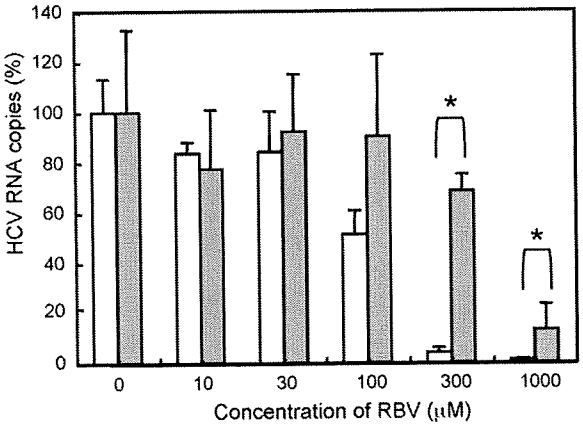

Fig. 1. Inhibitory effect of RBV on HCV RNA levels in genotype 2a replicon cells after long-term treatments with RBV. The replicon cells were serially passaged in 0 or 200 μM RBV for 20 weeks. The cells were then split and incubated with fresh RBV at various concentrations in the absence of G418 for 3 days, followed by the determination of HCV RNA. Clear bars, passage in the absence of RBV: gray bars, passage in the presence of RBV. HCV RNA copies per microgram of total RNA were normalized as percentages of those of untreated (RBV 0 μM). Each data point is presented as the mean of three independent determinations with standard deviation. $^*p < 0.05$.

cells; the EC_{50} values for the variant and wild-type replicon cells were 470 and 102 μM, respectively (Fig. 1). Comparable cytotoxic effects of RBV were observed against wild-type and variant replicon cells, with the CC_{50} (50% cytotoxicity concentration) values of 151 and 156 μM, respectively (data not shown).

3.2. Mapping RBV resistance to cell line or replicon RNA

To test whether reduced susceptibility to RBV in the variant cells observed as above was due to the appearance of mutations within the viral RNA or was cell-derived, total RNAs from the variant and wild-type replicon cells were extracted and used for retransfection of naïve Huh7 cells. Retransfected cells resistant to G418 were established after 4 weeks of cultures in the presence of 1 mg/ml G418 and were assessed for HCV RNA replication sensitivity to RBV (Fig. 2A). HCV RNA levels in the cells obtained from the wild-type replicon were inhibited by 56, 89 and 97% with 100, 300 and 1000 μM RBV, respectively. By contrast, the culture retransfected with RNA derived from the variant replicon cells exhibited inhibition levels of 13, 29 and 89% with the corresponding concentrations of RBV. EC_{50} values were calculated to be 93 and 449 μM, respectively. We confirmed the presence of replicon mutations, as described below, in the cells retransfected with RNA derived from the variant replicon cells.

In order to explore the possibility for cell-derived resistance, both wild-type and variant replicon cells were cured of viral RNAs by IFN treatment; cells were passaged with media containing 100 IU/mL IFN-α in the absence of G418 for 2 months. To compare RBV sensitivity, cured cells were transiently transfected with the wild-type JFH-1 subgenomic replicon RNA and were treated with various concentrations of RBV for 72 h. Similar anti-HCV effects of RBV were observed in the cured cells derived from wild-type and variant replicons, with the EC_{50} values of 147 and 118 μM, respectively (Fig. 2B). Thus, the results suggest that the RBV resistance observed may arise by mutations in the replicon rather than by changes in the cells.

3.3. HCV mutations in replicon variant with reduced susceptibility to RBV

It has been reported that mutations in RNA virus genomes responsible for RBV resistance are mostly present in the coding region for the viral RNA-dependent RNA polymerase (RdRp). On the other hand, it is known that RBV works as an RNA mutagen to generate rapidly mutating viral RNA and that NS5B RdRp and other nonstructural proteins in HCV are involved in the viral replication complex, playing key roles in genome replication. Therefore, we sequenced the coding regions for NS3 through NS5B proteins of the replicon molecules in order to determine whether mutations associated with RBV resistance were generated. As shown in Table 2, there were numerically more synonymous and non-synonymous mutations in the RBV-resistant variant replicon cells (RBV treatment) when compared with untreated replicative conditions (No-treatment) across most regions examined. Mutation frequencies of NS3, NS4B and NS5A regions of RBV treatment were significantly higher than those of No-treatment. The total number of synonymous mutations in the RBV-resistant variant replicon cells was 3 times higher than that under untreated replicative conditions, and the number of non-synonymous mutations in the RBV-resistant variant replicon cells was 1.5 times higher than that under untreated replicative conditions. The number of both synonymous and non-synonymous mutations (NS3, NS4B, NS5A and NS5B regions) in the RBV-resistant replicon cells was greater than that in the control cells. We also found a large number of transition

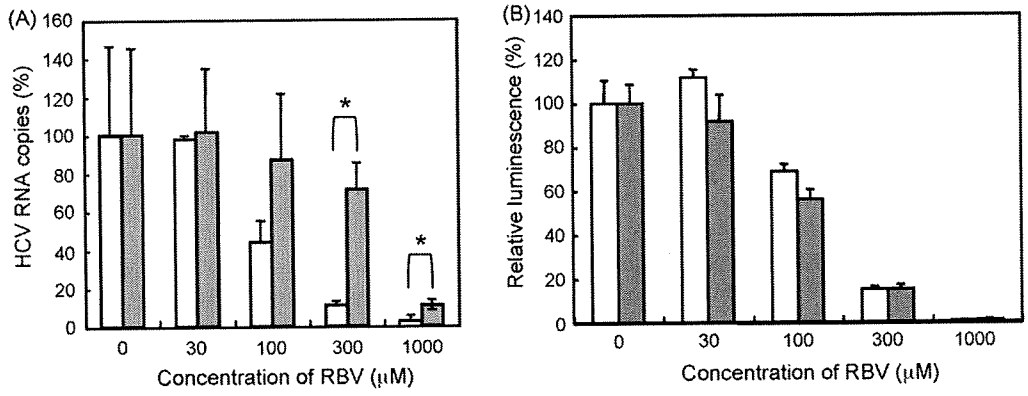

Fig. 2. Testing for replicon-derived resistance (A) or for cell-derived resistance (B). (A) Total RNA from RBV-resistant- or wild-type replicon cells was transfected into naïve Huh7 cells. After selection in 1 mg/ml G418 for 4 weeks, re-established replicon cells, wild-type derived (clear bars) and RBV resistance derived (gray bars), were treated with increasing concentrations of RBV in the absence of G418 for 3 days. HCV RNA copies per microgram total RNA were assessed and the levels from wild-type cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations. $^*p < 0.05$. (B) RBV-resistant- or wild-type replicon cells were cured by passage in IFN-α in the absence of G418. Cured cells were transiently transfected with the replicon RNA derived from pSGR-JFH1/luc. Transient replication assay of transfectants derived from wild-type (clear bars) and RBV resistance (gray bars) was performed after treatment with various concentrations of RBV for 72 h. The values for wild-type-derived cells without RBV treatment were set at 100%. Data are indicated as means with standard deviations.

Table 2
Mutation frequencies in HCV NS regions after 20-weeks culture with or without RBV treatment.

Region	nt length	No-treatment			RBV treatment		
		No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})	No. of non-synonymous mutations[a]	No. of synonymous mutations[a]	Mutation frequency (10^{-3})
NS3	1893	1.7 ± 2.1	2.3 ± 1.5	2.1	4.7 ± 2.4	6.5 ± 2.5	5.9[b]
NS4A	165	1.0 ± 1.0	0.3 ± 0.6	8.1	0.3 ± 0.5	0.5 ± 0.9	4.4
NS4B	780	1.3 ± 1.2	0.3 ± 0.6	2.1	2.3 ± 1.5	2.5 ± 1.2	4.7[c]
NS5A	1380	4.0 ± 1.2	2.0 ± 1.2	4.3	5.9 ± 1.2	6.2 ± 2.4	12.2[c]
NS5B	1773	4.5 ± 1.5	2.3 ± 1.5	3.8	4.8 ± 1.8	4.2 ± 1.1	9.0
NS3–NS5B	5991	12.5 ± 2.7	7.3 ± 2.7	–	17.8 ± 4.5	20.1 ± 4.6	–

[a] Values are means ± standard deviations.
[b] $p < 0.05$ relative to No-treatment by the unpaired t-test.
[c] $p < 0.01$ relative to No-treatment by the unpaired t-test.

mutations in RBV-resistant cells, particularly G-to-A and C-to-U transitions, as expected from previous studies. Although mutations were distributed throughout nonstructural regions, four major amino acid substitutions; T1134S in the NS3 region, P1969S in NS4B, V2405A in NS5A, and Y2471H in NS5B, not seen in wild-type cells were observed in most of the subclones among RBV-resistant replicon cells. T1134S, P1969S, V2405A, and Y2471H were present, respectively, in 7 of 11, 6 of 11, 8 of 13, and 7 of 13 PCR subclones sequenced.

3.4. Effects of T1134S, P1969S, V2405A, and Y2471H on RBV susceptibility

To test the possibility that any of the four mutations as identified confer resistance to RBV, we introduced these mutations individually into the JFH-1 subgenomic replicon containing a luciferase reporter gene. Cells transfected with mutant- or wild-type replicon RNA grown in the presence of various concentrations of RBV for 2 or 3 days. As demonstrated in Fig. 3A, the replication levels of all four mutant replicons (SGR-JFH1/Luc-T1134S, -P1969S, -V2405A, and -Y2471H) in the presence of 125 or 500 μM RBV were higher than those of the wild-type replicon. In particular, the Y2471H mutant significantly reduced susceptibility to RBV; replication levels of SGR-JFH1/Luc-Y2471H were 3–5-fold higher when compared to those of wild-type under the present assay conditions.

The relative replication activity of these mutant replicons was further determined in 3-day replication assay without drug treatment (Fig. 3B). All mutant replicons exhibited reduced efficiency relative to the wild-type replicon. Levels of the Y2471H-mutated replicon were approximately 30% of those of the wild-type, thus suggesting that replicon mutants with reduced sensitivity to RBV are associated with decreased replication fitness.

4. Discussion

It is generally accepted that, during chemotherapy against viral infection, high rates of viral replication and high frequencies of mutation lead to generation of drug-resistant mutants. Although several potential mechanisms for the inhibition of HCV replication by RBV have been proposed, the molecular mechanisms involved in the generation of RBV-resistant HCV remain poorly understood.

This study found that long-term treatment of HCV JFH-1-derived replicon cells with RBV leads to selection of preferential mutations in NS3 (T1134S), NS4B (P1969S), NS5A (V2405A) and NS5B (Y2471H) genes. Each mutation only required a single nucleotide change, and P1969S, V2405A and Y2471H are transition mutations, which are known to be commonly caused by incorporated RBV. Site-directed mutagenesis of these mutations into the replicon demonstrated that Y2471H plays a role in reduced susceptibility to RBV.

Crystal structure information revealed that HCV RdRp is organized into an arrangement with palm, fingers, and thumb subdomains (Lesburg et al., 1999). Residue 2471 (the 33rd position of NS5B) is present in the N-terminal loop region that bridges the fingers. Although this site is apparently distant from the active site of the polymerase in the palm region, it has been reported

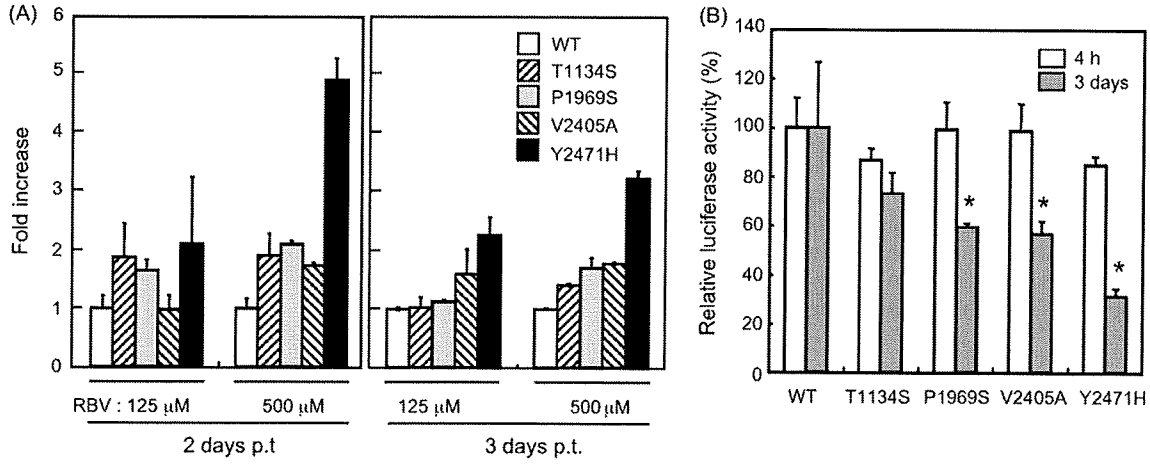

Fig. 3. Impact of major mutations in NS3–NS5B regions on RBV susceptibility (A) and replication capacity (B). Mutated replicons carrying single residue substitutions (T1134S, P1969S, V2405A, and Y2471H) were constructed and used for transient replication assay. Cells were transfected with either wild-type (WT) or with mutant replicon RNA in the absence or presence (125, 500 μM) of RBV. Luciferase activity was assessed at 4 h, 2 days and 3 days post-transfection (p.t.). (A) Luciferase activities of WT were set at 1, and the fold increases in the activities of mutants were plotted. (B) Luciferase activities in the absence of RBV at 4 h and 3 days post-transfection were shown. The activities of mutants were normalized as percentages of the WT activities. Data from triplicate samples were averaged and indicated with standard deviations. *$p < 0.05$ against WT.

that small molecules, such as benzimidazole compounds, are able to specifically bind the fingers-thumb interface and inhibit polymerase activity (Herlihy et al., 2008), thus suggesting that amino acid substitutions in the loop region may affect RNA polymerization. The involvement of tyrosine residue at position 415 of HCV NS5B in RBV resistance has been previously described for patients with genotype 1a infection and for the genotype 1b replicon (Young et al., 2003). Although the mechanism for resistance remains elusive, it has been hypothesized that RBV interacts with RdRp around this residue, which is located in the thumb subdomain, thus affecting RNA polymerization (Young et al., 2003).

Based on analysis of available sequences from Genbank, tyrosine at the 33rd residue of NS5B is conserved in all isolates of genotype 2a, but not in other genotypes. In genotype 1a and 1b isolates, 96% contain histidine and only a small population contains tyrosine or asparagine at the site. All the isolates of genotypes 3, 4, 5 and 6 contain histidine, whereas phenylalanine is conserved for genotype 2b. It should be noted that V2405 and P1969 are also completely conserved for genotype 2a but not for other genotypes. Therefore, it is likely that the identified HCV variants with reduced susceptibility to RBV are genotype-specific. It will be of interest to determine whether HCV genotype 2a is intrinsically more sensitive to RBV when compared with other genotypes.

At present, at least 4 mechanisms of action of RBV are proposed (Lau et al., 2002). They include (1) direct inhibition of the HCV replication machinery, (2) as an RNA mutagen that drives a rapidly mutating RNA virus over the threshold to "error catastrophe", (3) inhibition of the host enzyme inosine monophosphate dehydrogenase (IMPDH), and (4) enhancement of host T-cell-mediated immunity against viral infection. In addition to the direct inhibition, it is also possible that other mechanisms such as error-prone and IMPDH-inhibition are involved in HCV escape from RBV treatment. Further investigation of the interaction of HCV variants with the viral and cellular factors involved in viral resistance may improve understanding of the mechanism(s) of RBV resistance.

In conclusion, RBV encountered resistance from the HCV genotype 2a replicon largely mediated by mutations in the N-terminal region of NS5B. Although whether these mutagenic effects are also demonstrable in IFN-RBV combination therapy will require further studies, the mutations identified in this study represent the first drug-resistant variants belonging to HCV genotype 2a. The drug resistance patterns found in this study may be of benefit in prediction in vivo resistance profiles and the development of next-generation nucleoside analogues as anti-HCV drugs.

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Synthesis and anti-HCV activity of 2',5'-deoxy-5'-phenacyladenosine analogs

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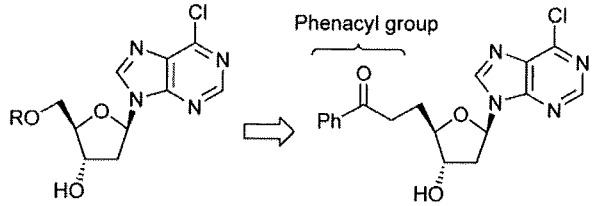
ABSTRACT

Several nucleoside analogs containing a methylene group instead of a 5'-*O* atom were synthesized to study the effect of the 5'-modification of nucleoside analogs on their anti-HCV activity. Among the analogs, a 5'-phenacyl analog exhibited good anti-HCV activity with an EC_{50} of 15.1 μM. This compound is hypothesized to function via a novel type of mechanism that does not involve the conventional 5'-*O*-triphosphorylation process.

INTRODUCTION

Hepatitis C virus (HCV) is the major causative agent of non-A and non-B hepatitis, and it is estimated to infect over 170 million individuals, that is, 3.5% of the world's population. HCV infection is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. However, current therapy based on the administration of pegylated interferon and ribavirin is often poorly tolerated and is effective in only 50% of patients. Therefore, it is important to prioritize the development of effective anti-HCV agents from the viewpoint of public health.[1]

In our previous study,[2] we revealed that several 5'-*O*-masked analogs of 6-chloropurine-2'-deoxyriboside, such as benzoate **1**, exhibit effective anti-HCV activity in a subgenomic replicon cell line and are more potent than the corresponding unmasked analog **2** (Fig. 1). Since it is generally accepted that most nucleoside antivirals exhibit their potency after being converted to the corresponding 5'-triphosphates,[1a] the unmasked 5'-hydroxyl group is indispensable for antiviral activity. In this respect, our result that the 5'-*O*-masking leads to an improvement in the anti-HCV activity appears to be inconsistent with established results. We presume that the anti-HCV activity of the 5'-*O*-masked analogs arises from a new type of mechanism that does not involve the 5'-*O*-triphosphorylation process. However, there is room for discussion on the new mechanism because certain C–O bonds, for example, the carboxylic ester bond of **1** (i.e., the benzoate moiety), are often hydrolyzed in cultured cells; in other words, it is possible that **1** simply functions as a prodrug of **2**. Therefore, in order to confirm the effectiveness of 5'-*O*-masking groups, particularly that of the benzoyl group of **1**, we synthesized phenacyl analog **3** and evaluated its anti-HCV activity; in this analog, the 5'-*O* atom was replaced with a methylene group to prevent the hydrolytic removal of the benzoyl group.



1: R = Bz (EC_{50} = 6.1 μM)
2: R = H (EC_{50} = 47.2 μM)
3

Fig. 1 Structures of 5'-modified analogs.

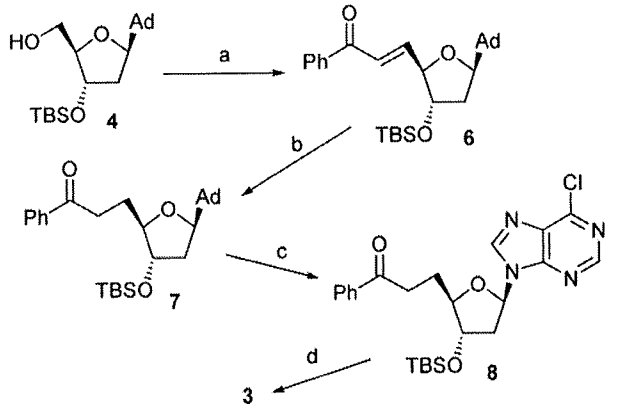
RESULTS AND DISCUSSION

Chemistry—Synthesis of **3** began with 3'-*O*-TBS-2'-deoxyadenosine (**4**),[3] as shown in Scheme 1. A one-pot oxidation/Wittig reaction[4] with Dess-Martin periodinane (DMP) and stabilized ylide **5a** afforded the desired compound **6** in 60% yield. The one-pot procedure was effective since the corresponding 5'-aldehyde was not sufficiently stable for isolation. The C–C double bond of the enone moiety in **6** was reduced with indium hydride generated in situ by using Bu_3SnH-$InCl_3$ to afford **7** in 93% yield.[5] After the substitution of the amino group of **7** with a chloro group, the resulting compound **8** was treated with tetrabutylammonium fluoride (TBAF) to afford the desired product **3** in 91% yield. The moderate yield (40%) of the chlorination step was mainly due to a competitive elimination of the nucleobase moiety.

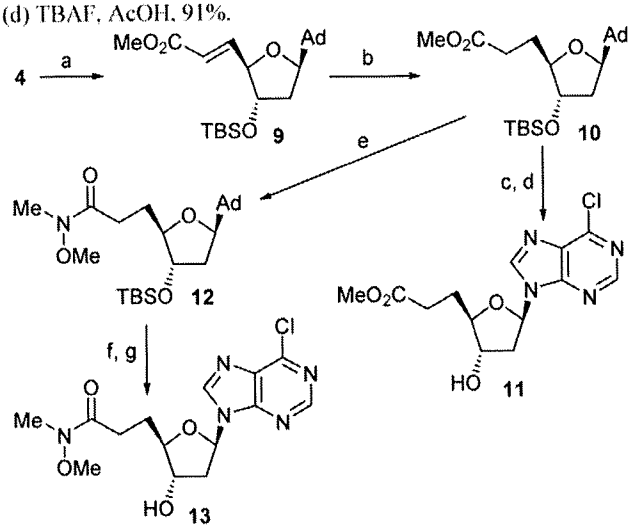
Since we are interested in the structure-activity relationship of the 5'-position, syntheses of other analogs were also attempted (Scheme 2). The one-pot oxidation/Wittig reaction is an attractive method for preparing analogs; thus, ylides **5b–d** (**5b**: Ph_3P=$CHCO_2Me$; **5c**: Ph_3P=CHCHO; **5d**: Ph_3P=CHC(O)N(OMe)Me) were subjected to the reaction instead of **5a**. However, the desired compound **9** was obtained (90% yield) only in the case of **5b**. The olefin moiety of **9** was reduced by

hydrogenation to afford **10**, which was subsequently converted to **11** in a manner similar to the conversion of **7** to **3**. Compound **10** was also used to synthesize an amide analog **13** via a three-step sequence.

To prepare various analogs, radical allylations between 5'-deoxy-5'-iodinated derivatives and allyltributylstannane, as well as Wittig reaction, were investigated. However, none of the attempts were successful in obtaining the desired products.



Scheme 1 *Reagents and conditions:* (a) DMP, Ph_3P=CHC(O)Ph (**5a**), 60%; (b) Bu_3SnH, $InCl_3$, 93%; (c) ^{t}BuONO, Et_4NCl, 40%; (d) TBAF, AcOH, 91%.



Scheme 2 *Reagents and conditions:* (a) DMP, Ph_3P=$CHCO_2Me$ (**5b**), 90%; (b) Pd-C, H_2, 94%; (c) ^{t}BuONO, Et_4NCl, 41%; (d) TBAF, AcOH, 96%; (e) Me(MeO)NH, BuLi, 96%; (f) ^{t}BuONO, Et_4NCl; (g) TBAF, AcOH, 24% from **12**.

Biological evaluations— Compounds **3**, **11**, and **13** were assayed for their ability to inhibit HCV RNA replication in a subgenomic replicon cell line.[6] As shown in Table 1, phenacyl analog **3** exhibited anti-HCV activity with an EC_{50} of 15.1 μM, which is nearly comparable to that of **1**. Interestingly, compounds **11** and **13** also exhibited anti-HCV activities.

From this result, it appears that the phenacyl group ($BzCH_2$-) at the C5' position as well as the benzoyloxyl group (BzO-) is an effective functional group for anti-HCV activity; this strongly supports our hypothesis that the 5'-*O*-masking group can contribute to anti-HCV activity not only as a part of the prodrug system but also as a part of the substrate.

Table 1 Anti-HCV activity (EC_{50}) and cytotoxicity (CC_{50}) of **1**, **3**, **11**, and **13** in HCV replicon assay.

Compounds	EC_{50} (μM)	CC_{50} (μM)
1	6.1	111
3	15.1	76.3
11	32.9	>200
13	40.4	>200

CONCLUSION

We have found that 5'-phenacyladenosine analog **3** exhibited good anti-HCV activity, which is nearly comparable to that of 5'-*O*-benzoyl analog **1**.[7] Thus, this result supports a new type of mechanism of the 5'-modified analogs, which does not involve the conventional 5'-*O*-phosphorylation process.

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Identification of cellular and viral factors related to anti-hepatitis C virus activity of cyclophilin inhibitor

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We have so far reported that an immunosuppressant cyclosporin A (CsA), a well-known cyclophilin (CyP) inhibitor (CPI), strongly suppressed hepatitis C virus (HCV) replication in cell culture, and that CyPB was a cellular cofactor for viral replication. To further investigate antiviral mechanisms of CPI, we here developed cells carrying CsA-resistant HCV replicons, by culturing the HCV subgenomic replicon cells for 4 weeks in the presence of CsA with G418. Transfection of total RNA from the isolated CsA-resistant cells to naïve Huh7 cells conferred CsA resistance, suggesting that the replicon RNA itself was responsible for the resistant phenotype. Of the identified amino acid mutations, D320E in NS5A conferred the CsA resistance. The replicon carrying the D320E mutation was sensitive to interferon-α, but was resistant to CsA and other CPIs including NIM811 and sanglifehrin A. Knockdown of individual CyP subtypes revealed CyP40, in addition to CyPA and CyPB, contributed to viral replication, and CsA-resistant replicons acquired independence from CyPA for efficient replication. These data provide important evidence on the mechanisms underlying the regulation of HCV replication by CyP and for designing novel and specific anti-HCV strategies with CPIs. (*Cancer Sci* 2009; 100: 1943–1950)

Hepatitis C virus (HCV) is a leading cause of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma (HCC), and affects an estimated 170 million people worldwide.[1] The current standard therapy for patients infected with HCV is the combination treatment with pegylated interferon and ribavirin.[2,3] However, approximately half of individuals infected with HCV are unable to reach sustained virological response following such treatment. In addition, several side effects have been reported, which hinder continued treatment and impair the regimen efficacy. Thus, the development of novel anti-HCV strategies is essential for the treatment of infected individuals.

We have previously reported that a well-known immunosuppressant cyclosporin A (CsA) strongly suppressed the replication of HCV *in vitro*, in a manner independent of the interferon (IFN) signal transduction pathway.[4] Cyclophilin B (CyPB), a cellular target of CsA, was subsequently revealed to facilitate viral replication via the regulation of the RNA binding ability of NS5B.[5] Thus CyP, in addition to viral proteins including NS3 protease and NS5B polymerase, can also prove useful as a molecular target for antiviral strategies. Indeed, the non-immunosuppressive CsA analogs NIM811, DEBIO-025, and SCY635 have been observed to exert strong inhibitory effects on HCV replication, and these compounds are now in clinical trial.[6–8] Thus, it is crucial to deepen understanding of the anti-HCV actions of cyclophilin inhibitor (CPI) in order to maximize the efficacy of the agent. CPIs also need to face challenges such as side effects and drug resistance, which was observed as barrier to successful treatment in cases of human immunodeficiency virus (HIV),[9–12] and further clarification of the mechanism of CPI's anti-HCV activities is vital for the development of stronger and more specific therapeutic drug types. For this purpose, we here established and characterized the resistant replicon to CPIs using the subgenomic replicon system. We found that D320E, a mutation in NS5A, conferred resistance to CsA on the replicon, while additional mutations in NS3, Q86R and I252T seen in our CsA-resistant clone affected the replication fitness positively and negatively, respectively. The CsA-resistant replicons with the D320E mutation showed cross-resistance to other CPIs, NIM811 and sanglifehrin A (SFA), which were thus verified to suppress HCV replication through targeting CyP, and those resistant replicons were inhibited by treatment with IFNα as effectively as the wild type. Knockdown of individual CyP subtypes in the wild-type and CsA-resistant replicon cells revealed that CyP40, besides CyPA and CyPB, played important roles in HCV replication, and CyPA was related to the CsA-resistance. These results are important for elucidating additional mechanisms of the regulation of HCV replication by CyP and also for designing novel and specific anti-HCV strategies with CPI.

Materials and Methods

Compounds. CsA and IFNα were purchased from Merck Biosciences (San Diego, CA, USA) and Otsuka Pharmaceutical (Tokyo, Japan), respectively. NIM811 and SFA were generously provided by Novartis (Basel, Switzerland).

Cell culture. MH14 cells were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carlsbad, CA, USA) with 10% fetal bovine serum, nonessential amino acids (Invitrogen), and L-glutamine (Invitrogen) in the presence of 700 μg/mL G418 (Invitrogen).

Establishment of cell clones. We established each cell clone along with the outline shown in Figure 1. CsR#4, CsR#10, and CsR#11 cells were established through the selection of MH14#12 cell colonies in the presence of 1000 μg/mL G418 and 2 μg/mL CsA. CsR#11-2 and CsR#11-3 cells were established from Huh7 cells transfected with total RNAs extracted from CsR#11 cells in the presence of 700 μg/mL G418. Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells were produced by 700 μg/mL G418 selection of Huh7 cells transfected with 5 μg RNA transcribed from pMH14 carrying the individual mutations Q86R in NS3 and D320E in NS5A, double mutations Q86R in NS3 and D320E in NS5A, and triple mutations Q86R in NS3, I252T in NS3, and D320E in NS5A, respectively.

Colony formation assay. MH14 cells were treated with either CsA or NIM811 in the presence of 700 μg/mL G418 for 4 weeks, followed by fixation and staining with crystal violet.

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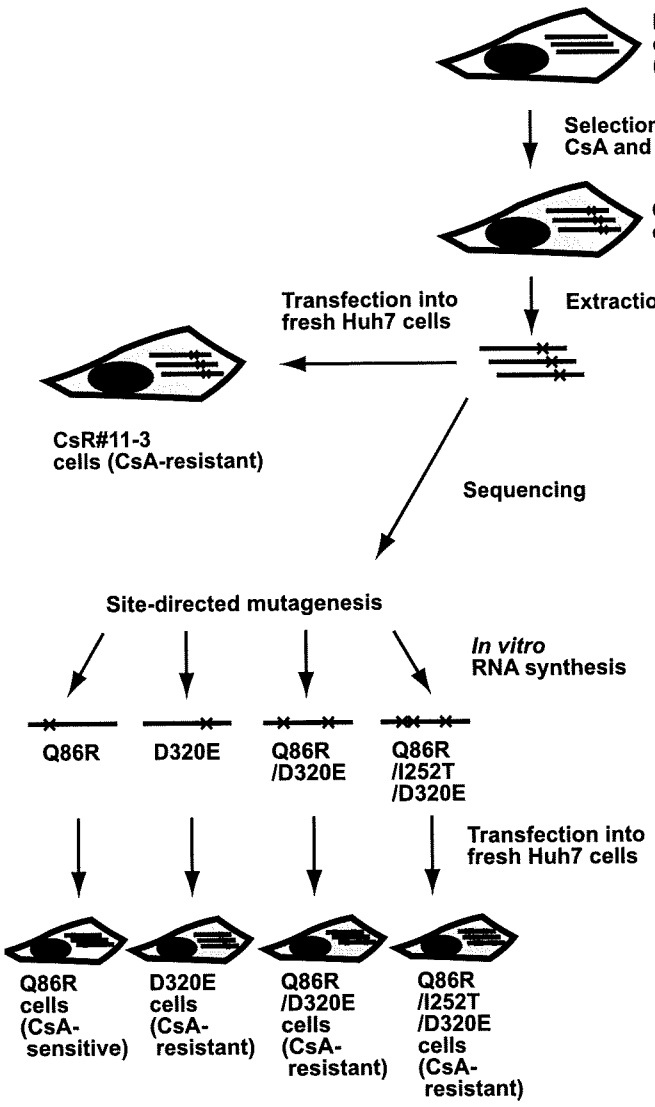

Fig. 1. Schematic diagram outlining the production of individual cell clones carrying hepatitis C virus (HCV) subgenomic replicons. MH14#12 cells, carrying wild-type HCV subgenomic repicon, were treated with 2 μg/mL cyclosporin A (CsA) in the presence of 1000 μg/mL G418 and CsR#11 cells were selected. Total RNA was extracted from CsR#11 cells and transduced into naïve Huh7 cells to select CsR#11-3 cells, and sequencing of the replicon RNA in CsR#11 cells identified mutations, Q86R in NS3, I252T in NS3, and D320E in NS5A. Site-directed mutagenesis followed by *in vitro* RNA synthesis generated HCV replicon RNA carrying Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E mutations. Transduction of the RNA into naïve Huh7 cells resulted in the production of Q86R, D320E, Q86R/D320E, and Q86R/I252T/D320E cells. The sensitivity of each replicon clone to CsA is presented as 'CsA-resistant' or 'CsA-sensitive'.

Real-time RT-PCR analysis. The 5′-non-translated region of HCV-RNA was quantified using an ABI Prism 7500 sequence detector (Applied Biosystems, Foster City, CA, USA), as previously described.[4]

Replicon sequencing. Total RNA from replicon cells was extracted with sepasol-RNA I Super (Nacalai Tesque, Kyoto, Japan) and subjected to RT-PCR reaction using super script III (Invitrogen). The products were then amplified by dividing the whole HCV region into approximately 300 bp using appropriate primer sets, and the sequence of the entire region encoding non-structural proteins was determined.

Plasmid construction. The Q86R and I252T mutations in NS3 and the D320E mutation in NS5A were generated via site-directed mutagenesis using the following primer sets: Q86R (S) 5′-AGGACCTCGTCGGCTGGCGGGCGCC-3′ plus Q86R (AS) 5′-GGCGCCCGCCAGCCGACGAGGTCCT-3′, I252T (S) 5′-AACACCAGAACTGGGGTAAGGACCA-3′ plus I252T (AS) 5′-TGGTCCTTACCCCAGTTCTGGTGTT-3′, and D320E (S) 5′-GAGTATAATCCTCCACTGCTAGAGC-3′ plus D320E (AS) 5′-GCTCTAGCAGTGGAGGATTATACTC-3′, respectively. The PCR products carrying either Q86R in NS3, I252T in NS3, or D320E in NS5A were inserted into the NotI-MluI and MluI-XbaI sites of pMH14, respectively. The resultant plasmids were termed pMH14 (Q86R), pMH14 (I252T), and pMH14 (D320E) respectively. The double mutant carrying both Q86R and D320E mutations was produced by exchanging the MluI-XbaI region of pMH14 (Q86R) with that of pMH14 (D320E), and termed pMH14 (Q86R/D320E). The triple mutant carrying Q86R, I252T, and D320E was produced by exchanging the Not-MluI region of pMH14 (D320E) with the fragments amplified by the primer set, I252T (S) plus I252T (AS), using pMH14 (Q86R) as templates for the PCR reaction. Sequence analysis of the resultant plasmids was also undertaken for confirmation of the mutations.

***In vitro* RNA synthesis.** Wild-type and mutant RNA of pMH14 was prepared by *in vitro* transcription using the MEGAscript T7 kit (Ambion, Austin, TX, USA), as described previously.[13]

Electroporation and colony formation. 8×10^6 cells suspended in 400 μL of cytomix buffer (120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4, 25 mM HEPES, 2 mM EGTA, and 5 mM $MgCl_2$, together with 2 mM ATP, 5 mM reduced form of glutathione, and 1.25 % DMSO) were electroporated at 250 V, 950 μF with either 100 μg total RNA extracted from replicon cells or 5 μg RNA transcribed *in vitro* from the HCV replicon construct cDNA. Cells were then treated with 1000 μg/mL G418 for 4 weeks following electroporation.

RNAi. Validated siRNAs against CyPB were purchased from Invitrogen. SiRNA duplexes against CyPA (siCyPA161, 5′-UCUGUGAAAGCAGGAACCCUU-3′; siCyPA285, 5′-GAUG

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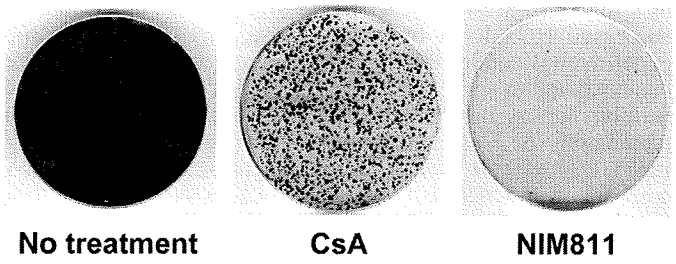

Fig. 2. Cyclophilin (CyP) inhibitor (CPI)-resistant colony emergence. MH14#12 cells were treated either without (left panel) or with 2 μg/mL CPIs, cyclosporin A (CsA) (middle panel), and NIM811 (right panel), in the presence of 700 μg/mL G418. Following 4 weeks in culture, cells were fixed and stained with crystal violet.

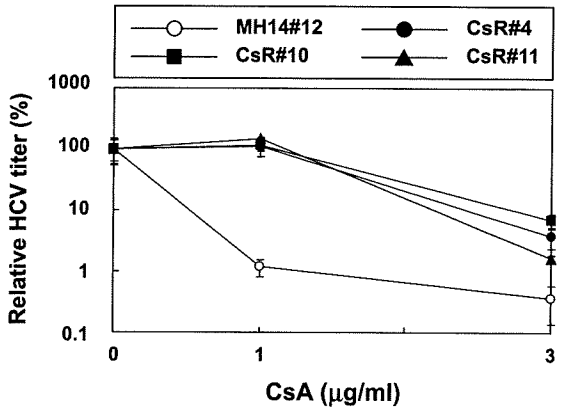

Fig. 3. Cyclosporin A (CsA) responses of the hepatitis C virus (HCV) replicon clones surviving the selection with CsA and G418. MH14#12 cells and three MH14#12-derived cell clones that survived double selection with G418 and CsA, CsR#4, CsR#10, and CsR#11, were treated with 1 and 3 μg/mL CsA for 7 days, and the HCV-RNA titers were measured by real-time RT-PCR. The data represent the percentage of HCV-RNA level in cells either untreated or treated with CsA, and the dots represent the means of three independent experiments.

CCAGGACCCGUAUGCUU-3′; siCyPA459, 5′-CUUCUUG CUGGUCUUGCCAUU-3′) were synthesized (Yahima Pure Chemicals, Osaka, Japan). SiRNAs against CyP40 were purchased from Invitrogen (siCyP40-3) and from Ambion (siCyP40-4). Pre-designed siRNAs, siCyPC, siCyPE, siCyPF, and siCyPG were obtained from Ambion. Transfection was performed using Lipofectamine RNAiMAX Transfection Reagent (Invitrogen) with 20 nM siRNAs in the absence of CsA according to the manufacturer's protocol.

Reverse transcription–polymerase chain reaction (RT-PCR) analysis. RT-PCR was performed as described previously[4] using the following primer sets: 5′-TGTTCTTCGACATTGCCGTC-3′ and 5′-CAGTCTTGGCAGTGCAGATG-3′ to detect mRNA for CyPA, 5′-TCTCCGAACGCAACATGAAG-3′ and 5′-CTGCGA TGATCACATCCTTC-3′to detect mRNA for CyPB, 5′-GGCGCA CTTGTGTTTTCTTC-3′ and 5′-TGCCATAGTGCTTCAGCTTG-3′ to detect mRNA for CyPC, 5′-TTTCGTGCACTGTGTACAGG-3′ and 5′-TTGGCTCTATCTGCTGTCTC-3′ to detect mRNA for CyP40, 5′-AGAGGAAGTGGACGACAAAG-3′ and 5′-GATGTCCATGTACACCTGAG-3′ to detect mRNA for CyPE, 5′-TGGAGCTGAAGGCAGATGTC-3′ and 5′-ACGTGACCG AACACAACATG-3′ to detect mRNA for CyPF, 5′-GAGTTGT CTCTTTCACAGAG-3′ and 5′-AACTGAGTATCCGTACCTCC-3′ to detect mRNA for CyPG, and 5′-ATGGGGAAGGTGAA GGTCGG-3′ and 5′-TGGAGGGATCTCGCTCCTGG-3′ to detect glyceraldehydes-3-phosphate dehydrogenase (GAPDH).

Results

Resistance emergence against individual CPIs. We have previously demonstrated the robust anti-HCV activities of CPIs, and it was reported that CPI significantly decreased HCV viral load in HCV-infected patients.[14,15] The problem of the drug-resistant HCV variants, hence, should be assessed *in vitro*, considering that practical efficacies of these inhibitors with long-term effectiveness are required in patients. In the first step of this study, we investigated the emergence of drug resistant replicon against CPIs. We treated MH14#12 cells, Huh7 cells carrying wild-type MH14 replicon with 2 μg/mL CsA, or the non-immunosuppressive analog NIM811 in the presence of 700 μg/mL G418 for 4 weeks. To visualize the appearance of drug-resistant clones, we stained cells after the selection. We observed colonies resistant to CsA, while we obtained few colonies under the treatment with the same concentration of NIM811 (Fig. 2).

Isolation and characterization of replicon cells resistant to CsA. To characterize the CsA-resistant HCV, we isolated the resistant clones following selection with 2 μg/mL CsA and 1000 μg/mL G418 for 4 weeks. We obtained several clones (named CsR cells), and examined their CsA responses. In contrast to the wild-type MH14#12 replicon cell, which showed an approximately 2-log reduction of HCV-RNA level by treatment with 1 μg/mL CsA for 7 days, all the clones isolated (the results of three representative clones, CsR#4, CsR#10, and CsR#11 cells are shown here) demonstrated resistant phenotypes against CsA with no significant reduction of HCV-RNA by CsA treatment at 1 μg/mL (Fig. 3). The resistance of these clones was thought to arise as a result of (1) mutations on the HCV-RNA genome or (2) alterations in cellular factors. To test the first possibility, we investigated whether HCV-RNA itself in CsR#11 could induce the CsA resistance to naïve cells. Fresh Huh7 cells were transfected with total RNA, including HCV replicon RNA, extracted from CsR#11 cells or MH14#12 cells as controls and cultured for 3 weeks in the presence of G418 (Fig. 1). The resulting colonies were isolated and propagated individually (named cell clones from total RNA of wild-type MH14#12, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and those from CsR#11, CsR#11-2, CsR#11-3, and CsR#11-5 cells). The HCV-RNA titer in MH14#12-derived cells was reduced approximately to 100th by treatment with 1 μg/mL CsA for 7 days (Fig. 4). In contrast, cell clones generated from CsR#11 cells retained a normal HCV titer level after treatment with CsA, indicating that they had lost their sensitivity to CsA. Thus, it was suggested that the CsA-resistant profile in CsR#11 cells was attributed to its HCV-RNA.

D320E mutation in NS5A confers HCV replicon resistance to CsA. In order to identify the mutation in the HCV genome that resulted in the resistance to CsA, HCV subgenomic RNA isolated from CsR#11 cells was sequenced across the subgenomic region encoding non-structural proteins. We found three specific base changes that resulted in amino acid alteration including changes from glutamine to arginine, and isoleucine to threonine at positions 86 (Q86R) and 252 (I252T) in NS3, respectively, and a change from aspartic acid to glutamic acid at position 320 (D320E) in NS5A. Given that all these three mutations, Q86R, I252T, and D320E, were retained in every replicon in CsR#11-2, CsR#11-3, and CsR#11-5 cells, it is likely that they are inherited from CsR#11 cells and are associated with the acquired CsA-resistant phenotype. To examine this possibility, we synthesized replicon RNA carrying all these three mutations and established cells carrying these replicons. The resultant cell clones were named Q86R/I252T/D320E-1 and -3 cells. Treatment of these cells with 1 μg/mL CsA decreased the HCV titer only

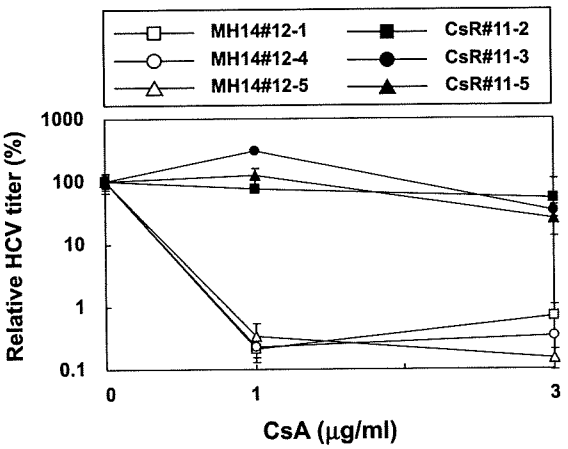

Fig. 4. Hepatitis C virus (HCV) RNA alteration contributed to cyclosporin A (CsA)-resistance. Total RNA extracted from CsA-resistant CsR#11 cells or that from wild-type MH14#12 cells as a control was transfected into Huh7 cells. Colonies established after 4-week selection with G418 were isolated, propagated individually, and tested for CsA response. Three cell clones derived from MH14#12 cells, MH14#12-1, MH14#12-4, and MH14#12-5 cells, and three cell clones from CsR#11 cells, CsR#11-2, CsR#11-3, and CsR#11-5 cells, were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments.

by 1 log, in contrast to the wild-type MH14 clone, in which CsA decreased HCV-RNA by more than 2 logs under the same experimental condition (Fig. 5b). Thus, these mutations were demonstrated to confer CsA resistance; in addition to this, some cellular factors in Huh7 cells may also play minor roles in modulating the CsA sensitivities, given the result that Q86R/I252T/D320E cell clones were relatively sensitive to CsA compared with CsR#11-derived cell clones as shown in Figure 4. We next aimed to determine which of the three mutations, Q86R/I252T/D320E, was responsible for the CsA resistant phenotype, and individual mutations were engineered back into the wild-type MH14 replicon and stable replicon cells were produced as described above. Among three single amino acid mutations, the I252T mutation in NS3 resulted in a significant reduction in replication fitness (Fig. 5a), and almost failed to produce cell colonies. Cell clones harboring MH14 with both Q86R and D320E mutations, Q86R/D320E-2 and Q86R/D320E-3 cells, showed reduced sensitivity to CsA that was comparable to the levels in Q86R/I252T/D320E cells, suggesting Q86R and/or D320E mutation(s) was enough to confer the resistance. Subsequently, we treated the replicon cell clones carrying MH14 with either Q86R or D320E mutation alone, Q86R (Q86R-1 and -4 cells) and D320E (D320E-1 and -2 cells) cells, with CsA for 1 week. The titer of Q86R replicons was reduced to less than 100th by CsA treatment at a concentration of 1 μg/mL, similar to the wild type. In contrast, HCV replicon with D320E mutation in NS5A exhibited reduced sensitivity to CsA, resulting in little reduction of HCV-RNA by the treatment with 1 μg/mL CsA (Fig. 5b). Q86R mutation considerably enhanced colony

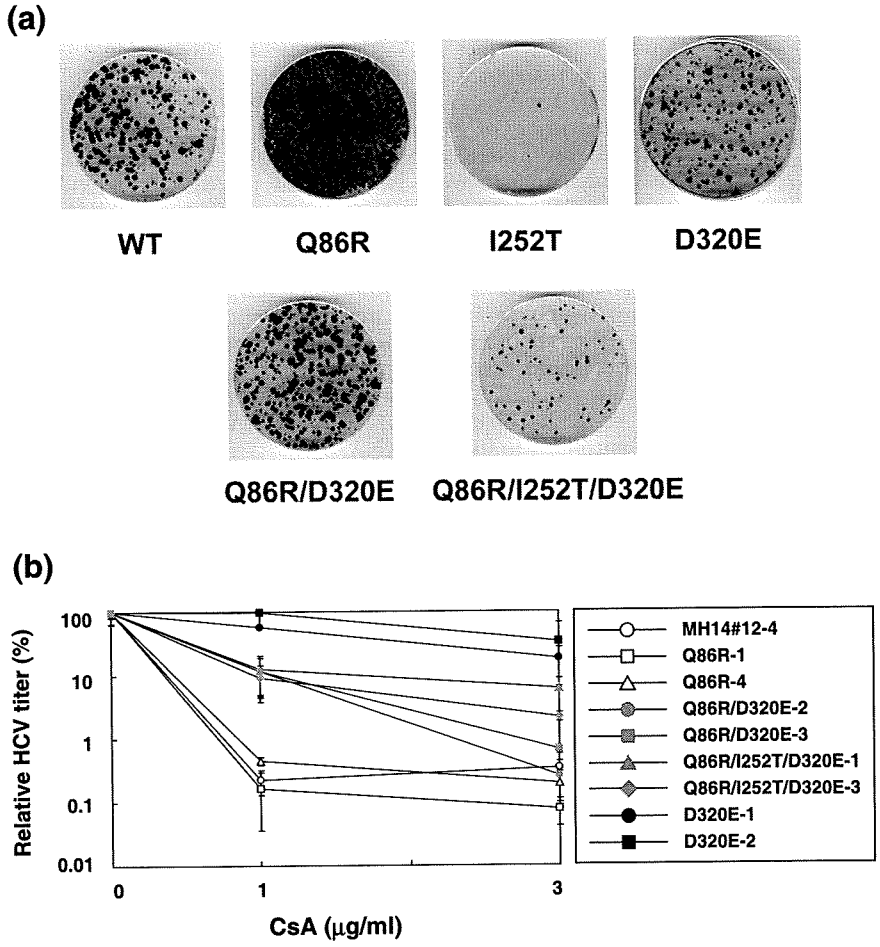

Fig. 5. The amino acid mutation D320E in NS5A conferred the cyclosporin A (CsA)-resistance to hepatitis C virus (HCV) replicons. (a) Colony formation assay for replicons carrying mutations. Five-microgram replicon RNA carrying the mutation(s), Q86R in NS3, I252T in NS3, D320E in NS5A, Q86R and D320E, or Q86R, I252T and D320E, or wild-type RNA transcribed *in vitro* were transduced into Huh7 cells. After culture with G418 for 4 weeks, colonies were stained with crystal violet. (b) Cell clones with replicons carrying indicated mutations were treated with 1 and 3 μg/mL CsA for 7 days. HCV-RNA titers were quantified by real-time RT-PCR analysis. The dots represent the means of three independent experiments. MH14#12-4, wild-type replicon; Q86R-1 and Q86R-4, replicon with Q86R mutation; D320E-1 and D320E-2, replicon with D320E mutation; Q86R/D320E-2 and Q86R/D320E-3, replicon with both Q86R and D320E mutations; Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3, replicon with all three mutations, Q86R, I252T, and D320E.

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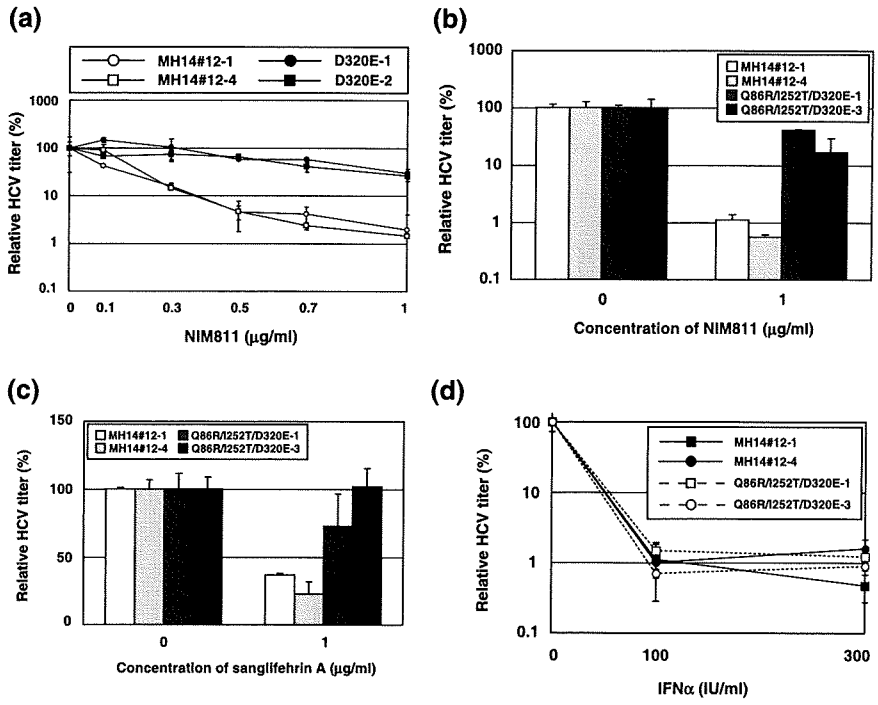

Fig. 6. Cyclosporin A (CsA)-resistant replicons demonstrated cross resistance to additional cyclophilin (CyP) inhibitors, NIM811 and sanglifehrin A (SFA), but not interferon (IFN)-α. MH14#12-1, MH14#12-4, D320E-1 and D320E-2 cells were treated with NIM811 at 0.1, 0.3, 0.5, 0.7 and 1 μg/mL (a), and MH14#12-1, MH14#12-4, Q86R/I252T/D320E-1, and Q86R/I252T/D320E-3 cells were treated with 1 μg/mL NIM811 (b), 1 μg/mL SFA (c), and 100 and 300 IU/mL IFN-α (d) for 7 days. The amounts of hepatitis C virus (HCV) RNA were quantified by real-time RT-PCR analysis. The data represent the means of three independent experiments.

formation efficiency and the D320E mutation showed little significant effect on the efficiency (Fig. 5a). Thus, the D320E mutation in NS5A was suggested as a sufficient factor to induce HCV replicon resistance to CsA, while the Q86R mutation was likely not to contribute to the resistance but to augment the efficiency of HCV replication itself.

The point mutation in NS5A conferred resistance to CPIs. Next, we examined cross-resistance between CsA and other CPIs or IFNα using the CsA-resistant replicon we produced above. Treatment with 0.1–1 μg/mL NIM811 for 7 days showed that the response to NIM811 of D320E-1 and -2 cells was less compared with that of MH14#12-1 and -4 cells, indicating that a CsA-resistant clone also acquired NIM811 resistance (Fig. 6a). A similar result was seen using Q86R/I252T/D320E cells (Fig. 6b). We then tested the anti-HCV activity of SFA, an additional CPI possessing distinct chemical backbone from those of cyclosporins.[16,17] Treatment with 1 μg/mL SFA reduced HCV replication in the wild-type cells, MH14#12-1, and -4 cells; however, it did not significantly reduce replication in Q86R/I252T/D320E cells (Fig. 6c). These results demonstrate that the CsA-resistant cells described in this study were also resistant to additional CPIs, confirming that these two compounds exerted anti-HCV effects via targeting CyP. Finally, we treated Q86R/I252T/D320E cell clones with 100 and 300 IU/mL IFNα for 7 days, and HCV-RNA titers were reduced by 2 logs in both clonal cell lines examined, Q86R/I252T/D320E-1 and Q86R/I252T/D320E-3 cells, as well as in wild-type MH14#12-derived cells, MH14#12-1, and MH14#12-4 cells (Fig. 6d). These results suggested no cross-resistance between CsA and IFNα, consistent with the previous report that the anti-HCV activity of CsA was independent of the IFNα signaling pathway.[18]

The role of CyP subtypes in HCV replication. We have previously reported that CyPB played a significant role in the efficient replication of HCV and CsA inhibited CyPB-mediated regulation of HCV replication. We have also suggested the involvement of other CyP subtypes in HCV replication.[19] To gain further insight into mechanisms underlying the anti-HCV properties of CPIs, we examined the roles of individual CyP subtypes in HCV replication in the wild-type MH14#12-1 and -4 replicon cells. In order to achieve this we knocked down CyPB with siRNAs (Fig. 7d), siCyPB-1 and -2, and found that this procedure reduced the amount of replicons to approximately half the initial level (Fig. 7c), a result consistent with the previous reports. Knockdown of CyPC, CyPE, CyPF, and CyPG (Fig. 7b) did not significantly affect the viral replication under these experimental conditions (Fig. 7a). Some groups have also suggested a role of CyPA in HCV replication.[20,21] Then, we synthesized individual siRNAs reported so far to be effective against CyPA, siCyPA-161, siCyPA-285, and siCyPA-459, and transfected them using a reagent with low cytotoxic activity to knock down endogenous CyPA (Fig. 7d). As shown in Figure 7c, the siRNAs directed against CyPA reduced HCV titers in MH14#12-1, and -4 cells. We previously observed that knockdown of CyPA little affected HCV replication in MH14 cells.[5] Here, by using a new transfection reagent with less cytotoxicity and higher knockdown efficiency, we observed the effect of CyPA knockdown on HCV replication, which suggests that CyPA-mediated regulation of HCV replication is strictly influenced by CyPA's expression level and cellular condition. Under this experimental condition, our RNAi experiments also displayed that knockdown of CyP40 (Fig. 7g), alternatively known as peptidylprolyl isomerase D (NM_005038), decreased the HCV titer (Fig. 7f) without significant cytotoxic effects, presenting CyP40 as additional cellular factor required for HCV replication.

CyPA was related to the CsA-resistant phenotype. We next asked which CyP subtype among CyPA, B, and 40 was related to the CsA resistance observed in our clones. To answer this question, we performed RNAi experiments in the CsA-resistant cell lines, CsR#11-2 and CsR#11-3 cells. Transfection of these cells with specific CyPB or CyP40 siRNAs resulted in the reduction of each subtype (Fig. 7d,g) and decreased the amount of HCV-RNA in CsR#11-derived cells and wild-type MH14#12-derived cells by approximately 50% (Fig. 7c,f). Thus, CyPB and CyP40 were likely to play roles in viral replication, even in the CsA-resistant cells. However, relative HCV titers were not reduced by CyPA knockdown in these CsA-resistant cells in contrast to the case with the wild-type replicon cells (Fig. 7c). A similar resistant phenotype to CyPA knockdown was observed in D320E

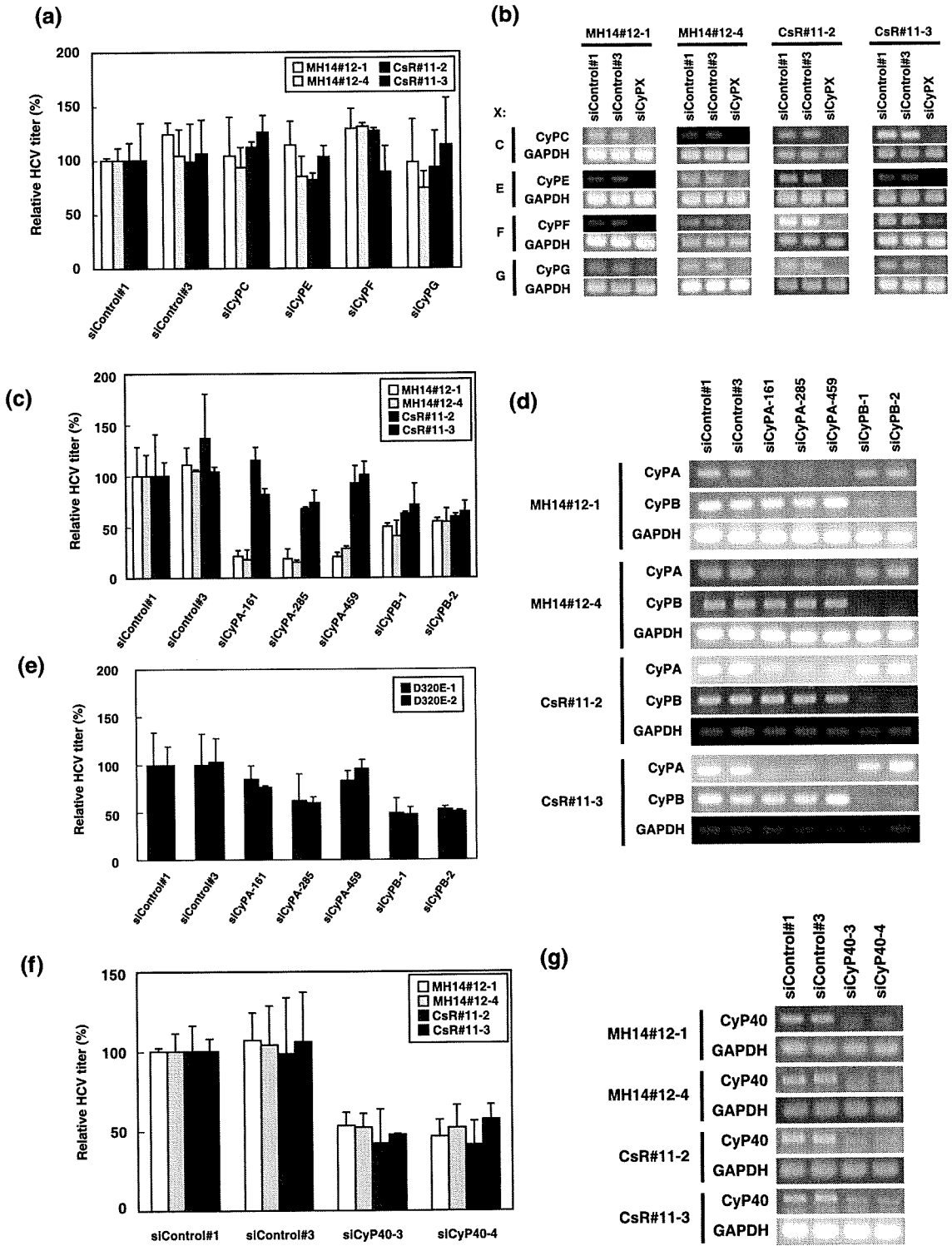

Fig. 7. Cyclophilin (CyP) subtypes related to anti-hepatitis C virus (HCV) effect of CyP inhibitor. MH14#12-derived cells, MH14#12-1 and MH14#12-4 cells, and CsR#11-2 and CsR#11-3 cells, were transfected with siRNAs specific for CyPC (siCyPC), CyPE (siCyPE), CyPF (siCyPF), and CyPG (siCyPG) (a); or those specific for CyPA (siCyPA-161, siCyPA-285, and siCyPA-459) and CyPB (siCyPB-1 and siCyPB-2) (c); or those specific for CyP40 (siCyP40-3 and siCyP40-4) (f); or randomized siRNA controls (siControl#1 and siControl#3). D320E cells were also transfected with the above siRNAs specific for either CyPA or CyPB (e). At 5 days post-transfection, the levels of HCV-RNA were quantified by real-time RT-PCR analysis. The mRNA levels of individual CyP subtypes, CyPC, CyPE, CyPF, and CyPG (CyPX corresponds to each CyP subtype indicated on the left side of the panels) (b), or CyPA and CyPB (d), or CyP40 (g) were measured using glyceraldehydes-3-phosphate dehydrogenase (GAPDH) as internal controls by RT-PCR analysis at 5 days post-transfection. The data represent the means of three independent experiments.

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cell clones (Fig. 7e), showing that CyPA was related to the CsA-resistance conferred by D320E mutation. The CsA-resistant clones obtained in this study were likely to have acquired CyPA independence for efficient HCV replication.

Discussion

Given that CPIs suppressed HCV viral load in cell culture and in patients with chronic hepatitis C,[14,15] CPIs are expected to be new anti-HCV agents. It is important to further reveal the factors related to CPI's anti-HCV activities, thinking over the practical use of CPIs with maximized efficacy and high specificity facing challenges such as side effects and the emergence of resistance to them in clinical settings. Here, we isolated and characterized a variant resistant to CPIs using a HCV subgenomic replicon system. A mutation in NS5A, D320E, was shown to confer the CPI-resistance to HCV replicon, resulting in CyPA independence for efficient viral replication. In addition, assessment of a wide range of CyP subtype knockdown experiments found CyP40 to be a new contributor to HCV replication.

Of the mutations identified, Q86R substitution in NS3 dramatically enhanced the capacity of replication. This mutation was observed as compensatory mutation[22] following the selection of replicons resistant to protease inhibitors SCH503034[23] and SCH6.[24] In addition, this mutation also appeared following the passaging of replicon cells in the absence of drug pressure.[25,26] In actuality, this mutation did not contribute to CsA resistance in the replicon cells (Fig. 5b), and thus was thought to be an adaptive mutation similar to that suggested in previous reports. I252T mutation in NS3, on the other hand, severely reduced the replicative fitness of HCV. The significance of I252T mutation under CsA treatment remains to be studied. The alteration of amino acid residue in NS5A, D320E, resulted in the conversion of HCV replicon to that of the CsA-resistant phenotype. There have been no reports of a link between NS5A and individual CyP subtype in the context of HCV replication, though mutations in NS5A were found to be keys for the acquisition of CsA resistance.[27] We have previously reported that CyPB was important for viral replication, but NS5A did not interact with CyPB in MH14 cells.[5] Indeed, in cells harboring replicons with D320E mutation, CyPB was found to contribute to viral replication but was not related to CsA resistance, as knockdown of CyPB diminished the viral titer to approximately half, similar to the case of the wild type. Therefore, other CyP molecules crucial for viral replication were suggested to be involved in the phenomenon of the CsA resistance. CyPA is another CyP subtype recently published to be critical for HCV replication in connection with viral polymerase.[20,21] Our CsA-resistant replicon cells displayed resistance to CyPA knockdown when compared to wild-type replicon, suggesting that CyPA participated in the replication process and the CsA resistance was due in part to resistance to CyPA inhibition. Therefore, it might be possible that NS5A functions coordinated with CyPA for viral replication and D320E mutation could contribute to enhancement of the relation. But NS5A was unable to bind CyPA *in vitro*.[5] NS5A might be regulated by CyPA associated with other cellular or viral factors during HCV replication. The fact that the D320 falls upon one of the two discontinuous domains needed for the interaction with NS5B to functionally modulate it[28,29] lead us to presume influence of NS5A on the reported NS5B–CyPA interaction.[21] In addition to CyPA and CyPB, which have been published to be cellular factors required for HCV replication, the results suggested that another CyP subtype, CyP40, contributed to viral replication. Acting as a molecular chaperone, it is conceivable that CyP40 directly interacts with viral proteins to boost their functions, similar to CyPA and CyPB. Heat shock protein (Hsp) 90 is a well-known chaperone forming complex with CyP40. Recently, Hsp90 was shown to be harnessed by HCV NS5A via the FK-506 binding protein 8 (FKBP8) bridge. FKBP8 is a homologous immunophilin of CyP40 that is required for viral replication.[30] This result led to the hypothesis that CyP40 serves as a linker between viral proteins and Hsp90. CyP40 is also known to associate with estrogen receptor (ESR) and we have published that ESRα escorted NS5B to replication complex (RC).[31] We also speculate CyP40 connected to ESRα may be important for the recruitment or functional reinforcement of viral and cellular factors for HCV replication in RC. Among these CyP subtypes, CyPA dependency was suggested to be one of the determinants of CsA sensitivity. Interestingly, CyPB and CyP40 play significant role in HCV replication even in CsA-resistant replicon cells. Another CPI, NIM811, is also likely to target CyPA, at least in part, to suppress HCV replication, given the cross-resistance of CsA-resistant replicon to MIN811. However, there is still also the possibility that other CyPs mediate anti-HCV effect of NIM811, which needs to be elucidated in future study.

Understanding the profile of CPI-resistance mutations in the HCV genome and the viral and cellular factors involved will aid in the progression of CPI-centered strategies preparing for the problem of drug resistance. In addition, the cells harboring CPI-resistant replicons established here may prove beneficial for further characterization of resistance mechanisms and for the screening of novel compounds with the potential of clinical application to defeat CPI-resistant variants. Also, CyP40 as a contributor to HCV replication could become another specific antiviral target. The information arising from this study is expected to contribute to the successful use of CPIs against a liver carcinogen, HCV.

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Strain-Dependent Viral Dynamics and Virus-Cell Interactions in a Novel *In Vitro* System Supporting the Life Cycle of Blood-Borne Hepatitis C Virus

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We developed an *in vitro* system that can be used for the study of the life cycle of a wide variety of blood-borne hepatitis C viruses (HCV) from various patients using a three-dimensional hollow fiber culture system and an immortalized primary human hepatocyte (HuS-E/2) cell line. Unlike the conventional two-dimensional culture, this system not only enhanced the infectivity of blood-borne HCV but also supported its long-term proliferation and the production of infectious virus particles. Both sucrose gradient fractionation and electron microscopy examination showed that the produced virus-like particles are within a similar fraction and size range to those previously reported. Infection with different HCV strains showed strain-dependent different patterns of HCV proliferation and particle production. Fluctuation of virus proliferation and particle production was found during prolonged culture and was found to be associated with change in the major replicating virus strain. Induction of cellular apoptosis was only found when strains of HCV-2a genotype were used for infection. Interferon-alpha stimulation also varied among different strains of HCV-1b genotypes tested in this study. *Conclusion:* These results suggest that this *in vitro* infection system can reproduce strain-dependent events reflecting viral dynamics and virus-cell interactions at the early phase of blood-borne HCV infection, and that this system can allow the development of new anti-HCV strategies specific to various HCV strains. (HEPATOLOGY 2009;50:689-696.)

Hepatitis C virus (HCV) is a serious problem worldwide, with 3% of the world's population chronically infected.[1] Chronic infection with HCV may lead to high rates of liver cirrhosis and hepatocellular carcinoma.[2] Because the HCV standard therapy is still insufficient for treating many patients,[3] the development of more effective and less toxic anti-HCV agents is desired. The virological studies required to reach this goal need reproducible and efficient HCV proliferation in cell culture. An *in vitro* infection system using recombinant HCV-JFH1 was developed. In this system, HuH7 cells transfected with *in vitro*-synthesized JFH1-RNA were

Abbreviations: 2D, two-dimensional; 2D-HuS-E/2, HuS-E/2 cells cultured in two-dimensional condition; 3D, three-dimensional; 3D/HF, 3D hollow fibers; 3D-HuS-E/2, HuS-E/2 cells cultured in three-dimensional condition in the hollow fibers; HCV, hepatitis C virus; IFN-α, interferon alpha; LDH, lactate dehydrogenase; p.i., postinfection; RFB, radial-flow bioreactor; RT-PCR, reverse transcription polymerase chain reaction.

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shown to secrete infectious viral particles.[4] This system, however, requires the combination of HuH-7-derived cell lines and JFH1-based constructs, limiting its usefulness for studying other HCV strains. Because HuH-7 cells cannot support the complete life cycle of blood-borne HCV (bbHCV) derived from clinical samples,[5] this system is insufficient for studying all the events related to bbHCV infection.

Many researchers have attempted to develop an *in vitro* system for bbHCV.[6-8] These current systems, however, are still insufficient due to their low efficiency for infectivity and replication of bbHCV. Working toward this same goal, we recently established immortalized primary human hepatocyte cell lines by transducing them with E6 and E7 genes from the human papilloma virus 18.[5,9] As expected, we observed improved infection and replication of bbHCV especially in one of these cell lines (HuS-E/2 cells) that showed a similar expression profile to that of human primary hepatocytes, but this strategy did not improve production of infectious particles.

Recently, a hybrid artificial liver support system was developed using animal hepatocytes cultured in a three-dimensional hollow fiber (3D/HF) system. This bioartificial liver showed several characteristic features of liver tissue for more than 4 months.[10-12] By growing our HuS-E/2 cells in a similar 3D culture[5] the gene expression profile was improved to more closely match that of human primary hepatocytes. Because the 3D cell culture condition more closely reproduces the *in vivo* environment of hepatocytes,[13] culturing these cells in this manner may support the entire HCV life cycle.

In this study we utilized this small 3D culture system and showed it to be ideal for culturing HuS-E/2 cells for the study of bbHCV infection. Using this system we are now able to study the variable patterns of the life cycle of different bbHCV strains as well as HCV-related cellular events.

Materials and Methods

Cell Culture. HuS-E/2 cells were cultured as previously described.[5] For the 3D/HF system, HuS-E/2 suspension was injected into the lumen of HF (Toyobo, Osaka, Japan) made from cellulose acetate and containing pores for nutrients and waste exchange (Supporting Fig. 1). The bundles were centrifuged to induce organoid formation. The cells in the fibers were cultured in 12-well plates (two capillary bundles per well) with gentle rotation using serum-free medium (Toyobo) in a CO_2 incubator at 37°C. The number of cells was adjusted to 3 × 105 cells per two-capillary bundle at the start of each experiment.

RNA Experiments. Total RNA was extracted from two-dimensional (2D) cultured cells, patient sera, or from 100 times concentrated culture medium as previously described.[4,5] For cells cultured in the 3D/HF, sterile scissors were used to cut each fiber into small pieces (1 mm^2 each), which were then solubilized in Sepasol RNA-1 (Nacalai Tesque, Kyoto, Japan). RNA was then extracted according to the manufacturer's protocol. Real-time reverse transcription polymerase chain reaction (RT-PCR) was performed as described.[5]

HCV Infection. HCV infection experiments were carried out using sera from HCV patients. The amount of each inoculum was adjusted so as to add similar amount of HCV-RNA to the medium of the cells. After 24 hours, the cells were washed three times with phosphate-buffered saline (PBS) and cultured for the designated times. To assess the passage of infectivity, 12 mL of culture medium from the primary infected cells was collected, concentrated 100 times by filtration through Amicon Ultra-15, Ultracel-10K filters (Millipore, Carrigtwohill, Cork, Ireland), and 40 μL concentrated medium/well was used to infect naïve HuS-E/2 cells. All experiments were done with approval of the Ethical Committee of Kyoto University. Informed consent from patients was required for this approval.

Cloning and Sequencing. To amplify the complementary DNA (cDNA) fragment corresponding to hypervariable region 1 (HVR-1),[14] a nested RT-PCR was performed using Superscript III (Invitrogen, Carlsbad, CA) and PrimeSTAR HS DNA Polymerase (Takara, Tokyo, Japan). Reaction conditions were adjusted according to the manufacturer's protocol. Primers used were previously described[15] and are shown in Supporting Table 1. PCR products were then purified and cloned using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen). Ten recombinant clones were randomly isolated for each PCR product and sequenced as described.[16]

Quantitative Detection of HCV Core and Interferon alpha (IFN-α) Protein by Enzyme-Linked Immunosorbent Assay (ELISA). The culture medium of infected cells was collected and concentrated 100 times as previously mentioned for the detection of HCV-core, or used directly for detection of IFN-α. HCV core protein was quantified using the Trak-C Core ELISA (Ortho Clinical Diagnostics, Neckargemünd, Germany). IFN-α was quantified using the Human IFN-A ELISA kit (PBL Biomedical Laboratories, Piscataway, NJ). Light absorbance was then measured using a Wallac 1420 multilabel counter (PerkinElmer Life Science, Waltham, MA).

Cytotoxicity Assay. Culture medium was collected from HCV-infected cells and used for measuring lactate dehydrogenase (LDH) levels using an LDH cytotoxicity

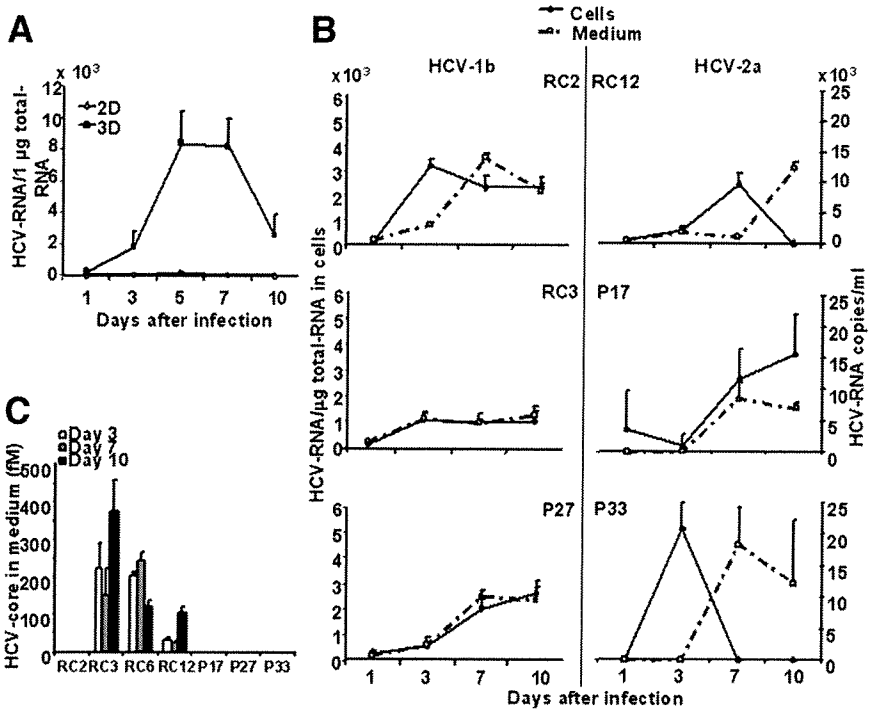

Fig. 1. Infection and proliferation of bbHCV in 3D-HuS-E/2 cells. (A) The quantity of HCV genomic RNA in 1 μg total RNA of 2D- or 3D-HuS-E/2 cells infected with HCV-RC6 was determined at each timepoint after infection by real-time RT-PCR analysis. (B) 3D-HuS-E/2 cells were infected with HCV-1b-containing sera: RC2, RC3, and P27; or HCV-2a-containing sera: 4: RC12, P17, P33. The quantity of HCV genomic RNA in the infected cells was determined as in (A). The culture medium from the last 2 days at each timepoint was collected, concentrated, and the amount of HCV-RNA (B) or HCV-core (C) was measured. Data represent the mean ± standard deviation (SD) of three independent experiments.

detection kit (Takara Biomedicals). Light absorbance was then measured as described above.

Sucrose Density Gradient. The culture medium of the infected cells was collected, concentrated 500 times, and loaded onto a 20%-50% (wt/vol) sucrose gradient containing 50 mM PBS, 100 mM NaCl, and 1 mM EDTA, followed by centrifugation at 100,000*g* for 16 hours at 4°C in a SW41Ti rotor (Beckman, Fullerton, CA). The gradient was fractionated into 31 fractions that were used for HCV-RNA and core detection and HCV infection into naïve cells as described above.

Electron Microscopy. The 1.12 g/mL fraction obtained by the sucrose density gradient showed the secondary infection activity as analyzed by transmission electron microscopy. The fraction was ultracentrifuged and the almost all supernatant was removed. The residual 10 μL of the solution was directly applied to a formvar-carbon grid for negative staining with 1% uranyl acetate solution and observed with an electron microscope (JEOL1010, JEOL, Tokyo, Japan).

Results

HuS-E/2 Cells Cultured in 3D/HF System Are Highly Permissive for Infection and Proliferation of bbHCV. We compared the ability of HuS-E/2 cells cultured in the 3D/HF system (3D-HuS-E/2 cells) to those cultured as a monolayer (2D-HuS/E2 cells) to reproduce infection by HCV genotype 1b (HCV-RC6), derived from patient serum (RC6). The HCV-RC6 RNA levels in the 3D-HuS/E2 cells were significantly higher at all timepoints (Fig. 1A), showing that the 3D/HF system greatly improves the proliferation of bbHCV in HuS-E/2 cells. We observed that both the early stages of infection and the continuous replication of HCV-RC6 in HuS-E/2 cells was improved by 3D/HF culture when the culture conditions were changed after the infection from 3D/HF to 2D and vice versa (Supporting Fig. 2).

As reported,[17] blocking CD81, an HCV-supposed entry receptor, during infection significantly impaired HCV proliferation into 3D-HuS-E/2 cells (Supporting Fig. 3), suggesting that CD81 is essential for HCV infectivity in 3D-HuS-E/2 cells. Although the expression level of CD81 mRNA in 3D-HuS-E/2 cells was observed, no significant change from 2D-HuS/E2 cells was found (data not shown), indicating that the quantity of CD81, at least, is not responsible for the improvement.

We then examined whether this system can be used for proliferation of six different bbHCV samples, three of which are HCV-1b (HCV-RC2, HCV-RC3, and HCV-P27) and three HCV-2a genotypes (HCV-RC12, HCV-P17, and HCV-P33) (Fig. 1B). Proliferation of HCV-RNA in the cells was seen in all six cases, suggesting that this system can be widely used for analysis of infection and proliferation of bbHCV strains. HCV-RNA and HCV-core were also detected in the culture medium (Fig. 1B). Different HCV strains showed variable patterns of proliferation and HCV-core secretion into the medium. Although HCV-core was detected from day 3 onward when

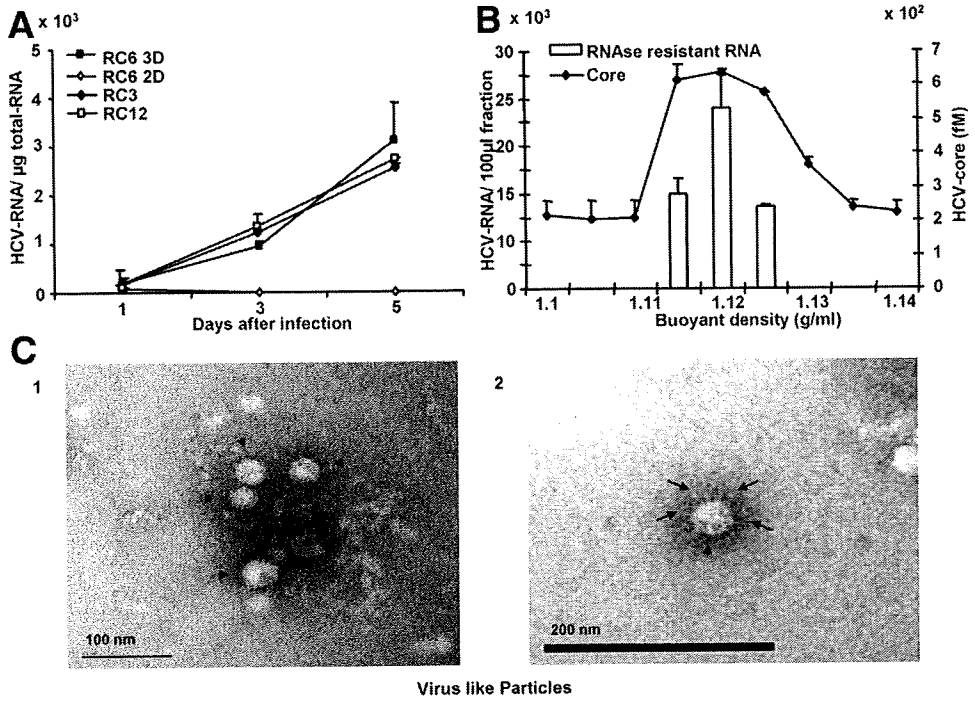

Fig. 2. Production of infectious virus-like particles from 3D-HuS-E/2 cells infected with different HCV strains. (A) The culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 or HCV-RC6 was collected from days 5 to 7 p.i. and for HCV-RC12 from days 23 to 25 p.i. The culture medium of 2D-HuS-E/2 cells infected with HCV-RC6 was also collected from days 5 to 7 p.i., and used to treat naïve 3D-HuS-E/2 cells. The quantity of HCV genomic RNA in 1 μg of total cellular RNA was determined as in Fig. 1. (B) The concentrated culture medium of 3D-HuS-E/2 cells infected with HCV-RC3 was collected from days 5 to 7 p.i., and fractionated by ultracentrifugation with a 20%-50% sucrose density gradient. HCV-core protein and the RNase A-resistant HCV-RNA in the different fractions were quantitatively analyzed using an HCV-core ELISA kit and real-time RT-PCR, respectively. Data represent the mean ± SD of three independent experiments. (C) Photomicrograph showing negatively stained virus-like particles from the culture medium of HCV-RC3-infected 3D-HuS-E/2 cells (arrowheads, panels 1 and 2). The arrows indicate the spike-like structures found on the surface of the virus-like particles (panel 2).

RC3, RC6, and RC12 were used for infection, it was undetectable when RC2, P17, P27, and P33 sera were used, similar to 2D-HuS-E/2 cells infected with HCV-RC6 (Fig. 1C).

Production of Infectious Particles from 3D-HuS-E/2 Cells Infected with bbHCV. The culture media from 2D or 3D-HuS/E2 cells infected with RC6 serum (Fig. 1A) were collected from days 5 to 7 postinfection (p.i.), concentrated, and inoculated into naïve 3D-HuS-E/2 cell culture media. HCV-RNA's proliferation in the infected cells was only detected when using the culture medium from 3D-HuS-E/2 cells and not 2D-HuS-E/2 cells (Fig. 2A). Media collected from HCV-RC3 at days 5 to 7 and from HCV-RC12 from days 23 to 25 p.i. were also able to infect naïve cells (Fig. 2A). These data suggested the production and secretion of infectious virus-like particles. To investigate this further, biophysical analysis was performed. The culture medium of HCV-RC3 infected 3D-HuS-E/2 cells at day 7 p.i. was fractionated using a sucrose density gradient after RNase A treatment. HCV core was detected in the 1.11 to 1.14 g/mL fractions; similarly, the nuclease-resistant HCV RNA peaked in the 1.12 g/mL fraction (Fig. 2B). Furthermore, only the 1.12 g/mL fraction was able to infect naïve cells as examined above (data not shown). This fraction was pelleted by ultracentrifugation and examined by electron microscopy with negative staining. We observed 33-nm to 45-nm diameter spherical particles (Fig. 2C, panel 1) with spike-like structures from 7-9 nm in length on the surface (Fig. 2C, panel 2), consistent with HCV morphology reported previously in HCV patients.[18] These were detected in the sample collected from HCV-RC3-treated but not mock-treated 3D-HuS-E/2 cells. These data suggest that production of infectious virus-like particles occurs in 3D-HuS-E/2 cells infected with some bbHCV strains. It is therefore likely that 3D-HuS-E/2 cells can be used to reproduce nearly all steps in the HCV life cycle.

Prolonged Culture of HCV-Infected Cells in the 3D Hollow Fiber System. For HCV-RC6-infected cells (Fig. 3A), the amount of HCV-RNA in the cells fluctuated during the 30-day culture period. The levels of both HCV-RNA and HCV-core in the medium showed a similar pattern of fluctuations that peaked on days 5 and 20 p.i. Unlike RC6, the pattern of HCV-RNA levels in the medium of RC12-infected cells showed a negative

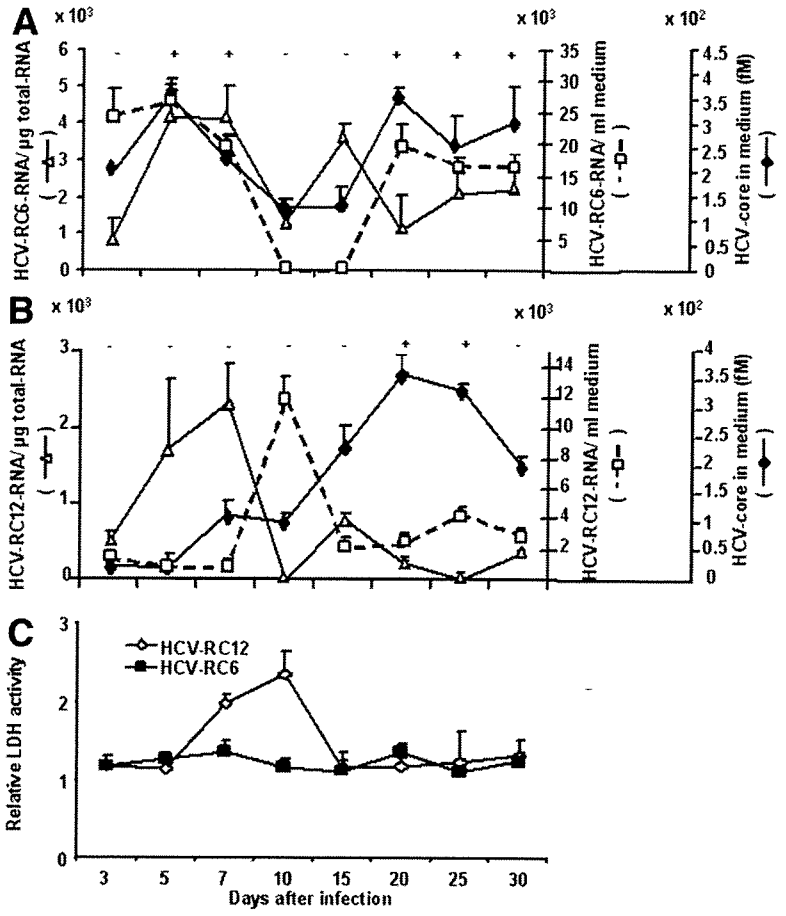

Fig. 3. Prolonged culture of HCV-infected cells in the 3D/HF system. After infection with HCV-RC6 (A) and HCV-RC12 (B), 3D-HuS-E/2 cells were cultured for 30 days with a medium change every 2 days. The HCV-RNA in the cells and medium as well as the HCV-core in the medium were quantitatively analyzed at the designated timepoints as in Fig. 1. Culture media were also used to treat naïve 3D-HuS-E/2 cells to examine the secondary infection as in Fig. 2. (+) and (−) indicate detection or no detection of secondary infection. (C) Culture media of HCV-RC6 and HCV-RC12 infected cells collected at each timepoint were used for the detection of LDH levels released from dead cells. LDH levels were normalized to uninfected cells cultured for the same time. Data represent the mean ± SD of three independent experiments.

correlation with that detected in the cells. This was clearly seen on day 10 p.i., when a sharp increase and decrease of HCV-RNA in the medium and the cells, respectively, was observed (Fig. 3B). Similarly, the amount of HCV-core detected in the medium throughout the culture was not correlated with RNA levels in the medium. Instead, core levels were very low in the first 10 days, at which time levels increased, reaching a peak on day 20 p.i. (Fig. 3B). Culture media from cells infected with HCV-RC6 from days 5 to 7 and 20 to 30 p.i. (Fig. 3A) and that from HCV-RC12 from days 20 to 25 p.i. showed passage of infectivity (Fig. 3B). All culture media showing infectivity appeared to have a high amount of HCV-core protein.

Clonal Changes in HCV During Prolonged Culture. In order to perform a populational analysis to understand the fluctuating pattern seen during HCV proliferation, two sera with limited HCV variants, HCV-RC6 (two major strains) and -RC12 (single major strain) from immunosuppressed liver transplantation patients with recurrent HCV were used in the previous prolonged infection experiment. The variants' composition was analyzed by single-strand confirmation polymorphism analysis for HCV-HVR1 (Supporting Fig. 4). RC6 serum (Fig. 4A) showed two different major sequences, HCV-RC6-1 and -2 strains, which constituted 60% and 40%, respectively, and shared 85% homology. In cells infected with HCV-RC6 the nucleotide sequence of HVR1 on day 5 showed 97% homology to HCV-RC6-1, and on day 20 p.i. it showed 97% homology to HCV-RC6-2. These data suggest selection of the dominant HCV strain in the cells over time. For RC12 (Fig. 4B), the nucleotide sequence on day 5 p.i. had only one nucleotide difference from that of the HCV from the original serum. The sequence from day 20 p.i. was four nucleotides different from that from the serum, and five different from the cells on day 5 p.i. These data indicated that each peak of HCV-RNA that appeared in the cells infected with RC12 serum included primarily a single HCV strain with a slightly different genomic sequence. This suggests that the periodic appearance of HCV-RNA peaks in the cells infected with a particular HCV strain is a result of selection and/or mutation of HCV strains during the prolonged culture period.

Cellular Response Induced by bbHCV Infection. At day 10 p.i., HCV-RNA levels in the culture medium rose and RNA levels in 3D-HuS-E/2 cells infected with HCV-RC12 dropped (Figs. 1B, 3B). To determine if this was caused by a cytotoxic effect of HCV infection, LDH levels were measured in the culture medium of HCV-RC6- and

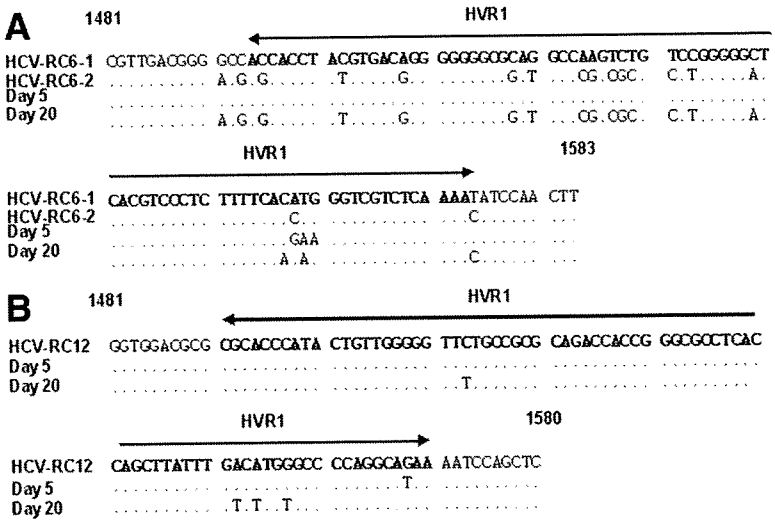

Fig. 4. Comparison of HCV-HVR1 sequences in the serum used for infection and the HCV replicating in the cells on days 5 and 20 after infection of HCV-RC6 (A) or HCV-RC12 (B). Nucleotide numbering was based on HCV-J1 sequence (GenBank Access. No. D10749). Three additional nucleotides were found at the 5′-terminal end of the E2 regions of all RC6 sequences. The major sequence present in the serum used for infection is shown in the upper row in each panel. Dots represent the identical nucleotides.

HCV-RC12-infected 3D-HuS-E/2 cells. LDH activity showed a strong correlation with HCV-RNA levels in the medium on day 10 p.i. in HCV-R12-infected cells (Fig. 3B), suggesting a cytotoxic effect of HCV-RC12 that was not observed in the case of HCV-RC6 (Fig. 3A,C). To determine if this HCV infection-mediated cytotoxicity is due to apoptosis, as with other viruses belonging to the Flaviviridae family,[19] the involvement of caspase was examined using the caspase inhibitor z-VAD-fmk. A significant dose-dependent reduction in HCV-RNA levels in the medium and LDH activity (Fig. 5A,B) was found, whereas no significant effect was observed on the viability of noninfected cells (Fig. 5B) or intracellular HCV-RNA levels (Fig. 5A). This suggested that the cytotoxic effect of HCV infection is mediated by apoptosis. It is noteworthy that HCV-induced cytopathicity was also found when HCV-P17 and HCV-P33 samples were used for infection (both are HCV-2a genotype) and was not reproduced in any of the HCV-1b genotype samples used in this work (Fig. 5C).

After infection with HCV-RC6, no cytotoxicity was detected that might have inhibited HCV-RC6-1 proliferation in the cells. However, HCV-RC6-2 RNA replaced HCV-RC6-1 RNA during prolonged culture. To assess a

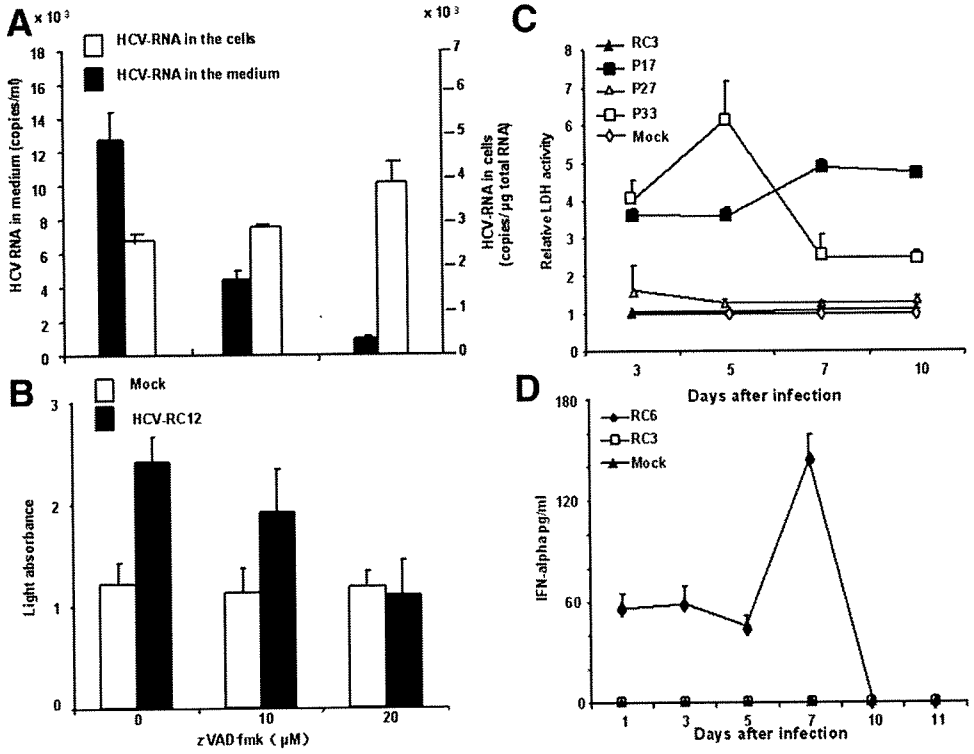

Fig. 5. Cellular response of 3D-HuS-E/2 cells infected with bbHCV. 3D-HuS-E/2 cells infected with HCV-RC12 and mock-treated cells were cultured for 10 days in the presence of z-VAD-fmk (0, 10, and 20 μM). (A) HCV-RNA in the cells and medium on day 10 was measured as in Fig. 1. (B) LDH levels in the medium on day 10 after infection with HCV-RC12 was measured as in Fig. 3. (C) Culture media of HCV-RC3, HCV-P17, HCV-P27, HCV-P33, and mock-infected cells collected at designated points were used for the detection of LDH levels. (D) IFN-α levels in the culture media of HCV-RC6, HCV-RC3, and mock-infected cells collected at each designated timepoint were measured by ELISA. Data represent the mean ± SD of three independent experiments.

possible role of the innate-immune response in this phenomenon, the production of IFN-α in the medium was measured during the first 11 days p.i. IFN-α production was detected as early as day 1 p.i., reached a peak at day 7 p.i., and was then rapidly lost (Fig. 5D). These data suggest that HCV-RC6-1 infection induced the innate-immune response of the cells, possibly leading to suppression of its proliferation. In contrast to HCV-RC6-1, HCV-RC3 did not show any stimulation of IFN-α production upon infection in the first 10 days, showing a possible strain-dependent evasion from the host defense within the same genotype.

Discussion

In this study we report the development of a novel system that reproduces bbHCV infection, proliferation, and production of infectious virus. The most recent models used in the study of the life cycle of HCV infection are based on subclones of HuH-7 cells infected with JFH1 recombinant virus or its derivatives.[4] HuH-7 cells and its subclones, however, do not support the entire life cycle of the bbHCVs present in patients' blood.[5] Moreover, HCV has considerable diversity and variability. It is generally classified into six major genotypes and more than 100 subtypes.[20] This huge pool of natural HCV variants causes a wide variety of diseases, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma.[21] JFH1, however, is a single isolate of HCV genotype 2a that was originally derived from a patient with rare fulminant hepatitis.[4] We suggest that our newly established system has an important advantage because it supports the entire life cycle of a variety of HCV strains and genotypes.

Due to the lack of some *in vivo* factors, including host immune response, *in vitro* systems may not completely reproduce the *in vivo* situation. However, *in vitro* experimental systems seem to be important to simplify particular events from the complex situation *in vivo*. From that standpoint, our cell culture system is likely reproducing the early event of HCV infection in the absence of host-immune responses and supporting whole life cycle of the blood-borne HCV. Several *in vitro* hepatocyte culture systems have been reported to be useful for studying the infection and replication of bbHCV.[5-8,22] Only the radial-flow bioreactor (RFB) 3D culture system demonstrated production of infectious viruses.[22] In our studies we observed not only the enhancement of HCV replication, but also the production of infectious HCV particles in the medium using the 3D/HF system. These data suggest that some structure of the cell mass formed by the 3D culture system, most likely the polar character, is essential for the life cycle of bbHCV. The RFB system is composed of a dedicated device containing 1×10^9 FLC4 cells with a culture area of 2.7 m^2.[22] It can only be used to study HCV particle production in the medium and not the cellular events that accompany the HCV life cycle. In contrast, because cells grown in our 3D/HF system are cultured in 12-well plates at a density of 3×10^5/fiber, it is much simpler to study both viral and cellular events.

The production of infectious particles was not detected with infection by different HCV strains, despite detecting equivalent levels of HCV-RNA in the cells (Fig. 1B,C). Delayed production of infectious particles was also observed in cells infected with HCV-RC12 after prolonged culture. A similar delay was also observed in the RFB system.[22] Considering the relative stability of HuS-E/2 cells[5] and the relatively high frequency of the change in HCV population in the cells,[16] it is likely that mutation of the HCV genome and/or selection of clones during prolonged culture improved the productivity of infectious particles. A marked improvement of infectious particle production by substitution of the structural proteins of the genome was also reported in the recombinant HCV production system.[23] The lack of production of infectious particles soon after infection may serve to avoid an early strong response from the host immune system, and demonstrates a novel mechanism of latent infection by HCV. Although they may not be associated with plasma components as those present *in vivo*, HCV virus-like particles produced by this system showed a close resemblance to those isolated from infected HCV patients because they showed the same size[18] and were within the fraction range.[24] They may help in the study of viral and cellular factors required for particle production and the possible receptors utilized for infection with different HCV strains.

Fluctuation in HCV proliferation was observed during the prolonged culture of 3D-HuS-E/2 cells infected with bbHCV (Fig. 3A,B), consistent with previous reports in other culture systems.[6,22] This fluctuation was associated with a change in viral quasispecies, suggesting that an HCV strain having a growth advantage proliferates selectively and dominantly in these culture conditions. Because the progressive emergence of each dominant strain was only temporary, it is highly likely that the infection and proliferation of such an HCV strain is suppressed by cellular mechanism(s). Our results suggest that there are actually two cellular mechanisms functioning to do this. The first is the involvement of the innate immune system, as evidenced by the secretion of IFN-α during the first week of infection (Fig. 5D). This is the first report of secretion of IFN-α from cultured cells infected with bbHCV. Although recent reports suggest that stimulation of the IFN pathway by HCV infection could be impaired by HCV NS3-4a proteinase-mediated cleavage of IPS-