCV infection, and the most effective currently available therapy is combination therapy with pegylated (PEG)-IFN and ribavirin (RBV).[3-5] Among HCV genotypes, genotype 1 is the most resistant to

Abbreviations: aa, amino acid; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HCV, hepatitis C virus; HSA, human serum albumin; IFN, interferon; IL28B, interleukin-28B; ISDR, interferon-sensitivity–determining region; ISG, interferon-stimulated gene; MxA, myxovirus resistance protein A; NVR, nonvirological response; OAS, oligoadenylate synthetase; PBS, phosphate-buffered saline; PEG, pegylated; PKR, RNA-dependent protein kinase; RBV, ribavirin; RT-PCR, reverse-transcription polymerase chain reaction; SCID, severe combined immunodeficiency; SNP, single-nucleotide polymorphism; SVR, sustained virological response; uPA, urokinase-type plasminogen activator.

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IFN therapy.[6] The limited success of combination therapy for genotype 1 HCV infection is because of the low response rate during therapy and high relapse rate after therapy.[7]

Recent studies have identified both viral and host factors predictive of IFN therapy. Among the viral factors, amino acid (aa) substitutions in the IFN-sensitivity–determining region (ISDR) (nucleotides 2209-2248 or aa positions 237-276 within the NS5A region) are associated with sustained virological response (SVR) after IFN treatment in HCV genotype 1b patients.[8,9] Akuta et al. reported that substitution of aa70 or 91 in the HCV core region are independent predictors of SVR and nonvirological response (NVR).[10-12] Recently, we[13] and another group[14] also reported that wild-type HCV core aa70 and two or more aa substitutions in the ISDR are effective predictors of SVR in patients with HCV genotype 1b.

Among host factors associated with SVR, many common genetic polymorphisms in the human genome have been identified, including single-nucleotide polymorphisms (SNPs).[15-19] More recently, an association between several linked SNPs in the interleukin-28B (IL28B) locus and the effect of combination therapy has been reported.[20-22]

We recently reported that the core aa wild type is significantly more likely to be found in patients with the eradication-favorable IL28B SNP genotype.[23-25] The underlying mechanism of this association as well as the reason for the differential response to therapy by viruses with core aa substitutions are unknown. This is partly because of the presence of HCV quasispecies in human serum samples and the difficulty of performing infection experiments in a small animal model.

The severe combined immunodeficient (SCID) urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice able to develop HCV viremia after injection of serum samples positive for the virus.[26] We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating anti-HCV drugs, such as IFN-alpha and NS3-4A protease inhibitor.[27,28] We have further improved the replacement levels of the human hepatocytes in this mouse model,[29] which enabled us to perform infection experiments more easily because highly repopulated mice (defined as human serum albumin [HSA] levels well above 1 mg/mL) successfully develop viremia more often than poorly repopulated mice.[30] Using this mouse model, we developed a reverse genetics system for HCV.[31,32] This system is useful for studying characteristics of HCV strains with various substitutions of interest, because the effects of quasispecies can be minimized. Furthermore, as there is no adaptive immune system in this mouse model, we are able to examine the replication of HCV and the effect of therapy while avoiding the influence of the immunological response. In the present study, we investigated effects of viral and host factors on HCV infectivity, replication ability, and IFN susceptibility using genetically engineered genotype 1b HCV-infected mice that underwent transplantation with hepatocytes having eradication-favorable or eradication-unfavorable IL28B SNP genotypes.

Table 1. Characteristics of Donors for Transplanted Human Hepatocytes

Donor	A	B	C	D
Sex	Female	Male	Female	Male
Age	10	2	5	2
Ethnic group	Caucasian	Caucasian	African American	Hispanic
rs8099917	TG	TT	TG	TT
rs8109886	AA	CC	AA	CC
rs12979860	TT	CC	TT	CC
rs11882871	GG	AA	GG	AA
rs73930703	TT	CC	TT	CC
rs8107030	AG	AA	AG	AA
rs28416813	GG	CC	GG	CC
rs8103142	CC	TT	CC	TT
rs11881222	GG	AA	GG	AA
rs4803217	AA	CC	AA	CC

Materials and Methods

Animal Treatment. Generation of the $uPA^{+/+}$/$SCID^{+/+}$ mice and transplantation of human hepatocytes were performed as described previously.[29] All animal protocols described in this study were performed in accord 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 HSA, which serve as useful markers of the extent of repopulation, were measured as previously described.[29] Mice underwent transplantation with frozen human hepatocytes obtained from four different human donors (Table 1). Genotyping of IL28B SNPs of human hepatocytes was performed using the Invader assay as described previously.[33,34] We used 1000 IU/g/day of IFN-alpha (Dainippon Sumitomo Pharma Co., Tokyo, Japan) for 2 weeks. This dosage was selected based on a previous report showing that this regimen reduced mouse serum

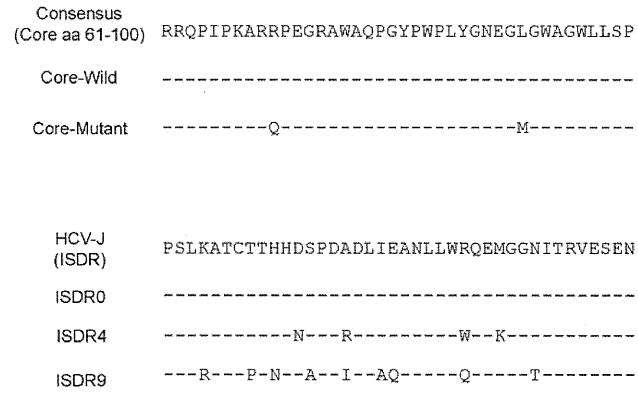

Fig. 1. The aa sequences of infectious genotype 1b HCV clones, Core-Wild, Core-Mutant (substitutions at aa70 and aa91), and ISDR variants (with 0, 4, and 9 substitutions).

HCV RNA levels by 0.5-2 log copies/mL during therapy.[31]

HCV RNA Transcription and Inoculation into Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9, that was obtained from a Japanese patient with severe acute hepatitis (GenBank accession no. AB435162).[32] Ten micrograms of plasmid DNA, linearized by digestion with *Xba*I (Promega, Madison, WI), was transcribed in a 100-μL reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and then analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μL of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.[32] The HCV-KT9 clone has aa substitutions at aa70 and 91 (arginine to glutamine and leucine to methionine, respectively) in the core region (Core-Mutant), compared to the consensus sequence,[10-12] and no aa substitutions in the ISDR (ISDR0),[8] relative to the prototype sequence (HCV-J).[35] Using the original HCV-KT9 clone, we created two additional HCV clones having wild-type core aa70 and 91 (Core-Wild) and four (ISDR4) and nine (ISDR9) aa substitutions in the ISDR, respectively (Fig. 1). To introduce the aa substitutions, site-directed mutagenesis was performed with a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA).

Human Serum Samples. Human serum samples containing a high titer of genotype 1b HCV (2.2×10^6 copies/mL) were obtained from a patient with chronic hepatitis after obtaining written informed consent. Aliquots of serum were stored in liquid nitrogen until use. Core 70 and 91 aas were Gln and Leu, respectively, and only one aa substitution was present in the ISDR. The study protocol involving human subjects conformed to the ethical guidelines of the 1975 Declaration of Helsinki and was approved by the institutional review committee.

Quantitation of HCV RNA and IFN-stimulated gene-expression levels. RNA was extracted from mice serum and liver samples by Sepa Gene RV-R (Sankojunyaku, Tokyo, Japan), dissolved in 8.8 μL of ribonuclease-free H_2O, and reverse transcribed using random primer (Takara Bio Inc., Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, TOYOBO Co., Osaka, Japan) in 20 μL of reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostics, Tokyo, Japan) were performed as previously described.[32] Quantitation of IFN-stimulated genes (ISGs) (myxovirus resistance protein A [MxA], oligoadenylate synthetase [OAS], and RNA-dependent protein kinase [PKR]) was performed using real-time PCR Master Mix (Toyobo, Kyoto, Japan) 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 minute at 95°C, followed by 40 cycles of denaturation at 95°C for 15 seconds and annealing/extension at 60°C for 1 minute. ISG messenger RNA expression levels were expressed relative to the endogenous RNA levels of the housekeeping reference gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Statistical Analysis. The HCV infectious ratio of chimeric mice was assessed using the chi-square test. Mice serum HCV RNA titers, HSA concentrations, and ISG expression levels were compared using the Mann-Whitney U test. A P value less than 0.05 was considered statistically significant.

Results

Influence of aa Substitutions in the HCV Core Region and ISDR on HCV Infectivity and Replication Ability. We investigated the influence of aa substitutions in the core region and ISDR on HCV infectivity and replication ability in mice that underwent transplantation with human hepatocytes obtained from donor A (Table 1). Each 30 μg of *in vitro*–transcribed RNA was inoculated into the livers of mice. Six weeks after inoculation, serum HCV RNA titers increased above the detectable limit (1000 copies/mL) in 11 of 12 (92%) mice infected with Core-Wild-ISDR0 and in 14 of 16 (88%) mice with Core-Mutant-ISDR0 (Fig. 2A). HCV RNA titers in Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice increased to the same levels (Fig. 2B). In contrast, serum HCV

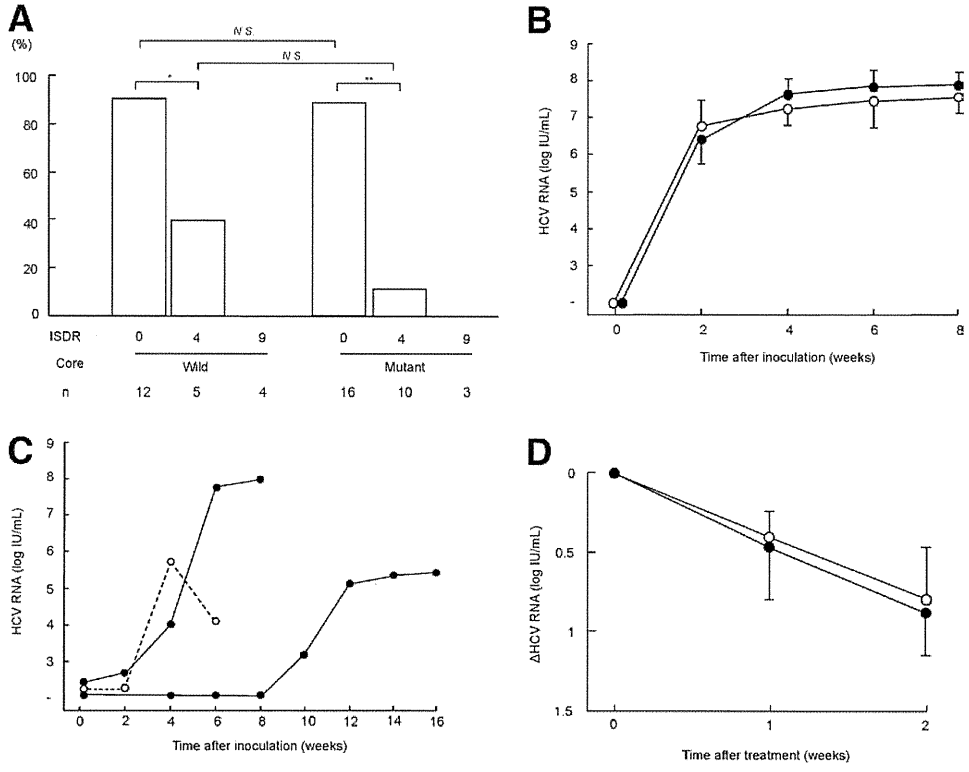

Fig. 2. Infectivity and replication ability of HCV clones. Mice that underwent transplantation with hepatocytes obtained from donor A were inoculated with 30 μg of *in vitro*-transcribed RNAs of indicated clones. (A) Proportion of HCV-infected mice. Infection was defined as serum HCV RNA titer above the detection limit (1000 copies/mL) 6 weeks after inoculation. aa sequences of the core (Wild or Mutant) and number of substitutions in the ISDR are noted below the graph. (B) Time course of serum HCV RNA levels in mice inoculated with either Core-Wild-ISDR0 (closed circles, n = 11) or Core-Mutant-ISDR0 (open circles, n = 14) HCV clones. Data are represented as mean ± standard deviation. (C) Time course of serum HCV RNA levels in two Core-Wild-ISDR4-infected mice (closed circles) and a Core-Mutant-ISDR4-infected mouse (open circles). Serum HCV RNA levels were measured until the mice died. (D) Core-Wild-ISDR0- (closed circles, n = 8) and Core-Mutant-ISDR0 (open circles, n = 4)-infected mice were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Mice serum HCV RNA titers were measured at the indicated times. $*P < 0.05$, $**P < 0.01$; NS, not significant.

RNA titer increased above the detection limit in only two of five (40%) Core-Wild-ISDR4 mice and in only 1 of 10 (10%) Core-Mutant-ISDR4 mice, and the titers in these mice were lower than in mice with ISDR0 (Fig. 2C). HCV RNA titers failed to increase above the detection limit in mice with Core-Wild-ISDR9 and Core-Mutant-ISDR9 (Fig. 2A).

Influence of Core aa Substitutions on the Effect of IFN. To investigate the influence of aa substitutions in the core region on the effect of IFN, Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in a 0.84 ± 0.3 log IU/mL reduction of HCV RNA titer in Core-Wild-ISDR0–infected mice and a 0.79 ± 0.34 log IU/mL reduction in Core-Mutant-ISDR0–infected mice (Fig. 2D).

We also investigated the influence of aa substitutions in the core region on the effect of IFN plus RBV combination therapy. Core-Wild-ISDR0– and Core-Mutant-ISDR0–infected mice were treated with 1000 IU/g of human IFN-alpha and 20 mg/kg of RBV daily for 2 weeks. The treatment resulted in similar HCV RNA reductions in all treated mice. However, as with IFN monotherapy, there were no significant differences in HCV reductions among mice with different aa substitutions in the core region (data not shown). The dose of ribavirin used was relatively small, however, because of the drug's toxicity in mice.

HCV Infectivity, Replication Levels, and IFN Susceptibility by Core aa Substitutions and Genetic Variation in the IL28B Locus. We investigated the influence of IL28B genotypes on HCV infectivity, replication ability, and IFN susceptibility. *In vitro*–transcribed RNA (30 μg) was inoculated into the livers of mice with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) or donor B (rs8099917 TT and rs12979860 CC). Eight weeks after inoculation, serum HCV RNA titers increased above the detection limit in 22 of 25 (88%) mice with hepatocytes from donor A and in 20 of 23 (87%) mice with hepatocytes from donor B (Fig. 3A). Serum HCV RNA levels were

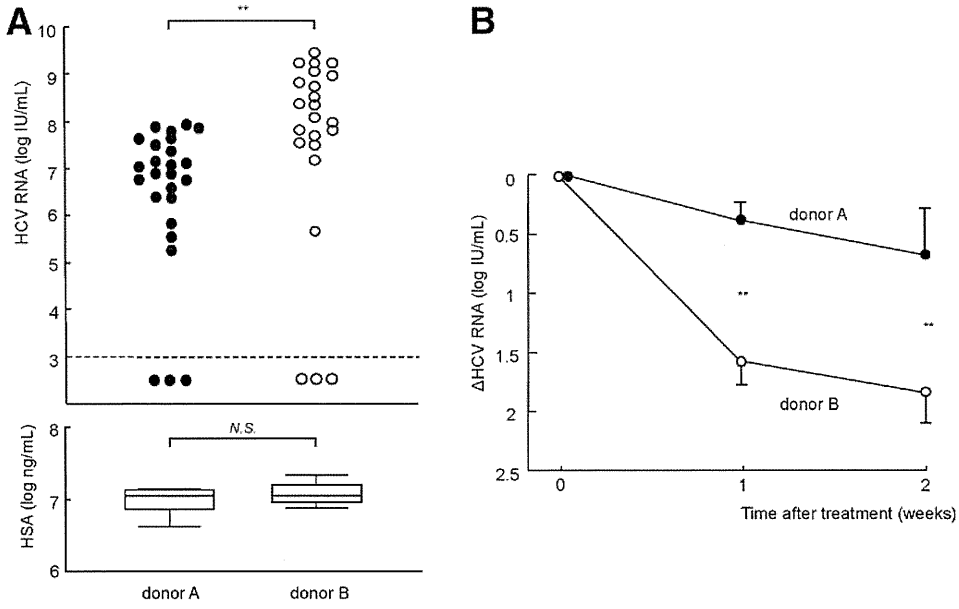

Fig. 3. HCV infectivity, replication ability, and IFN susceptibility in HCV-KT9-injected mice. Mice that underwent transplantation with hepatocytes from donor A (rs8099917 TG and rs12979860 TT) (closed circles, n = 25) or B (rs8099917 TT and rs12979860 CC) (open circles, n = 23) were intrahepatically inoculated with RNA transcribed from either Core-Wild-ISDR0 or Core-Mutant-ISDR0 clones. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. (B) HCV-infected mice with hepatocytes from donor A (closed circles, n = 12) or B (open circles, n = 8) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. Data are represented as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

significantly higher in mice with hepatocytes from donor B than from donor A ($P < 0.001$). HCV-infected mice were treated with 1000 IU/g of human IFN-alpha daily for 2 weeks. The treatment resulted in 0.65 ± 0.38 and 1.84 ± 0.23 log IU/mL reductions in HCV RNA titer in mice with hepatocytes from donors A and B, respectively ($P < 0.01$) (Fig. 3B). Interestingly, despite the higher serum HCV RNA levels, reduction levels of HCV were higher in mice that underwent transplantation with hepatocytes obtained from donor B than in mice that underwent transplantation with hepatocytes obtained from donor A.

To confirm an association between IL28B SNP genotype and HCV RNA titer, we compared HCV RNA titers using mice with hepatocytes from an additional pair of donors with the favorable (donor C) and unfavorable (donor D) SNP genotypes. To determine whether results obtained by clonal infection would be comparable to results obtained using the more natural serum injection, which should have contained more complex viral species, mice were injected with genotype 1b HCV obtained from a human patient with core and ISDR substitutions, as described above. Mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) or donor D (rs8099917 TT and rs12979860 CC) were inoculated intravenously with 10^5 copies of HCV. Eight weeks after inoculation, serum HCV RNA titer increased above the detection limit in 13 of 14 (93%) mice with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) and in 12 of 12 (100%) mice with hepatocytes from donor D (rs8099917 TT and rs12979860 CC) (Fig. 4A). With results similar to those found for the mice inoculated with transcribed HCV RNA, serum HCV RNA levels were significantly higher in mice with hepatocytes from donor D than from donor C ($P < 0.001$), and the effect of IFN was also greater in donor D mice than in donor C mice (Fig. 4B); however, statistical significance using these donors was only achieved at week 1, probably resulting from fluctuation of HCV RNA titers and the small number of animals analyzed.

Expression Levels of ISGs in Mouse Livers. ISG expression levels in mice livers were measured after 2 weeks of IFN treatment (Fig. 4B). MxA, OAS, and PKR levels were significantly higher in mice with human hepatocytes from donor D than from donor C (Fig. 4C).

Discussion

In this study, we investigated the effect of substitutions at core protein aa70 and 91 and within the

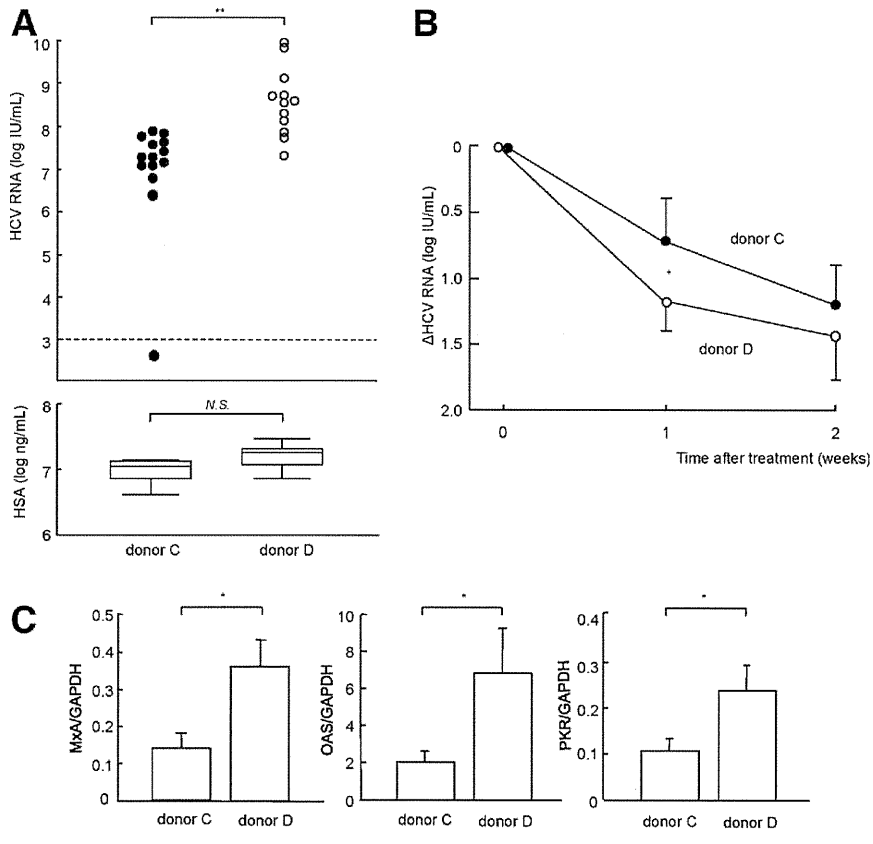

Fig. 4. HCV infectivity, replication ability, and IFN susceptibility in HCV-infected mice. Mice that underwent transplantation with hepatocytes from donor C (rs8099917 TG and rs12979860 TT) (closes circles, n = 14) or D (rs8099917 TT and rs12979860 CC) (open circles, n = 12) were intravenously injected with HCV-infected patient serum samples. (A) Eight weeks after infection, serum HCV RNA titers (upper panel) and HSA concentrations (lower panel) were measured. The horizontal dotted line indicates the HCV RNA titer detection limit (1000 copies/mL). In these box-and-whisker plots, lines within the boxes represent median values; the upper and lower lines of the boxes represent the 75th and 25th percentiles, respectively; the upper and lower bars outside the boxes represent the 90th and 10th percentiles, respectively. HCV-infected mice with hepatocytes from donor C (closed circles, n = 5) or D (open circles, n = 4) were treated daily with 1000 IU/g/day of IFN-alpha for 2 weeks. (B) Changes in mice serum HCV RNA titers measured after 1 and 2 weeks are shown. (C) Intrahepatic ISG expression levels in the IFN-treated mice with donor C (n = 4) or D (n = 3) were measured and expressed relative to GAPDH messenger RNA. Data are reported as mean ± standard deviation. $*P < 0.05$, $**P < 0.01$; NS, not significant.

ISDR, which have been reported to be associated with the outcome of IFN plus ribavirin combination therapy.[8-14] Clones with core aa70 and 91 substitutions showed comparable infection and replication abilities, whereas clones with substitutions in the ISDR showed reduced infectivity and replication rates. It has been reported that patients infected with HCV strains with multiple substitutions in the ISDR have lower viral titers than those with wild-type ISDR, and that these patients respond well to IFN therapy.[8,9] We showed, in this study, that infectivity and replication ability of HCV are apparently impaired in ISDR mutants (Fig. 2A,C). This may explain, at least partially, the better effect of IFN therapy in patients with multiple ISDR mutations. However, why aa substitutions in this particular region are associated with the effect of IFN still remains to be elucidated. In contrast, aa substitutions in the core, which more profoundly affect the outcome of combination therapy,[10-13] did not influence the infectivity and replication ability of the virus (Fig. 2A,B). This suggests that aa substitutions in this region affect response to therapy in a way that is independent of the replication level of the virus. A recent report by Eng et al.[36] showed that a mutation in core aa91 results in the production of minicore protein, which might alter the effect of IFN. The presence of minicore protein and its effect on IFN therapy should be further investigated using the chimeric mouse model.

In contrast to these viral substitutions, host IL28B genotype significantly affected viral replication levels (Figs. 3A and 4A). Curiously, replication levels of the virus are higher in mice with human hepatocytes from donors with rs8099917 TT and rs12979860 CC genotypes, even though these genotypes are associated with successful response to the therapy.[20-22] This result is consistent with clinical observation of higher viral loads in patients with the rs12979860 CC genotype.[20] The favorable IL28B genotype is associated not only with successful response to IFN treatment, but also to spontaneous clearance of the virus.[37,38] However, the incidence of HCV infection was similar in mice with hepatocytes from donors with rs8099917 TT and rs8099917 TG (Figs. 3A and 4A), suggesting that spontaneous clearance was rare. The fact that our animal model was immunodeficient suggests that spontaneous clearance of HCV might require the involvement of the adaptive immune system. The wild-type core protein, aa70, is reported to be found more often in patients with the rs8099917 TT genotype,[23,24] even though patients with this genotype are more likely to be able to eradicate the virus without therapy during

the natural course of infection.[37,38] These data suggest that core aa70 wild-type virus can be eradicated more easily in the natural course of infection, especially in patients with rs8099917 TT or rs12979860 CC genotypes; but once the infection is established, core aa70 wild type replicates more effectively than core aa70 mutant strains.

The effect of IFN on reduction of the virus did not differ between core aa70 wild-type and mutant strains, which showed similar replication levels (Fig. 2D). This is in contrast to clinical observations that the effect of therapy on viral reduction is more prominent in patients with wild-type core protein.[13,25] One of the differences between the mouse model and human patients is term of infection. Long-term HCV infection results in alteration of lipid metabolism and accumulation of lipids in hepatocytes.[39] Patients with fatty change of the liver often fail to respond to therapy.[40] We observed no severe fatty change in mouse livers, suggesting that such long-term change might be absent in this mouse model (data not shown).

On the other hand, the effect of IFN was significantly greater in mice with hepatocytes with the eradication-favorable IL28B genotype (rs8099917 TT and rs12979860 CC) (Figs. 3B and 4B), despite the higher replication rate of the virus. This suggests that the IL28B genotype affects the outcome of therapy based on a different mechanism than viral replication. Because of strong linkage disequilibrium, genotypes of the SNPs around the two IL28B landmark SNPs (rs8099917 and rs12979860) were identical between donors A and C as well as between B and D (data not shown). Further study using human hepatocytes with various IL28B SNP genotypes will identify a primary SNP that directly affects the outcome of therapy. Response to IFN was associated with higher expression levels of ISGs, including MxA, OAS, and PKR (Fig. 4C). This is in agreement with previous studies showing that SVR is associated with stronger induction of ISG expression.[41] However, we observed no statistically significant differences in ISG expression levels from the IL28B SNP genotype before therapy (data not shown). This may result from lower ISG expression levels before therapy and the relatively small number of mice examined. Because there is no adaptive immune system in this mouse model, such differences primarily involve individual hepatocytes, although whether the presence of immune cells enhances this difference should be investigated further.

In summary, we demonstrated that viral infectivity and replication ability are associated with hepatocyte IL28B genotype and are not associated with viral substitutions in the core protein or ISDR. Understanding the mechanism underlying the higher, more prolonged expression of antiviral genes in response-favorable hepatocytes will help us to develop improved therapeutic regimens to eradicate HCV more effectively.

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References

1. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis, and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. HEPATOLOGY 1990;12:671-675.
2. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hürter D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.
3. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Gonçales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for patients with chronic hepatitis C virus infection. N Engl J Med 2002;347:975-982.
4. Hoofnagle JH, Ghany MG, Kleiner DE, Doo E, Heller T, Promrat K, et al. Maintenance therapy with ribavirin in patients with chronic hepatitis C who fail to respond to combination therapy with interferon alfa and ribavirin. HEPATOLOGY 2003;38:66-74.
5. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958-965.
6. Fried MW, Shiffman M, Sterling RK, Weinstein J, Crippin J, Garcia G, et al. A multicenter, randomized trial of daily high-dose interferon-alfa 2b for the treatment of chronic hepatitis C: pretreatment stratification by viral burden and genotype. Am J Gastroenterol 2000;95:3225-3229.
7. McHutchison JG, Gordon SC, Schiff ER, Shiffman ML, Lee WM, Rustgi VK, et al. Interferon alfa-2b alone or in combination with ribavirin as initial treatment for chronic hepatitis C. N Engl J Med 1998;339:1485-1492.
8. Enomoto N, Sakuma I, Asahina Y, Kurosaki M, Murakami T, Yamamoto C, et al. Mutations in the nonstructural protein 5A gene and response to interferon in patients with chronic hepatitis C virus 1b infection. N Engl J Med 1996;334:77-81.
9. Chayama K, Tsubota A, Kobayashi M, Okamoto K, Hashimoto M, Miyano Y, et al. Pretreatment virus load and multiple amino acid substitutions in the interferon sensitivity-determining region predict the outcome of interferon treatment in patients with chronic genotype 1b hepatitis C virus infection. HEPATOLOGY 1997;25:745-749.
10. Akuta N, Suzuki F, Sezaki H, Suzuki Y, Hosaka T, Someya T, et al. Association of amino acid substitution pattern in core protein of hepatitis C virus genotype 1b high viral load and non-virological response to interferon-ribavirin combination therapy. Intervirology 2005;48:372-380.
11. Akuta N, Suzuki F, Kawamura Y, Yatsuji H, Sezaki H, Suzuki Y, et al. Predictive factors of early and sustained responses to peginterferon plus ribavirin combination therapy in Japanese patients infected with hepatitis C virus genotype 1b: amino acid substitutions in the core region and low-density lipoprotein cholesterol levels. J Hepatol 2007;46:403-410.
12. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. A matched case-controlled study of 48 and 72 weeks of peginterferon plus ribavirin combination therapy in patients infected with HCV genotype 1b in Japan: amino acid substitutions in HCV core region as predictor of sustained virological response. J Med Virol 2009;81:452-458.

13. Mori N, Imamura M, Kawakami Y, Saneto H, Kawaoka S, Takaki S, et al. Randomized trial of high-dose interferon-alpha-2b combined with ribavirin in patients with chronic hepatitis C: correlation between amino acid substitutions in the core/NS5A region and virological response to interferon therapy. J Med Virol 2009;81:640-649.
14. Okanoue T, Itoh Y, Hashimoto H, Yasui K, Minami M, Takehara T, et al. Predictive values of amino acid sequences of the core and NS5A regions in antiviral therapy for hepatitis C: a Japanese multi-center study. J Gastroenterol 2009;44:952-963.
15. Welzel TM, Morgan TR, Bonkovsky HL, Naishadham D, Pfeiffer RM, Wright EC, et al. Variants in interferon-alpha pathway genes and response to pegylated interferon-alpha2a plus ribavirin for treatment of chronic hepatitis C virus infection in the hepatitis C antiviral long-term treatment against cirrhosis trial. HEPATOLOGY 2009;49:1847-1858.
16. Hijikata M, Ohta Y, Mishiro S. Identification of a single nucleotide polymorphism in the MxA gene promoter (G/T at nt -88) correlated with the response of hepatitis C patients to interferon. Intervirology 2000;43:124-127.
17. Knapp S, Yee LJ, Frodsham AJ, Hennig BJ, Hellier S, Zhang L, et al. Polymorphisms in interferon-induced genes and the outcome of hepatitis C virus infection: roles of MxA, OAS-1, and PKR. Genes Immun 2003;4:411-419.
18. Matsuyama N, Mishiro S, Sugimoto M, Furuichi Y, Hashimoto M, Hijikata M, et al. The dinucleotide microsatellite polymorphism of the IFNAR1 gene promoter correlates with responsiveness of hepatitis C patients to interferon. Hepatol Res 2003;25:221-225.
19. Naito M, Matsui A, Inao M, Nagoshi S, Nagano M, Ito N, et al. SNPs in the promoter region of the osteopontin gene as a marker predicting the efficacy of interferon-based therapies in patients with chronic hepatitis C. J Gastroenterol 2005;40:381-388.
20. Ge D, Fellay J, Thompson AJ, Simon JS, Shianna KV, Urban TJ, et al. Genetic variation in IL28B predicts hepatitis C treatment-induced viral clearance. Nature 2009;461:399-401.
21. Suppiah V, Moldovan M, Ahlenstiel G, Berg T, Weltman M, Abate ML, et al. IL28B is associated with response to chronic hepatitis C interferon-alpha and ribavirin therapy. Nat Genet 2009;41:1100-1104.
22. Tanaka Y, Nishida N, Sugiyama M, Kurosaki M, Matsuura K, Sakamoto N, et al. Genome-wide association of IL28B with response to pegylated interferon-alpha and ribavirin therapy for chronic hepatitis C. Nat Genet 2009;41:1105-1109.
23. Abe H, Ochi H, Maekawa T, Hayes CN, Tsuge M, Miki D, et al. Common variation of IL28 affects gamma-GTP levels and inflammation of the liver in chronically infected hepatitis C virus patients. J Hepatol 2010;53:439-443.
24. Akuta N, Suzuki F, Hirakawa M, Kawamura Y, Yatsuji H, Sezaki H, et al. Amino acid substitution in hepatitis C virus core region and genetic variation near the interleukin 28B gene predict viral response to telaprevir with peginterferon and ribavirin. HEPATOLOGY 2010;52:421-429.
25. Hayes CN, Kobayashi M, Akuta N, Suzuki F, Kumada H, Abe H, et al. HCV substitutions and IL28B polymorphisms on outcome of peg-interferon plus ribavirin combination therapy. Gut 2011;60:261-267.
26. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927-933.
27. Kneteman NM, Weiner AJ, O'Connell J, Collett M, Gao T, Aukerman L, et al. Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. HEPATOLOGY 2006;43:1346-1353.
28. Kamiya N, Iwao E, Hiraga N, Tsuge M, Imamura M, Takahashi S, et al. Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3-4A protease inhibitor. J Gen Virol 2010;91:1668-1677.
29. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901-912.
30. Vanwolleghem T, Libbrecht L, Hansen BE, Desombere I, Roskams T, Meuleman P, et al. Factors determining successful engraftment of hepatocytes and susceptibility to hepatitis B and C virus infection in uPA-SCID mice. J Hepatol 2010;53:468-476.
31. Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. FEBS Letts 2007;581:1983-1987.
32. Kimura T, Imamura M, Hiraga N, Hatakeyama T, Miki D, Noguchi C, et al. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. J Gen Virol 2008;89:2108-2113.
33. Ohnishi Y, Tanaka T, Ozaki K, Yamada R, Suzuki H, Nakamura Y. A high-throughput SNP typing system for genome-wide association studies. J Hum Genet 2001;46:471-477.
34. Suzuki A, Yamada R, Chang X, Tokuhiro S, Sawada T, Suzuki M, et al. Functional haplotypes of PADI4, encoding citrullinating enzyme peptidylarginine deiminase 4, are associated with rheumatoid arthritis. Nat Genet 2003;34:395-402.
35. Kato T, Matsumura T, Heller T, Saito S, Sapp RK, Murthy K, et al. Production of infectious hepatitis C virus of various genotypes in cell cultures. J Virol 2007;81:4405-4411.
36. Eng FJ, Walewski JL, Klepper AL, Fishman SL, Desai SM, McMullan LK, et al. Internal initiation stimulates production of p8 minicore, a member of a newly discovered family of hepatitis C virus core protein isoforms. J Virol 2009;83:3104-3114.
37. Thomas DL, Thio CL, Martin MP, Qi Y, Ge D, O'Huigin C, et al. Genetic variation in IL28B and spontaneous clearance of hepatitis C virus. Nature 2009;461:798-801.
38. Montes-Cano MA, García-Lozano JR, Abad-Molina C, Romero-Gómez M, Barroso N, Aguilar-Reina J, et al. Interleukin-28B genetic variants and hepatitis virus infection by different viral genotypes. HEPATOLOGY 2010;52:33-37.
39. Lonardo A, Adinolfi LE, Loria P, Carulli N, Ruggiero G, Day CP. Steatosis and hepatitis C virus: mechanisms and significance for hepatic and extrahepatic disease. Gastroenterology 2004;126:586-597.
40. Poynard T, Ratziu V, McHutchison J, Manns M, Goodman Z, Zeuzem S, et al. Effect of treatment with peginterferon or interferon alfa-2b and ribavirin on steatosis in patients infected with hepatitis C. HEPATOLOGY 2003;38:75-85.
41. Sarasin-Filipowicz M, Oakeley EJ, Duong FH, Christen V, Terracciano L, Filipowicz W, et al. Interferon signaling and treatment outcome in chronic hepatitis C. Proc Natl Acad Sci U S A 2008;105:7034-7039.

Rapid Emergence of Telaprevir Resistant Hepatitis C Virus Strain from Wildtype Clone *In Vivo*

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Telaprevir is a potent inhibitor of hepatitis C virus (HCV) NS3-4A protease. However, the emergence of drug-resistant strains during therapy is a serious problem, and the susceptibility of resistant strains to interferon (IFN), as well as the details of the emergence of mutant strains *in vivo*, is not known. We previously established an infectious model of HCV using human hepatocyte chimeric mice. Using this system we investigated the biological properties and mode of emergence of mutants by ultra-deep sequencing technology. Chimeric mice were injected with serum samples obtained from a patient who had developed viral breakthrough during telaprevir monotherapy with strong selection for resistance mutations (A156F [92.6%]). Mice infected with the resistant strain (A156F [99.9%]) developed only low-level viremia and the virus was successfully eliminated with interferon therapy. As observed in patients, telaprevir monotherapy in viremic mice resulted in breakthrough, with selection for mutations that confer resistance to telaprevir (e.g., a high frequency of V36A [52.2%]). Mice were injected intrahepatically with HCV genotype 1b clone KT-9 with or without an introduced resistance mutation, A156S, in the NS3 region, and treated with telaprevir. Mice infected with the A156S strain developed lower-level viremia compared to the wildtype strain but showed strong resistance to telaprevir treatment. Although mice injected with wildtype HCV showed a rapid decline in viremia at the beginning of therapy, a high frequency (11%) of telaprevir-resistant NS3 V36A variants emerged 2 weeks after the start of treatment. *Conclusion:* Using deep sequencing technology and a genetically engineered HCV infection system, we showed that the rapid emergence of telaprevir-resistant HCV was induced by mutation from the wildtype strain of HCV *in vivo*. (HEPATOLOGY 2011;54:781-788)

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 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 loads.[3-5] Given the low

Abbreviations: HCV, hepatitis C virus; HSA, human serum albumin; PEG-IFN, peg-interferon; RBV, ribavirin; RT-PCR, reverse transcript-polymerase chain reaction; SCID, severe combined immunodeficiency; SVR, sustained viral response; uPA, urokinase-type plasminogen activator.

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effectiveness of the current therapy, many molecules have been screened for antiviral activity against HCV for use in development of novel anti-HCV therapies. A number of new selective inhibitors of HCV proteins, the so-called STAT-C (specifically targeted antiviral therapy for HCV) inhibitors, are currently under development. Telaprevir is a reversible, selective, specific inhibitor of the HCV NS3-4A protease that has shown potent antiviral activity in HCV replicon assays.[6] Although the antiviral effect of telaprevir is quite potent, monotherapy using these drugs results in rapid emergence of drug-resistant strains.[7,8] Accordingly, these drugs are used in combination with pegylated-IFN and ribavirin for chronic hepatitis C patients. Because the HCV virus replicates rapidly and RNA polymerase lacks a proofreading system, HCV viral quasispecies can emerge *de novo*, and some of these variants may confer resistance. Although a resistant variant is initially present at low frequency, it may quickly emerge as the dominant species during antiviral treatment.[9,10] Resistant clones against HCV NS3-4A protease inhibitors have reportedly been induced in replicon systems.

The immunodeficient urokinase-type plasminogen activator (uPA) mouse permits repopulation of the liver with human hepatocytes, resulting in human hepatocyte chimeric mice that are able to develop HCV viremia after injection of serum samples positive for the virus.[11] We and other groups have reported that the human hepatocyte chimeric mouse is useful for evaluating the effect of NS3-4A protease inhibitor.[12,13] Using this mouse model, we developed a reverse genetics systems for HCV.[14,15] This system is useful to study characteristics of HCV strains with various substitutions of interest because the confounding effects of quasispecies can be minimized. Using ultra-deep sequencing technology, we demonstrate the rapid emergence of telaprevir resistance in HCV as a result of mutation from wildtype strain using genetically engineered HCV-infected human hepatocyte chimeric mice.

Materials and Methods

Animal Treatment. Generation of the uPA$^{+/+}$/SCID$^{+/+}$ mice and transplantation of human hepatocytes were performed as described recently by our group.[16] All mice were transplanted with frozen human hepatocytes obtained from the same donor. Mice received humane care and all animal protocols were performed in accordance with the guidelines of the local committee for animal experiments. Infection, extraction of serum samples, and sacrifice were performed under ether anesthesia. Mice were injected either intravenously with HCV-positive human serum samples or intrahepatically with *in vitro*-transcribed genotype 1b HCV RNA. HCV-infected mice were administered either perorally with 200-300 mg/kg of telaprevir (VX950; MP424; Mitsubishi Tanabe Pharma, Osaka, Japan) twice a day or intramuscularly with 1,500 IU/g of IFN-alpha (Dainippon Sumitomo Pharma, Tokyo). The telaprevir dose was determined in a previous study in which this dosage range was found to yield serum concentrations equivalent to treated human patients.[13]

Human Serum Samples. After obtaining written informed consent, human serum samples containing genotype 1b HCV were obtained from two patients with chronic hepatitis. The individual serum samples were divided into aliquots and stored separately in liquid nitrogen until use. The study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved *a priori* by the Institutional Review Committee.

HCV RNA Transcription and Inoculation into Chimeric Mice. We have previously established an infectious genotype 1b HCV clone HCV-KT9 derived from a Japanese patient with severe acute hepatitis (GenBank access. no. AB435162).[15] We cloned this HCV complementary DNA (cDNA) into plasmid pBR322 under a T7 RNA promoter to create the plasmid pHCV-KT9. Ten μg of plasmid DNA, linearized by *Xba*I (Promega, Madison, WI) digestion, were transcribed in a 100 μL reaction volume with T7 RNA polymerase (Promega) at 37°C for 2 hours and analyzed by agarose gel electrophoresis. Each transcription mixture was diluted with 400 μL of phosphate-buffered saline (PBS) and injected into the livers of chimeric mice.[15] The QuikChange site-directed mutagenesis kit (Stratagene, Foster City, CA) was used to introduce a substitution at amino acid 156 of the NS3 region (A156S).

RNA Extraction and Amplification. RNA was extracted from serum samples by Sepa Gene RV-R (Sankojunyaku, Tokyo), dissolved in 8.8 μL RNase-free H_2O, and reverse transcribed using a random primer (Takara Bio, Shiga, Japan) and M-MLV reverse transcriptase (ReverTra Ace, Toyobo, Osaka, Japan) in a 20-μL reaction mixture according to the instructions provided by the manufacturer. Nested polymerase chain reaction (PCR) and quantitation of HCV by Light Cycler (Roche Diagnostic, Japan, Tokyo) were performed as reported.[15]

Ultra-Deep Sequencing. We adapted multiplex sequencing-by-synthesis to simultaneously sequence

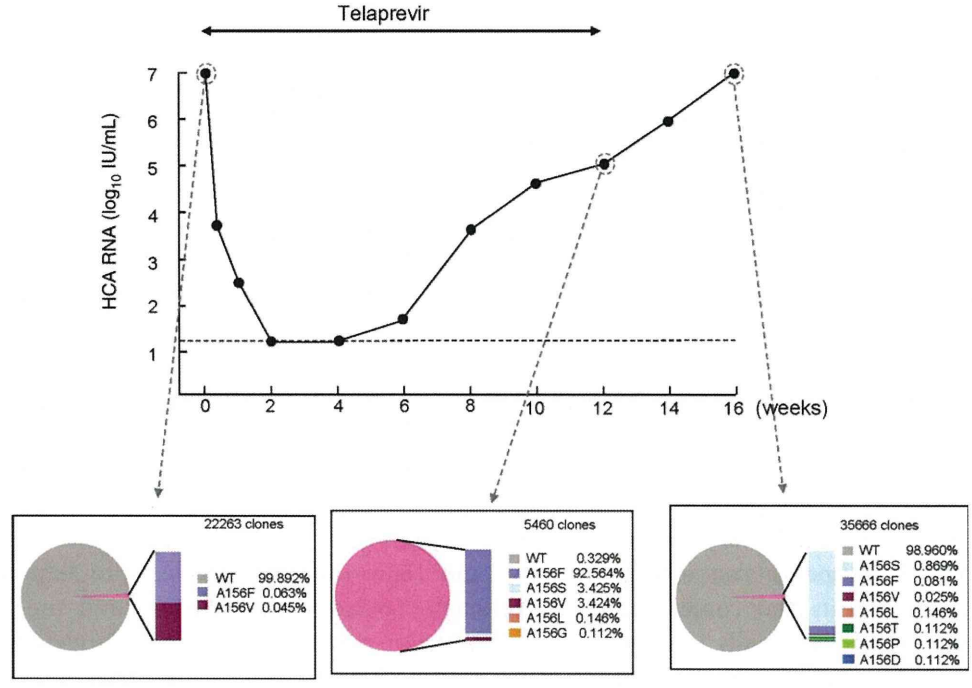

Fig. 1. Changes in serum HCV RNA levels in a telaprevir-treated chronic hepatitis C patient. A 55-year-old woman infected with genotype 1b HCV was treated with 750 mg of telaprevir every 8 hours for 12 weeks. Serum HCV RNA (upper panel) and the amino acid (aa) frequencies at aa156 in the HCV NS3 region by ultra-deep sequencing at the indicated times are shown. The horizontal dotted line indicates the detectable limit (1.2 log copy/mL).

multiple genomes using the Illumina Genome Analyzer. Briefly, cDNA was fragmented using sonication and the resultant fragment distribution was assessed using the Agilent BioAnalyzer 2100 platform. A library was prepared using the Multiplexing Sample Preparation Kit (Illumina, CA). Imaging analysis and base calling were performed using Illumina Pipeline software with default settings.[17-23] The N-terminal 543 nucleotides of NS3 protease were analyzed. This technique revealed an average coverage depth of over 1,000 sequence reads per basepair in the unique regions of the genome. Read mapping to a reference sequence was performed using Bowtie.[24] Because of the short 36 nucleotide read length, mapping hypervariable regions with multiple closely spaced variants against a reference sequence yields poor coverage. Therefore, common variants were identified by relaxing the mismatch settings as well as using *de novo* assembly using ABySS.[25] Multiple alternative reference sequences were included to improve coverage in variable regions. Codon counts were merged and analyzed using R v. 2.12.

Results

Emergence of a Telaprevir-Resistant Variant in a Hepatitis C Patient Treated with Telaprevir and Analysis of the A156F Mutation. A 55-year-old woman infected with genotype 1b HCV was treated with 750 mg of telaprevir every 8 hours for 12 weeks (Fig. 1). After 1 weeks of treatment, serum HCV RNA titer decreased below the detectable limit (1.2 log copy/mL). However, HCV RNA titer became positive by week 4. By week 12, HCV RNA titer had increased to 4.8 log copy/mL and telaprevir treatment was discontinued. Because direct sequence analysis showed an A156F mutation in the NS3 region in the serum samples at 12 weeks, we performed ultra-deep sequence analysis and confirmed the high frequency (92.5%) of A156F mutation. Four weeks after cessation of treatment (at 16 weeks), sequence analysis revealed that the major strain had reverted to wildtype (99%). To analyze the replication ability and the susceptibility of the A156F mutation to telaprevir, 100 μL serum samples containing 10^4 copies of HCV obtained at week 12 were injected into human hepatocyte chimeric mice. Two wildtype HCV-inoculated mice became positive for HCV RNA 2 weeks after inoculation and serum HCV RNA titer increased to high levels (7.6 and 7.8 log copy/mL, respectively) at 6 weeks after inoculation (Fig. 2). In contrast to wildtype HCV-infected mice, a mouse inoculated with serum containing the A156F mutant developed measurable viremia at 4 weeks postinoculation, although serum HCV RNA titer remained low at 6 weeks (5.2 log copy/mL). Eight weeks after inoculation ultra-deep sequence analysis showed a high frequency (99.9%) of A156F mutation. From this point the mouse was administrated 200 mg/kg of telaprevir perorally twice a day for 4 weeks. However, this treatment resulted in no reduction in serum HCV RNA level. During the observation period the A156F mutation remained at

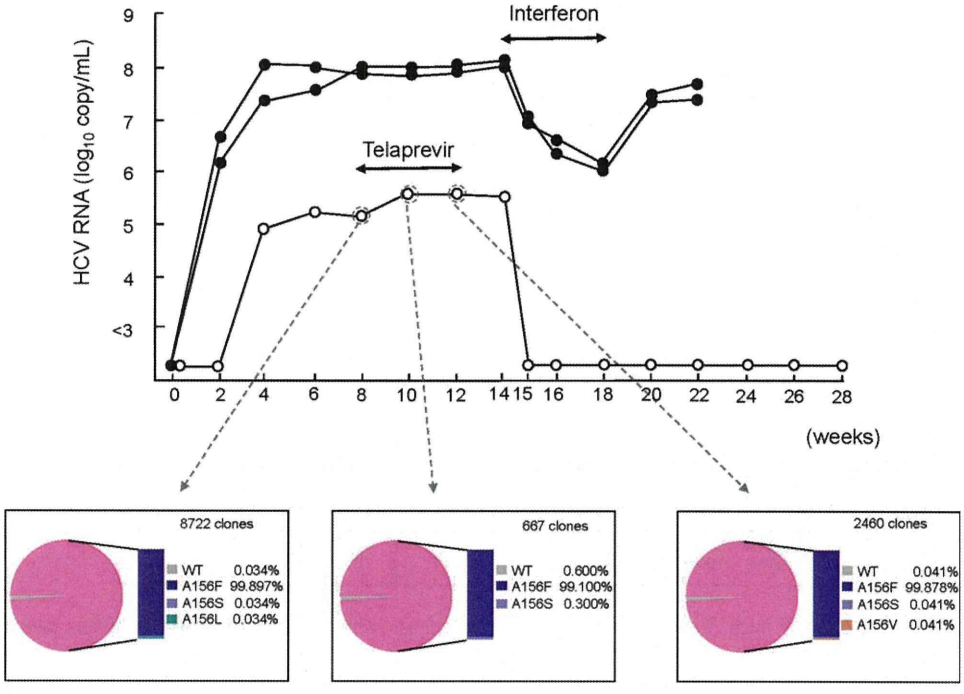

Fig. 2. Changes in serum virus titers in HCV-infected mice. Mice were injected with either wildtype (closed circles) or A156F-mutated HCV serum samples (obtained from an HCV-infected patient who received telaprevir monotherapy for 12 weeks; see Fig. 1) (open circles). Six weeks after injection the A156F mutant mouse was treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks and injected intramuscularly with 1,500 IU/g/day of interferon-alpha for 4 weeks. Serum HCV RNA (upper panel) and amino acid (aa) frequencies at aa156 in the HCV NS3 region by ultra-deep sequencing at the indicated times are shown.

high frequency (>99%). To analyze the susceptibility of the A156F mutation to IFN, wildtype or A156F-mutated HCV-infected mice were treated with 1,500 IU/g/day of IFN-alpha for 4 weeks. Treatment resulted in only a two log reduction in HCV RNA level in wildtype HCV-infected mice. In contrast, serum HCV RNA titer decreased below the detectable limit 1 week after treatment in an A156F-infected mouse. Ten weeks after cessation of IFN-treatment (at week 28), HCV RNA in the mouse serum remained undetectable, suggesting that HCV RNA was eliminated. These results demonstrate that the A156F variant is associated with telaprevir-resistance, but the mutant has low replication ability and a high susceptibility to IFN.

Effect of Telaprevir on HCV-Infected Mice and Sequence Analysis of NS3 Region. Next we investigated the effect of telaprevir on wildtype HCV-infected mice. Two chimeric mice were inoculated intravenously with serum samples containing 10^5 copies of HCV obtained from an HCV-positive patient (Fig. 3). Six weeks after inoculation both mice were administered 200 mg/kg of telaprevir perorally twice a day for 4 weeks. Serum HCV RNA titer in both mice rapidly decreased; however, in one of the mice HCV RNA titer increased again 3 weeks after the start of treatment. Ultra-deep sequence analysis of the NS3 region showed that following the start of telaprevir administration the frequency of the V36A mutation increased from 18% at 2 weeks to 52% at 4 weeks, at which point it was accompanied by an increase in the HCV RNA titer. Two weeks after cessation of telaprevir treatment (at week 12), ultra-deep sequence analysis revealed that the frequency of the V36A mutant had decreased to 13% and the frequency of the wildtype HCV had increased to 84%, although the HCV RNA titer increased only slightly.

Intrahepatic Injection of HCV-KT9-Wild RNA and KT9-NS3-A156S RNA into Human Hepatocyte Chimeric Mice. We previously established an infectious genotype 1b HCV clone, HCV-KT9 (HCV-KT9-wild).[15] We created a telaprevir-resistant HCV clone by introducing an A156S amino acid substitution in the NS3 region of HCV-KT9 (KT9-NS3-A156S) (Fig. 4A). Using wildtype and telaprevir-resistant clones we investigated the replication ability *in vivo*. Mice were injected intrahepatically with 30 μg of *in vitro*-transcribed HCV-KT9-wild RNA or KT9-NS3-A156S RNA. Mice injected with HCV-KT9-wild developed measurable viremia at 2 weeks postinoculation and by 4 weeks postinoculation HCV RNA had reached 10^7 copy/mL (Fig. 4B). On the other hand, mice injected with KT9-NS3-A156S developed measurable viremia at 4 weeks postinoculation but maintained only low levels of viremia. These results suggest that the telaprevir-resistant HCV clone has a lowered replication ability compared to the wildtype HCV clone *in vivo*.

Treatment with Telaprevir and Analysis of Mutagenesis in Mice. Two mice infected with HCV-KT9-wild and one mouse infected with KT9-NS3-A156S were treated with 200 mg/kg of telaprevir twice a day for 2 weeks (Fig. 5A), resulting in 1.4 and 2.7 log

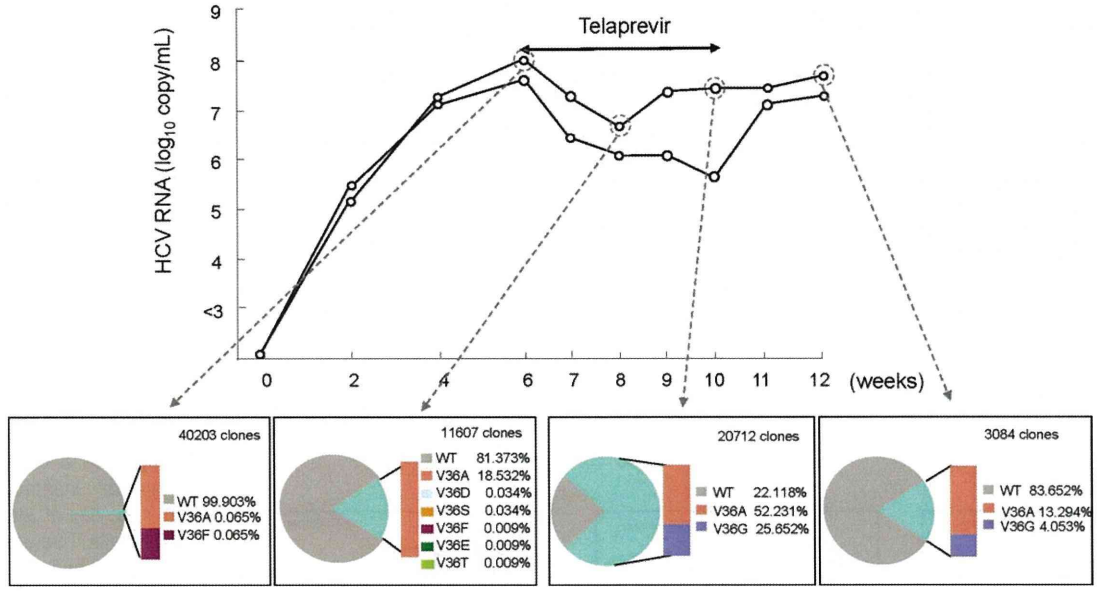

Fig. 3. Treatment with telaprevir in wildtype HCV-infected mice. Two mice were injected intravenously with 50 μL of HCV-positive human serum samples. Six weeks after HCV injection mice were treated with 200 mg/kg of telaprevir orally twice a day for 4 weeks. Serum HCV RNA (upper panel) and amino acid (aa) frequencies at aa36 in the HCV NS3 region by ultra-deep sequencing at the indicated times are shown.

reductions in HCV RNA level in the two wildtype HCV-infected mice. In contrast, only a 0.6 log reduction was observed in the KT9-NS3-A156S-infected mouse. These results demonstrate that our human hepatocyte chimeric mouse model infected with *in vitro*-transcribed HCV RNA provides an effective system for analysis of the susceptibility of HCV mutants to antiviral drugs. Interestingly, ultra-deep sequence analysis showed a rapid emergence of a V36A variant in the NS3 region in mouse serum 2 weeks after treatment (Fig. 5B). Four weeks after cessation of treatment (at week 6) the frequency of the V36A variant had decreased. Mice were then treated with 300 mg/kg of telaprevir twice a day for 4 weeks, which resulted in an elevated frequency of V36A variants at 1 (at week 7, 5.4%) and 4 weeks (at 10 week, 41.8%) after treatment and no reduction in serum HCV RNA level. These results suggest that telaprevir-resistant mutations emerged *de novo* from the wildtype strain of HCV, presumably through error-prone replication and potent selection for telaprevir escape mutants. During the telaprevir treatment period no increases of HCV RNA titers in these mice were observed, probably due to the low frequency of the resistant strain.

Discussion

Telaprevir is a peptidomimetic inhibitor of the NS3-4A serine protease that is currently undergoing clinical evaluation. Despite its effectiveness against HCV, some patients have shown a rapid viral breakthrough during the first 14 days of treatment.[26] Population sequencing of the viral NS3 region identified a number of mutations near the NS3 protease catalytic

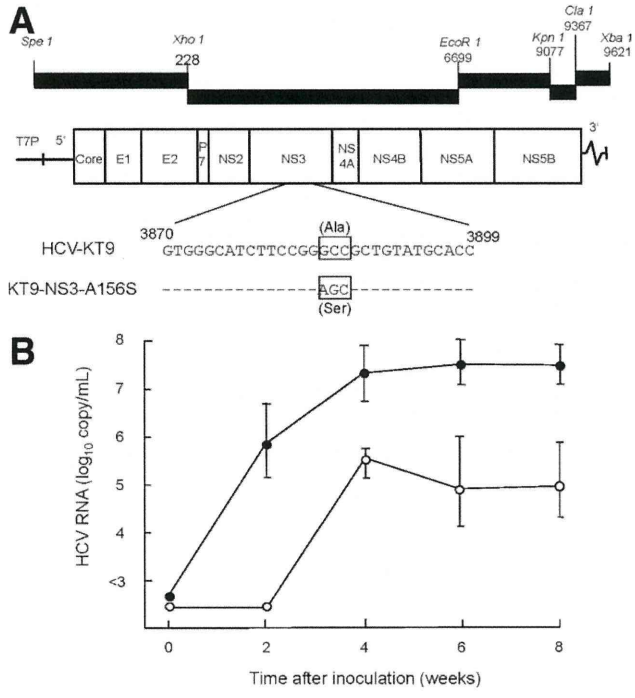

Fig. 4. Intrahepatic injection of *in vitro* transcribed HCV-KT9 RNA and KT9-NS3-A156S RNA into human hepatocyte chimeric mice. (A) The schematic of infectious genotype 1b HCV clones, HCV-KT9 and KT9-NS3-A156S. Boxes indicate codons at amino acid 156 in HCV NS3 region. Ala, alanine; Ser, serine. (B) Changes in serum levels of HCV RNA in mice intrahepatically injected with either HCV-KT9 RNA (closed circles) or KT9-NS3-A156S RNA (open circles). Data are represented as the mean ± SD of three mice.

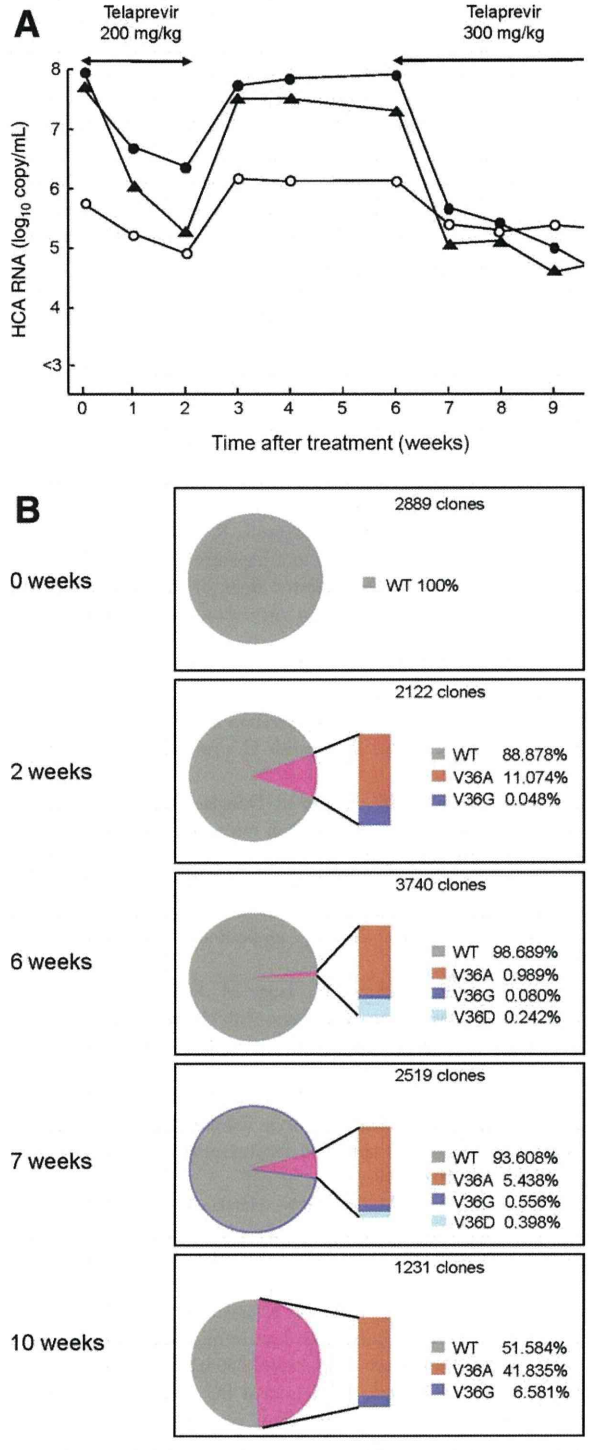

Fig. 5. The effect of telaprevir on mice infected with *in vitro*-transcribed HCV. Mice were injected with *in vitro*-transcribed HCV-KT9 RNA (closed circles and closed triangles) or KT9-NS3-A156S RNA (open circles). Six weeks after HCV RNA injection, mice were treated perorally with 200 mg/kg of telaprevir twice a day for 2 weeks. Four weeks after cessation of treatment mice were treated with 300 mg/kg of telaprevir twice a day for 4 weeks. (A) Mice serum HCV RNA titers at the indicated times are shown. Serum samples obtained from one of two HCV-KT9-infected mice (closed triangles) were used for ultra-deep sequencing. (B) Amino acid (aa) frequencies at aa36 in the HCV NS3 region based on ultra-deep sequencing are shown.

domain.[26] In particular, variants at NS3 residues 36, 54, 155, and 156 were shown to confer reduced sensitivity to telaprevir.[27]

In this study we analyzed the association between the antiviral efficacy of telaprevir and sequence variants within the NS3 region using chimeric mice infected with serum samples obtained from an HCV genotype 1b-infected patient. One of two HCV-infected mice had a viral breakthrough during the dosing period (Fig. 3). Ultra-deep sequence analysis of the NS3 region showed an increase of the V36A mutant, which has been reported to confer telaprevir resistance.[26] Consequently, our results show evidence of emergence of a telaprevir-resistant variant previously detected in human clinical trials.

We detected an A156F mutant in the HCV NS3 region in a chronic hepatitis patient who had experienced viral breakthrough during telaprevir monotherapy (Fig. 1). Likewise, HCV RNA titer in mice infected with the A156F variant showed no reduction following 2 weeks of telaprevir treatment (Fig. 2). However, 2 weeks of treatment with IFN-alpha rapidly suppressed serum HCV RNA titer below the detectable limit. These results demonstrate that A156F is telaprevir-resistant but has a high susceptibility to IFN.

Interestingly, ultra-deep sequencing revealed that the wildtype strain was present at low frequency (0.3%) in the serum inoculum (Fig. 2). However, the frequency of the wildtype failed to increase over time (Fig. 3), suggesting that the very small number of wildtype viral RNA (about 30 copies) may be incomplete or defective, as a large proportion of viral genomes are thought to be defective due to the virus's high replication and mutation rates.[9] Further analysis is necessary in order to interpret the significance of the presence of very low frequency variants detected by ultra-deep sequencing.

The short read lengths used in next generation sequencing also complicates the detection of rare variants, especially when variants are clustered within a region smaller than an individual read length (e.g., 36 basepairs). Relaxing the matching criteria allows mapping of more diverse reads but increases the error rate, whereas default settings may be geared toward more genetically homogenous haploid or diploid genomes. In this study we used *de novo* assembly to identify more diverse variants that failed to map to the reference sequence. Examining the variation in codon frequencies among samples, we created alternative reference sequences containing a sufficient range of variants to provide more uniform coverage of variable regions.

Using our previously established infectious HCV-KT9 genotype 1b HCV clone, we investigated the antiviral efficacy of telaprevir and the effect of

resistance mutations on viral replication. HCV RNA titer in mice infected with the telaprevir-resistant strain KT9-NS3-A156S was lower than in mice infected with the wildtype strain HCV-KT9-wild (Fig. 4B). HCV NS proteins include proteases for sequential processing of the polyprotein and are thought to be important in viral replication.[28] Our results suggest that differences in viral fitness underlie the differences in viral replication capacity. We analyzed the antiviral efficacy of telaprevir and the sequence of the NS3 region using HCV-infected mice treated with telaprevir. Although telaprevir treatment suppressed serum HCV RNA titer in mice infected with HCV-KT9, the decline of HCV RNA titer was only 0.6 log copy/mL in a mouse infected with KT9-NS3-A156S under the same treatment (Fig. 5A). These results suggest that our genetically engineered HCV-infected mouse model is useful for analyzing HCV escape mutants associated with antiviral drugs. Interestingly, treatment with telaprevir resulted in selection for V36A variants in the NS3 region in an HCV-KT9-infected mouse (Fig. 5B). There are a few controversial reports proposing that resistant variants may already be present at low frequency (<1%) within the quasispecies population in treatment-naïve patients,[29] consistent with their rapid emergence only days after treatment initiation.[26,30] This might well occur, due to the large number of mutated HCV clones. However, our results provide evidence in support of *de novo* emergence of telaprevir resistance induced by viral mutation followed by selection. HCV has both a high replication rate (10^{12} particles per day) and a high mutation rate (10^{-3} to 10^{-4}),[9,10] suggesting that the viral quasispecies population is likely to represent a large and genetically diverse substrate for immune selection.

In summary, we established an infection model of a genotype 1b HCV clone using the human hepatocyte chimeric mouse model. Using this model we demonstrate rapid emergence of *de novo* telaprevir-resistant HCV quasispecies from wildtype HCV.

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References

1. Kiyosawa K, Sodeyama T, Tanaka E, Gibo Y, Yoshizawa K, Nakano Y, et al. Interrelationship of blood transfusion, non-A, non-B hepatitis and hepatocellular carcinoma: analysis by detection of antibody to hepatitis C virus. HEPATOLOGY 1990;12:671-675.
2. Niederau C, Lange S, Heintges T, Erhardt A, Buschkamp M, Hurter D, et al. Prognosis of chronic hepatitis C: results of a large, prospective cohort study. HEPATOLOGY 1998;28:1687-1695.
3. Manns MP, McHutchison JG, Gordon SC, Rustgi VK, Shiffman M, Reindollar R, et al. Peginterferon alfa-2b plus ribavirin compared with interferon alfa-2b plus ribavirin for initial treatment of chronic hepatitis C: a randomised trial. Lancet 2001;358:958-965.
4. Fried MW, Shiffman ML, Reddy KR, Smith C, Marinos G, Goncales FL Jr, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med 2002;347:975-982.
5. Hoofnagle JH, Ghany MG, Kleiner DE, Doo E, Heller T, Promrat K, et al. Maintenance therapy with ribavirin in patients with chronic hepatitis C who fail to respond to combination therapy with interferon alfa and ribavirin. HEPATOLOGY 2003;38:66-74.
6. Perni RB, Almquist SJ, Byrn RA, Chandorkar G, Chaturvedi PR, Courtney LF, et al. Preclinical profile of VX-950, a potent, selective, and orally bioavailable inhibitor of hepatitis C virus NS3–4A serine protease. Antimicrob Agents Chemother 2006;50:899-909.
7. Lin C, Gates CA, Rao BG, Brennan DL, Fulghum JR, Luong YP, et al. In vitro studies of cross-resistance mutations against two hepatitis C virus serine protease inhibitors, VX-950 and BILN 2061. J Biol Chem 2005;280:36784-36791.
8. Mo H, Lu L, Pilot-Matias T, Pithawalla R, Mondal R, Masse S, et al. Mutations conferring resistance to a hepatitis C virus (HCV) RNA-dependent RNA polymerase inhibitor alone or in combination with an HCV serine protease inhibitor in vitro. Antimicrob Agents Chemother 2005;49:4305-4314.
9. Bartenschlager R, Lohmann V. Replication of hepatitis C virus. J Gen Virol 2000;81:1631-1648.
10. Rong L, Dahari H, Ribeiro RM, Perelson AS. Rapid emergence of protease inhibitor resistance in hepatitis C virus. Sci Transl Med 2010;2:30ra32.
11. Mercer DF, Schiller DE, Elliott JF, Douglas DN, Hao C, Rinfret A, et al. Hepatitis C virus replication in mice with chimeric human livers. Nat Med 2001;7:927-933.
12. Kneteman NM, Weiner AJ, O'Connell J, Collett M, Gao T, Aukerman L, et al. Anti-HCV therapies in chimeric scid-Alb/uPA mice parallel outcomes in human clinical application. HEPATOLOGY 2006;43:1346-1353.
13. Kamiya N, Iwao E, Hiraga N, Tsuge M, Imamura M, Takahashi S, et al. Practical evaluation of a mouse with chimeric human liver model for hepatitis C virus infection using an NS3–4A protease inhibitor. J Gen Virol 2010;91:1668-1677.
14. Hiraga N, Imamura M, Tsuge M, Noguchi C, Takahashi S, Iwao E, et al. Infection of human hepatocyte chimeric mouse with genetically engineered hepatitis C virus and its susceptibility to interferon. FEBS Lett 2007;581:1983-1987.
15. Kimura T, Imamura M, Hiraga N, Hatakeyama T, Miki D, Noguchi C, et al. Establishment of an infectious genotype 1b hepatitis C virus clone in human hepatocyte chimeric mice. J Gen Virol 2008;89:2108-2113.
16. Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, et al. Near completely humanized liver in mice shows human-type metabolic responses to drugs. Am J Pathol 2004;165:901-912.
17. Cronn R, Liston A, Parks M, Gernandt DS, Shen R, Mockler T. Multiplex sequencing of plant chloroplast genomes using Solexa sequencing-by-synthesis technology. Nucleic Acids Res 2008;36:e122.
18. Mitsuya Y, Varghese V, Wang C, Liu TF, Holmes SP, Jayakumar P, et al. Minority human immunodeficiency virus type 1 variants in antiretroviral-naive persons with reverse transcriptase codon 215 revertant mutations. J Virol 2008;82:10747-10755.
19. Margeridon-Thermet S, Shulman NS, Ahmed A, Shahriar R, Liu T, Wang C, et al. Ultra-deep pyrosequencing of hepatitis B virus quasispecies from nucleoside and nucleotide reverse-transcriptase inhibitor (NRTI)-treated patients and NRTI-naive patients. J Infect Dis 2009;199:1275-1285.
20. Szpara ML, Parsons L, Enquist LW. Sequence variability in clinical and laboratory isolates of herpes simplex virus 1 reveals new mutations. J Virol 2010;84:5303-5313.

21. Wright CF, Morelli MJ, Thebaud G, Knowles NJ, Herzyk P, Paton DJ, et al. Beyond the consensus: dissecting within-host viral population diversity of foot-and-mouth disease virus by using next-generation genome sequencing. J Virol 2011;85:2266-2275.
22. Verbinnen T, Van Marck H, Vandenbroucke I, Vijgen L, Claes M, Lin TI, et al. Tracking the evolution of multiple in vitro hepatitis C virus replicon variants under protease inhibitor selection pressure by 454 deep sequencing. J Virol 2010;84:11124-11133.
23. Wang GP, Sherrill-Mix SA, Chang KM, Quince C, Bushman FD. Hepatitis C virus transmission bottlenecks analyzed by deep sequencing. J Virol 2010;84:6218-6228.
24. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and memory-efficient alignment of short DNA sequences to the human genome. Genome Biol 2009;10:R25.
25. Simpson JT, Wong K, Jackman SD, Schein JE, Jones SJ, Birol I. ABySS: a parallel assembler for short read sequence data. Genome Res 2009;19:1117-1123.
26. Sarrazin C, Kieffer TL, Bartels D, Hanzelka B, Muh U, Welker M, et al. Dynamic hepatitis C virus genotypic and phenotypic changes in patients treated with the protease inhibitor telaprevir. Gastroenterology 2007;132:1767-1777.
27. Kuntzen T, Timm J, Berical A, Lennon N, Berlin AM, Young SK, et al. Naturally occurring dominant resistance mutations to hepatitis C virus protease and polymerase inhibitors in treatment-naive patients. HEPATOLOGY 2008;48:1769-1778.
28. Hijikata M, Mizushima H, Tanji Y, Komoda Y, Hirowatari Y, Akagi T, et al. Proteolytic processing and membrane association of putative nonstructural proteins of hepatitis C virus. Proc Natl Acad Sci U S A 1993;90:10773-10777.
29. Lu L, Mo H, Pilot-Matias TJ, Molla A. Evolution of resistant M414T mutants among hepatitis C virus replicon cells treated with polymerase inhibitor A-782759. Antimicrob Agents Chemother 2007;51:1889-1896.
30. Kieffer TL, Sarrazin C, Miller JS, Welker MW, Forestier N, Reesink HW, et al. Telaprevir and pegylated interferon-alpha-2a inhibit wild-type and resistant genotype 1 hepatitis C virus replication in patients. HEPATOLOGY 2007;46:631-639.

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Prolonged recurrence-free survival following OK432-stimulated dendritic cell transfer into hepatocellular carcinoma during transarterial embolization

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Summary

Despite curative locoregional treatments for hepatocellular carcinoma (HCC), tumour recurrence rates remain high. The current study was designed to assess the safety and bioactivity of infusion of dendritic cells (DCs) stimulated with OK432, a streptococcus-derived anti-cancer immunotherapeutic agent, into tumour tissues following transcatheter hepatic arterial embolization (TAE) treatment in patients with HCC. DCs were derived from peripheral blood monocytes of patients with hepatitis C virus-related cirrhosis and HCC in the presence of interleukin (IL)-4 and granulocyte-macrophage colony-stimulating factor and stimulated with 0·1 KE/ml OK432 for 2 days. Thirteen patients were administered with 5×10^6 of DCs through arterial catheter during the procedures of TAE treatment on day 7. The immunomodulatory effects and clinical responses were evaluated in comparison with a group of 22 historical controls treated with TAE but without DC transfer. OK432 stimulation of immature DCs promoted their maturation towards cells with activated phenotypes, high expression of a homing receptor, fairly well-preserved phagocytic capacity, greatly enhanced cytokine production and effective tumoricidal activity. Administration of OK432-stimulated DCs to patients was found to be feasible and safe. Kaplan–Meier analysis revealed prolonged recurrence-free survival of patients treated in this manner compared with the historical controls ($P = 0{\cdot}046$, log-rank test). The bioactivity of the transferred DCs was reflected in higher serum concentrations of the cytokines IL-9, IL-15 and tumour necrosis factor-α and the chemokines CCL4 and CCL11. Collectively, this study suggests that a DC-based, active immunotherapeutic strategy in combination with locoregional treatments exerts beneficial anti-tumour effects against liver cancer.

Keywords: dendritic cells, hepatocellular carcinoma, immunotherapy, recurrence-free survival, transcatheter hepatic arterial embolization

Introduction

Many locoregional therapeutic approaches including surgical resection, radiofrequency ablation (RFA) and transcatheter hepatic arterial embolization (TAE) have been taken in the search for curative treatments of hepatocellular carcinoma (HCC). Despite these efforts, tumour recurrence rates remain high [1,2], probably because active hepatitis and cirrhosis in the surrounding non-tumour liver tissues causes *de novo* development of HCC [3,4]. One strategy to reduce tumour recurrence is to enhance anti-tumour immune responses that may induce sufficient inhibitory effects to prevent tumour cell growth and survival [5,6]. Dendritic cells (DCs) are the most potent type of antigen-presenting cells in the human body, and are involved in the regulation of both innate and adaptive immune responses [7]. DC-based immunotherapies are believed to contribute to the eradication of residual and recurrent tumour cells.

To enhance tumour antigen presentation to T lymphocytes, DCs have been transferred with major histocompatibility complex (MHC) class I and class II genes [8] and co-stimulatory molecules, e.g. CD40, CD80 and CD86 [9,10], and loaded with tumour-associated antigens, including tumour lysates, peptides and RNA transfection [11]. To induce natural killer (NK) and natural killer T (NK T) cell activation, DCs have been stimulated and modified to

Table 1. Patient characteristics.

Patient no.	Gender	Age (years)	HLA	TNM stages	No. of tumours	Largest tumour (mm)	Child–Pugh	KPS	Post-TAE Rx
1	M	60	A11 A33	III	5	35	B	100	RFA
2	M	57	A11 A24	III	1	21	B	100	RFA
3	M	57	A11 A31	III	2	39	B	100	RFA
4	M	77	A2 A24	III	2	35	A	100	RFA
5	F	83	A11 A24	III	3	29	B	100	RFA
6	F	74	A2 A24	II	1	35	A	100	RFA
7	F	72	A24 A33	III	3	41	B	100	RFA
8	F	65	A2 A11	II	4	12	B	100	RFA
9	M	71	A2 A11	II	4	16	A	100	RFA
10	M	79	A11 A24	III	2	40	A	100	RFA
11	M	71	A2 A24	II	1	28	A	100	RFA
12	M	56	A2 A26	III	2	25	B	100	RFA
13	M	64	A2 A33	III	2	37	B	100	RFA

M, male; F, female; TNM, tumour–node–metastasis; Child–Pugh, Child–Pugh classification; KPS, Karnofsky performance scores; TAE, transcatheter arterial embolization; Rx, treatment; HCC, hepatocellular carcinoma; HLA, human leucocyte antigen; RFA, percutaneous radiofrequency ablation.

produce larger amounts of cytokines, e.g. interleukin (IL)-12, IL-18 and type I interferons (IFNs)[10,12]. Furthermore, DC migration into secondary lymphoid organs could be induced by expression of chemokine genes, e.g. C-C chemokine receptor-7 (CCR7) [13], and by maturation using inflammatory cytokines [14], matrix metalloproteinases and Toll-like receptor (TLR) ligands [15].

DCs stimulated with OK432, a penicillin-inactivated and lyophilized preparation of *Streptococcus pyrogenes*, were suggested recently to produce large amounts of T helper type 1 (Th1) cytokines, including IL-12 and IFN-γ and enhance cytotoxic T lymphocyte activity compared to a standard mixture of cytokines [tumour necrosis factor-α (TNF-α), IL-1β, IL-6 and prostaglandin E_2 (PGE_2)] [16]. Furthermore, because OK432 modulates DC maturation through TLR-4 and the β_2 integrin system [16,17] and TLR-4-stimulated DCs can abrogate the activity of regulatory T cells [18], OK432-stimulated DCs may contribute to the induction of anti-tumour immune responses partly by reducing the activity of suppressor cells. Recently, in addition to the orchestration of immune responses, OK432-activated DCs have themselves been shown to mediate strong, specific cytotoxicity towards tumour cells via CD40/CD40 ligand interactions [19].

We have reported recently that combination therapy using TAE together with immature DC infusion is safe for patients with cirrhosis and HCC [20]. DCs were infused precisely into tumour tissues and contributed to the recruitment and activation of immune cells *in situ*. However, this approach by itself yielded limited anti-tumour effects due probably to insufficient stimulation of immature DCs (the preparation of which seems closely related to therapeutic outcome [21,22]). The current study was designed to assess the safety and bioactivity of OK432-stimulated DC infusion into tumour tissues following TAE treatment in patients with cirrhosis and HCC. In addition to documenting the safety of this approach, we found that patients treated with OK432-stimulated DCs displayed unique cytokine and chemokine profiles and, most importantly, experienced prolonged recurrence-free survival.

Patients and methods

Patients

Inclusion criteria were a radiological diagnosis of primary HCC by computed tomography (CT) angiography, hepatitis C virus (HCV)-related HCC, a Karnofsky score of $\geq$ 70%, an age of $\geq$ 20 years, informed consent and the following normal baseline haematological parameters (within 1 week before DC administration): haemoglobin $\geq$ 8·5 g/dl; white cell count $\geq$ 2000/μl; platelet count $\geq$ 50 000/μl; creatinine $<$ 1·5 mg/dl and liver damage A or B [23].

Exclusion criteria included severe cardiac, renal, pulmonary, haematological or other systemic disease associated with a discontinuation risk; human immunodeficiency virus (HIV) infection; prior history of other malignancies; history of surgery, chemotherapy or radiation therapy within 4 weeks; immunological disorders including splenectomy and radiation to the spleen; corticosteroid or anti-histamine therapy; current lactation; pregnancy; history of organ transplantation; or difficulty in follow-up.

Thirteen patients (four women and nine men) presenting at Kanazawa University Hospital between March 2004 and June 2006 were enrolled into the study, with an age range from 56 to 83 years (Table 1). Patients with verified radiological diagnoses of HCC stage II or more were eligible and enrolled in this study. In addition, a group of 22 historical controls (nine women and 13 men) treated with TAE without DC administration between July 2000 and September 2007 was included in this study. All patients received RFA therapy to increase the locoregional effects 1 week later [24].

They underwent ultrasound, computed tomography (CT) scan or magnetic resonance imaging (MRI) of the abdomen about 1 month after treatment and at a minimum of once every 3 months thereafter, and tumour recurrences were followed for up to 360 days. The Institutional Review Board reviewed and approved the study protocol. This study complied with ethical standards outlined in the Declaration of Helsinki. Adverse events were monitored for 1 month after the DC infusion in terms of fever, vomiting, abdominal pain, encephalopathy, myalgia, ascites, gastrointestinal disorder, bleeding, hepatic abscess and autoimmune diseases.

Preparation and injection of autologous DCs

DCs were generated from blood monocyte precursors, as reported previously [25]. Briefly, peripheral blood mononuclear cells (PBMCs) were isolated by centrifugation in Lymphoprep™ Tubes (Nycomed, Roskilde, Denmark). For generating DCs, PBMCs were plated in six-well tissue culture dishes (Costar, Cambridge, MA, USA) at $1{\cdot}4 \times 10^7$ cells in 2 ml per well and allowed to adhere to plastic for 2 h. Adherent cells were cultured in serum-free media (GMP CellGro® DC Medium; CellGro, Manassas, VA, USA) with 50 ng/ml recombinant human IL-4 (GMP grade; CellGro®) and 100 ng/ml recombinant human granulocyte–macrophage colony-stimulating factor (GM-CSF) (GMP grade; CellGro®) for 5 days to generate immature DC, and matured for a further 2 days in 0·1 KE/ml OK432 (Chugai Pharmaceuticals, Tokyo, Japan) to generate OK-DC. On day 7, the cells were harvested for injection, 5×10^6 cells were suspended in 5 ml normal saline containing 1% autologous plasma, mixed with absorbable gelatin sponge (Gelfoam; Pharmacia & Upjohn, Peapack, NJ, USA) and infused through an arterial catheter following Lipiodol (iodized oil) (Lipiodol Ultrafluide, Laboratoire Guerbet, Aulnay-Sous-Bois, France) injection during selective TAE therapy. Release criteria for DCs were viability > 80%, purity > 30%, negative Gram stain and endotoxin polymerase chain reaction (PCR) and negative in process cultures from samples sent 48 h before release. All products met all release criteria, and the DCs had a typical phenotype of $CD14^-$ and human leucocyte antigen (HLA)-DR^+.

Flow cytometry analysis

The DC preparation was assessed by staining with the following monoclonal antibodies for 30 min on ice: anti-lineage cocktail 1 (lin-1; CD3, CD14, CD16, CD19, CD20 and CD56)-fluorescein isothiocyanate (FITC), anti-HLA-DR-peridinin chlorophyll protein (PerCP) (L243), anti-CCR7-phycoerythrin (PE) (3D12) (BD PharMingen, San Diego, CA, USA), anti-CD80-PE (MAB104), anti-CD83-PE (HB15a) and anti-CD86-PE (HA5.2B7) (Beckman Coulter, Fullerton, CA, USA). Cells were analysed on a fluorescence activated cell sorter (FACS0Calibur™ flow cytometer. Data analysis was performed with CELLQuest™ software (Becton Dickinson, San Jose, CA, USA).

DC phagocytosis

Immature DCs and OK432-stimulated DCs were incubated with 1 mg/ml FITC dextran (Sigma-Aldrich, St Louis, MO, USA) for 30 min at 37°C and the cells were washed three times in FACS buffer before cell acquisition using a FACS-Calibur™ cytometer. Control DCs (not incubated with FITC dextran) were acquired at the same time to allow background levels of fluorescence to be determined.

Enzyme-linked immunosorbent assay (ELISA)

DCs were seeded at 200 000 cells/ml, and supernatant collected after 48 h. IL-12p40 and IFN-γ were detected using matched paired antibodies (BD Pharmingen) following standard protocols.

Cytotoxicity assays

The ability of DCs to exert cytotoxicity was assessed in a standard ^{51}Cr release assay [19]. We used the HCC cell lines Hep3B and PLC/PRF/5 [American Type Culture Collection (ATCC), Manassas, VA, USA] and a lymphoblastoid cell line T2 that expresses HLA-A*0201 (ATCC) as target cells. Target cells were labelled with ^{51}Cr. In a 96-well plate, $2{\cdot}5 \times 10^3$ target cells per well were incubated with DCs for 8 h at different effector/target (E/T) ratios in triplicate. Percentage of specific lysis was calculated as follows: (experimental release – spontaneous release)/(maximum release – spontaneous release) × 100. Spontaneous release was always < 20% of the total.

NK cell activity

NK cell cytotoxicity against K562 erythroleukemia target cells was measured by using ^{51}Cr-release assay, according to previously published methods [26], with PBMCs obtained from the patients. All experiments were performed in triplicate. Percentage of cytotoxicity was calculated as follows: {[experimental counts per minute (cpm) – spontaneous cpm]/[total cpm – spontaneous cpm]} × 100.

Intracellular cytokine expression

Freshly isolated PBMCs were stimulated with 25 ng/ml phorbol 12-myristate 13-acetate (PMA; Sigma-Aldrich) and 1 μg/ml ionomycin (Sigma-Aldrich) at 37°C in humidified 7% CO_2 for 4 h. To block cytokine secretion, brefeldin A (Sigma) [27] was added to a final concentration of 10 μg/ml. After addition of stimuli, the surface staining was performed with anti-CD4-PC5 (13B8·2), anti-CD8-PerCP (SK1) and anti-CD56-PC5 (N901) (Beckman