inhibited HCV replication. We found that Hsp90 was an important host-derived factor that regulates HCV replication in an HCV replicon system. Using HCV-infected chimeric mice with humanized liver, which have recently been developed as a practical small animal model of HCV infection [12–14], we also demonstrated inhibition of native HCV replication in human liver cells by Hsp90 inhibitors.

Materials and methods

HCV replicon cells. Two types of human hepatoma HuH-7 cells carrying an HCV subgenomic replicon, FLR 3-1 (genotype 1b, Con-1) [15] and R6FLR-N (genotype 1b, strain N) [16], were maintained in Dulbecco's modified Eagle's medium supplemented with GlutaMAX™ I (Invitrogen, Carlsbad, CA, USA) and 0.5 mg/ml of G418 at 37 °C in 5% CO_2. The replicons were constructed as shown in Fig. 1A.

Infection of chimeric mice with HCV. Chimeric mice harboring a functional human liver cell xenograft were purchased from PhenixBio (Hiroshima, Japan). The chimeric mice were generated by transplanting human primary hepatocytes into severe combined immunodeficient (SCID) mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter. The chimeric mice used in this study had been improved over the original mice described by Tateno et al. [14], and they had a higher human hepatocyte substitution rate. Six weeks after hepatocyte transplantation, we intravenously injected each mouse with an HCV-infected patient's serum containing 10^6 copies of HCV genotype 1b (HCR6; Accession No. AY045702). The protocols for animal experiments were approved by the Ethics Committee of The Tokyo Metropolitan Institute of Medical Science. The animals received humane care according to the guidelines of the National Institutes of Health. The HCV-infected patient who provided the serum gave written informed consent before blood sampling.

Administration of 17-DMAG and/or PEG-IFN. Starting on day 0, HCV-infected chimeric mice with humanized liver received intravenous injections of 4 mg/kg of 17-DMAG and/or subcutaneous injections of 30 μg/kg PEG-IFNα-2a (Chugai, Tokyo, Japan) according to the schedule shown in Table 1.

Quantification of HCV RNA by real-time PCR. Total RNA was purified from 1 μl of serum from chimeric mice with humanized liver by the acid guanidinium–phenol–chloroform method, and HCV RNA was quantified by real-time PCR as described by Takeuchi et al. [17].

Results

Effect of Hsp90 inhibitors on HCV replication in HCV replicon cells

We have developed two modified HCV replicon cell lines, FLR 3-1 (genotype 1b, Con-1) [15] and R6FLR-N (genotype 1b, strain N) [16]. The HCV replicons are composed of the HCV 5′-untranslated region (UTR) containing an internal ribosomal entry site (IRES), the first 45 nucleotides of the core protein gene, fusion genes for luciferase and neomycin phosphotransferase, the encephalomyocarditis virus (EMCV) IRES, HCV NS3 through NS5B, and the HCV 3′-UTR (Fig. 1A). The construct allows quantification of replication levels by measuring luciferase activity. Taking advantage of this feature, we performed random screening of potential anti-HCV compounds using FLR 3-1 cells. Geldanamycin and radicicol, both of which are well-known Hsp90 inhibitors, were included among the hits. To assess the effects of Hsp90 inhibition on the intracellular replication of HCV in more detail, we treated the two different types of HCV replicon cells (FLR 3-1 and R6FLR-N) with each of four different Hsp90 inhibitors, 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), geldanamycin, herbimycin A, and radicicol. All of the Hsp90 inhibitors suppressed replication of the HCV replicon in both cell types in a dose-dependent manner. The half-maximal inhibitory concentration (IC_{50}) for HCV replication in FLR 3-1 cells was 4.4 nM for 17-DMAG, 5.5 nM for geldanamycin, 50 nM for herbimycin A, and 133 nM for radicicol (Fig. 1B, upper panel). In R6FLR-N cells, the IC_{50} was 8.1 nM for 17-DMAG, 7.8 nM for geldanamycin, 180 nM for herbimycin A, and 125 nM for radicicol (Fig. 1B, lower panel). We also assessed the levels of HCV NS3 protein, which plays a key role in HCV replication, by Western blotting and immunostaining. Hsp90 inhibitors brought about a dose-dependent decrease in the levels of this protein (Fig. 1C and D).

Effect of Hsp90 knockdown by siRNA on HCV replication in HCV replicon cells

To examine whether suppression of the replication of the HCV replicon by Hsp90 inhibitors resulted from functional inhibition of Hsp90, we used small-interfering RNA (siRNA) to perform knockdown of Hsp90. In mammalian cells there are two Hsp90 isoforms, Hsp90α and Hsp90β, which are encoded by separate genes, so we transfected siRNA targeting Hsp90α, Hsp90β, or both Hsp90α and Hsp90β into FLR 3-1 cells. As a positive control, we used siE-R7 [16], an siRNA that is sequence specific for HCV IRES. As a negative control we used sip53m [16], an siRNA that is sequence specific for p53 mRNA except for two nucleotides. Replication of the HCV replicon was suppressed by siRNA against either Hsp90α or Hsp90β in a dose-dependent manner with no effect on cell viability (Fig. 2A and B). Double knockdown of both Hsp90α and Hsp90β suppressed replication of the HCV replicon more effectively than knockdown of either Hsp90α or Hsp90β alone. HCV replication was reduced to 50–60% of control levels when cells were treated with 70 nM siRNA targeting either Hsp90α or Hsp90β, but it fell to 30% of control when cells were treated with 35 nM siRNA against Hsp90α combined with 35 nM siRNA against Hsp90β (Fig. 2A). To confirm the functionality of the siRNAs, cells were treated with 100 nM siRNA and the expression of Hsp90 protein was assessed by Western blotting. siRNA against Hsp90α or Hsp90β specifically inhibited the expression of the corresponding Hsp90 protein (Fig. 2C). It was also observed that the expression of NS3 protein was downregulated in cells transfected with siRNA targeting either Hsp90α or Hsp90β (Fig. 2C). Moreover, when cells were cotransfected with siRNA against Hsp90α and siRNA against Hsp90β (each at a concentration of 50 nM), the expression of NS3 protein was more effectively downregulated than when cells were transfected with 100 nM of siRNA targeting either Hsp90α or Hsp90β alone (Fig. 2C).

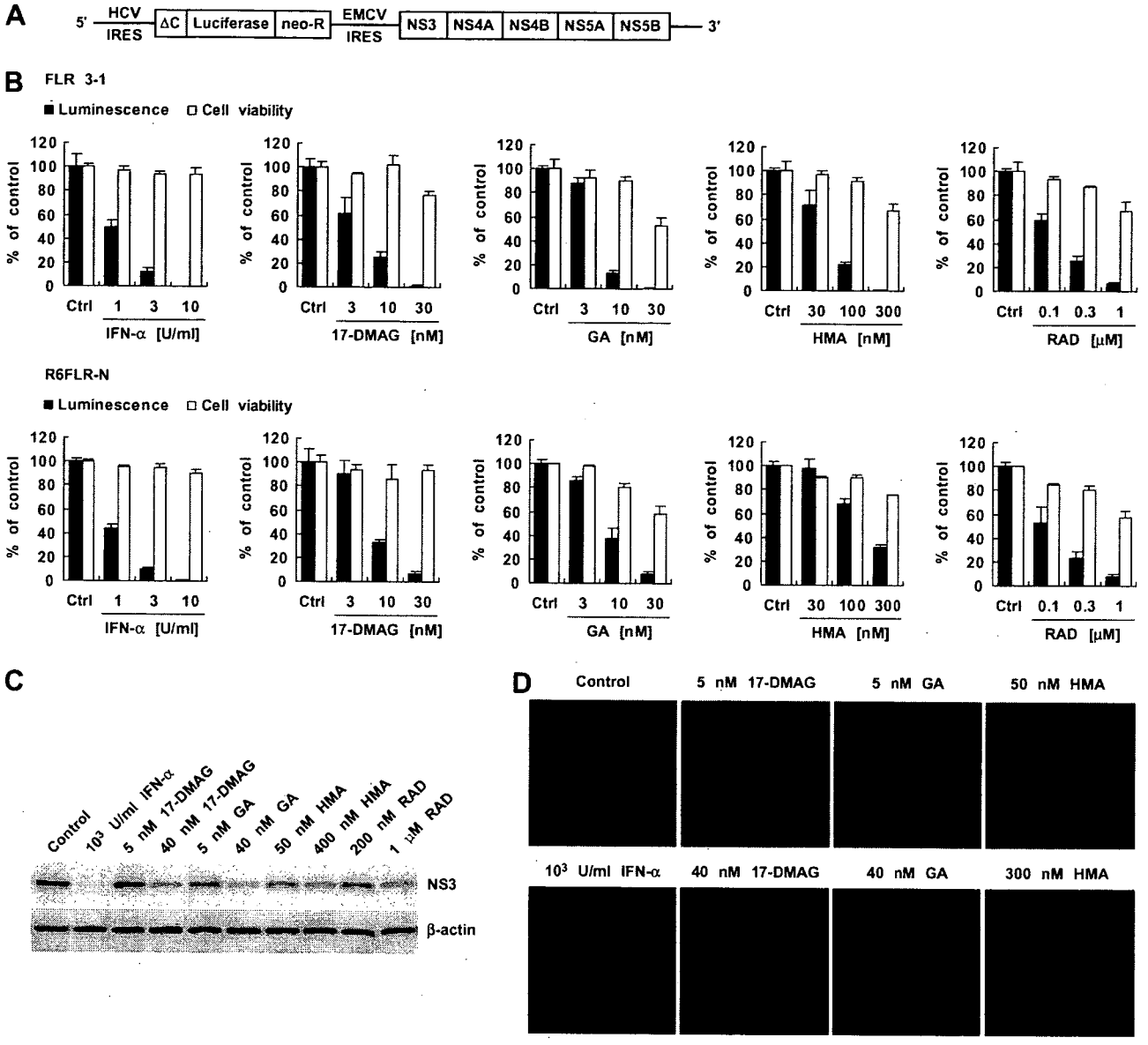

Fig. 1. Effect of Hsp90 inhibitors on HCV replication and cell viability in HCV replicon cells. (A) Schematic representation of the HCV subgenomic replicons used in this study. ΔC, first 45 nucleotides of HCV core protein gene; neo-R, neomycin phosphotransferase gene. (B) Inhibition of replication of the HCV replicon by the Hsp90 inhibitors 17-(dimethylaminoethylamino)-17-demethoxygeldanamycin (17-DMAG), geldanamycin (GA), herbimycin A (HMA), and radicicol (RAD) in FLR 3-1 replicon cells (upper panels) and R6FLR-N replicon cells (lower panels). Interferon-α (IFN-α) was used as a positive control. Seventy-two hours after treatment, HCV replication was determined by luciferase assay and cell viability by WST-8 assay. The data represent the mean of four values and the bars indicate the standard deviation. (C) FLR 3-1 cells were treated with IFN-α or Hsp90 inhibitors for 72 h. Saline was added to control cells. The levels of NS3 protein or β-actin were assessed by Western blotting. (D) FLR 3-1 cells were immunostained with anti-NS3 antibody (green) and diamidino-2-phenylindole (red). Cells were treated with saline (control) or with 10^3 U/ml IFN-α, 5 nM 17-DMAG, 40 nM 17-DMAG, 5 nM GA, 40 nM GA, 50 nM HMA or 300 nM HMA as indicated.

Table 1
Schedule of blood sampling and drug administration for chimeric mice infected with HCV

Day	−1	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14
Collection of blood	B					B										B
17-DMAG		D	D	D	D	D	D	D	D	D	D	D	D	D	D	
PEG-IFN		I			I				I			I				
17-DMAG + PEG-IFN		D/I	D	D	D/I	D	D	D	D/I	D	D	D/I	D	D	D	

B indicates sampling of blood; D, intravenous injection of 17-DMAG (4 mg/kg); I, subcutaneous injection of PEG-IFN (30 μg/kg).

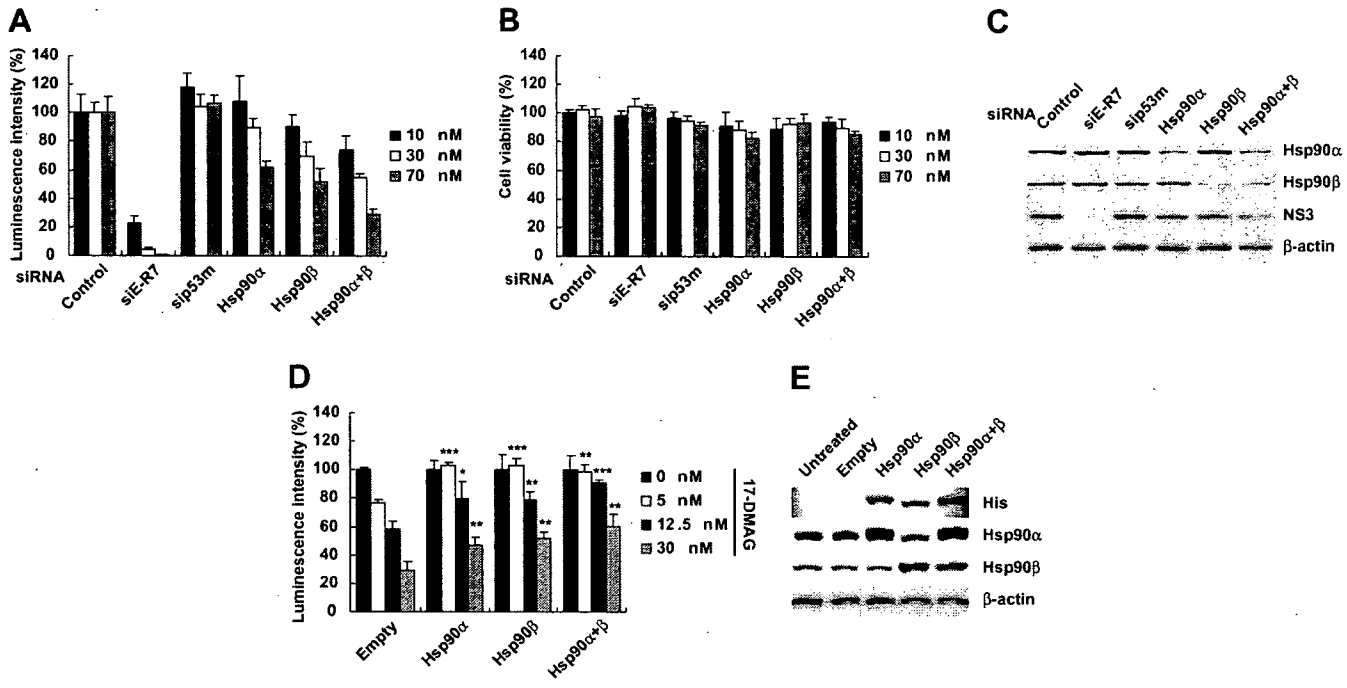

Fig. 2. Effect of knockdown or overexpression of Hsp90 on replication of the HCV replicon. (A,B) FLR 3-1 replicon cells were transfected with HCV-directed siRNA (siE-R7), negative-control siRNA (sip53m) or Hsp90-directed siRNA. Seventy-two hours after siRNA transfection, HCV replicon levels were determined by luciferase assay (A) and cell viability was determined by WST-8 assay (B). Saline was added to control cells. The data represent the mean of four values and the bars indicate the standard deviation. (C) Seventy-two hours after siRNA transfection, NS3, Hsp90α, and Hsp90β protein were quantified by Western blotting. (D) FLR 3-1 replicon cells were transfected with an expression vector encoding His-tagged Hsp90α, Hsp90β, or both Hsp90α and Hsp90β, or control empty vector and treated with 17-DMAG for 6 h. Forty-eight hours after treatment with 17-DMAG, HCV replicon levels were determined by luciferase assay. The data represent mean of four values and the bars indicate the standard deviation. The asterisks indicate significant differences between the Hsp90 expression vector and control vector groups at the corresponding concentration ($^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$; Student's t-test). (E) Seventy-two hours after transfection with expression vector encoding His-tagged Hsp90, His-tag, Hsp90α, and Hsp90β protein were quantified by Western blotting.

Effect of complementation with Hsp90

We tested whether the reduction in HCV replicon replication caused by Hsp90 inhibitors could be prevented by transfection with an expression vector encoding Hsp90. FLR 3-1 cells were transfected with an expression vector encoding histidine (His)-tagged Hsp90α, Hsp90β, or both Hsp90α and Hsp90β, or an control empty vector (Fig. 2E), and pulse-stimulated with 17-DMAG for 6 h. As expected, replication of the HCV replicon in cells transfected with control vector was suppressed by 17-DMAG in a dose-dependent manner. In contrast, replication of the HCV replicon in cells transfected with an expression vector encoding Hsp90α, Hsp90β, or both Hsp90α and Hsp90β was significantly higher than in cells transfected with control empty vector, when cells were treated with 17-DMAG (Fig. 2D).

Anti-HCV effects of 17-DMAG and PEG-IFN in HCV-infected chimeric mice with humanized liver

The anti-HCV effect of Hsp90 inhibitors was investigated in chimeric mice with humanized liver injected with an HCV-infected patient's serum. Four weeks after infection, HCV RNA levels had reached 2.3×10^6–1.4×10^7 copies/ml. We then administered 17-DMAG intravenously and/or PEG-IFN subcutaneously over a period of 14 days according to the schedule shown in Table 1. Blood samples were collected before, during, and after this period (Table 1). In the group treated with 30 μg/kg of PEG-IFN, a dose 10-fold larger than that used in clinical treatment, HCV genome RNA levels had decreased in the serum 8.8-fold by day 4 and 11-fold by day 14 (Fig. 3A). Combined treatment with 4 mg/kg of 17-DMAG and 30 μg/kg of PEG-IFN reduced HCV genome RNA levels significantly more than did PEG-IFN monotherapy. HCV genome RNA levels in the coadministration group had decreased 25-fold by day 4 and 45-fold by day 14 (Fig. 3A). In the group treated with 4 mg/kg of 17-DMAG, no reduction in HCV genome RNA levels was observed at this dose. We monitored the concentration of human albumin over the same period and observed no suppression of this protein during or after administration of 17-DMAG (Fig. 3B).

Discussion

In an effort to develop safer and more effective drug treatments, new antiviral agents, including inhibitors of

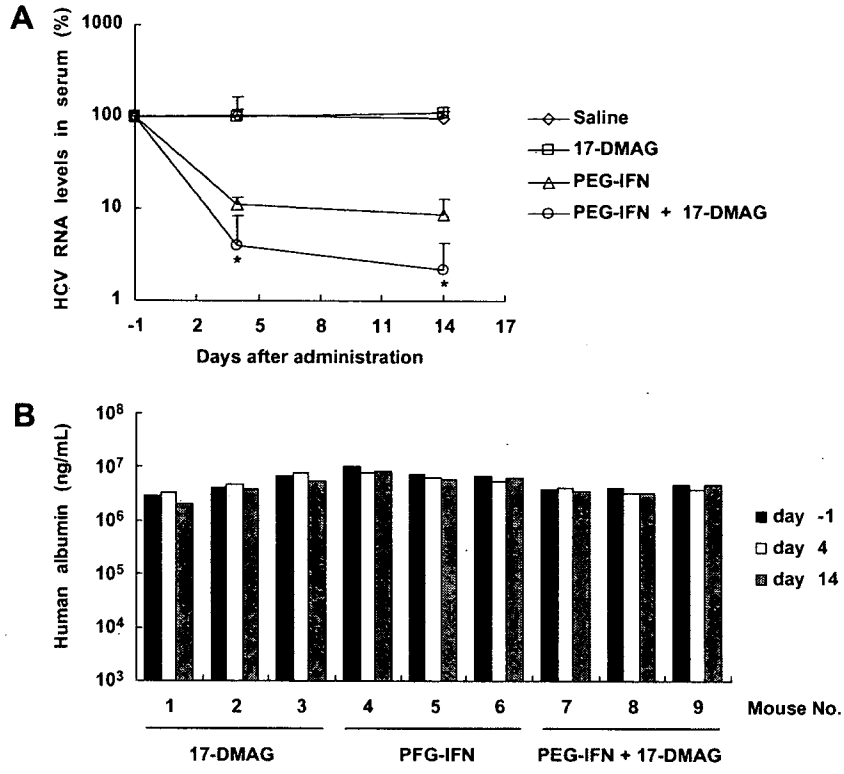

Fig. 3. Anti-HCV effect of 17-DMAG in HCV-infected chimeric mice. (A) HCV genome RNA levels in the serum of chimeric mice were determined by real-time PCR. (B) Human albumin concentrations in the sera of chimeric mice. The numbers 1–9 represent individual mice. Numbers 1, 2, and 3 were in the group treated with 4 mg/kg 17-DMAG, numbers 4, 5, and 6 were in the group treated with 30 μg/kg polyethylene glycol-conjugated interferon (PEG-IFN), and numbers 7, 8, and 9 were in the group treated with 4 mg/kg 17-DMAG and 30 μg/kg PEG-IFN. Asterisks indicate significant differences at the $P < 0.1$ level between the coadministration and PEG-IFN monotherapy groups on the same date.

the HCV NS3/4A serine protease and the HCV NS5B RNA-dependent RNA polymerase, are currently under clinical investigation [18]. However, it is likely that viral resistance will develop against antiviral agents that directly target viral enzymes, because of the error-prone nature of the virus reverse transcriptase and RNA-dependent RNA polymerase [19,20]. Therefore, we believe that anti-HCV therapies that target host-derived factors may be preferable to those that target a viral protein.

Recently, several host-derived factors that regulate replication of the HCV replicon or genome have been identified. Inhibitors of serine palmitoyltransferase have been found to suppress HCV replication in cultured cells and chimeric mice infected with HCV [15,21]. They act by preventing *de novo* synthesis of sphingolipids, major components of the lipid raft where HCV NS proteins associate. In other studies the immunosuppressant cyclosporin A has been shown to inhibit replication of the HCV genome [22,23], and cyclophilin B has been identified as the host-derived target of cyclosporin A [24,25].

In the present study, we tested the effects of Hsp90 inhibitors on HCV replication in cells carrying an HCV subgenomic replicon. All of the Hsp90 inhibitors we tested had potent anti-HCV activity. Using NS2/3 translated in a rabbit reticulocyte lysate and expressed in Jurkat cells, Waxman et al. [26] demonstrated a role for Hsp90 in promoting the cleavage of HCV NS2/3 protein . Because the replicon cells used in our study genetically lacked NS2, our results suggest that Hsp90 may directly or indirectly interact with any of the proteins NS3 through NS5B to regulate replication of the HCV replicon. Recently, Okamoto et al. [27] have reported that FKBP8, an Hsp90 partner protein, directly interacts with NS5A and regulates HCV RNA replication.

We also performed siRNA knockdown of Hsp90 and found suppression of HCV replication, consistent with the results of our experiment with Hsp90 inhibitors. Moreover, the HCV replication suppressed by Hsp90 inhibitors was rescued by the expression of Hsp90. These results indicate that Hsp90 is critical for efficient replication of the HCV replicon and that both Hsp90 isoforms (Hsp90α and Hsp90β) participate in replication.

Although the HCV replicon system is useful for screening compounds with potential anti-HCV activity, the inhibitory effects on replication are not always consistent with those found for HCV genome RNA in HCV-infected human liver. To bridge the gap between the replicon system and native HCV replication in the human liver, we tested the anti-HCV effect of Hsp90 inhibitors in HCV-infected chimeric mice with humanized liver. Of the Hsp90 inhibitors we tested, we chose to administer 17-DMAG to the mice because it is highly specific and therefore less toxic, and also because it

is currently under clinical investigation for the treatment of cancer [28,29]. We found that 17-DMAG exerted an apparently synergistic anti-HCV effect when used in combination with PEG-IFN to treat HCV-infected chimeric mice with humanized liver. Furthermore, 17-DMAG did not alter the levels of human albumin, indicating that it did not cause appreciable damage to the human hepatocytes. These results suggest that 17-DMAG may be a promising agent for the treatment of HCV infection.

In conclusion, our results demonstrate that Hsp90 supports HCV RNA replication both in an HCV replicon system and in a humanized liver mouse model infected with HCV. In addition, our results suggest that inhibition of Hsp90 may provide a feasible therapeutic strategy for the treatment of HCV infection. The precise molecular mechanism by which Hsp90 participates in the replication of HCV RNA remains to be elucidated and is under investigation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.12.117.

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Evaluation of a Cyclophilin Inhibitor in Hepatitis C Virus–Infected Chimeric Mice *In Vivo*

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Cyclosporin A (CsA) inhibits replication of the HCV subgenomic replicon, and this effect is believed to not be mediated by its immunosuppressive action. We found that DEBIO-025, a novel non-immunosuppressive cyclophilin inhibitor derived from CsA, inhibited HCV replication *in vitro* more potently than CsA. We also examined the inhibitory effect of DEBIO-025 on naive HCV genotypes 1a or 1b *in vivo* using chimeric mice with human hepatocytes. These mice were treated for 14 days with DEBIO-025, pegylated-interferon α−2a (Peg-IFN), a combination of either drugs, or CsA in combination with Peg-IFN. In mice treated with Peg-IFN, serum HCV RNA levels decreased approximately 10-fold whereas DEBIO-025 treatment alone did not induce any significant change. In mice treated with both DEBIO-025 and Peg-IFN, HCV RNA levels decreased more than 100-fold. All mice treated with Peg-IFN combined with CsA died within 4 days. The combination treatment of DEBIO-025 and Peg-IFN reduced HCV RNA levels and core protein expression in liver, indicating that the HCV RNA levels reduction in serum was attributable to intrahepatic inhibition of HCV replication. *Conclusion:* We demonstrated that DEBIO-025 was better tolerated than CsA, and that its anti-HCV effect appeared to be synergistic in combination with Peg-IFN *in vivo*. (HEPATOLOGY 2007;45:921-928.)

Hepatitis C virus is a small enveloped RNA virus that belongs to the *Flaviviridae* family.[1] A hallmark of HCV infection is its high propensity to establish a persistent infection that evades the host immune response, leading to chronic liver disease, chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[2,3] Although approximately 170 million individuals are infected with HCV worldwide, drugs that are specifically active against hepatitis C are not yet available.

Currently, the main therapy for chronic hepatitis C is a combination of pegylated interferon alpha (Peg-IFN) and ribavirin. In the intention-to-treat analysis, this combination therapy led to a sustained virological response in approximately 55%[4,5] of patients infected with any HCV genotype and in 42%[4] to 46%[5] of patients with genotype 1. The results of clinical trials were based on selected patients. The proportion of elderly patients was low, and patients with HBV or HIV coinfection, renal disease, post-transplantation status, or hematological disorders were excluded.[4-8] Because approximately 50% of patients show a poor response to combined treatment with Peg-IFN and ribavirin, effective therapies are urgently needed.

We previously reported that combination therapy of interferon (IFN) α-2b and cyclosporin A (CsA) for 24 weeks produced a sustained virological response in 42% of patients with both HCV genotype 1b and high viral levels.[9] High blood levels of CsA correlate with virological response during treatment for HCV, but occasionally can cause adverse events related to immunosuppression.[10] CsA also suppresses HCV replication *in vitro*, by inhibiting the interaction between HCV nonstructural protein 5B and cyclophilin.[11]

Abbreviations: CsA, cyclosporin A; Peg-IFN, pegylated-interferon α-2a.

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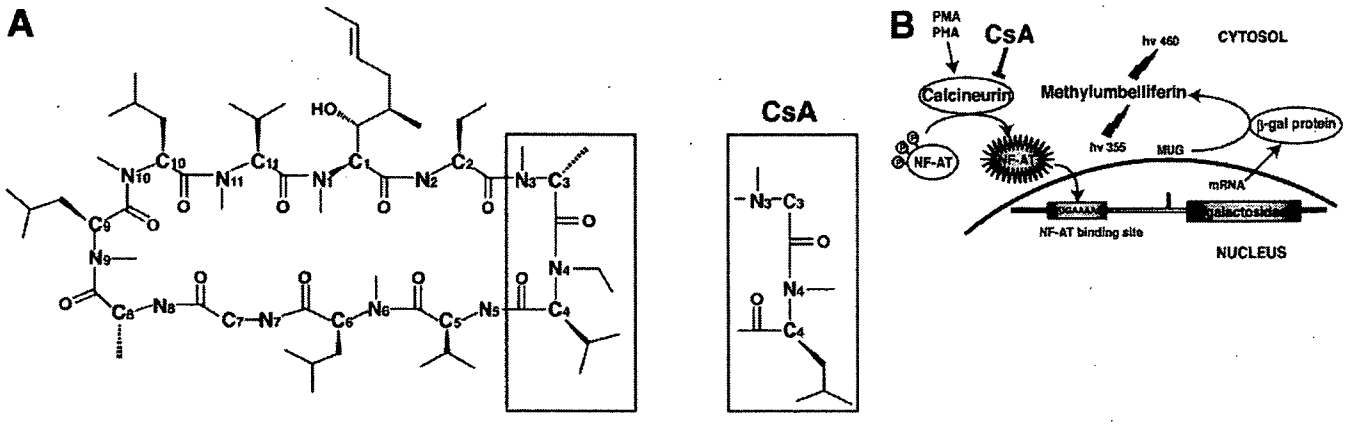

Fig. 1. (A) Structure of DEBIO-25, which was derived from CsA by substitution of amino acids at positions 3 and 4. (B) Scheme for IL-2 reporter gene assay. Nuclear factor of activated T cells (NF-AT), phorbol-12-myristate-13-acetate (PMA), phytohemagglutinin (PHA), 4-methyl umbelliferyl-β-D-galactoside (MUG).

CsA is an immunosuppressive agent widely used to improve graft survival after organ transplantation.[12] It was isolated as a metabolite from *Beauveria nivea* and consists of a cyclic polypeptide of 11 amino acids.[13] DEBIO-025 is a synthetic compound showing a more potent cyclophilin inhibitory activity as compared with CsA[14] and differing from CsA by the substitution of 2 amino acids (Fig. 1A; see Materials and Methods).[15] DEBIO-025 lacks immunosuppressive effects, although it still has remarkable inhibitory effects on HCV replication *in vitro*.[16]

We report the *in vivo* effectiveness and tolerability of DEBIO-025 administered in combination with Peg-IFN in chimeric mice with human hepatocytes that were infected with HCV genotypes 1a or 1b.

Materials and Methods

Compounds. DEBIO-025 is a synthetic compound derived from CsA. Sarcosine (*N*-methyl-D-glycine) at position 3 and *N*-methyl-D-leucine at position 4 are substituted for *N*-methyl-D-alanine and *N*-ethyl-D-valine, respectively (Fig. 1A).[16] DEBIO-025 was obtained from Debiopharm (Lausanne, Switzerland). CsA was purchased from Fluka Chemie (Buchs, Switzerland), and Peg-IFN was purchased from Chugai Pharmaceutical Co. (Tokyo, Japan).

Anti-HCV Assay in HuH-7 Cells Harboring Subgenomic Replicons. We used 2 HCV subgenomic replicon cell lines, FLR3-1[17] and R6FLR-N,[18] which were constructed as shown in Fig. 2A. They were seeded at a density of 5×10^3 per well in 96-well tissue culture plates, in complete Dulbecco's modified Eagle's medium GlutaMax I (DMEM-GlutaMaxI; Invitrogen, Carlsbad, CA) and containing 5% fetal bovine serum (Invitrogen).[17,18] The genome of the 2 replicons was genotype 1b. After incubation for 24 hours at 37°C (5% CO_2), the medium was removed, and serial dilutions of DEBIO-025 or CsA in growth medium were added. After 72 hours, luciferase activity was determined using the Bright-Glo luciferase assay kit (Promega Madison, WI). The luciferase signal was measured in triplicate using an LB940 luminometer (Berthold, Freiburg, Germany), and the results were expressed as the average percentage of control. IC_{50} values of DEBIO-025 and CsA were calculated by nonlinear curve fitting following the equation: $Y = 100 - (Y_{Bottom} \times X/(IC_{50} + X))$, where Y represents percentage inhibition and X represents the concentration of the agent. The viability of replicon cells was measured using the WST-8 cell counting kit according to the manufacturer's instructions (Dojindo, Kumamoto, Japan).

Western Blot Analysis of HCV NS3 and β-Actin. HCV replicon cells (1×10^6) were lysed with 100 μl of lysis buffer (1% SDS, 0.5% Nonidet P-40, 150 mmol/l NaCl, 0.5 mmol/l EDTA, 1 mmol/l dithiothreitol, and 10 mmol/l Tris, pH 7.4). Five micrograms total protein was electrophoresed on a 10% SDS-polyacrylamide gel and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Billerica, MA). Nonstructural protein 3 (NS3) of HCV was detected using the rabbit anti-NS3 (R212) polyclonal antibody that was prepared in our laboratory. Beta actin was detected using anti–β-actin monoclonal antibody (Sigma, St. Louis, MO).

***Immunosuppressive Activity of DEBIO-025 and CsA by Interleukin-2 Reporter Gene Assay* In Vitro.** We examined the immunosuppressive activities of DEBIO-025 and CsA using a nuclear factor of activated T cells–dependent IL-2 reporter gene assay (Fig. 1B).[19] We used Jurkat T-cells stably expressing lac-Z controlled by the IL-2 promoter. The cells were grown in RPMI-1640 medium containing 10% fetal bovine serum, 2

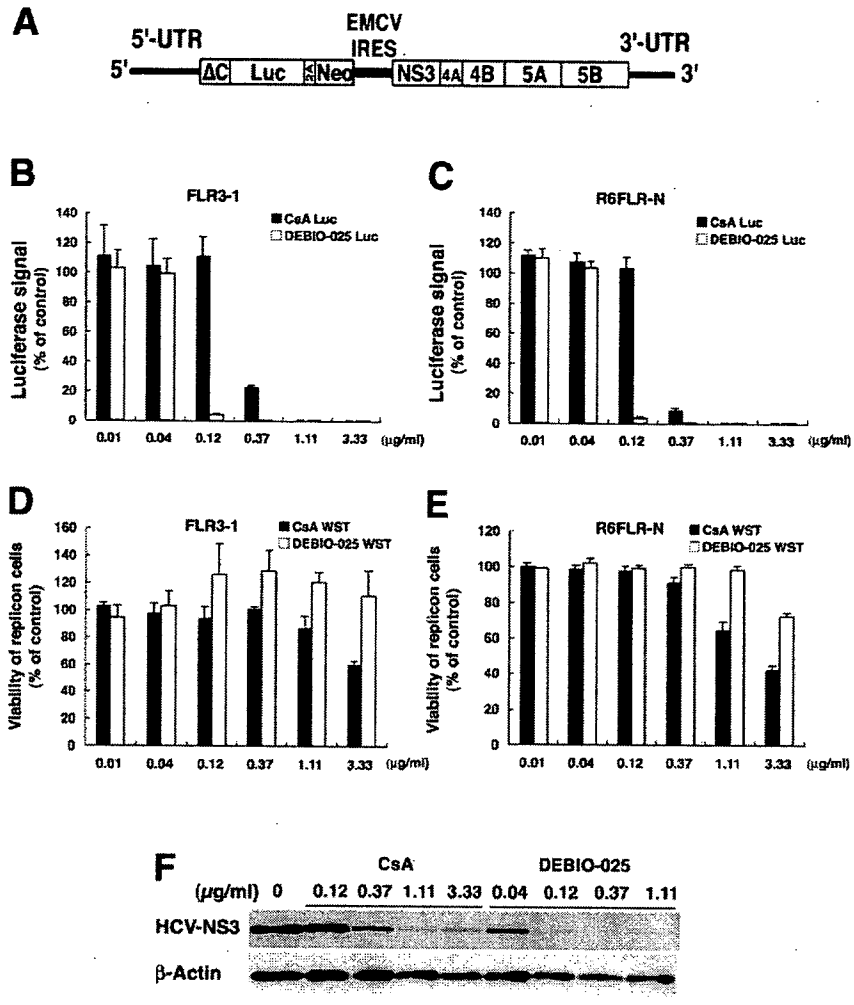

Fig. 2. (A) Structure of HCV replicon genome. FLR3-1 and R6FLR-N were of similar construction. Encephalomyocarditis virus (EMCV), internal ribosomal entry site (IRES). untranslated region (UTR). (B, C) Effect of DEBIO-025 or cyclosporin A (CsA) on HCV replication, as monitored in triplicate by luciferase signal in the 2 HCV replicon systems. Data are expressed as percentages of the untreated control. Error bars indicate SD. (D,E) Effect of DEBIO-025 or CsA on viability of replicon-containing cells, as measured in triplicate by WST-8. Data are expressed as percentages of the untreated control. Error bars indicate SD. (F) Effect of DEBIO-025 or CsA on HCV NS3 protein or β-actin expression, shown by western blotting.

mmol/l glutamine, 50 μM 2-mercaptoethanol, and 100 U/ml hygromycin B. Jurkat T-cells were stimulated with phorbol-12-myristate-13-acetate (2.4 μM) and phytohemagglutinin (75 μg/ml) in the presence or absence of DEBIO-025 or CsA (10^{-9} to 2×10^{-5} mol/l). After incubation at 37°C for 20 hours, cells were harvested by lysis buffer (50 mmol/l Na_2HPO_4, pH 9.0, 10 mmol/l KCl, 1 mmol/l $MgSO_4$, and 1% Triton X-100), and then β-galactosidase activity in the lysate was measured using 4-methyl umbelliferyl-β-D-galactoside (0.5 mmol/l; Sigma).

HCV Infection into Chimeric Mice. We purchased chimeric mice from PhenixBio (Hiroshima, Japan). The chimeric mice were generated by transplanting human primary hepatocytes into severe combined immunodeficient (SCID) mice carrying the urokinase plasminogen activator transgene controlled by an albumin promoter.[20] The chimeric mice used in this study were improved from the original ones, as described by Tateno et al.,[21] and had a high substitution rate of human hepatocytes. Six weeks after hepatocyte transplantation, we intravenously injected each mouse with patient serum containing 10^6 copies of HCV genotype 1a (HCG9) or 1b (HCR6).[22] HCV inoculations, drug administration, blood collection, and killing were performed under ether anesthesia. Blood samples were taken from the orbital vein and sera were immediately isolated. The protocols for animal experiments were approved by the local ethics committee. The animals received humane care according to NIH guidelines. Patients gave written informed consent before sampling.

Measurement of Human Serum Albumin. Human serum albumin in the blood of chimeric mice was measured with a commercially available kit according to the manufacturer's instructions (Alb-II kit; Eiken Chemical, Tokyo, Japan).

Schedule for Administration of Agents into Chimeric Mice Infected with HCV Genotype 1b or 1a. Treatment was started 12 weeks after HCV inoculation and continued during 14 days (Fig. 3A and Fig. 4A). Each treatment group comprised 3 animals. Peg-IFN and DEBIO-025 in mice with HCV genotype 1a or 1b were administered as follows: either Peg-IFN (30 μg/kg) was injected subcutaneously twice weekly alone or DEBIO-

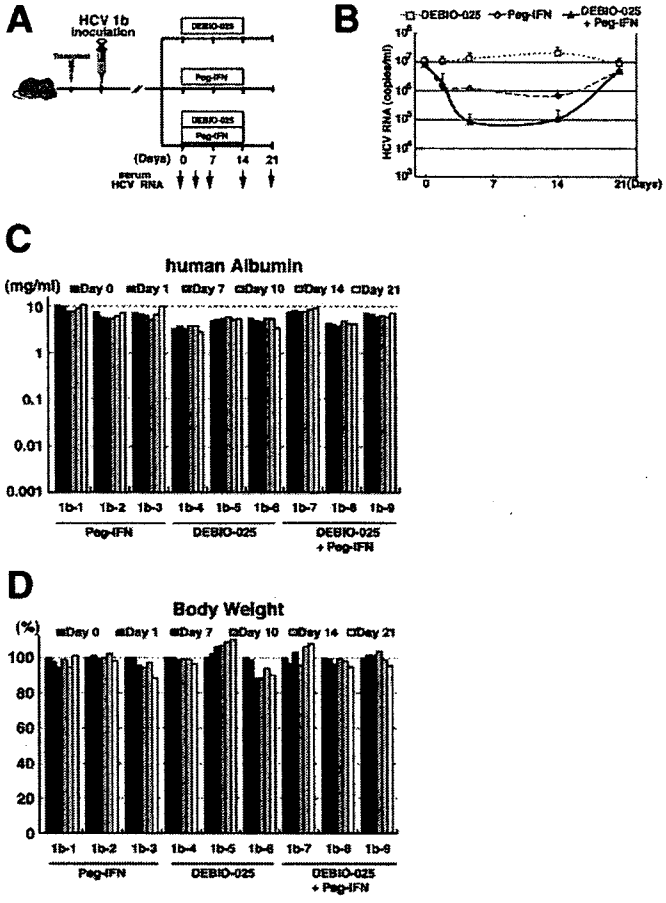

Fig. 3. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1b. The mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Pegylated-interferon $\alpha-2a$ (Peg-IFN) 30 μg/kg subcutaneously twice weekly, or DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly. (B) Time course of serum HCV RNA levels in mice treated with DEBIO-025 (open squares), Peg-IFN (gray diamonds), or DEBIO-025 with Peg-IFN (closed triangles). Error bars indicate SD. (C) Human albumin concentrations in the sera of individual mice during the experimental period. (D) Body weight of individual mice during the experimental period.

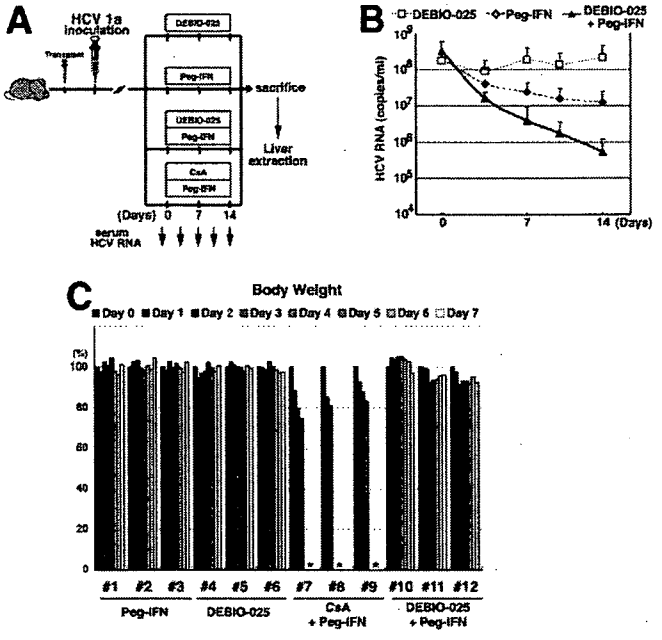

Fig. 4. (A) Schedule of experiments using chimeric mice infected with HCV genotype 1a. The mice were treated for 14 days with DEBIO-025 100 mg/kg/day orally, Peg-IFN 30 μg/kg subcutaneously twice weekly, DEBIO-025 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly, or CsA 100 mg/kg/day orally combined with Peg-IFN 30 μg/kg subcutaneously twice weekly. (B) Time course of serum HCV RNA levels in mice treated with DEBIO-025 (open squares), Peg-IFN (gray diamonds), or DEBIO-025 with Peg-IFN (closed triangles). Error bars indicate SD. (C) Body weight of individual mice during the first 7 days of the experimental period. All mice treated with CsA combined with Peg-IFN died within 4 days.

025 (100 mg/kg) was given orally every day alone, or a combination of both drugs was given. CsA (100 mg/kg) was given orally every day combined with Peg-IFN (30 μg/kg) subcutaneously twice weekly only to chimeric mice inoculated with genotype 1a.

Measurement of HCV Core Protein in Liver. Liver tissues were homogenized in lysis buffer (10 mM Tris pH 7.5, 1% SDS, 0.5% NP-40, and 150 mM NaCl) and centrifuged for 60 seconds at 16,000 g. HCV core protein was quantified using a commercially available kit (Ortho Clinical Diagnostics, Tokyo, Japan).[23]

Quantification of HCV RNA by Real-Time Reverse Transcription PCR. HCV RNA in serum or liver tissue was extracted using the acid guanidinium-phenol-chloroform method. Quantification of HCV RNA was performed using real-time reverse transcription PCR based on TaqMan chemistry, as described.[24]

Immunohistochemistry. Liver tissues obtained from mice were embedded in OCT compound (Ted Pella, Redding, CA). The frozen tissues were cut into thin sections (6 μm) and placed on glass slides. The sections were fixed in 10% buffered formalin and then treated with 0.1% Triton X-100. To detect HCV protein, the slides were incubated with rabbit anti-core protein IgG and then donkey anti-rabbit IgG polyclonal antibody [Fab fragment, labeled with horseradish peroxidase; Dako, Glostrup, Denmark]. The horseradish peroxidase label was amplified with FITC-conjugated tyramide according to the manufacturer's instructions (Molecular Probes, Eugene, OR). To detect human hepatocytes, liver sections were probed by anti-human hepatocyte monoclonal antibody (Dako), followed by anti-mouse IgG-Alexa 546 (Molecular Probes). Nuclei were stained by DAPI (Molecular Probes). Normal rabbit IgG was used as a control.

Results

Antiviral Activity of DEBIO-025 in HCV Subgenomic Replicon Cells. The anti-HCV effects of DEBIO-025 and CsA were initially confirmed using

HCV replicon cells. Both inhibited the replication of HCV replicon RNA in a concentration-dependent manner. The IC_{50} values of DEBIO-025 and CsA against replicon cell line of FLR3-1 were 0.06 μg/ml and 0.31 μg/ml respectively (Fig. 2B). The IC_{50} values of DEBIO-025 and CsA against replicon cell line of R6FLR-N were 0.07 μg/ml and 0.27 μg/ml, respectively (Fig. 2C). The inhibitory effect of DEBIO-025 was approximately 5-fold greater than that of CsA. When cell viabilities were monitored using WST-8, DEBIO-025 differed from CsA by showing a reduction of cell viability only in R6FLR-N cells (CsA reduced cell viability in both types of replicon cells; Fig. 2D-E). In R6FLR-N cells, DEBIO-025 at 3.33 μg/ml reduced cell viability by an average of 27.8%, whereas CsA at the same concentration reduced cell viability by an average of 57.2% (Fig. 2E). Western blotting of FLR3-1 cells showed that expression levels of NS3 protein, but not β-actin, were decreased by treatment with DEBIO-025 or CsA (Fig. 2F).

Immunosuppressive Activity of DEBIO-025. To examine the immunosuppressive activity of DEBIO-025, we used an nuclear factor of activated T cells–dependent IL-2 reporter gene assay. DEBIO-025 showed only a slight inhibitory effect on this system, with an activity that was 7,000-fold lower than that of CsA (data not shown). This indicates that the substitution of 2 amino acids in CsA to produce DEBIO-025 resulted in a greatly reduced immunosuppressive activity.

Human Albumin Levels in Mouse Serum After Transplantation of Human Hepatocytes. The concentration of human albumin in the serum of the chimeric mice was measured to provide an index of the substitution rate of mouse to human hepatocytes after transplantation.[21] The concentration measured 20 days after transplantation of human hepatocytes was 3.5 to 6.0 mg/ml, indicating that human hepatocytes had settled into the chimeric mice. At 6 weeks after transplantation, we inoculated the mice with patient serum containing HCV genotypes 1a or 1b. We repeatedly measured the concentrations of human albumin after inoculation and found that they reached a plateau at approximately 6.5 mg/ml. Although the mice were infected with HCV, significant reductions of the human albumin concentrations were not observed (Fig. 5A-B).

Persistent Infection of HCV in Chimeric Mice. To determine whether the chimeric mice were persistently infected with HCV, we measured HCV RNA levels in serum weekly after the inoculation. HCV RNA disappeared at the first week and was then detected from 2 weeks after the inoculation. Four weeks after infection, HCV RNA levels reached 10^8 to 10^9 copies/ml in the genotype 1a group (Fig. 5C) and 10^6 to 10^7 copies/ml in the genotype 1b group (Fig. 5D). These results showed that our patient sera containing HCV had infected the chimeric mice. Furthermore, the increase of HCV levels in the serum was time dependent, indicating that HCV replicated and accumulated in the human hepatocytes of the chimeric mice.

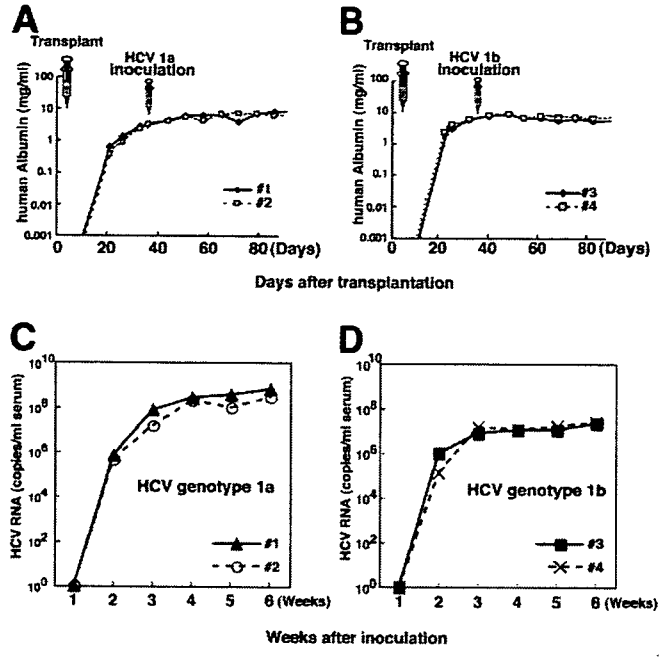

Fig. 5. Time course studies in 4 mice inoculated with human serum samples positive for HCV genotypes 1a or 1b. (A,B) Human albumin concentrations in mouse serum after transplantation of hepatocytes. (C,D) HCV RNA levels in mouse serum after inoculation.

Effect on HCV RNA Levels of DEBIO-025 and/or Peg-IFN in Mice Infected with HCV Genotype 1b. DEBIO-025 alone did not inhibit HCV replication, but Peg-IFN alone reduced serum HCV RNA levels approximately 10-fold from day 3 to day 14 (Fig. 3B). A 100-fold reduction was observed with the combined treatment (Fig. 3B). These results indicated an effect of DEBIO-025 that appeared to be synergistic with Peg-IFN against genotype 1b. The concentration of human serum albumin and the body weight of the mice did not change significantly during this period (Fig. 3C, D). After cessation of treatment, HCV RNA levels returned to 10^7 copies/ml.

Comparison of DEBIO-025 and CsA Effect in Chimeric Mice Infected with HCV Genotype 1a. The serum HCV RNA levels with the administration of DEBIO-025 or Peg-IFN alone seemed to be similar at day 7 and at day 14 as compared with those seen in mice infected with genotype 1b (Fig. 4B). The combined administration of DEBIO-025 with Peg-IFN resulted in a 600-fold reduction of HCV RNA levels at day 14 (Fig. 4B). The combined administration of CsA and Peg-IFN resulted in the death of all treated mice within 4 days. The

body weight of all CsA-treated mice was reduced by more than 20% during this period (Fig. 4C). The concentration of human serum albumin in the mice treated with CsA did not change significantly (data not shown). This toxicity was not observed with DEBIO-025 and Peg-IFN.

Quantification of Hepatic HCV RNA and Core Protein Levels and Immunohistochemistry at the End of Treatment in Chimeric Mice Infected with Genotype 1a. At the end of treatment, hepatic HCV RNA was quantified by real-time reverse transcription PCR, and core protein levels were quantified by enzyme-linked immunosorbent assay (Fig. 6A,B). DEBIO-025 monotherapy (1a-3 mouse) reduced HCV RNA by 3-fold compared with the nontreated mouse (1a-4 mouse). Peg-IFN reduced both HCV RNA and core protein levels by approximately 10-fold (1a-2 mouse). Combined treatment with DEBIO-025 and Peg-IFN resulted in an approximately 100-fold reduction in HCV RNA and HCV core protein levels (1a-1 mouse). Moreover, immunohistochemistry was performed. In 1a-4 mouse, HCV core protein was detected in human hepatocytes. In 1a-1 mouse, HCV core protein was not detected by immunohistochemistry; however, reduced HCV core protein was quantified by enzyme-linked immunosorbent assay, which is more sensitive than immunohistochemistry (Fig. 6C, D).

Discussion

Development of new anti-HCV drugs has been significantly impeded by the lack of a suitable cell culture model for the propagation of HCV in laboratories. This obstacle has been partially overcome by the development of the replicon system, which can be used for evaluating the *in vitro* anti-HCV effect of compounds. However, because adaptive mutation into the replicon genome and host permissiveness enable particularly efficient replication in cultured hepatoma cell lines,[25] evaluation of HCV drugs using replicon systems alone is considered insufficient. The only animal species readily infected with HCV has been the chimpanzee, which is labor-intensive and expen-

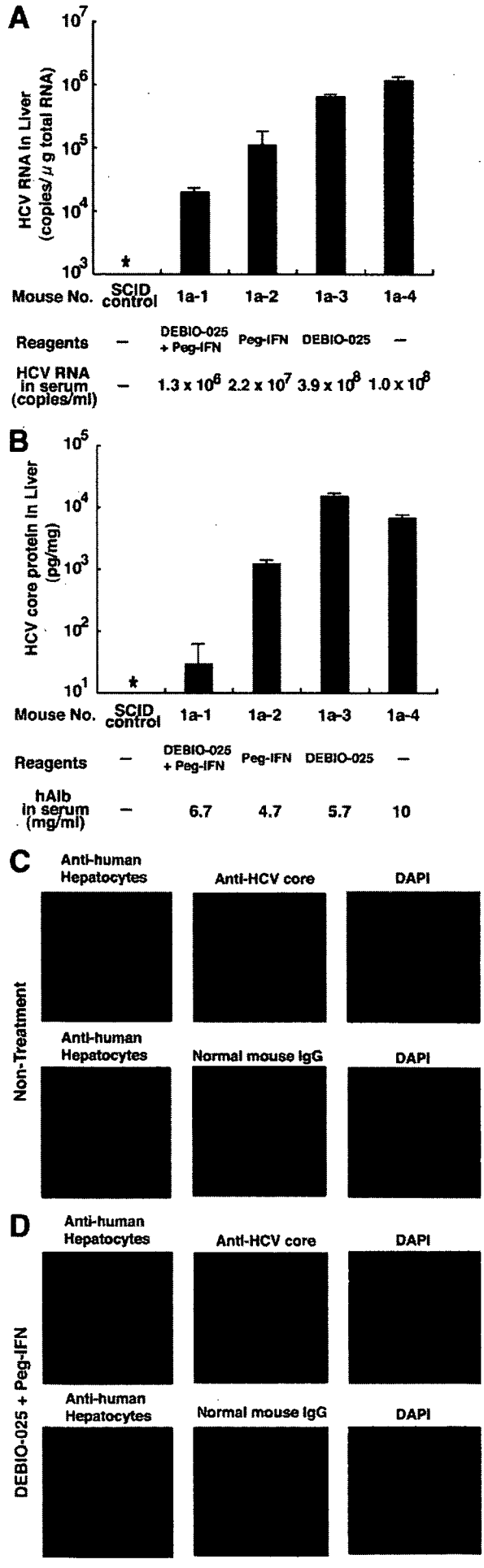

Fig. 6. Analysis of liver tissue from chimeric mice infected with HCV genotype 1a. (A) HCV RNA, and (B) HCV core protein, measured in triplicate in the livers of mice undergoing different treatment protocols. Severe combined immunodeficient (SCID) control: noninfected SCID mouse; 1a-1, mouse treated with DEBIO-025 combined with Peg-IFN; 1a-2, mouse treated with Peg-IFN; 1a-3, mouse treated with DEBIO-025; 1a-4, nontreated mouse infected with HCV. (C,D) Immunofluorescent labeling of human hepatocytes and HCV core protein, and fluorescent staining of nuclei. HCV core protein was labeled in human hepatocytes of nontreated chimeric mouse (C), but was not apparent in chimeric mouse treated with DEBIO-025 combined with Peg-IFN (D).

sive to use, and is associated with ethical problems. The chimeric mouse with human hepatocytes has recently been developed as a practical small animal model that can be infected with HCV.[20] This model is promising for the evaluation of new anti-HCV drugs because the mice are easy to handle, grow rapidly, and are well characterized genetically and immunologically. In this study, we used chimeric mice to bridge the gap between the replicon system and naive HCV replication in human liver, and to examine the anti-HCV effect of DEBIO-025, a novel cyclophilin inhibitor and non-immunosuppressive cyclosporin.

We found that HCV from our patient sera were able to infect the chimeric mice and persistently replicate over several weeks. HCG9 (1a) and HCR6 (1b) reached 10^8 to 10^9 copies/ml and 10^6 to 10^7 copies/ml, respectively, resulting in HCV RNA levels in serum that were higher than those previously reported.[20] This was probably because of a high substitution rate of human hepatocytes in the chimeric mice. When Mercer et al.[20] initially developed chimeric mice infected with HCV, they reported that human albumin concentrations in sera of the mice reached 2 mg/ml and that the substitution rate of liver from mouse to human was approximately 50%. In our study, the human albumin concentration in the chimeric mice reached 6.5 mg/ml, which would be consistent with a higher substitution rate of 80% to 90%.[21] In addition, our findings also indicate that the plateau point of HCV RNA in serum depends on the type of inoculum, because the HCV RNA levels were different for HCG9 and HCR6. Taken together, the results suggest that our chimeric mice propagated large amounts of HCV in their livers.

Although DEBIO-025 strongly inhibited replication of the HCV replicon, it did not affect the replication of naive HCV *in vivo* when given as monotherapy. These results probably indicate differences between the replication of naive HCV *in vivo* and the replicon system. The sensitivity of HCV strains to CsA and non-immunosuppressive cyclosporins was variable, depending on their cyclophilin requirement for their replication.[26] Cyclophilin polymorphism and its role in HCV replication will be the focus of future study.

The HCV RNA levels are known to decline biphasically in most patients treated with IFN.[27] During the first phase, there is a rapid drop in viremia that reflects the direct inhibition of HCV replication. During the second phase, there is a slower decline in serum HCV RNA levels, which appears to reflect the elimination of infected cells by host immune responses. In chimeric mice, the second-phase decline is not obvious, because they lack T cells and B cells (being SCID). Thus, it appears that DEBIO-025 accelerates the decline in HCV RNA levels induced by Peg-IFN during the first phase. There is no evidence that DEBIO-025 enhances the interferon pathway. Also, recent *in vitro* findings show that cyclosporins do not modify the IFN-α signal transduction pathway as assessed by 2′, 5′-oligoadenylate synthetase (2′, 5′-OAS) levels.[28] It therefore seems likely that the apparent synergistic effect of DEBIO-025 seen in our *in vivo* model is not solely related to the antiviral effect mediated by IFN. The DEBIO-025 inhibition of cyclophilin may produce a proper anti-HCV effect by interacting with the RNA-dependent RNA polymerase.[11]

CsA was originally used as an immunosuppressive agent, and we previously demonstrated in clinical trials that CsA has an anti-HCV effect.[9] However, CsA is not devoid of adverse effects, such as hypertension, neurotoxicity, and nephrotoxicity, limiting its therapeutic usefulness against HCV.[29] The immunosuppressive action of CsA occurs by inhibition of calcineurin. Our findings showing that DEBIO-025 exhibits a 7,000-fold lower immunosuppressive activity than CsA suggest that it has less affinity to calcineurin and may lead to fewer adverse effects in patients.

In conclusion, our results indicate that naïve HCV replication *in vivo* is inhibited by the combined administration of the cyclophilin inhibitor DEBIO-025 and Peg-IFN. These findings support further evaluation of DEBIO-025 as a promising drug for the treatment of chronic hepatitis C.

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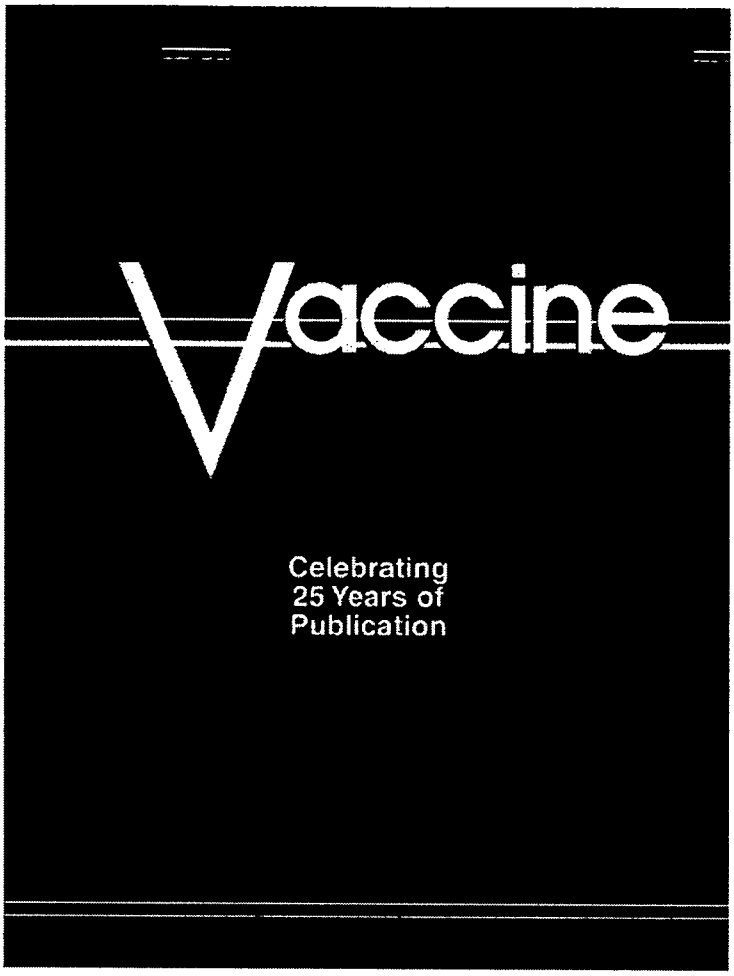

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SARS-CoV spike protein-expressing recombinant vaccinia virus efficiently induces neutralizing antibodies in rabbits pre-immunized with vaccinia virus

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Abstract

A vaccine for severe acute respiratory syndrome (SARS) is being intensively pursued against its re-emergence. We generated a SARS coronavirus (SARS-CoV) spike protein-expressing recombinant vaccinia virus (RVV-S) using highly attenuated strain LC16m8. Intradermal administration of RVV-S into rabbits induced neutralizing (NT) antibodies against SARS-CoV 1 week after administration and the NT titer reached 1:1000 after boost immunization with RVV-S. Significantly, NT antibodies against SARS-CoV were induced by administration of RVV-S to rabbits that had been pre-immunized with LC16m8. RVV-S can induce NT antibodies against SARS-CoV despite the presence of NT antibodies against VV. These results suggest that RVV-S may be a powerful SARS vaccine, including in patients previously immunized with the smallpox vaccine.

Keywords: SARS coronavirus; Recombinant vaccinia virus; LC16m8

1. Introduction

In November 2002, an influenza-like acute pneumonia designated as severe acute respiratory syndrome (SARS) by the World Health Organization, first emerged in China and spread to 29 countries within a few months. By July 2003, 8098 probable cases with 774 deaths were reported (www.cdc.gov/mmwr/mguide_sars.html). The etiologic agent of SARS was identified as a novel type of coronavirus (CoV) that was genetically distinct from previously characterized members of the Coronaviridae family [1–3]. Like other coronaviruses, SARS-CoV is a positive stranded RNA virus with an approximately 30 kb genome encoding non-structural proteins as well as structural proteins, including spike, envelope, membrane and nucleocapsid. Spike protein is a type I transmembrane glycoprotein that mediates binding to the host cell receptor using an amino-terminal S1

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domain and membrane fusion using a carboxyl-terminal S2 domain [4]. Angiotensin-converting enzyme 2 (ACE2) binds to the S1 domain of SARS-CoV spike protein and functions as a receptor for SARS-CoV [5]. CoV spike protein is a major target of protective immunity [6], and neutralizing (NT) antibodies and cytotoxic T lymphocytes against SARS-CoV spike protein have been detected in SARS patients [7,8]. These findings indicate that SARS-CoV spike protein is an appropriate target for vaccines and therapy.

The SARS epidemic broke in May 2003. However, several cases of SARS were reported in China in 2004. Although the civet cat and bats are suspected to be the natural hosts of SARS-CoV, the reservoir of SARS-CoV has yet to be identified [9–11]. In addition, the precise mechanism underlying the development of SARS is not clear and the therapeutic guidelines for SARS have not been established. It has been reported that prophylactic and therapeutic treatment with pegylated IFN-α reduces viral replication and excretion in SARS-CoV infected macaques [12]. Although pegylated IFN-α may eventually become a good therapeutic agent for SARS after infection, it cannot provide long-term protection when used as a prophylactic agent. Therefore, the development of a SARS vaccine is imperative. Several groups have reported a number of SARS vaccine candidates, including inactivated SARS-CoV vaccines [13,14], DNA vaccines [15,16] and recombinant viral vaccines [17–19] expressing one or more SARS-CoV structural proteins. Recombinant live viral vaccines can generally induce strong and long-term immunity, similar to an attenuated live vaccine, and can be abundantly manufactured in a short period of time. More importantly, a safe vaccine can be developed using an attenuated strain that has already been proven safe.

Vaccinia virus (VV) is a double stranded DNA virus with an approximately 180 kb genome, and attenuated strains have been used as the smallpox vaccine. A long DNA fragment is able to be inserted into the VV genome by homologous recombination without damaging viral integrity, as the VV genome is large and contains genes non-essential for viral replication. In fact, recombinant VV can express various proteins encoded by the transduced gene, including the glycosylated proteins of pathogens, some of which have been evaluated as candidates for prophylactic and therapeutic vaccines [20]. Lister is the attenuated VV strain that was used in the worldwide smallpox eradication program. However, additional attenuated strains were generated from Lister due to its side effects, which included erythema, fever and encephalitis. LC16m8 was isolated from Lister via the intermediate strains, LC16 and LC16mO, by multiple plaque purification in primary rabbit kidney cells. LC16m8 is characterized by temperature sensitivity and the formation of small pocks [21]. No serious side effects were observed among the over 100,000 people who were immunized with LC16m8, while the immunogenicity of LC16m8 is similar to that of Lister [22]. Therefore, LC16m8 was authorized as the vaccine against smallpox by the Japanese Ministry of Health and Welfare in 1975.

Recombinant VV expresses proteins encoded by transduced genes under the control of its own promoters. Highly efficient hybrid promoters have been developed and are composed of poxvirus A-type inclusion body (ATI) late promoter and tandem repeats of mutated 7.5 kDa protein (p7.5) early promoter [23]. The protein expressed under the control of the ATI/p7.5 hybrid promoter strongly induces both humoral and cellular immunity [24]. In the present study, we generated a recombinant VV expressing SARS-CoV spike protein (RVV-S) under the control of the ATI/p7.5 hybrid promoter, using LC16m8, and examined whether RVV-S could be used as a SARS vaccine.

2. Materials and methods

2.1. Viruses and cells

SARS-CoV (Vietnam/NB-04/2003 strain), which was isolated from nasal and throat swabs from 1 patient in Hanoi, has been previously described [25]. LC16m8 and LC16mO were kindly provided by the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan). The RK13 cell line (ATCC: CCL-37) and VERO E6 cell line (ATCC: CRL-1586) were cultured in MEM (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 5% fetal bovine serum.

2.2. Generation of recombinant vaccinia virus

The pSFJ1-10 vector contains the ATI/p7.5 hybrid promoter within the hemagglutinin (HA) gene region of VV [23]. Full length cDNA encoding the SARS-CoV spike protein gene was cloned from SARS-CoV viral RNA by RT-PCR, and then inserted downstream of the ATI/p7.5 hybrid promoter of pSFJ1-10; final designation: pSFJ1-10-SARS-S. pSFJ1-10-SARS-S was then transfected into RK13 cells that had been infected with LC16m8 at a multiplicity of infection (moi) of 10 plaque forming units (PFU)/cell. At 24 h after transfection, the virus was harvested. HA negative plaques were cloned as described previously [26]. Briefly, the harvested virus was re-infected into RK13 cells. At 96 h after infection, cells were washed with PBS (+) twice, and then incubated with chicken erythrocytes for 30 min at 30 °C. Following washing again with PBS (+), white plaques were isolated. Isolated viruses were cloned by three serial rounds of plaque purification using erythrocyte agglutination and then propagated in RK13 cells. Insertion of the SARS-CoV spike protein gene into LC16m8 genome was confirmed by direct PCR and expression was detected by Western blotting. The viral titer of RVV-S was determined using the standard plaque assay.

2.3. Western blotting

RK13 cells were infected with RVV-S or LC16m8 at moi 10. After 24 h infection, cells were lysed with RIPA

Table 1
Immunization schedule of RVV-S and LC16m8

Rabbit #	0 week		6 weeks		12 weeks		18 weeks	
	Virus	Dose (PFU)	Virus	Dose (PFU)	Virus	Dose (PFU)	Virus	Dose (PFU)
R1–R3	RVV-S	10^8	RVV-S	10^8				
R4–R6	LC16m8	10^8	LC16m8	10^8	RVV-S	10^8	RVV-S	10^8
R7–R9	RVV-S	10^6	RVV-S	10^6				
R10–R12	RVV-S	10^7	RVV-S	10^7				
R13–R15	LC16m8	10^7	RVV-S	10^7	RVV-S	10^7		

buffer (10 mM Tris, pH 7.4, 150 mM NaCl, 1% SDS and 0.5% Nonidet-P40), and 30 μg of total protein was subjected to 7.5% SDS-PAGE and was transferred to a polyvinylidene difluoride membrane (Immobilon-P, Millipore, Bedford, MA). The membrane was blocked in 5% skim milk in TBS containing 0.1% Tween-20 (TBS-T) and then washed with TBS-T. Polyclonal antibodies against spike protein were used as the primary antibody. These were prepared from rabbit sera immunized with a KLH-conjugated spike protein peptide (amino acid residues 559–570 or 1236–1248) and the IgG fraction purified using the Ampure PA kit (Amersham Bioscience, Piscataway, NJ). Antigen-antibody interaction was detected by horseradish peroxidase (HRP)-conjugated donkey anti-rabbit polyclonal antibodies (Amersham Bioscience) and visualized using the ECL system (Amersham Bioscience).

2.4. Immunofluorescence analysis

RK13 cells seeded on slide-glass were infected with RVV-S or LC16m8 at moi 5. At 12 h after incubation at 30 °C, cells were fixed in cold acetone/methanol and then blocked in 1% BSA in PBS (−) for 1 h at room temperature. Following removal of the blocking buffer, cells were incubated with polyclonal antibodies against spike protein, which recognize the C-terminal peptide of spike protein (amino acid residues 1236–1248), for 1 h at room temperature. Following three washes with PBS containing 0.05% Tween-20 (PBS-T), cells were incubated with Alexa 488-conjugated anti-rabbit IgG (Invitrogen, Carlsbad, CA) for 1 h at room temperature. After washing again with PBS-T, the slide-glasses were mounted in Permafluor (Beckman Coulter, Fullerton, CA) containing 1 μg/ml 6-diamidino-2-phenylindole (DAPI) and analyzed using a confocal microscope (LSM510, Carl Zeiss, Oberkochen, Germany).

2.5. Immunization of rabbits

Groups of three New Zealand White rabbits, which were purchased from SLC (Hamamatsu, Japan), were intradermally immunized with one of several doses (10^6, 10^7 or 10^8 PFU) of RVV-S, or with 10^8 PFU of LC16m8, at 0 and 6 weeks. The LC16m8 immunized group was further immunized with 10^8 PFU of RVV-S at 12 and 18 weeks. Another group of three rabbits was immunized with 10^7 PFU of LC16m8 at 0 week, and then immunized with 10^7 PFU of RVV-S at 6 and 12 weeks. A summary of the immunization schedule is shown in Table 1. Sera were collected every week, and used for enzyme linked immunosorbent assay (ELISA) and the *in vitro* neutralization (NT) assay below. All animal experiments were approved by The Tokyo Metropolitan Institute of Medical Science Animal Experiment Committee and were performed in accordance with the animal experimentation guidelines of The Tokyo Metropolitan Institute of Medical Science.

2.6. ELISA

Full length recombinant SARS-CoV spike protein containing a six-histidine tag (His) was expressed in RK13 cells by RVV-S-His, which was generated from LC16mO, and purified using Nickel sepharose (Amersham Bioscience). Peptides from the N-terminal (mixture of three peptides, amino acid residues 12–53, 90–115 and 171–203), middle position (mixture of two peptides, amino acid residues 408–470 and 540–573) and C-terminal (mixture of three peptides, amino acid residues 1051–1076, 1121–1154 and 1162–1190) of the spike protein, which respond to sera from SARS-infected individuals, were purchased from ProSpec-Tany TechnoGene Ltd. (Rehovot, Israel). These three peptide mixtures or full length spike protein were coated onto the 96 well plates at 4 °C. The plates were blocked with 1% BSA in PBS (−) containing 0.5% Tween-20 and 2.5 mM EDTA, and then incubated with serial dilutions of sera from the rabbits immunized with RVV-S or LC16m8. After extensive washing, the plates were incubated with HRP-conjugated donkey anti-rabbit polyclonal antibodies (Amersham Bioscience). Antigen–antibody interactions were detected using 3,3′,5,5′-tetramethylbenzidine solution as the substrate (Becton Dickinson, San Jose, CA), and the binding activity was measured by the absorbance at 450 nm.

2.7. In vitro NT assay for SARS-CoV

Serial dilutions of heat-inactivated sera were mixed with equal volumes of 100 $TCID_{50}$ of SARS-CoV and incubated at 37 °C for 1 h. VERO E6 cells were then infected with the virus/sera mixtures in 96 well plates. At 120 h after infection, the NT titer was determined as the maximum dilution of sera that inhibited the SARS-CoV induced cytopathic effect by more than 50%. All experiments with SARS-CoV were performed in a biosafety containment level III facility.

2.8. In vitro NT assay for VV

Serial dilutions of heat-inactivated sera were mixed with equal volumes of 100 PFU of LC16mO, and incubated at 37 °C for 1 h, followed by incubation at 4 °C for 16 h. RK13 cells were then infected with the virus/sera mixtures in 6 well plates. At 48 h after infection, the NT titer was determined as the maximum dilution of sera that inhibited plaque formation by more than 50%.

2.9. Statistical analysis

All data were expressed as mean ± S.E.M. Data for RVV-S dose dependent effect were statistically analyzed by one-way ANOVA followed by Turkey test. Data for LC16m8 pre-immunization effect were statistically analyzed by Student's or by Welch's t-test. $p < 0.05$ was considered to be statistically significant.

3. Results

3.1. Generation and characterization of RVV-S

The full length SARS-CoV spike protein gene was inserted by homologous recombination into the HA gene region of LC16m8, which was located downstream of the powerful ATI/p7.5 hybrid promoter (Fig. 1A). Recombination between pSFJ1-10-SARS-S and LC16m8 results in inactivation of the HA gene. We screened for RVV-S using the erythrocyte agglutination assay, and insertion of the transduced gene was then confirmed by PCR. To confirm the expression of SARS-CoV spike protein, Western blotting was performed. Two kinds of rabbit polyclonal antibodies that recognized different epitopes, amino acid residues 559–570 and 1236–1248 of SARS-CoV spike protein, were used as the primary antibody. In RVV-S but not LC16m8 infected cells, both antibodies detected an approximately 180 kDa protein (Fig. 1B and C), which is consistent with the molecular mass of spike protein [18]. SARS-CoV spike protein is reported to be highly glycosylated, and thus the molecular mass on SDS-PAGE is larger than that predicted from the gene sequence [18]. Expression of spike protein following infection with RVV-S was also confirmed by immunofluorescence analysis. RVV-S infected VERO E6 cells were stained with antibody against spike protein, whereas no staining was observed in the cells infected with LC16m8 or the uninfected control cells (Fig. 1D).

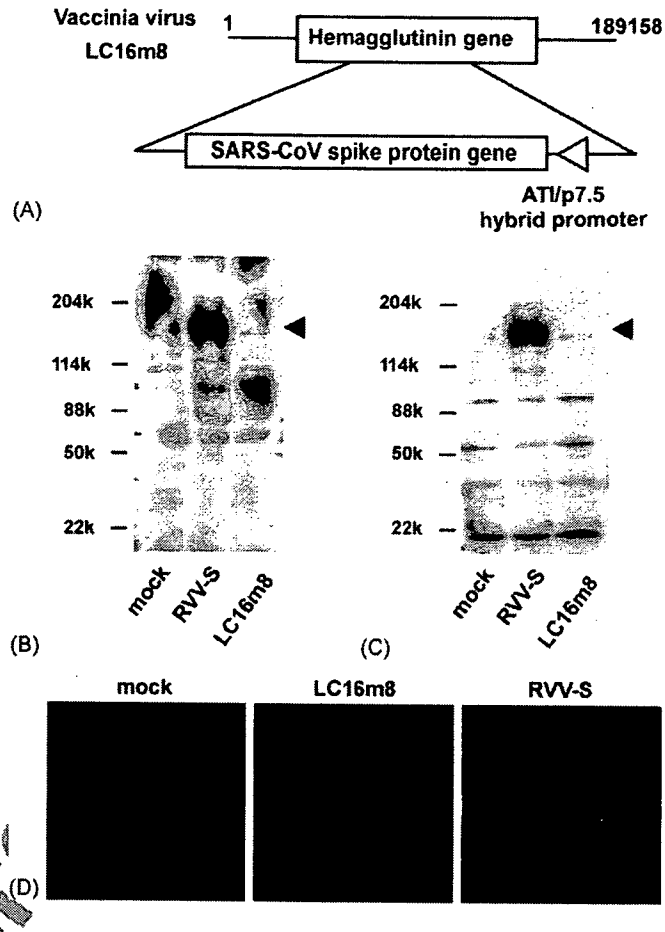

Fig. 1. Characteristics of SARS-CoV spike protein-expressing recombinant vaccinia virus (RVV-S) derived from LC16m8. (A) The full length SARS-CoV spike protein gene was inserted into the HA gene region of the LC16m8 genome. The ATI/p7.5 hybrid promoter regulates expression of spike protein. (B and C) RK13 cells were infected with RVV-S or LC16m8 at moi 10. At 24 h after infection, cells were harvested and analyzed. Two kinds of anti-SARS-CoV spike protein polyclonal antibodies, which recognize different epitopes, namely amino acid residues 559–570 (B) and 1236–1248 (C) of spike protein, were used as the primary antibodies. The molecular masses of marker proteins in kDa are shown on the left and the position of the spike protein is indicated by an arrowhead on the right. (D) Indirect immunofluorescence staining of spike protein. Expression of spike protein was visualized by staining with anti-SARS-CoV spike polyclonal antibodies, followed by Alexa 488-conjugated anti-rabbit IgG (green). Nuclei were stained with DAPI (red).

3.2. Induction of binding antibodies against spike protein in RVV-S-immunized rabbits

To investigate whether RVV-S induces binding antibodies against spike protein, 10^8 PFU of RVV-S or LC16m8 (as the control) was intradermally injected into rabbits at 0 and 6 weeks. Rabbits immunized with either RVV-S or LC16m8 did not exhibit weight loss or any clinical signs except for regional skin reactions, such as erythema and induration. The skin reaction induced by RVV-S was comparable to that induced by LC16m8 (data not shown). Binding antibodies against full length spike protein were detected by ELISA in the sera from rabbits immunized with RVV-S (Fig. 2A). Next, we investigated the binding activities of immunized sera against different epitopes of the spike protein. RVV-S-immunized sera reacted with all three regions of spike protein. The sera bound to the C-terminal peptides, which contained the heptad repeat 2 (HR2) region, reported to be the NT epitope of SARS-CoV (Fig. 2B) [27] and to the middle peptides,

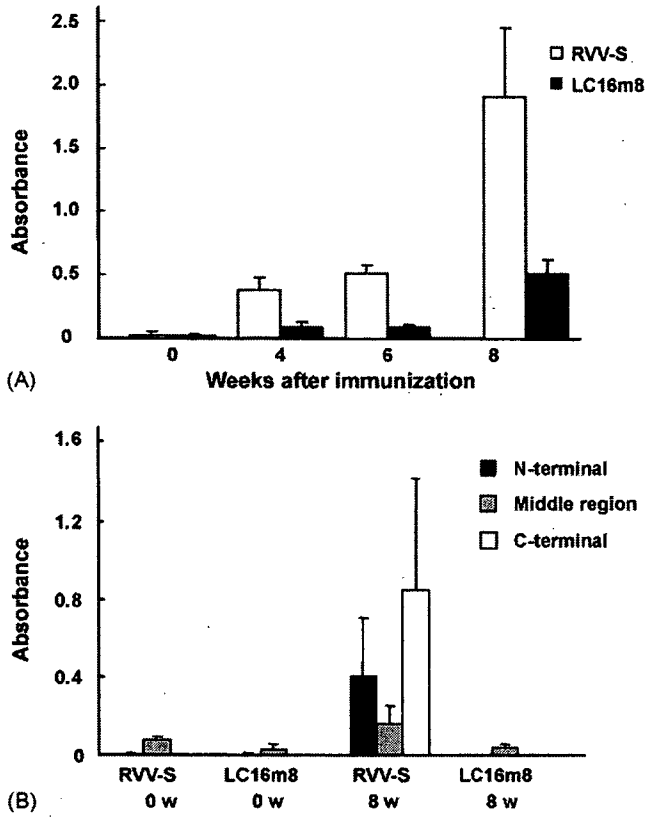

Fig. 2. Induction of binding antibodies against SASR-CoV spike protein. New Zealand White rabbits ($n = 3$) were immunized with 10^8 PFU of RVV-S (R1-3; open symbols) or LC16m8 (R4–6; closed symbols) at 0 and 6 weeks. The binding activity of 10^3- or 10^4-fold dilutions of immunized sera was assessed using His-tagged full length spike protein (A), or one of three kinds of spike protein partial peptides (B), as the capture antigen.

in which a receptor binding domain, another NT epitope of SARS-CoV, was included [28].

3.3. Induction of NT antibodies against SARS-CoV in RVV-S-immunized rabbits

Next, to determine whether the antibodies induced by immunization with RVV-S have NT activity against SARS-CoV, we performed an *in vitro* NT assay against SARS-CoV using immunized sera. Interestingly, the sera obtained from all three rabbits in this group showed NT activity against SARS-CoV, even at 1 week after immunization with 10^8 PFU of RVV-S (Fig. 3A). The NT titer reached 1:100 at 3 weeks, and increased 10-fold further by boost immunization. In contrast, sera obtained from rabbits immunized with LC16m8 did not show any NT activity against SARS-CoV (Fig. 3A). Next, to determine the minimum dose that can induce NT antibodies against SARS-CoV by single immunization, rabbits were immunized with lower doses of RVV-S. All three rabbits that underwent single immunization with 10^7 PFU of RVV-S generated NT antibodies against SARS-CoV (Fig. 3A). The NT titer further increased by boost immunization with 10^7 PFU of RVV-S and reached a comparable level to that induced by 10^8 PFU of RVV-S (Fig. 3B). On the other hand, NT activity was induced by single immunization with 10^6 PFU of RVV-S at 2 and 4 weeks after immunization in all three rabbits, but then decreased below the detection limit in one rabbit at 6 weeks (Fig. 3A). However, the NT titer increased to approximately 1:300 in the group immunized with 10^6 PFU of RVV-S by boost immunization with the same dose of RVV-S (Fig. 3B).

3.4. RVV-S induces NT antibodies against SARS-CoV in the presence of NT antibodies against VV

Induction of NT antibodies against VV by RVV-S was next examined. The *in vitro* NT assay against VV revealed that LC16m8 and RVV-S equally induced NT antibodies against VV in the rabbits (Fig. 3C). NT activity against VV was induced by 10^8 PFU of RVV-S at 1 week after immunization, similar to SARS-CoV. The NT titer against VV, which reached 1:10,000 at 2 weeks after boost immunization with 10^8 PFU of RVV-S, was similar to that induced by 10^8 PFU of LC16m8. These results suggest that the epitopes of the NT antibodies against VV were preserved in RVV-S. Since VV has been used as a smallpox vaccine in humans, we were concerned that RVV-S might be eliminated by the host's immune response before inducing effective immunity against a protein encoded by the transduced gene. Therefore, to assess whether RVV-S can induce NT antibodies against SARS-CoV in rabbits that had NT antibodies against VV, RVV-S was injected into rabbits which had been pre-immunized with LC16m8. NT antibodies against VV were induced in the rabbits by single immunization with 10^7 PFU of LC16m8 and the NT titer reached 1:64–256 (Fig. 4A). By following immunization with an equal dose of RVV-S (10^7 PFU), NT antibodies against SARS-CoV were induced in all three rabbits, although induction of NT antibodies was delayed in one rabbit (R14). Although the induction of NT antibodies against SARS-CoV was partially suppressed in the LC16m8 pre-immunized rabbits, the NT titer further increased in all three rabbits by boost immunization with RVV-S (Fig. 4C). These results suggest that RVV-S can induce NT antibodies in individuals who have been previously immunized with a smallpox vaccine. Next, we examined whether RVV-S induced NT antibodies against SARS-CoV in rabbits with a high titer of NT antibodies against VV. The NT titer against VV in rabbits that had been immunized twice with 10^8 PFU of LC16m8 was sustained at approximately 1:4000 (Fig. 4B). Although these rabbits had an extremely high titer of NT antibodies against VV, NT antibodies against SARS-CoV were induced in all three rabbits upon a booster injection with 10^8 PFU of RVV-S (Fig. 4B). Surprisingly, the NT titer of these rabbits increased to levels comparable to those of the non-pre-immunized rabbits (Fig. 4C). These results indicate that an immune response against a protein encoded by a transduced gene can be induced by immunization with 10^8 PFU of RVV in spite of the pre-existence of NT antibodies against VV.