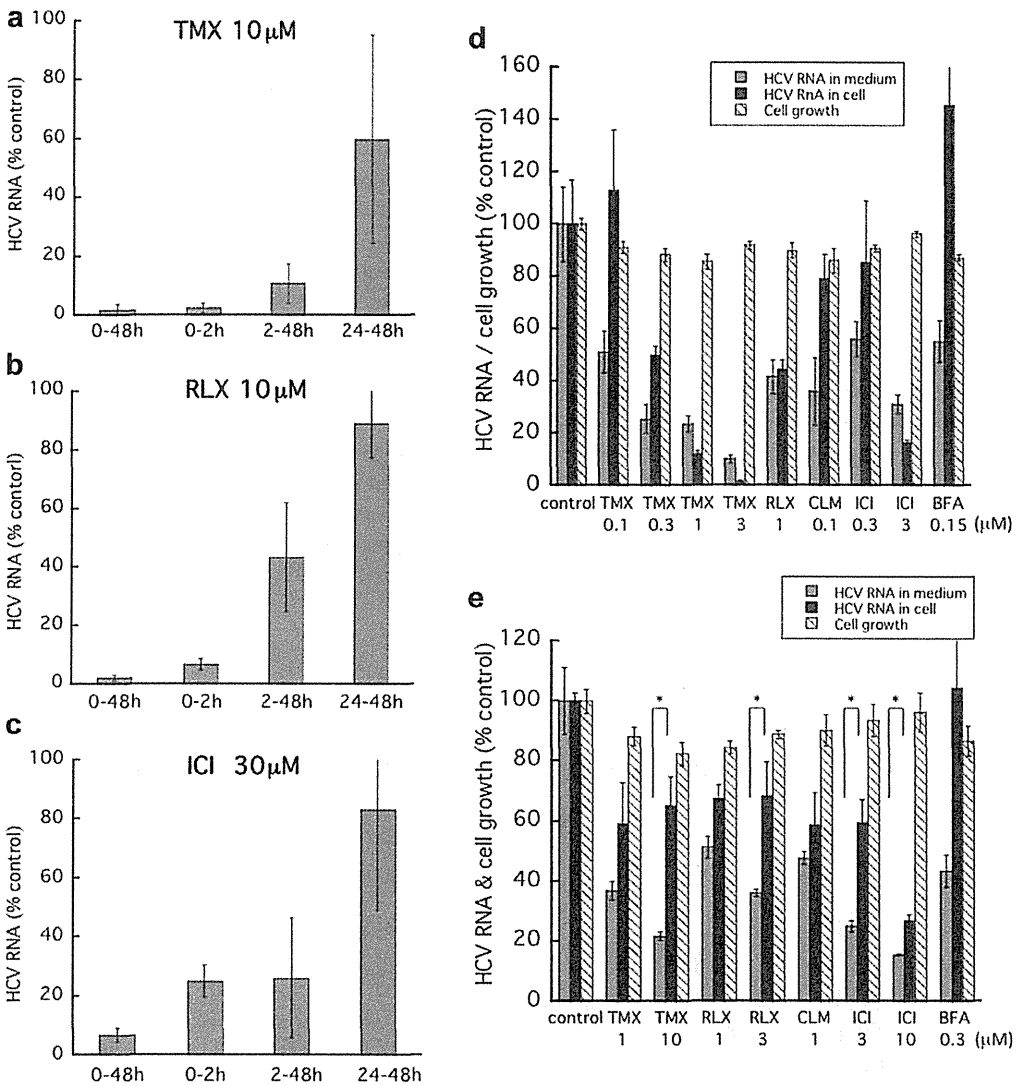

Fig. 2. Time-of-addition experiments (a–c) and the effect of SERMs on extracellular and intracellular HCV RNA in simultaneous infection (d) and in persistent infection (e). Huh 7.5.1 cells were treated with tamoxifen (TMX) (10 μM, a), raloxifene (RLX) (10 μM, b), or ICI 182,780 (ICI) (30 μM, c) during the following time periods: 0–2 h, 2–48 h, or 24–48 h after JFH-1 infection (moi 0.1). Forty-eight hours after infection, the culture supernatant was harvested, and HCV RNA was extracted and subjected to quantitative real-time RT-PCR to determine the number of copies of the JFH-1 genome. The data are the averages of three independent experiments and the standard deviation. d) Effect of treatment with SERMs for 3 days on extra- and intra-cellular HCV RNA levels. Huh 7.5.1 cells were infected with JFH-1 (moi 0.1) just after addition of the SERMs. Three days later, RNA was extracted from the cells and from the culture supernatant. The amount of HCV RNA was measured by quantitative real-time RT-PCR. Brefeldin A (BFA) was used as a positive control. e) Huh 7.5.1 cells were infected at a moi of 0.01, 3 days before addition of drugs. The infected cells were treated with SERMs for 48 h. RNA was subsequently extracted from the cells and the culture supernatant to determine the viral genome copy number. The results are presented as the percentage of control cells without drug. The data are the averages of triplicates and the error bars represent standard deviation. *P-value < 0.05. One representative experiment of two independent experiments is shown.

Next we treated this (#4-1, genotype 2a) and another replicon (#5-15, genotype 1b) [5] with the SERMs for 3 days and examined the effect of the compounds on the HCV NS5A protein levels by western blotting. As shown in Fig. 3b, the SERMs except ICI 182,780 reduced the level of NS5A in accordance with the results in Fig. 3a. ICI 182,780 seemed to slightly reduce NS5A protein in #5-15 replicon cell. The SERMs did not reduce the protein levels of GAPDH in the subgenomic replicon cells (Fig. 3b). These results indicated that SERMs, at least tamoxifen, raloxifene and clomifene,

control cell growth are indicated by solid lines and dotted lines, respectively. b) Effect of the following ERα antagonists: ICI 182,780 (closed triangles), ZK164015 (closed rectangles), and MPP (open rectangles). c) Effect of the following ERα agonists: 17β-estradiol (closed triangles), diethylstilbestrol (open rectangles), and PPT (closed circles). The results are presented as percentages of the control cells that were not treated with drugs. Values are the averages of triplicates, and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown. d) Huh 7.5.1 cells were infected (moi 0.01) in the presence of tamoxifen (TMX), clomifene (CLM), raloxifene (RLX), or ICI 182,780 (ICI) and incubated for 5 days. Cell lysates were blotted with anti-core and anti-GAPDH antibodies as described in the Section Materials and methods.

were effective not only against HCV genotype 2a but also HCV genotype 1b and that the compounds inhibited a HCV replication step. The growth of the replicon cells was suppressed by treatment with 10 μM of clomifene. Clomifene at concentrations less than 10 μM and tamoxifen, raloxifene and ICI 182,780 at 10 μM concentration or lower did not inhibit cell growth (Fig. 3c).

3.4. SERMs inhibited entry of HCVpp but not VSVpp

To further examine the inhibition of early viral processes by the SERMs, we used infectious HCV pseudo-particles (HCVpp). Because HCVpp enter into cell dependent on HCV envelope protein but replicate dependent on retroviral system in the cell, we can exclude other effects of the drug except effect on HCV entry system. Pseudo-particles with the viral envelope glycoprotein mimic the entry of the parental virus, and this system has been used for investigation of HCV entry [7,8,18,20,21]. The infectious titer is determined by luciferase activity. We added tamoxifen to HCVpp- or VSVpp-containing medium and incubated Huh 7.5.1 cells with this medium for 3 h. After washing the cells, fresh medium was added, and the cells were incubated for 3 days. Treatment with tamoxifen reduced the luciferase activity of the cells that were infected with HCVpp in a dose-dependent fashion. In contrast, the luciferase activity caused by VSVpp was not reduced by the same concentrations of tamoxifen (Fig. 4a). We also examined the effect of other SERMs, such as clomifene, raloxifene, ICI 182,780, ZK164015, and MPP, on HCVpp infection. All of these SERMs inhibited the luciferase activity caused by HCVpp but not the activity caused by VSVpp (Fig. 4b). ICI 182,780 showed a weaker effect compared to tamoxifen, clomifene and raloxifene. Next, we examined the effects of these drugs on various genotypes of HCVpp. Although the extent of inhibition was varied, the compounds inhibited all of the genotypes that were examined (Fig. 4c). At a concentration of 10 μM, ICI 182,780 inhibited all of the genotypes of HCVpp other than genotype 2a. These results suggested that the SERMs inhibit entry of all genotypes of HCV.

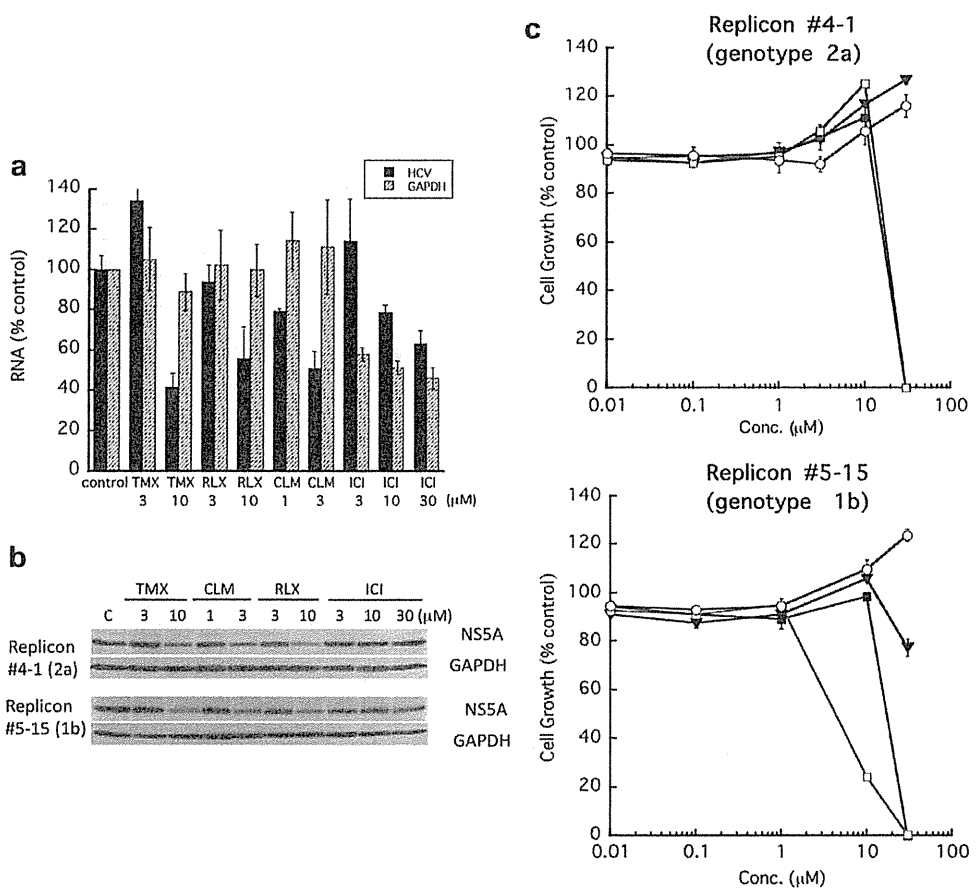

Fig. 3. The effect of SERMs on cells that harbored a subgenomic replicon. A subgenomic-replicon-harboring cell line clone #4-1 (genotype 2a) was treated with SERMs for 48 h. The total RNA was extracted from the cells, and amount of HCV RNA genome was measured. As an internal control, relative amount of GAPDH RNA was measured and indicated as percentage of control cells without drug (a). Another subgenomic-replicon-harboring cell line, clone #5-15 (genotype 1b) was treated with SERMs for 3 days. Cell lysates were subjected to western blotting with an anti-NS5A antibody or an anti-GAPDH antibody (b). Cells that were grown for 3 days in the presence of tamoxifen (closed rectangles), clomifene (open rectangles), raloxifene (closed triangles), or ICI 182,780 (open circles) were measured using the MTT assay. Cell growth is expressed as a percentage of control cells without drug (c). The values are the average of triplicate and the error bars represent the standard deviation of the mean. One representative experiment of two independent experiments is shown.

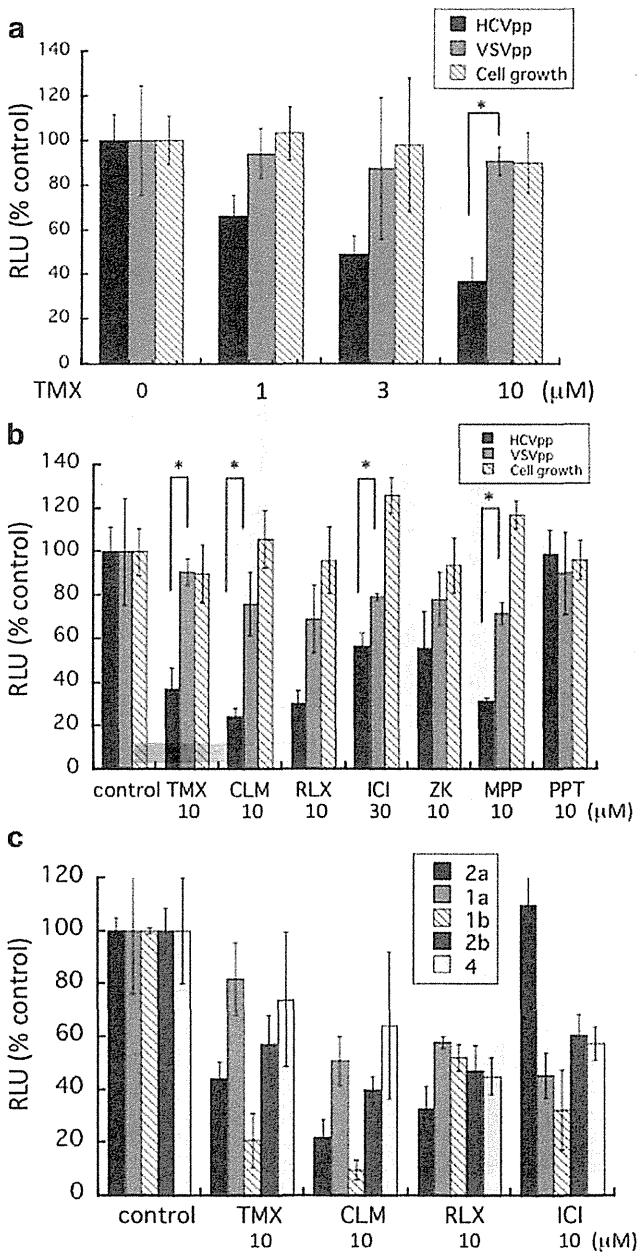

Fig. 4. Effect of SERMs on HCV pseudo-particle (HCVpp) infection. a) Huh 7.5.1 cells were incubated with pseudo-particles (HCVpp or VSVpp) in the presence or absence of tamoxifen for 3 h. The supernatants were removed, medium was added back to the cell cultures, and the cells were incubated for another 3 days. The VSVpp preparation was diluted 600 times so it was infected at similar RLU activity levels compared to HCVpp (approximately 5000 RLU). A parallel culture without pseudo-particles was analyzed using the MTT assay to evaluate the effect of the drugs on cell growth. b) Effects of various SERMs on HCVpp and VSVpp infection. c) Effects of SERMs on the various genotypes of HCVpp infection. The control luciferase activities were approximately 5000 RLU (genotype 2a), 3000 RLU (genotype 1a), 2400 RLU (genotype 1b), 3900 RLU (genotype 2b), and 860 RLU (genotype 4). The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars are the standard deviation of the mean. *P-value < 0.05. One representative experiment of three independent experiments is shown.

3.5. Effect of tamoxifen on the attachment and entry steps

To better understand how tamoxifen blocks HCV entry, we performed an experiment to discriminate between the inhibition of HCV attachment to cells and the inhibition of post-binding entry events. HCV attaches to several cellular receptors via its E1 and E2 envelope proteins and enters via clathrin-mediated endocytosis [14–16]. We used HCVpp because infection with HCVpp is thought to simulate HCV entry [7,17,18] and the entry is independent of HCV replication. HCVpp binding to the cellular receptors was performed at 4 °C for 1.5 h. Under these conditions, HCVpp bind to the cells but entry is not efficient. The inoculum was removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. In protocol I, the drug was administered during the binding step at 4 °C. After the shift to 37 °C, treatment with the drug was performed during first hour (protocol II) or after 1 h at 37 °C (protocol III) to distinguish between the inhibition of early and late post-binding events (Fig. 5a). The inoculum was removed after treatment, and fresh medium was added to the cells. We used chloroquine, a lysosome-tropic agent, as a control inhibitor for early entry (protocol II) [19]. We also used an anti-CD81 antibody that specifically inhibits HCV entry through the inhibition of the HCV cellular receptor protein CD81 at early entry [20,21]. As expected, chloroquine inhibited luciferase activity when the cells were treated during the early post-binding step (protocol II). This result suggested that endocytosis occurred primarily during the first post-binding period (protocol II). Anti-CD81 markedly inhibited luciferase activity during protocol II as reported [18,19]. Tamoxifen treatment did not result in clear differences between the protocols and the compound displayed similar activity regardless of the treatment period (Fig. 5b left). As a control, the same experiment was performed using VSVpp. Chloroquine inhibited the early entry step of VSVpp, but anti-CD81 and tamoxifen did not show any inhibition (Fig. 5b right).

Tamoxifen is a lipophilic weak base and inhibits acidification intracellularly [22]. Therefore, we examined whether the inhibition of the endocytosis of HCVpp by tamoxifen was dependent on its function as a weak base. Chloroquine is a weak base and inhibits endosome acidification. The pH sensitivity is considered a good indication of clathrin-dependent endocytosis. Previous reports have indicated that chloroquine inhibited HCVcc and HCVpp infection [14,19]. We adjusted the medium to pH 5.5 and incubated the cells in this acidic medium in the presence or absence of tamoxifen for 2 h post-binding. The acidification of the medium did not affect either the entry of HCVpp or the cell growth (Fig. 5c). Treatment with tamoxifen in the medium with a normal pH (pH 7.1) reduced HCVpp entry, and treatment with the drug in the acidic medium also reduced entry to a similar extent. In contrast, chloroquine treatment in regular medium reduced HCVpp entry, but entry was restored in the acidic medium (Fig. 5c). These results indicate that the inhibitory effect of tamoxifen was not dependent on the function of this compound as a base, unlike the effects of chloroquine.

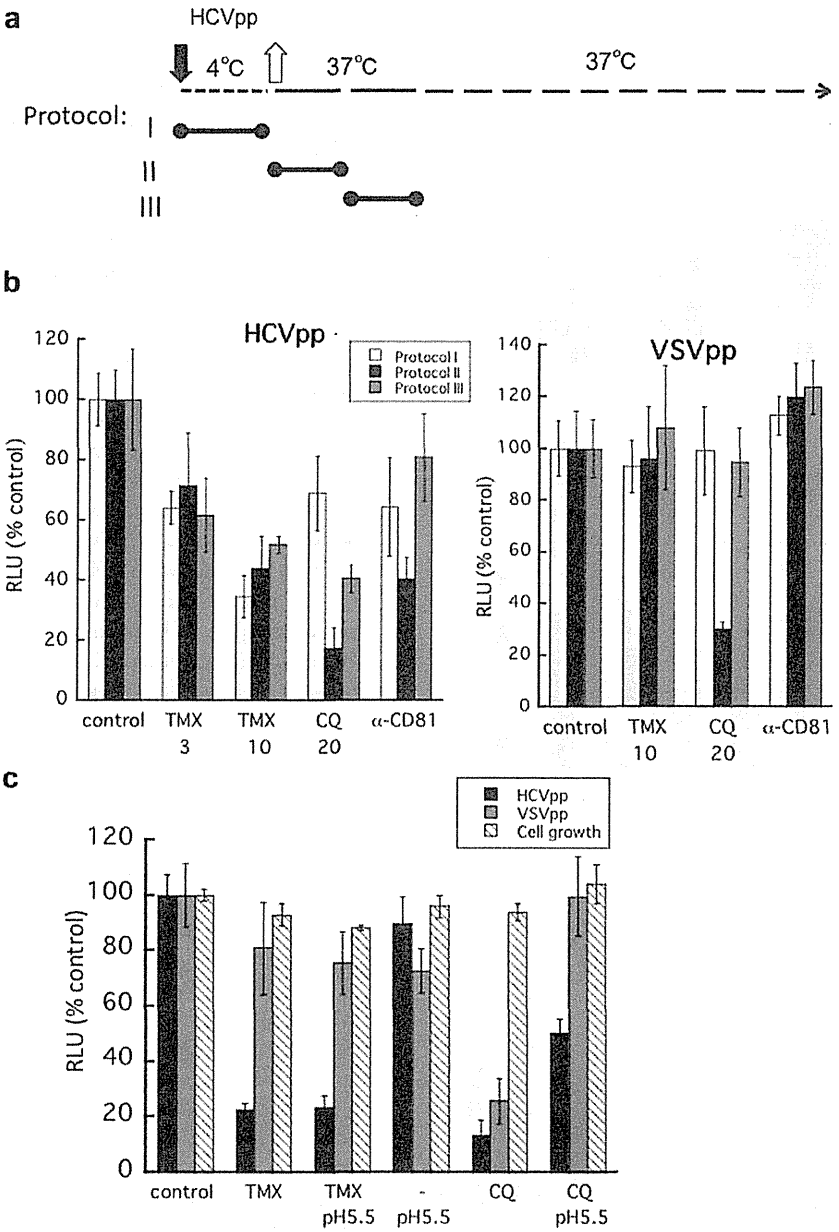

Fig. 5. Effect of tamoxifen on the attachment and endocytosis of HCVpp. a) Experimental design. HCVpp attachment to cells was performed at 4 °C for 1.5 h in the presence or absence of drug. Under these conditions, HCVpp bind to the cells but do not efficiently enter the cells. The inoculum was then removed, and fresh medium was added to the cells. The cells were subsequently incubated at 37 °C. The cells were treated with drug during the binding period at 4 °C (protocol I), during first hour after the shift to 37 °C (protocol II), or 1 h after the shift to 37 °C (protocol III). The drug-containing medium was removed for every treatment, and new medium was supplied to the cells. b) Effects of tamoxifen (TMX) (3 and 10 μM), chloroquine (CQ) (20 μM) and anti-CD81 antibody (20 μg/ml) on HCVpp attachment (protocol I) and post-binding events (protocol II and III) (left). Effects of tamoxifen, chloroquine and anti-CD81 on VSVpp (right). c) Effects of exposure to low pH on the inhibition of HCVpp entry by tamoxifen and chloroquine. The cells were incubated with HCVpp at 4 °C for 1.5 h in the absence of drug. After removing the inoculum, regular (pH 7.1) or acidic medium that was adjusted with HCl to pH 5.5, either with or without drug (tamoxifen, 10 μM, chloroquine, 20 μM), was added to the cells. The cells were subsequently incubated at 37 °C. The drug-containing medium was removed after 2 h of incubation, and the cells were incubated for an additional 3 days with fresh, regular medium. The values are expressed as the percentage of control cells without drug. The data are the averages of three wells and the error bars represent the standard deviation of the mean. One representative experiment of three independent experiments is shown.

4. Discussion

We screened for HCV inhibitors using the JFH-1-Huh 7.5.1 cell culture system and found that tamoxifen and ERα antagonists, but not ERα agonists, inhibited HCV JFH-1 infection. Although there are some reports about the HCV inhibitory effects of tamoxifen and other SERMs, we presented further information about the inhibitory effects of these substances. The time-of-addition experiments (Fig. 2a–c) suggested that these SERMs inhibit the entry and replication

steps in the HCV life cycle. These SERMs, except ICI 182,780, reduced level of HCV genome (genotype 2a) and NS5A (genotypes 1b and 2a) in the subgenomic replicon cells (Fig. 3), which supports the hypothesis that the inhibitory effect of the SERMs occurred during the replication steps. Further we observed that SERMs preferentially reduced extracellular HCV RNA compare to intracellular HCV RNA in the newly (Fig. 2d) and persistently infected cells (Fig. 2e). It suggests that the SERMs also target post replication step(s) in the viral life cycle, such as assembly and release. A low concentration of tamoxifen (0.1 μM) accumulated intracellular HCV RNA (Fig. 2d), which suggests that SERMs target post replication step(s) more efficiently than replication steps. Additionally, these compounds inhibited HCVpp infection (Fig. 4), which supports an inhibitory effect during the entry step. The inhibition of entry was mediated through the inhibition of viral binding to cells and through the inhibition of a post-binding event (Fig. 5b). Taken together, SERMs seemed to target multiple steps of the HCV viral life cycle.

Among the SERMs, ICI 182,780 did not exhibit distinct inhibition of replication in the replicon cells (Fig. 3a and b), although the compound seemed to inhibit entry and replication steps according to the result of time-of-addition experiment (Fig. 2c). The replicon cells are derived from Huh 7 cell. Although viral sensitivity to the SERMs might be dependent on the cell that HCV infect, it remains unclear whether ICI 182,780 inhibits replication step or not. However, the compound affected post replication step in a similar manner to other SERMs (Fig. 2d and e). It is unlikely that ICI 182,780 is an inhibitor with different mechanisms.

The inhibitory effect of tamoxifen on HCV replication has been previously reported by Watashi et al. [23]. They also demonstrated that ICI 182,780 inhibited HCV replication. This effect was discovered using a cell line that harbored a subgenomic replicon (genotype 1b). Additionally, these researchers reported that RNA interference-mediated knockdown of ERα, not ERβ, reduced HCV replication, but the reduction was not related to ERE-mediated transactivation activity. They suggested that ERα interacts with the HCV viral polymerase NS5B and that ERα promotes the participation of NS5B with the HCV replication complex. Using the Huh 7.5.1-JFH-1 screening system, Gastaminza et al. and Chockalingam et al. found that toremifene and raloxifene, respectively, function as HCV inhibitors. Gastaminza et al. [24] reported that toremifene inhibited HCV infection by inhibiting both the entry and release steps of the viral life cycle. Chockalingam et al. [25] determined that raloxifene inhibited the entry and replication steps, as we also observed. Our results are in accordance with these previous reports and other information about the inhibitory effects of SERMs.

Pseudo-particle experiments confirmed that SERMs affected the entry step of HCV viral life cycle (Fig. 4b), although the inhibitory effects were lower compare to those against HCVcc (Fig. 2a–c). The reason for the difference in sensitivity may account for some difference in the two entry systems. Otherwise, in the treatment with the drugs for the first 2 h of HCVcc infection, some amount of the drugs might enter the cell and remain affecting the other steps. The SERMs affected not only genotype 2a but also other genotypes of HCVpp that were examined, suggests that these chemicals have effects on various genotypes of HCV. Although the SERMs appeared to inhibit multiple steps of the HCV life cycle, the primary target step in the viral life cycle might be the entry step. In the time-of-addition experiments, treatment with tamoxifen or raloxifene during the first 2 h was more effective than treatment during the subsequent 2–48 h (Fig. 2a). These SERMs are thought to primarily prevent viral entry and to inhibit post replication step and replication at higher concentrations.

As shown in Fig. 5, an experiment that could discriminate an effect on viral attachment from an effect at the post-binding processes indicated that tamoxifen inhibited both steps. The inhibition of endocytosis by tamoxifen was not rescued by exposure to a low pH. This suggests that the observed inhibition is the result of a mechanism that is independent of the compound's function as a base. HCV entry is a highly complicated process that involves numerous viral and cellular factors. Tamoxifen is thought to target multiple steps that are involved in the attachment and entry steps of the HCV life cycle, which results in high levels of inhibition.

At present, the mechanism of the entry inhibition by SERMs is not clear. It is possible that tamoxifen targets viral molecules, but we have no evidence to support this hypothesis. ERα might be a target molecule because all of the antagonists of ERα that were examined had an inhibitory effect. Watashi et al. indicated that ERα is involved in HCV replication [23]. ERα is thought to be present in the cytoplasm, which is where HCV replicates. However, it is doubtful that ERα is present on the cell surface where viral entry occurs. The addition of 17β-estradiol with tamoxifen did not prevent the inhibitory effect of tamoxifen in the HCVpp experiment (data not shown). This result suggests that tamoxifen does not compete with 17β-estradiol for the target molecules involved in HCV entry. Additionally, a pure ERα antagonist, ICI 182,780, was a less effective inhibitor of the entry step. Based on these results, it is thought that the molecule responsible for HCV entry that is targeted by SERMs is not ERα.

Tamoxifen has various targets other than ERα, such as P-glycoprotein (GPR30), calmodulin, and protein kinase C [26]. GPR30 (G protein-coupled receptor protein 30) is a membrane-associated estrogen receptor that is distinct from the classical ER [27]. Tamoxifen and ICI 182,780 are agonists of GPR30 [28]. We examined the effect of a specific GPR30 agonist, G-1, and a GPR30 antagonist, G-15, on HCVpp infection [29]. G-1 and G-15 did not inhibit HCVpp infection. Conversely, HCVpp infection was observed to increase upon addition of these compounds (data not shown). This result suggested that GPR30 is not involved in the inhibition of HCV entry.

We previously reported that a typical PKC inhibitor, bisindolylmaleimide I (BIM I), inhibited HCV replication [2]. BIM I (10 μM) inhibited both HCVpp and VSVpp infection in a similar manner by approximately 50% (data not shown). This suggests that BIM I has a different mechanism for the

inhibition of entry compared to tamoxifen. PKC is not thought to be involved in the HCV-specific inhibition of entry by SERMs.

There were few reports of HCV entry inhibitors until the development of the cell-culture JFH-1 infection system. It has recently been reported that fluphenazine, trifluoperazine and related chemicals exhibit a strong, dose-dependent inhibition of HCV entry without significantly affecting the entry of VSVpp [24,25]. These compounds are structurally similar to chlorpromazine, which is an inhibitor of the clathrin-coated pit formation that is required for HCV entry [14]. Interestingly, these compounds and the SERMs have a common structural characteristic: planar, multiple aromatic rings with a tertiary amine side chain. Tamoxifen, raloxifene and ERα antagonists all have this structure, but the ERα agonists do not have these structures. Fluphenazine and related chemicals may inhibit HCV entry through a mechanism that is similar to tamoxifen.

In summary, we observed a significant HCV inhibitory effect of various SERMs using the Huh 7.5.1 cell-JFH-1 infection system. Additionally, we demonstrated that SERMs could be useful for the treatment of HCV. Because it takes a great deal of time and money to develop a new drug from a novel chemical compound, it may be easier to use previously developed drugs that can be used for new applications. Tamoxifen, toremifene, and raloxifene are all drugs that have been in use for an extended period of time. In our present *in vitro* study, the effective concentrations for the HCV inhibitory effects of the SERMs were approximately 0.1–10 μM. In the case of tamoxifen, 20 mg per day, administered for 8 weeks resulted in plasma concentrations of approximately 0.5 μM. These concentrations could be sufficient to exert an anti-HCV effect. SERMs should be investigated to determine their efficacy for treating HCV clinically. Further examination of the mechanism of the entry inhibition mediated by SERMs would produce significant new data relevant to the understanding of HCV entry.

Acknowledgments

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BRIEF REPORTS

Replication of Hepatitis C Virus Genotype 3a in Cultured Cells

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See Covering the Cover synopsis on page 1; see editorial on page 13.

Hepatitis C virus (HCV) genotype 3a is widespread worldwide, but no replication system exists for its study. We describe a subgenomic replicon system for HCV genotype 3a. We determined the consensus sequence of an HCV genome isolated from a patient, and constructed a subgenomic replicon using this clone. The replicon was transfected into HuH-7 cells and RNA replication was confirmed. We identified cell culture-adaptive mutations that increased colony formation multiple-fold. We have therefore established a genotype 3a replicon system that can be used to study this HCV genotype.

Keywords: Virology; Experimental Model; HCVGT3; In Vitro Culture System.

Hepatitis C virus (HCV) infection leads to chronic infection and advanced liver diseases in most infected adults.[1] Of the 6 major HCV genotypes, genotypes 1 and 2 are the most prevalent in North America, Europe, and Japan,[2,3] and are the most highly studied. However, other genotypes display specific characteristics. For example, genotype 3a infection can result in hepatic steatosis[4] and telaprevir and boceprevir are less effective against genotype 3a.[5] Therefore, the pathogenesis and inhibitor sensitivity of all HCV genotypes should be studied. Although HCV subgenomic replicons are useful for understanding viral/host factors involved in HCV replication and inhibitor sensitivity, only HCV replicons for genotypes 1a, 1b, and 2a have been established.[6–9] Here, we report on the robust genotype 3a replication system.

An almost complete HCV genome was recovered from the serum of a patient with post-transplantation recurrent HCV infection. This serum exhibited higher infectivity than other tested sera toward primary human hepatocytes (Supplementary Figure 1*A*). The isolate, named S310, contained the following structural elements: a 5′UTR (nt 1-339), an open reading frame encoding 3021 aa (nt 340-9402), and a 3′UTR (nt 9403-9654). Only the last 44 nt of the X-region (nt 9611-9654) could not be recovered. Two major virus populations were found; S310/A contained Ala, Thr, Thr, and Ile, and S310/B contained Thr, Ala, Ala, and Thr, at the 7th, 151st, 431st, and 472nd aa of the NS3 protein, respectively. S310 was clustered into genotype 3a by phylogenetic analysis (Supplementary Figure 1*B*). The complexity of the virus quasispecies in the serum was analyzed by sequencing the hypervariable region. Identical amino acid sequences in all 10 hypervariable region clones indicated a very low degree of diversity. The hypervariable region sequence of the JFH-1 strain also exhibited monoclonality,[10] which can be important for efficient replication in cultured cells.

Subgenomic replicons SGR-S310/A and SGR-S310/B were constructed and their replication efficiency was evaluated by G418-resistant colony-formation assay. After 3 weeks, a small number of colonies were visible for both replicons (Figure 1*A*). Because more colonies were observed in SGR-S310/A than in SGR-S310/B, we focused on SGR-S310/A (henceforth called SGR-S310). Ten cell colonies of SGR-S310 were isolated and analyzed for HCV replication. The mean RNA titer was $9.1 \times 10^7 \pm 4.6 \times 10^7$ copies/μg total RNA (Figure 1*B*). HCV RNA (approximately 8 kb) was detected by Northern blotting (Supplementary Figure 2*A*). Viral proteins in the replicon cells were detected by immunofluorescence and Western blotting (Supplementary Figure 2*B* and 2*C*). To determine whether the G418 resistance of the cells was transmissible by cellular RNA transfection, we electroporated total cellular RNA isolated from 4 replicon clones into naïve HuH-7 cells. Multiple G418-resistant colonies appeared after transfection of the RNA isolated from the replicon clones (Supplementary Figure 3*A*), but not from the naïve HuH-7 cells. These results indicate that the replicon RNA in the parental colonies could replicate in naïve cells. Thus, the G418-resistant colonies that were isolated from cells electroporated with SGR-S310 synthetic RNA contained replicating viral RNA.

Replicating genomes have been shown to accumulate cell culture adaptive mutations, which increase their replication potential. To examine whether SGR-S310 acquired mutations, the complete HCV sequences from 10 replicon clones were sequenced. At least one nonsynonymous mutation was detected in the NS3-NS5B region of each replicon clone (Figure 1B). The following mutations were identified: T1286I in the NS3 helicase (6 of 10

Abbreviation used in this paper: HCV, hepatitis C virus.

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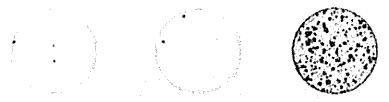
A



SGR-S310/A 10 μg | SGR-S310/B 10 μg | SGR-JFH1 0.1 μg

B

Replicon cell clone	Nucleotide mutation	Amino acid substitution	Affected protein	Replicon RNA amounts (copies/μg RNA)
1	5272 (6932) G→A	2198 R→H	NS5A	2.1×10^8
2	7362 (9022) A→G	2895 R→G	NS5B	9.9×10^7
3	2536 (4196) C→T	1286 T→I	NS3	9.5×10^7
4	2536 (4196) C→T	1286 T→I	NS3	9.9×10^7
5	2536 (4196) C→T	1286 T→I	NS3	5.6×10^7
6	2536 (4196) C→T	1286 T→I	NS3	4.0×10^7
7	2536 (4196) C→T	1286 T→I	NS3	8.3×10^7
8	2536 (4196) C→T	1286 T→I	NS3	8.7×10^7
9	6166 (7826) C→T	2496 T→I	NS5B	9.3×10^7
	7363 (9023) G→A	2895 R→K	NS5B	
10	5241 (6901) A→G	2188 T→A	NS5A	5.1×10^7

C

Replicon constructs	Protein	Colony formation efficiency (cfu/μg RNA)	Transient replication efficiency (72h/4h†)
SGR-S310/A	None	0.4×10^0	4.4×10^{-3}
T1286I	NS3	2.9×10^3	6.5×10^{-3}
T2188A	NS5A	2.3×10^2	4.9×10^{-3}
R2198H	NS5A	3.7×10^3	6.0×10^{-2}
S2210I	NS5A	6.2×10^2	1.1×10^{-2}
T2496I	NS5B	0.2×10^0	5.0×10^{-3}
R2895K	NS5B	2.3×10^3	7.9×10^{-2}
R2895G	NS5B	9.8×10^2	2.6×10^{-2}
T2496I+R2895K	NS5B	4.1×10^3	2.6×10^{-1}
Con1-NK5.1	NS3+NS5A	9.1×10^2	6.3×10^0
N	None	NA††	7.1×10^{-2}
SGR-JFH1	None	5.3×10^5	1.5×10^2

Figure 1. S310 subgenomic replicon analysis. (*A*) Three million HuH-7 cells were electroporated with 10 μg RNA from SGR-S310/A or SGR-S310/B or 0.1 μg RNA from SGR-JFH1. G418-selected colonies were fixed and stained after 3 weeks. (*B*) Non-synonymous mutations identified in the replicon genomes and HCV RNA titers in the replicon clones. Nucleotide positions within the S310 subgenomic replicon and within the full-length S310 genome (in parentheses) are given. (*C*) Replication potential of the adaptive mutants as determined by the colony-formation assay using Neo-replicons and by the transient replication assay using Fluc-replicons. †72 h/4 h, transient replication efficiency was determined as a ratio of luciferase activity in the transfected cells between 72 h and 4 h post transfection. ††NA, not available.

clones); T2188A or R2198H in NS5A (2 clones); an R2895G substitution in NS5B (1 clone); and T2496I in NS5A plus R2895K in NS5B (1 clone). These mutations and the S2210I mutation (corresponding to S2204I in genotype 1 replicon)[7,8] were introduced, individually or in combination, into the parental SGR-S310 and the colony-formation efficiencies of the mutant replicons were tested. All mutations, except T2496I, increased the colony formation, indicating an adaptive phenotype (Figure 1*C*, Supplementary Figure 3*B*). Transient replication efficiency was also tested using firefly luciferase reporter replicons. SGR-S310/Luc did not replicate in Huh-7.5.1 cells, whereas the adaptive mutants displayed varying degrees of replication (Figure 1*C*, Supplementary Figure 3*C*). Adaptive mutations T2496I and R2895K, when combined together, most efficiently enhanced the colony formation as well as transient replication (Figure 1*C*). Interestingly, T1286I and R2895G found in our study correspond to the Con1 adaptive mutations T1280I and R2884G, respectively.[11,12] T2188A or R2198H in NS5A were identified in 2 replicon clones and are located close to S2210I. Indeed, S2210I also enhanced SGR-S310 replication, suggesting that this region might be important for HCV replication. S310 replicons with adaptive mutations were compared with genotype 1b (Con1 and N) and 2a (JFH-1) replicons. Colony-formation efficiencies of most S310 adaptive replicons were at levels comparable with Con1 and JFH-1 (Figure 1*C*, Supplementary Figure 3*B*). In contrast, S310 adaptive replicons replicated less efficiently than Con1-NK5.1 and JFH-1 replicons in transient replication assays. However, genotype 1b N replicon replicated at a level similar to some S310 adaptive replicons (Figure 1*C*, Supplementary Figure 3*C*). Future studies will dissect the detailed mechanisms that underlie the effects of these mutations.

Successful generation of a genotype 3a replicon provided a unique opportunity to compare the susceptibility of genotype 3a (SGR-S310), 1b (Con1[13]), and 2a (JFH-1/4-1[13]) replicons to HCV inhibitors. Interferon-alfa dose-dependently decreased the replication of all tested genotypes (Figure 2*A*), whereas a protease inhibitor, BILN-2061, was more effective against replicons from genotypes 1b and 2a than 3a (Figure 2*B*). The non-nucleoside polymerase inhibitor JTK-109 was more potent against genotype 1b and 3a (Figure 2*C*). However, the nucleoside polymerase inhibitor, PSI-6130, equally inhibited all genotypes (Figure 2*D*).

In conclusion, we established a subgenomic replicon for genotype 3a, which should be useful for understanding the specific characteristics of this genotype and for the screening of antiviral chemicals that are effective against this genotype. Construction of a full-length infectious S310 clone is in progress.

Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2012.09.017.

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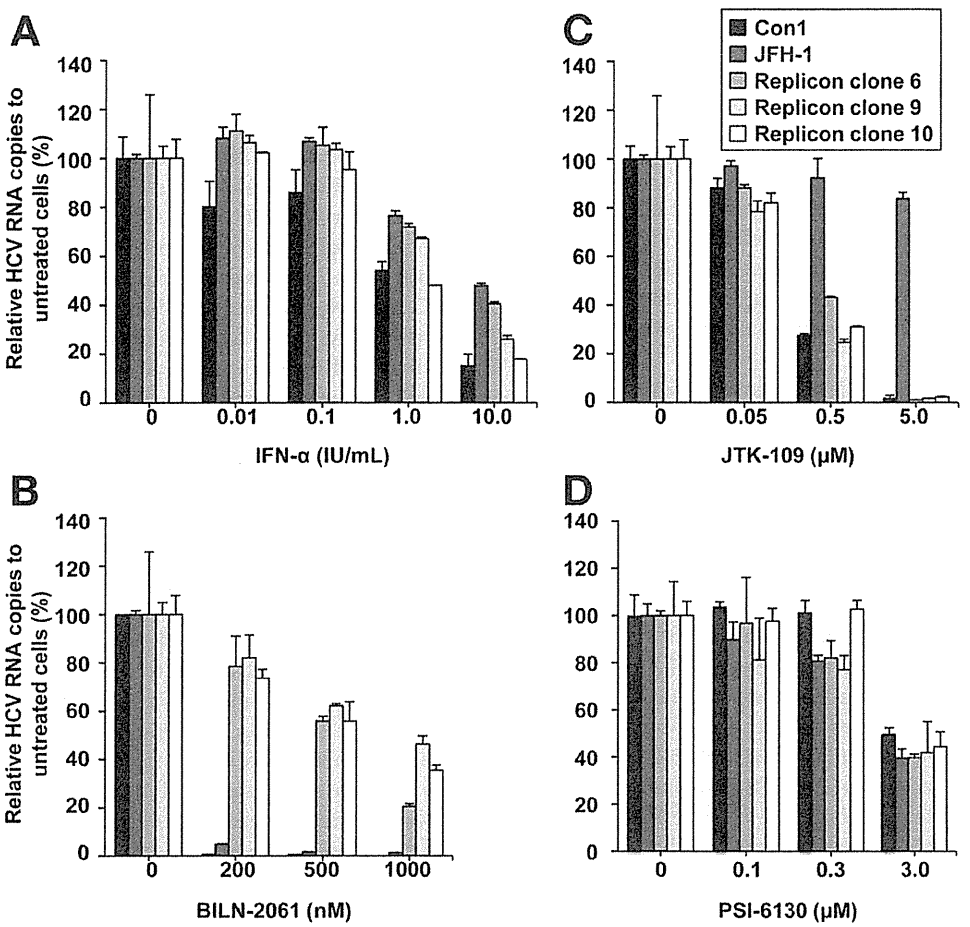

Figure 2. Effects of antiviral inhibitors on the replication of S310 subgenomic replicons. Three cell clones (clone 6, 9 and 10) carrying genotype 3a S310 replicons and one cell clone each harboring genotype 1b Con1 and genotype 2a JFH-1 replicons were treated with the indicated concentrations of (A) interferon alpha, (B) HCV protease inhibitor BILN-2061, (C) the non-nucleoside polymerase inhibitor JTK-109, and (D) the nucleoside polymerase inhibitor PSI 6130 for 72 hours and replication levels were measured by quantifying intracellular HCV RNA. Results are means ± standard deviations of 3 replicates.

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Reprint requests

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DDBJ/EMBL/GenBank accession numbers: S310/A: AB691595, S310/B: AB691596, SGR-S310/A: AB691597, SGR-S310/B: AB691598, SGR-S310/Luc: AB691599.

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Conflicts of interest

The authors disclose no conflicts.

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Supplementary Methods

Cell Culture

The human hepatoma cell line HuH-7[1] and its derivative cell line Huh-7.5.1[2] were maintained in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum, minimal essential medium nonessential amino acids, 100 U/mL penicillin, 100 μg/mL streptomycin, 10 mM HEPES, and 1 mM sodium pyruvate at 37°C in a 5% CO_2 incubator.

Primary human hepatocytes (PHH) were isolated from an encapsulated liver sample.[3] Isolated PHH were seeded in 12-well plates and cultured at 37°C in Lanford medium before infection.

PHH Infection With HCV-Positive Sera

Three days post seeding, PHH were inoculated with HCV-positive sera. After 16 h of inoculation, monolayers were washed with William's E medium and fresh Lanford medium was added. Cells were harvested at 72 h post infection. Total RNA was isolated using a guanidinium isothiocyanate solution (RNAble; Eurobio, Courtaboeuf, France) and intracellular levels of HCV RNA were quantified using the SuperScript III Platinium One-Step quantitative reverse transcription polymerase chain reaction (RT-PCR) system (Invitrogen, Carlsbad, CA) and a LightCycler480 real-time PCR system (Roche Diagnostics, Meylan, France).

HCV Genotype 3a Clone

Clone S310 was isolated from a 71-year-old female patient suffering from post liver transplantation HCV recurrence. She was diagnosed with HCV genotype 3a infection at the age of 59 years and underwent liver transplantation 4 years later due to liver cirrhosis. HCV-RNA titer was 2.8×10^6 copies/mL. Total RNA extracted from 100 μL serum using the acid-guanidinium isothiocyanate-phenol-chloroform method (Isogen-LS; Nippon Gene, Tokyo, Japan) was precipitated with isopropanol, washed with ethanol, and dissolved in 10 μL nuclease-free water. An aliquot of 4 μL was subjected to reverse transcription using random hexamers and Moloney murine leukemia virus reverse transcriptase (Superscript III; Invitrogen) at 42°C for 50 min and then at 50°C for 10 min.

Isolation of HCV

The sequences of 4 isolates of genotype 3a (accession numbers AF046866, D28917,[4] X76918, and D17763[5]) that were obtained from the HCV database (http://hcv.lanl.gov/content/sequence/HCV/ToolsOutline.html) were aligned and PCR primers were designed based on the conserved sequences. These primers were used to amplify the complementary DNA (cDNA) of S310 into 9 overlapping fragments by nested PCR (nt 1–370, nt 127–1284, nt 1117–1997, nt 1704–3352, nt 3152–5080, nt 4869–6842, nt 6601–8129, nt 7988–9145, and nt 9082–9576; nucleotide numbers refer to the positions on S310, with nt 1 being the first nucleotide of the 5′ UTR). The sequence of these primers is shown in Supplementary Table 1. Two microliters of cDNA was subjected to PCR using Pyrobest DNA polymerase (Takara Bio, Kyoto, Japan) and the outer set of primers, and this first-round PCR product (2 μL) was further amplified by a second round of PCR using the inner set of primers. PCR conditions for the first and second rounds of PCR consisted of 35 cycles each of denaturation at 98°C for 20 s, annealing at 55°C for 1 min, and extension at 72°C for 3 min. A fragment encompassing the 5′ end of the viral genome (nt 1–370) was amplified by 5′RACE. Briefly, cDNA was synthesized with a 5′ UTR primer (antisense), tailed with a dCTP homopolymer by using terminal deoxynucleotidyl transferase, and amplified by PCR (5′ RACE System for Rapid Amplification of cDNA Ends; Invitrogen) using TaKaRa LA Taq polymerase (Takara Bio). The PCR products of all fragments were separated by agarose gel electrophoresis, cloned into the pGEM-T EASY vector (Promega, Madison, WI) and sequenced using the Big Dye Terminator Mix and an automated DNA sequencer. The consensus sequence of 5 to 9 isolated cDNA clones was adopted for each fragment. Two major populations of the virus were identified in the patient's serum that differed in 4 amino acids in the NS3 protein (aa 1039, 1183, 1463, and 1504), and these populations were designated as S310/A and S310/B (DDBJ/EMBL/GenBank accession number: AB691595 and AB691596, respectively). To assess the complexity of the HCV population in the patient's serum, the hypervariable region sequences of 10 clones were determined.

Computer Analysis

A phylogenetic tree was constructed using the neighbor-joining method to examine the relationship between the polyprotein region of S310 and that of other HCV genotype 3a isolates available in the database. In order to analyze the diversity in each subgenomic region, the genetic distance was calculated between all possible pairs of genotype 3a isolates and between S310/A and other isolates using MacVector software (MacVector, Inc., Cary, NC). The ratios of these 2 values (mean genetic distance between S310/A and other isolates/mean genetic distance among all genotype 3a isolates) were compared.

Construction of Replicons

Based on the consensus sequence of S310, we assembled pS310/A and pS310/B, which contained the full-length S310/A and S310/B cDNA, respectively, downstream of the T7 RNA polymerase promoter. Briefly the 9 amplicons described here were combined by overlapping PCR and ligated with pGEM-T EASY vectors to generate 6 plasmids (A through F) in such a way that each plasmid contained a unique restriction enzyme

cleavage site toward the 3′ end of the viral fragment, which overlapped with the 5′ end of the next fragment. For this purpose, we took advantage of the EcoRI restriction site that is present in the polycloning site of the plasmid toward the 5′ end of the viral fragment. Plasmid A contained the T7 promoter sequence followed by one G-nucleotide and nt 1-3352 of S310, while plasmids B, C, D, and E contained nt 1704-4307, nt 4044-6013, nt 5424-7755, and nt 7276-9425, respectively. Plasmid F contained the fragment constructed by combining the C-terminal end of NS5B (nt 9182-9402) and the variable and poly U/UC regions of the S310/A 3′UTR (nt 9403-9610) with the last 44 nucleotides of JFH-1. Restriction sites for EcoRI and XbaI were introduced upstream of the T7 promoter sequence and downstream of the conserved region, termed the *X-region*, of the 3′UTR, respectively, and the restriction sites of these enzymes that were present within the cDNA were removed by PCR-based mutagenesis. In the neomycin-based subgenomic replicons (SGR-S310/A and SGR-S310/B, accession number: AB691597 and AB691598, respectively), the cassette containing the neomycin phosphotransferase gene and the EMCV IRES replaced the region of S310 that encompasses amino acids 20-1032. Firefly luciferase–based subgenomic replicons (SGR-S310/Luc, accession number: AB691599) were generated from SGR-S310/A by replacing amino acids 20-1032 of S310/A with the cassette containing firefly luciferase and the EMCV IRES from pSGR-JFH1/Luc.[6]

RNA Synthesis

RNA was synthesized by in vitro transcription as described previously.[7] Briefly, the plasmids carrying the cDNA described here were linearized with the XbaI restriction enzyme and 5′ overhangs were removed by treating with mung bean nuclease. Reaction mixtures were further incubated at 50°C for 1 h with 2 μL 20 mg/mL proteinase K and 10 μL 10% sodium dodecyl sulfate to degrade nucleases, and templates were purified with 2 rounds of phenol-chloroform extraction and ethanol precipitation. Three micrograms of templates were subjected to in vitro transcription using a MEGAscript T7 kit (Ambion, Austin, TX) according to the manufacturer's recommendations. Synthesized RNA was treated with DNase I (Ambion) and then purified using ISOGEN-LS (Nippon Gene). The quality of the synthesized RNA was examined by agarose gel electrophoresis.

RNA Transfection

In vitro transcribed RNA or total cellular RNA isolated from replicon cells was introduced into cells by electroporation. Trypsinized cells were washed twice with serum-free Opti-MEM I (Invitrogen) and 3.0×10^6 cells were resuspended in 400 μL cytomix buffer.[8] RNA was delivered into cells by a single pulse of 260 V and 950 μF using the Bio-Rad Gene Pulser II apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately suspended in culture medium and transferred to the appropriate plates. For G418 selection of colonies, the transfected cells were seeded in 10-cm dishes, each containing 8 mL culture medium. G418 (500 μg/mL; Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 24 h after transfection. Culture medium supplemented with G418 was replaced every 3 days. Three weeks after transfection, cells were fixed with buffered formalin and stained with crystal violet or replicon colonies were picked and expanded.

Analysis of G418-Resistant Cells

G418-resistant colonies were collected and used for further analysis. Colonies were independently isolated using cloning cylinders (Asahi Techno Glass Co., Tokyo, Japan) and were expanded until they were 80%–90% confluent in 10-cm dishes. Expanded cells were harvested for nucleic acid and protein analysis. Total RNA was isolated from the cells using the ISOGEN reagent (Nippon Gene). Another aliquot of the cell pellet was dissolved in RIPA buffer containing 0.1% sodium dodecyl sulfate for Western blot analysis. For immunofluorescence analysis of viral proteins, cells were seeded on 12-well slides.

Quantification of HCV RNA by Real-Time RT-PCR

Copy numbers of HCV RNA were determined by real-time detection RT-PCR, as described previously,[9] using the ABI Prism 7700 Sequence Detector System (Applied Biosystems Japan, Tokyo, Japan). The concentration of total RNA in the cells was determined using a Nanodrop Spectrophotometer ND-1000 (Thermo Scientific, Rockford, IL).

Northern Blot Analysis

Isolated RNAs (3 μg) from replicon cells were separated on a 1% agarose gel containing formaldehyde, transferred to a positively charged nylon membrane (Hybond-N+; GE Healthcare UK Ltd., Buckinghamshire, UK) and immobilized using a FUNA-UV-LINKER (Funakoshi, Tokyo, Japan). Hybridization was carried out with a [α-^{32}P]dCTP-labeled DNA probe using Rapid-Hyb buffer (GE Healthcare UK Ltd.). The DNA probe was synthesized from a BsrGI-MfeI fragment of the S310 clone that contained NS3-5B genes using the Megaprime DNA labeling system (GE Healthcare UK Ltd.).

Indirect Immunofluorescence

Untransfected HuH-7 cells or S310 replicon-replicating cells were grown on a glass slide for 24 h and fixed in acetone-methanol (1:1 [vol/vol]) for 10 min at −20°C. Cells were then incubated in immunofluorescence buffer (phosphate-buffered saline, 1% bovine serum albumin, 2.5 mM EDTA). S310 patient serum was added at

a dilution of 1:200 in immunofluorescence buffer. After incubation for 1 h at room temperature, cells were washed and then incubated with an Alexa Fluor488–conjugated goat anti-human IgG antibody (Invitrogen) in immunofluorescence buffer. The glass slide was washed and a cover glass was mounted using PermaFluor mounting solution (Thermo Scientific, Cheshire, UK). Cells were examined under a fluorescence microscope (Olympus, Tokyo, Japan).

Western Blot Analysis of HCV Proteins

The protein samples were separated on 12.5% polyacrylamide gels and subsequently transferred to a polyvinylidene difluoride membrane (Immobilon; Millipore, Bedford, MA). Transferred proteins were incubated with 2% skim milk. Anti-NS3 mouse monoclonal antibody (clone 8G2, Abcam, Cambridge, UK) and peroxidase-labeled sheep anti-mouse IgG (Cell Signaling Technology, Danvers, MA) were used to detect HCV proteins. The signals were detected with a chemiluminescence system (ECL Prime; GE Healthcare UK Ltd.).

Identification of Mutations

cDNA was synthesized from total RNA that was extracted from replicon-expressing cells at 2 different times. These cDNAs were amplified into 5 overlapping fragments that spanned the 5′UTR and the NS3-NS5B region using LA Taq DNA polymerase (Takara Bio) and the primers described in Supplementary Table 1. The sequence of each amplified DNA was determined. The mutations identified were subsequently introduced into SGR-S310/A and SGR-S310/Luc by PCR-mediated mutagenesis.

Luciferase Assay

Five micrograms of RNA, prepared by in vitro transcription of S310/SG-FLuc constructs with or without adaptive mutations, were introduced into 3.0×10^6 Huh-7.5.1 cells by electroporation. Cells were harvested with Cell Culture Lysis Reagent (Promega) at 4, 24, 72, and 96 h post electroporation, and luciferase activity was determined by use of a Luciferase Assay System (Promega) and the Lumat LB9507 luminometer (EG & G Berthold, Bad Wildbad, Germany).

Inhibition of S310 Replicon Replication by Specific Inhibitors

S310 replicon cell clones 6, 9, and 10 and the genotype1b Con1 and 2a JFH-1 replicon cells,[10] were seeded into 24-well plates at a density of 5.0×10^4 cells/well. On the next day, the culture medium was replaced with medium containing 0.1% dimethyl sulfoxide with or without various concentrations of interferon alfa (Dainippon-Sumitomo, Osaka, Japan), the specific NS3 protease inhibitor, BILN-2061 (Boehringer Ingelheim Ltd., Québec, Canada), or the NS5B inhibitors, JTK-109 (Japan Tobacco, Inc., Osaka, Japan) and PSI-6130 (Pharmasset, Inc., Princeton, NJ). After 72-h incubation, cells were harvested and HCV RNA was quantified as described.

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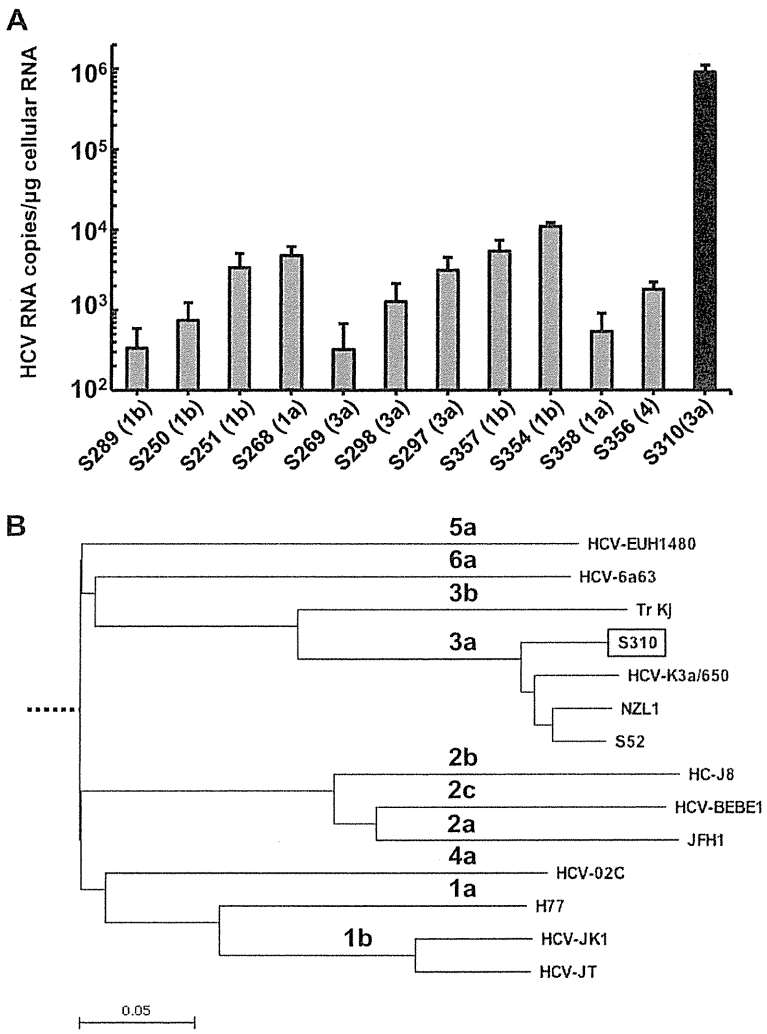
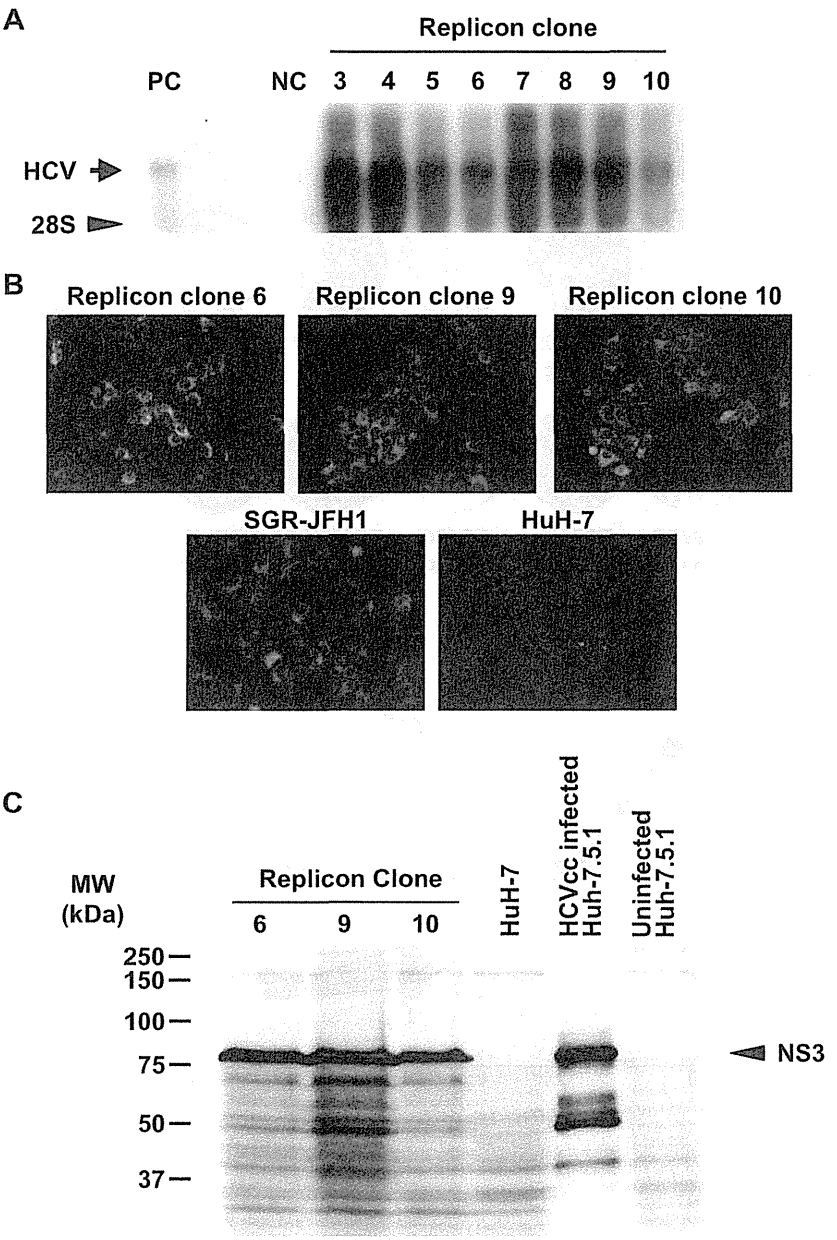
Supplementary Table 1. Primers for Amplification of the S310 HCV Strain

Fragment			Primer sequence (5′→3′)
1 (5′ RACE)[a]	Outer	Antisense	CTTGACGTCCTGTGGGCGA
	Inner	Antisense	TTTTTCTTTGGGGTTTAGG
2	Outer	Sense	GTCTTCACGCGGAAAGCGC
		Antisense	CACCCAAACCACCGACCAC
	Inner	Sense	CCGGGAGAGCCATAGTGGTC
		Antisense	TCCTGAAAGATGGCCTGGGTA
3	Outer	Sense	CTTGGCCCCTCTATGGTAA
		Antisense	GATGTTTCCTGAAGCAGTCG
	Inner	Sense	AGTCATGTGGACCTATTAGT
		Antisense	CACCCAAACCACCGACCAC
4	Outer	Sense	ATGGCTCGTGGCACATCAA
		Antisense	TAGTCATCAGCAGGTCCCAA
	Inner	Sense	GCTCAGCAGCTGCAAGCCCAT
		Antisense	CGCAAAGAATATCTCCGCAAG
5	Outer	Sense	ATTTTTGACATCACTAAGCTAC
		Antisense	AGTGTTGGCTTAAGCCGCA
	Inner	Sense	AATACTTCCAGATGATCATACT
		Antisense	GTGACAGAAAGTGGGCAT
6	Outer	Sense	GTTTCCCGCAGCCAACGT
		Antisense	GTCTCTCAACATCGAGGT
	Inner	Sense	CGGTGAAAGACCGTCTGGA
		Antisense	CAGGGGAGTTGAGATCCT
7	Outer	Sense	GGCCGCGTACATGTGCTAAC
		Antisense	CCGCAGACAAGAAAGTCCGGGT
	Inner	Sense	CTATGGCGCGTGGCTGCCA
		Antisense	ACCCCCAGGTCAGGGTACAC
8	Outer	Sense	CATAACCTAGTCTATTCAACG
		Antisense	TGGTCTTGGTGCGTACCG
	Inner	Sense	GCTCCGTCTGGGAGGACTTGC
		Antisense	CTCGTGCCCGATGTCTCCAA
9	Outer	Sense	TGCTCCTCCAACGTCTCCGT
		Antisense	GCGGCTCACGGACCTTTCAC
	Inner	Sense	GTCGCGGGGACACTCAGGAA
		Antisense	ACTAGGGCTAAGATGGAGCC

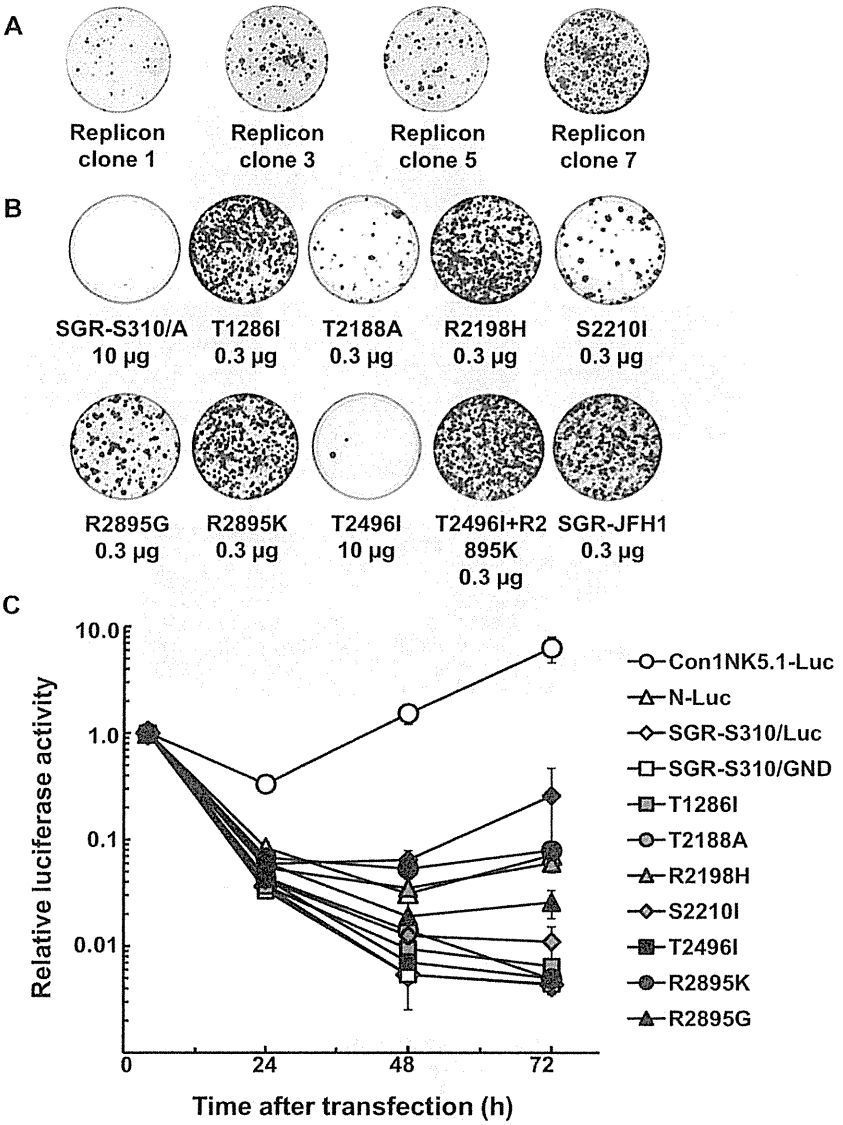
RACE, rapid amplification of complementary DNA ends.
[a]Forward primers used were those in the 5′RACE kit (Abridged Universal Amplification Primer (AUAP) for the first round of PCR and Universal Amplification Primer (UAP) for the second round of PCR).



Supplementary Figure 1. Infection of PHH with HCV patient sera and phylogenetic tree analysis of the S310 strain. (*A*) PHH were exposed to sera of patients infected with genotype 1b (S289, S250, S251, S357, S354), 1a (S268, S358), 3a (S269, S298, S297, S310), and 4 (S356) for 16 h (25 µL/well, except for S310, 10 µL). Intracellular HCV RNA was quantified 72 h post inoculation. Experiments were done in triplicate and data are presented as means ± standard deviation. (*B*) The phylogenetic tree was constructed using the polyprotein region of S310 and HCV strains of different genotypes. The HCV strains analyzed and their corresponding GenBank accession numbers are: K3a/650; D28917, NZL1; NC_009824, S52; GU814263, EUH1480, HCV-6a63; DQ480514, Tr Kj; D49374, HC-J8; D10988, BEBE1; D50409, JFH-1; AB047639, HCV-02C; DQ418784, H77; AF009606, HCV-JK1; X61596 and HCV-JT; D11168. The root of the tree was tentatively taken as the midpoint of the longest path. The length of the horizontal bar indicates the number of nucleotide substitutions per site.



Supplementary Figure 2. Detection and quantification of HCV RNA and proteins in replicon cells. (*A*) Total RNA (3 μg) from replicon cells was analyzed by Northern blot; 5.0 × 10^7 copies of in vitro–transcribed RNA were loaded in parallel as a positive control (PC), while total RNA from untransfected HuH-7 cells served as the negative control (NC). Replicon RNA was detected using a [α-^{32}P]dCTP-labeled DNA probe. Arrow and arrowhead indicate the positions of the replicon RNA and 28S ribosomal RNA, respectively. (*B*) Subcellular localization of viral proteins determined by immunofluorescence. S310 replicon cell clones, JFH-1 replicon cells, and untransfected HuH-7 cells were grown on glass slides for 24 h. After fixation, cells were incubated with patient serum. (*C*) Western blot analysis. Cell lysates were prepared from replicon clones 6, 9, and 10, untransfected HuH-7, and HCVcc (J6/JFH1)-infected Huh-7.5.1 cells and uninfected Huh-7.5.1 cells. Protein (10 μg) was resolved by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and viral nonstructural protein NS3-specific bands were detected using an anti-NS3 mouse monoclonal antibody (clone 8G2). Arrow indicates the position of NS3.



Supplementary Figure 3. Analysis of the effect of mutations on the colony-forming efficiency and transient replication of the subgenomic replicon S310. (*A*) Total RNA was isolated from the indicated replicon cell clones and 10 µg RNA was introduced into 3 million naïve HuH-7 cells by electroporation. After 3 weeks of G418 selection (500 µg/mL), colonies were stained. (*B*) Three million HuH-7 cells were electroporated with the indicated amounts of transcribed RNA and colonies were selected by a 3-week G418 selection. The JFH-1 subgenomic RNA was included as a positive control. (*C*) Huh-7.5.1 cells were transfected with the transcribed RNA from pSGR-S310/Luc and pSGR-S310/Luc constructs with mutations (GND mutation in NS5B, T1286I, T2188A, R2198H, S2210I, T2496I, R2895K, R2895G, and T2496I+R2895K) and Con1-NK5.1/Luc and N/Luc replicon. Transfected cells were harvested at the indicated time points and at 4 h post transfection. Relative luciferase activity (arbitrary units) was measured in the cell lysate and was normalized to the activity at 4 h post transfection. Assays were performed in triplicate, and data are presented as means ± standard deviation.

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Human Blood Dendritic Cell Antigen 3 (BDCA3)$^+$ Dendritic Cells Are a Potent Producer of Interferon-λ in Response to Hepatitis C Virus

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The polymorphisms in the interleukin (*IL)-28B* (interferon-lambda [IFN]-λ3) gene are strongly associated with the efficacy of hepatitis C virus (HCV) clearance. Dendritic cells (DCs) sense HCV and produce IFNs, thereby playing some cooperative roles with HCV-infected hepatocytes in the induction of interferon-stimulated genes (ISGs). Blood dendritic cell antigen 3 (BDCA3)$^+$ DCs were discovered as a producer of IFN-λ upon Toll-like receptor 3 (TLR3) stimulation. We thus aimed to clarify the roles of BDCA3$^+$ DCs in anti-HCV innate immunity. Seventy healthy subjects and 20 patients with liver tumors were enrolled. BDCA3$^+$ DCs, in comparison with plasmacytoid DCs and myeloid DCs, were stimulated with TLR agonists, cell-cultured HCV (HCVcc), or Huh7.5.1 cells transfected with HCV/JFH-1. BDCA3$^+$ DCs were treated with anti-CD81 antibody, inhibitors of endosome acidification, TIR-domain-containing adapter-inducing interferon-β (TRIF)-specific inhibitor, or ultraviolet-irradiated HCVcc. The amounts of IL-29/IFN-λ1, IL-28A/IFN-λ2, and IL-28B were quantified by subtype-specific enzyme-linked immunosorbent assay (ELISA). The frequency of BDCA3$^+$ DCs in peripheral blood mononuclear cell (PBMC) was extremely low but higher in the liver. BDCA3$^+$ DCs recovered from PBMC or the liver released large amounts of IFN-λs, when stimulated with HCVcc or HCV-transfected Huh7.5.1. BDCA3$^+$ DCs were able to induce ISGs in the coexisting JFH-1-positive Huh7.5.1 cells. The treatments of BDCA3$^+$ DCs with anti-CD81 antibody, cloroquine, or bafilomycin A1 reduced HCVcc-induced IL-28B release, whereas BDCA3$^+$ DCs comparably produced IL-28B upon replication-defective HCVcc. The TRIF-specific inhibitor reduced IL-28B release from HCVcc-stimulated BDCA3$^+$ DCs. In response to HCVcc or JFH-1-Huh7.5.1, BDCA3$^+$ DCs in healthy subjects with IL-28B major (rs8099917, TT) released more IL-28B than those with IL-28B minor genotype (TG). *Conclusion*: Human BDCA3$^+$ DCs, having a tendency to accumulate in the liver, recognize HCV in a CD81-, endosome-, and TRIF-dependent manner and produce substantial amounts of IL-28B/IFN-λ3, the ability of which is superior in subjects with IL-28B major genotype. (HEPATOLOGY 2013;57:1705-1715)

Hepatitis C virus (HCV) infection is one of the most serious health problems in the world. More than 170 million people are chronically infected with HCV and are at high risk of developing liver cirrhosis and hepatocellular carcinoma. Genome-wide association studies have successfully identified the genetic polymorphisms (single nucleotide polymorphisms, SNPs) upstream of the promoter region of the

Abbreviations: Ab, antibody; HCV, hepatitis C virus; HCVcc, cell-cultured hepatitis C virus; HSV, herpes simplex virus; IHL, intrahepatic lymphocyte; INF-λ, interferon-lambda; IRF, interferon regulatory factor; ISGs, interferon-stimulated genes; JEV, Japanese encephalitis virus; Lin, lineage; mDC, myeloid DC; MOI, multiplicity of infection; PBMC, peripheral blood mononuclear cell; pDC, plasmacytoid DC; Poly IC, polyinosine-polycytidylic acid; RIG-I, retinoic acid-inducible gene-I; SNPs, single nucleotide polymorphisms; TLR, Toll-like receptor; TRIF, TIR-domain-containing adapter-inducing interferon-β.

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interleukin (IL)-28B / interferon-lambda 3 (IFN-λ3) gene, which are strongly associated with the efficacy of pegylated interferon-α (PEG-IFN-α) and ribavirin therapy or spontaneous HCV clearance.[1-4]

IFN-λs, or type III IFNs, comprise a family of highly homologous molecules consisting of IFN-λ1 (IL-29), IFN-λ2 (IL-28A), and IFN-λ3 (IL-28B). In clear contrast to type I IFNs, they are released from relatively restricted types of cells, such as hepatocytes, intestinal epithelial cells, or dendritic cells (DCs). Also, the cells that express heterodimeric IFN-λ receptors (IFN-λR1 and IL-10R2) are restricted to cells of epithelial origin, hepatocytes, or DCs.[5] Such limited profiles of cells expressing IFN-λs and their receptors define the biological uniqueness of IFN-λs. It has been shown that IFN-λs convey anti-HCV activity by inducing various interferon-stimulated genes (ISGs),[5] the profiles of which were overlapped but others were distinct from those induced by IFN-α/β. Some investigators showed that the expression of IL-28 in PBMC was higher in subjects with IL-28B major than those with minor; however, the levels of IL-28 transcripts in liver tissue were comparable regardless of IL-28B genotype.[2,6]

At the primary exposure to hosts, HCV maintains high replicative levels in the infected liver, resulting in the induction of IFNs and ISGs. In a case of successful HCV eradication, it is postulated that IFN-α/β and IFN-λ cooperatively induce antiviral ISGs in HCV-infected hepatocytes. It is of particular interest that, in primary human hepatocytes or chimpanzee liver, IFN-λs, but not type I IFNs, are primarily induced after HCV inoculation, the degree of which is closely correlated with the levels of ISGs.[7] These results suggest that hepatic IFN-λ could be a principal driver of ISG induction in response to HCV infection. Nevertheless, the possibility remains that DCs, as a prominent IFN producer in the liver, play significant roles in inducing hepatic ISGs and thereby suppressing HCV replication.

DCs, as immune sentinels, sense specific genomic and/or structural components of pathogens with various pattern recognition receptors and eventually release IFNs and inflammatory cytokines.[8] In general, DCs migrate to the organ where inflammation or cellular apoptosis occurs and alter their function in order to alleviate or exacerbate the disease conditions. Therefore, the phenotypes and/or capacity of liver DCs are deemed to be influenced in the inflamed liver. In humans, the existence of phenotypically and functionally distinct DC subsets has been reported: myeloid DC (mDC) and plasmacytoid DC (pDC).[9] Myeloid DCs predominantly produce IL-12 or tumor necrosis factor alpha (TNF-α) following proinflammatory stimuli, while pDCs release considerable amounts of type I IFNs upon virus infection.[9] The other type of mDCs, mDC2 or BDCA3$^+$(CD141) DCs, have been drawing much attention recently, since human BDCA3$^+$ DCs are reported to be a counterpart of murine CD8a$^+$ DCs.[10] Of particular interest is the report that BDCA3$^+$ DCs have a potent capacity of releasing IFN-λ in response to Toll-like receptor 3 (TLR3) agonist.[11] However, it is still largely unknown whether human BDCA3$^+$ DCs are able to respond to HCV.

Taking these reports into consideration, we hypothesized that human BDCA3$^+$ DCs, as a producer of IFN-λs, have crucial roles in anti-HCV innate immunity. We thus tried to clarify the potential of BDCA3$^+$ DCs in producing type III IFNs by using cell-cultured HCV (HCVcc) or hepatoma cells harboring HCV as stimuli. Our findings show that BDCA3$^+$ DCs are quite a unique DC subset, characterized by a potent and specialized ability to secrete IFN-λs in response to HCV. The ability of BDCA3$^+$ DCs to release IL-28B upon HCV is superior in subjects with IL-28B major (rs8099917, TT) to those with minor (TG or GG) genotype, suggesting that BDCA3$^+$ DCs are one of the key players in IFN-λ-mediated innate immunity.

Patients and Methods

Subjects. This study enrolled 70 healthy volunteers (male/female: 61/9) (age: mean ± standard deviation [SD], 37.3 ± 7.8 years) and 20 patients who underwent surgical resection of liver tumors at Osaka University Hospital (Supporting Table 1). The study was approved by the Ethical Committee of Osaka University Graduate School of Medicine. Written informed consent was obtained from all of them. All healthy volunteers were negative for HCV, hepatitis B virus (HBV), and human immunodeficiency virus (HIV) and had no apparent history of liver, autoimmune, or malignant diseases.

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Additional Supporting Information may be found in the online version of this article.

Reagents. The specifications of all antibodies used for FACS or cell sorting TLR-specific synthetic agonists, pharmacological reagents, and inhibitory peptides are listed in the Supporting Materials.

Separation of DCs from PBMC or Intrahepatic Lymphocytes. We collected 400 mL of blood from each healthy volunteer and processed them for PBMCs. Noncancerous liver tissues were obtained from patients who underwent resection of liver tumors (Supporting Table 1). For the collection of intrahepatic lymphocytes (IHLs), liver tissues were washed thoroughly with phosphate-buffered saline to remove the peripheral blood adhering to the tissue and ground gently. After Lin-negative ($CD3^-$, $CD14^-$, $CD19^-$, and $CD56^-$) cells were obtained by the MACS system, each DC subset with the defined phenotype was sorted separately under FACS Aria (BD). The purity was more than 98%, as assessed by FACS Canto II (BD). Sorted DCs were cultured at 2.5×10^4/well on 96-well culture plates.

Immunofluorescence Staining of Human Liver Tissue. Tissue specimens were obtained from surgical resections of noncancerous liver from the patients as described above. Briefly, the 5-mm sections were incubated with the following antibodies: mouse biotinylated antihuman BDCA3 antibody (Miltenyi-Biotec), and mouse antihuman CLEC9A antibody (Biolegend) and subsequently with secondary goat antirabbit Alexa Fluor488 or goat antimouse Alexa Fluor594 (Invitrogen, Molecular Probes) antibodies. Cell nuclei were counterstained with Dapi-Fluoromount-GTM (Southern Biotech, Birmingham, AL). The stained tissues were analyzed by fluorescence microscopy (Model BZ-9000; Keyence, Osaka, Japan).

Cells and Viruses. The *in vitro* transcribed RNA of the JFH-1 strain of HCV was introduced into FT3-7 cells[12] or Huh7.5.1 cells. The stocks of HCVcc were generated by concentration of the medium from JFH-1-infected FT3-7 cells. The virus titers were determined by focus forming assay.[13] The control medium was generated by concentration of the medium from HCV-uninfected FT3-7 cells. Infectious JEVs were generated from the expression plasmid (pMWJEATG1) as reported.[14] HSV (KOS) was a generous gift from Dr. K. Ueda (Osaka University). Huh7.5.1 cells transduced with HCV JFH-1 strain was used for the coculture with DCs. The transcripts of ISGs in Huh7.5.1 were examined by reverse-transcription polymerase chain reaction (RT-PCR) methods using gene-specific primers and probes (Applied Biosystems, Foster City, CA).

Secretion Assays. IL-28B/IFN-λ3 was quantified by a newly developed chemiluminescence enzyme immunoassay (CLEIA) system.[15] IL-29/IFN-λ1, IL-28A/IFN-λ2, and IFN-β were assayed by commercially available enzyme-linked immunosorbent assay (ELISA) kits (eBioscience, R&D, and PBL, respectively). IFN-α was measured by cytometric beads array kits (BD) according to the manufacturer's instructions.

Statistical Analysis. The differences between two groups were assessed by the Mann-Whitney nonparametric *U* test. Multiple comparisons between more than two groups were analyzed by the Kruskal-Wallis nonparametric test. Paired *t* tests were used to compare differences in paired samples. All the analyses were performed using GraphPad Prism software (San Diego, CA).

Results

Human BDCA3$^+$ DCs Are Phenotypically Distinct from pDCs and mDCs. We defined BDCA3$^+$ DCs as $Lin^-HLA\text{-}DR^+BDCA3^{high+}$ cells (Fig. 1A, left, middle), and pDCs and mDCs by the patterns of CD11c and CD123 expressions (Fig. 1A, right). The level of CD86 on pDCs or mDCs is comparatively higher than those on BDCA3$^+$ DCs (Fig. 1B). The expression of CD81 is higher on BDCA3$^+$ DCs than on pDCs and mDCs (Fig. 1B, Supporting Fig. S1). CLEC9A, a member of C-type lectin, is expressed specifically on BDCA3$^+$ DCs as reported elsewhere,[16] but not on pDCs and mDCs (Fig. 1B).

Liver BDCA3$^+$ DCs Are More Mature than the Counterparts in the Periphery. BDCA3$^+$ DCs in infiltrated hepatic lymphocytes (IHLs) are all positive for CLEC9A, but liver pDCs or mDCs are not (data not shown). The levels of CD40, CD80, CD83, and CD86 on liver BDCA3$^+$ DCs are higher than those on the peripheral counterparts, suggesting that BDCA3$^+$ DCs are more mature in the liver compared to those in the periphery (Fig. 1C).

In order to confirm that BDCA3$^+$ DCs are localized in the liver, we stained the cells with immunofluorescence antibodies (Abs) in noncancerous liver tissues. Liver BDCA3$^+$ DCs were defined as BDCA3$^+$ CLEC9A$^+$ cells (Fig. 1D). Most of the cells were found near the vascular compartment or in sinusoid or the space of Disse of the liver tissue.

BDCA3$^+$ DCs Are Scarce in PBMCs but More Abundant in the Liver. The percentages of BDCA3$^+$ DCs in PBMCs were much lower than those of the other DC subsets (BDCA3$^+$ DCs, pDCs and mDCs, mean $\pm$ SD [%], 0.054 ± 0.044, 0.27 ± 0.21 and 1.30 ± 0.65) (Fig. 2A). The percentages of BDCA3$^+$ DCs in IHLs were lower than those of the others (BDCA3$^+$ DCs, pDCs, and mDCs, mean $\pm$ SD [%],