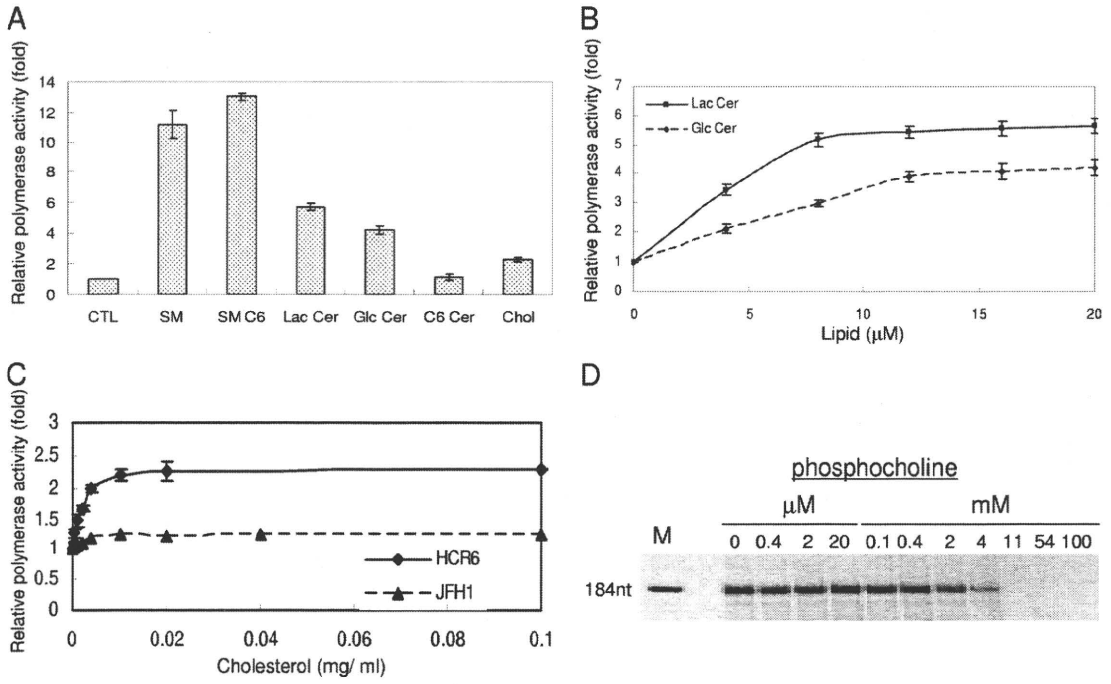

FIG. 3. HCV RNA polymerase activation effect of lipids. (A) Lipid activation of HCR6 (1b) RdRp wt. HCV HCR6 (1b) RdRp wt (100 nM) was incubated with or without (control [CTL]) 0.01 mg/ml egg yolk sphingomyelin (SM), 2 μM hexanoyl sphingomyelin (SM C6), 8 μM C_8-lactosyl(β) ceramide (Lac Cer), 12 μM C_8-β-D-glucosyl ceramide (Glc Cer), 12 μM C_6-ceramide (C6 Cer), or 0.02 mg/ml cholesterol (chol). (B) Activation kinetics of C_8-lactosyl(β) ceramide (Lac Cer) and C_8-β-D-glucosyl ceramide (Glc Cer) on HCR6 (1) RdRp. (C) Activation kinetics of cholesterol on HCR6 (1b) and JFH1 (12a) RdRps. (D) The effect of phosphocholine on HCR6 (1b) RdRp. The mean ± standard deviation of the activation ratio was calculated from three independent experiments.

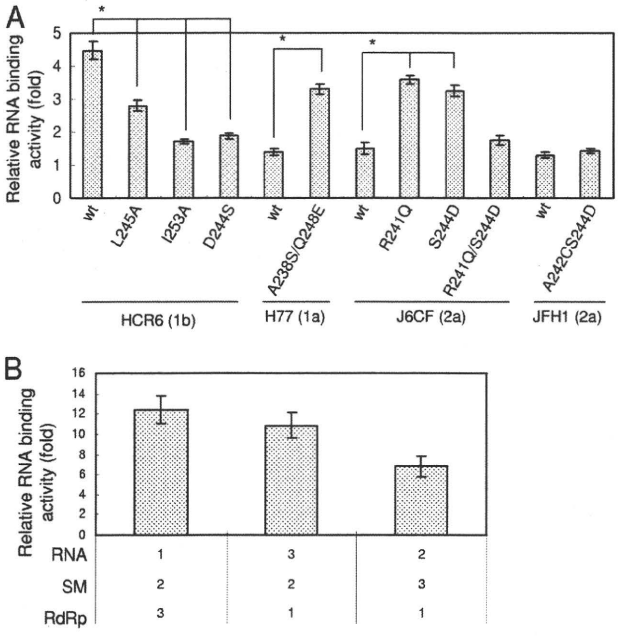

FIG. 4. Sphingomyelin activation of the RNA binding activity of HCV RNA polymerase. (A) Sphingomyelin activation of RNA filter binding of HCV RdRps (RdRp names are indicated below the graph). RdRps and ^{32}P-labeled RNA template (SL12-1S) were incubated with or without egg yolk sphingomyelin (SM), before filtration. (B) Effect of the order of sphingomyelin treatment. Numbers below the graph indicate the order in which the reagents were added. The graph represents the ratio to RNA binding without sphingomyelin. The mean ± standard deviation of the activation ratio was calculated from three independent experiments. *, $P < 0.01$.

function mutants H77 (1a) NS5B(A238S/Q248E) and JFH1 (2a) NS5B(A242C/S244D) were compared with 5 and 50 nM myriocin treatment for 72 h (Fig. 5).

First, HCV replicon activity was compared as the relative luciferase activity (Fig. 5A). Both JFH1 (2a) wt and NS5B(A242C/S244D) replicons showed similar and strong replicon activity ($133 \times 10^3 \pm 12 \times 10^3$ and $138 \times 10^3 \pm 8.5 \times 10^3$, respectively). JFH1 (2a) wt replicon was resistant to myriocin treatment, as reported by Aizaki et al. using other SPT inhibitors (3). The JFH1 (2a) NS5B(A242C/S244D) replicon became sensitive to myriocin but still showed higher replicon activity than NN (1b) or H77 (1a) replicons even at 50 nM myriocin.

To analyze the effect of mutations precisely, the replicon activity relative to each wt strain was compared (Fig. 5B). The JFH1 (2a) wt replicon with 50 nM myriocin showed the same luciferase activity as the wt without myriocin (102% ± 9.6%). JFH1 (2a) NS5B(A242C/S244D) replicon activity was the same as that of the wt without myriocin (103% ± 12%); with 5 nM myriocin it was 84.1% ± 6.6% of the wt level, but with 50 nM myriocin it was 70.3% ± 5.3% of the wt level, which was significantly lower ($P < 0.01$). NN (1b) wt replicon activity was 45.3% ± 6.6% with 5 nM myriocin and 21.7% ± 2.9% with 50 nM myriocin relative to the wt level without myriocin. NN (1b) NS5B(D244S) replicon activity was 72.2% ± 12% without myriocin ($P < 0.05$), 44.0% ± 7.4% with 5 nM myriocin, and 38.1% ± 4.2% with 50 nM myriocin relative to wt level without myriocin, which was significantly higher ($P < 0.01$). Thus, NN (1b) NS5B(D244S) showed lower replicon activity than the wt

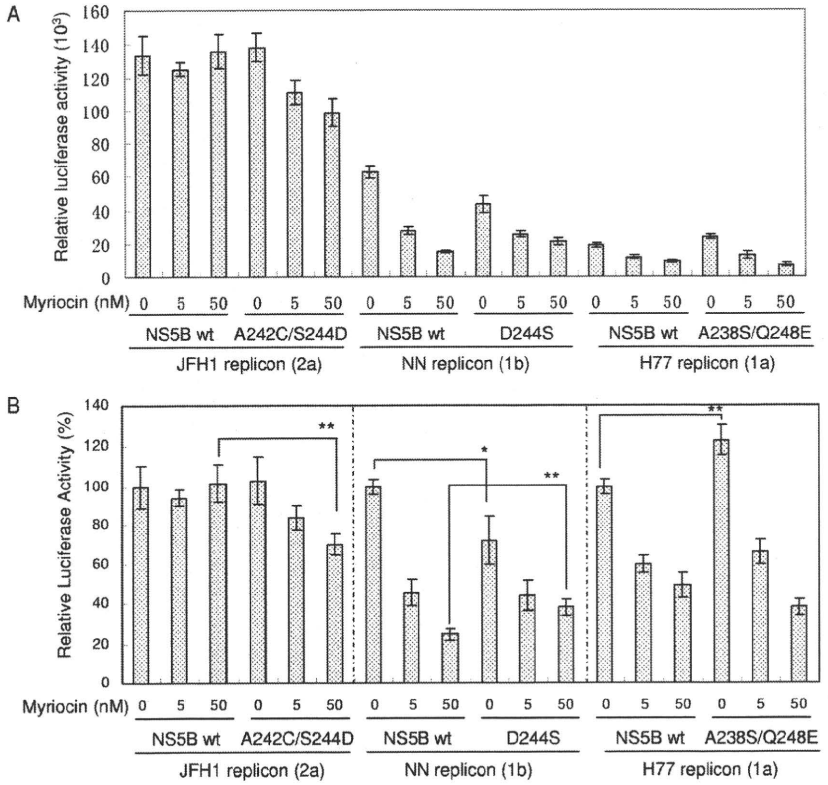

FIG. 5. Myriocin inhibition of HCV replicon activity. Huh7.5.1 cells were incubated with myriocin after transfection with the HCV replicons indicated below the graphs. Means ± standard deviations of the relative luciferase activity at 72 h after myriocin treatment compared to activity at 4 h after transfection (A) and to that of each wt without myriocin (B) were calculated from three independent measurements. *, $P < 0.05$; ** $P < 0.01$.

and was less sensitive to myriocin than the wt. H77 (1a) wt replicon activity was 59.9% ± 4.2% with 5 nM myriocin and 49.2% ± 6.4% with 50 nM myriocin relative to the wt level without myriocin. H77 (1a) NS5B(A238S/Q248E) replicon activity was 123% ± 7.1% without myriocin ($P < 0.01$), 66.1% ± 6.3% with 5 nM myriocin, and 38.0% ± 4.1% with 50 nM myriocin relative to wt level without myriocin. Both H77 (1a) wt and NS5B(A238S/Q248E) replicons were sensitive to myriocin, and the replicon activity of NS5B(A238S/Q248E) was higher than that of the wt.

JFH1 (2a) RdRp(A242C/S244D) localized in the DRM fractions. Myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicon indicates the importance of 244D in JFH1 NS5B for sphingomyelin binding. To further confirm the role of 244D for recruitment of HCV RdRp to the detergent-resistant membrane (DRM), where the HCV replication complex exists, we compared the distribution of NS5A and NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) in their replicon cells by sucrose density gradient centrifugation of the DRM (Fig. 6). NS5A proteins of both JFH1 (2a) wt and NS5B(A242C/S244D) replicons localized in the DRM fraction where caveolin-2 was present (11, 27), but most of NS5B wt localized in the Triton-soluble fractions. NS5B of JFH1 (2a) NS5B(A242C/S244D) replicon was shifted to the DRM fraction from the soluble fraction. The shift of NS5B(A242C/S244D) localization into the DRM demonstrated that SBD was the DRM localization domain of NS5B and that residue 244D was important for this localization.

DISCUSSION

Hepatitis C virus is an envelope virus, and the lipid components of the virion play important roles in HCV infectivity and virion assembly (3, 15, 20, 24). HCV replication complexes localize in lipid raft structures/DRMs in the membrane frac-

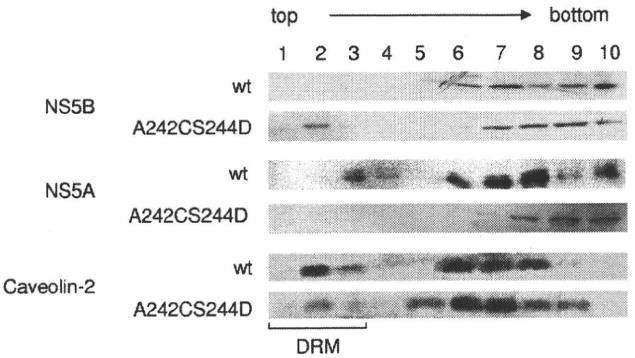

FIG. 6. Membrane floating assay of JFH1 wt and NS5B(A242C/S244D) replicon cells. The PNS fractions of HCV JFH1 (2a) wt and NS5B(A242C/S244D) replicon cells were treated with 1% Triton X-100 in TNE buffer for 30 min at 4°C and subjected to 10 to 40% sucrose gradient centrifugation in TNE buffer. Each fraction was subjected to 10% SDS-PAGE, followed by Western blotting with anti-NS5A, -NS5B, and -caveolin-2 antibodies. Fractions are numbered as indicated at the top of the panel. The DRM fractions (fractions 1 to 3) are indicated.

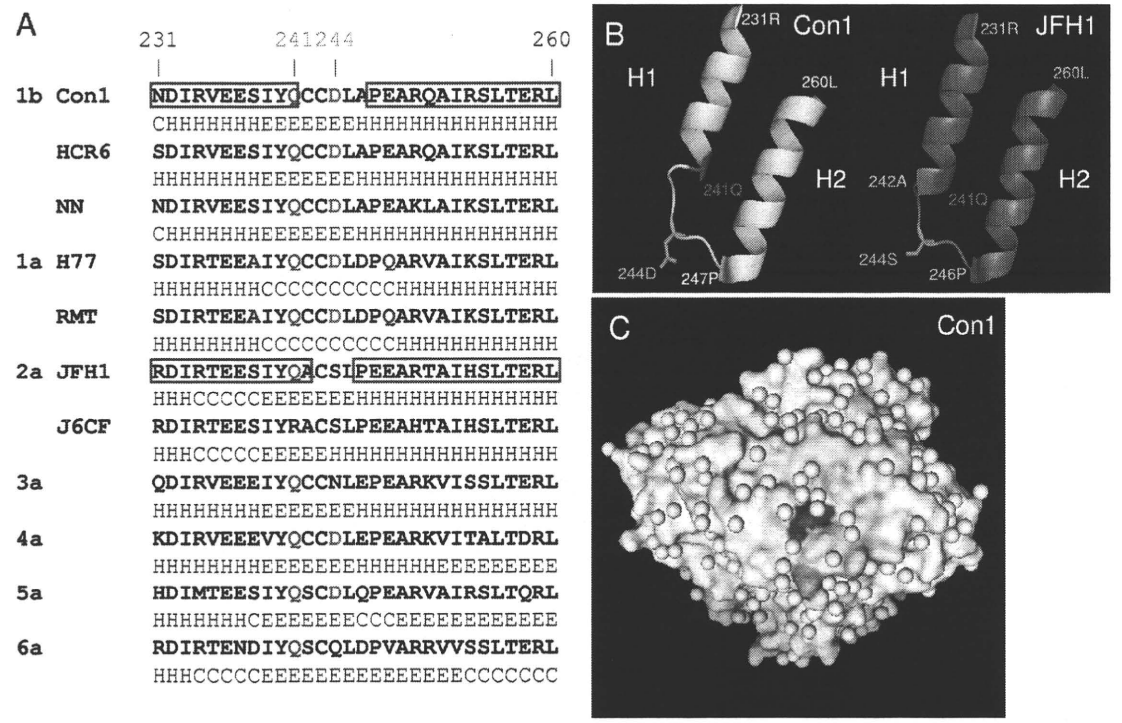

FIG. 7. Sphingomyelin binding domain (SBD) of HCV RNA polymerase. (A) The SBDs (231N to 260L) of HCV RdRps are aligned together with their secondary structure predicted by the Chou-Fasman program (10). The predicted secondary structure is indicated below the sequence as follows: H, α-helix; E, β-sheet; and C, coil. The α-helix structures of HCV Con1 (1b) RdRp and JFH1 (2a) RdRp are boxed in red. Residues 241Q and 244D are indicated in red and green, respectively. The 238A and 248E of the H77 and RMT (1a) RdRps are indicated in purple. GenBank accession numbers of HCV genotypes 3a, 4a, 5a, and 6a are GU814263 (12), GU814265 (12), Y13184 (8), and Y12083 (1), respectively. (B) Comparison of the SBDs of HCV Con1 (1b) (yellow) and JFH1 (2a) RdRps (magenta). The starting and ending amino acids of H1 and H2 are indicated. The sphingomyelin binding site, 241Q, is indicated in red, and 244D of Con1 (1b) and 244S of JFH1 (2a) RdRp are indicated in green. (C) Surface model of HCV Con1 (1b) RdRp. SBD is indicated in yellow, and 241Q and 244D are indicated in red and green, respectively. The structures of the Con1 and JFH1 RdRps were constructed by PyMOL, version 1.1.1 (http://www.pymol.org/). PDB numbers of Con1 (1b) RdRp and JFH1 (2a) RdRp are 3FQL (14) and 3I5K (31), respectively.

tions of subgenomic replicon cells (30). Lipid rafts are composed mainly of sphingomyelin, cholesterol, and glycosphingolipids. Most reports regarding the relationship between lipids and HCV have examined virion assembly, infectivity, and the localization of HCV, but their biochemical interactions have not been reported. Our findings clearly demonstrate that sphingomyelin plays an important role not only in HCV replication complex formation and its localization but also in HCV RdRp activity.

The helix-turn-helix structure of the SBD (residues 230 to 263), which is located between RNA polymerase motifs A and B, has been proposed as the sphingomyelin binding domain of HCV RdRp (29). We compared the SBD of Con1 (1b) (Protein Data Bank [PDB] 3FQL) (14) and JFH1 (2a) (PDB 3I5K) (31) and the secondary structure of the amino acids (201 to 290) in the SBD predicted by the Chou-Fasman program (10) (Fig. 7; see also Fig. S5 in the supplemental material) because the helix structures of the SBD of Con1 (helix 1 [H1], 231N to 241Q; helix 2 [H2], 247A to 260L) and JFH1 (H1, 231R to 242A; H2, 246P to 260L) RdRp fit with those predicted by the Chou-Fasman program. The structures contributing to sphingomyelin binding and activation are H1 and H2 and the junction (turn) between the two helix structures that are similar to the human immunodeficiency virus (HIV) gp120 V3 domain, prion protein (PrP), and β-amyloid peptide (13, 22). Although Con1 (1b) RdRp has a shorter helix structure than JFH1 (2a) RdRp (Fig. 6B), the structures of their SBDs are very similar (Fig. 7; see also Fig. S5). When the helix-turn-helix structure of the SBD was destroyed (HCR6 genotype 1b RdRp mutants L245A and I253A), the RdRp lost sphingomyelin binding activity and lost its activation (Fig. 2).

In order to study the structure-function relationship of the SBD and sphingomyelin, we compared the SBD of genotype 1a, 1b and 2a RdRps and particularly focused on residue 244D in the turn and residues 241Q and 238S/248E in the helix domains. The polar amino acid 241Q and the negatively charged 244D of Con1 (1b) RdRp located on the surface of the RdRp molecule bind and interact with the positively charged choline residue of sphingomyelin (Fig. 7C; see also Fig. S5 in the supplemental material). The positively charged 241R repels the choline residue of sphingomyelin, and as a result, J6CF (a) RdRp wt did not bind to sphingomyelin. J6CF (2a) RdRp(R241Q) showed almost the same sphingomyelin binding activity as HCR6 (1b) RdRp wt. This ionic interaction between SBD and sphingomyelin agrees with the activation of lipids with different sphingosine structures and fatty acid chains (Fig. 3A). JFH1 (2a) RdRp does not interact well with sphingomyelin because it does not have the negatively charged

amino acids at the tip of its turn structure. Once its 244S was changed to D, more sphingomyelin bound to JFH1 (2a) RdRp and activated the RdRp (Fig. 2A and C). The reason for the low activation of J6CF (2a) RdRp(R241Q/S244D) is not clear. Sometimes mutations affect the entire conformation of the molecule. In conclusion, from the comparison of sphingomyelin binding and activation of HCR6 (1b), J6CF (2a), and JFH1 (2a) RdRp SBD mutants, 241Q is the essential amino acid for sphingomyelin binding in the SBD. Amino acid 244D enhanced both binding and RdRp activation.

The *in vitro* sphingomyelin binding and RdRp activation experiments indicate that sphingomyelin binding and its RdRp activation are different biochemical reactions because we found controversial activation rates for sphingomyelin binding and RdRp activation among J6CF (2a) RdRp mutants (Fig. 2). The relationship between sphingomyelin binding and the activation of polymerase activity was studied by comparing genotype 1b and 1a RdRps, both of which bind to sphingomyelin (Fig. 2). However, 1a RdRp is not activated by sphingomyelin because both of the helix structures of 1a RdRp are probably terminated at 238A and 248Q, making its helix structures shorter than those of 1b RdRp (Fig. 6A). The length of the helix structure may be essential for sphingomyelin activation because RdRp changes its structure to bind to template RNA when sphingomyelin binds to SBD (Fig. 4).

HCV RdRp changes its conformations at the early stages of transcription initiation, including the template RNA binding step (6, 9). Sphingomyelin binding is likely to change the conformation of 1b RdRp to recruit template RNA and initiate transcription efficiently. Comparison of the activation ratio of RNA binding and polymerase activity of 1b RdRp, J6CF (2a) RdRp wt and R241Q and S244D mutants, and JFH1 (2a) RdRp wt and mutant A242C/S244D suggests that steps other than RNA binding are also likely to be activated by sphingomyelin.

From a kinetic analysis of sphingomyelin activation (Fig. 1C and D), 20 sphingomyelin molecules are estimated to interact with the SBD of RdRp and activate it because sphingomyelin activation plateaued at 20 sphingomyelin molecules per HCV RdRp molecule. It is not clear whether 20 sphingomyelin molecules form a micelle or a layer structure. However, the structure of sphingomyelin is important for the activation of HCV RdRp because phosphocholine did not activate the RdRp (Fig. 3D).

To confirm these biochemical findings in HCV replication, we tested the effect of SBD mutations in HCV replicon systems with the SPT inhibitor myriocin (Fig. 5) (4, 33) because NA255 was not available. The loss-of-function mutant, HCV NN (1b) NS5B(D244S), showed lower replicon activity than NN (1b) wt and more resistance to 50 nM myriocin, which did not affect the viability of cells (4, 33), than the wt. The gain-of-function mutant, H77 (1a) NS5B(A238S/Q248E), showed higher replicon activity than H77 wt and retained myriocin sensitivity because it had the sphingomyelin binding sites 241Q and 244D. At 50 nM myriocin, another gain-of-function mutant, JFH1 (2a) NS5B(A242C/S244D), was inhibited although its activity was the same as that of JFH1 (2a) wt without myriocin because the JFH1 wt replicon had high replicon activity without myriocin (Fig. 5A). The JFH1 replicon activity may be maximal in the system; therefore, the JFH1 (2a) NS5B(A242C/S244D) replicon did not show higher activity than JFH1 (2a) wt without myriocin while H77 (1a) NS5B(A238S/Q248E) showed higher replicon activity than H77 wt.

The binding and RdRp activation activity of the amino acid 244 mutants by sphingomyelin did not differ greatly from the wt *in vitro*. However, the myriocin sensitivity of JFH1 (2a) NS5B(S244D) was demonstrated clearly. That of H77 (1a) NS5B(A238S/Q248E) indicated that sphingomyelin binding was the target of myriocin inhibition, not the sphingomyelin activation of RdRp. These data confirm the importance of 241Q, 244D, and the helix structure in SBD for HCV replication in the cells.

Sphingomyelin is the major component of the lipid raft structure/DRM where the HCV genome replicates. To confirm that the SBD is the membrane binding site of HCV RdRp, we analyzed the localization of NS5B of JFH1 (2a) wt and NS5B(A242C/S244D) replicons by membrane floating assay (Fig. 6). JFH1 (2a) NS5B wt did not localize in the DRM. However, the localization of NS5B of the JFH1 (2a) NS5B(A242C/S244D) replicon shifted to the DRM from the soluble fractions. Previously, HCV NS5B was believed to localize in the DRM by its C-terminal hydrophobic sequences (21). However, our data demonstrate that the SBD is the membrane localization domain of HCV NS5B, which agrees with the myriocin sensitivity of JFH1 (2a) NS5B(A242C/S244D) replicons (Fig. 5) and the release of HCV 1b NS5B from the DRM by another SPT inhibitor, NA255 (29).

This is the first report of RNA polymerase activation by lipids. Twenty sphingomyelin molecules interact with SBD, particularly with residues 241Q and 244D of HCV (1b) RdRp, and change the conformation of the RdRp in order to recruit RNA templates. At the same time, HCV RdRp molecules may be aligned on the sphingomyelin layer formed via interactions between the hydrocarbon chains of sphingosine and fatty acids via placement of their SBD into the layer (Fig. 7C). Consistent with previous research (3, 23, 37), our findings explain why the inhibitors of the sphingolipid biosynthetic pathway influence subgenomic replicons derived from HCV genotypes 1a and 1b but not those derived from JFH1 (2a) (Fig. 5). Most HCV isolates have 241Q in NS5B, and some of them also have 244D (Fig. 7A). These sphingomyelin interactions are new targets for the treatment of HCV.

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3D cultured immortalized human hepatocytes useful to develop drugs for blood-borne HCV

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ABSTRACT

Due to the high polymorphism of natural hepatitis C virus (HCV) variants, existing recombinant HCV replication models have failed to be effective in developing effective anti-HCV agents. In the current study, we describe an *in vitro* system that supports the infection and replication of natural HCV from patient blood using an immortalized primary human hepatocyte cell line cultured in a three-dimensional (3D) culture system. Comparison of the gene expression profile of cells cultured in the 3D system to those cultured in the existing 2D system demonstrated an up-regulation of several genes activated by peroxisome proliferator-activated receptor alpha (PPARα) signaling. Furthermore, using PPARα agonists and antagonists, we also analyzed the effect of PPARα signaling on the modulation of HCV replication using this system. The 3D *in vitro* system described in this study provides significant insight into the search for novel anti-HCV strategies that are specific to various strains of HCV.

Infection with Hepatitis C virus (HCV) is a serious health problem worldwide and leads to high rates of liver cirrhosis and hepatocellular carcinoma [1]. Given that the standard HCV therapy remains insufficient for the successful treatment of many patients [2], the development of more effective and less toxic anti-HCV agents is required. *In vitro* systems like the HCV replicon-bearing cells and the infectious particle-producing JFH1 system, has contributed to the discovery of new targets for anti-HCV therapy. However, these recombinant HCV genomes only proliferate in sublines of HuH-7 cells, which do not permit infection or proliferation of blood-borne HCV. Due to the high polymorphism of natural HCV, data from recombinant HCV systems could be evaluated by studying the therapeutic response of a variety of naturally occurring HCVs. However, the current systems available for such study remain insufficient due to the low infection and replication efficiency of the natural HCV strains.

More recently, production and secretion of infectious HCV particles has been reported in two independent three-dimensional (3D) cell culture systems, termed the radial-flow bioreactor (3D/RFB) and the thermoreversible gelatin polymer (3D/TGP) systems. These results were not observed in monolayer cultures [3], suggesting that hepatocytes cultured in 3D more closely resemble liver cells *in vivo* [4] and thus support HCV proliferation. In addition, analysis of gene expression levels in 3D cultured cells revealed that the newly established immortalized human hepatocyte (HuS-E/2 cells) gene profile was altered to more closely resemble that of human liver tissue when the cells were cultured in 3D/TGP [5].

In the current study, we cultured HuS-E/2 cells in 3D/TGP and demonstrated efficient proliferation of natural HCV. Furthermore, gene expression analysis of these cells demonstrated the activation of the peroxisome proliferators-activated receptor α (PPARα) signaling pathway, suggesting an important role for this pathway in the replication of natural HCV. Thus, the *in vitro* system described appears to be a useful tool for the study of HCV infection and proliferation as well as for the development of effective anti-viral agents against various natural HCVs.

Materials and methods

Cell culture. Immortalized human hepatocytes (HuS-E/2) and LucNeo#2 replicon cells [6] were cultured as previously described [5,7]. For the 3D-TGP culture system, 1×10^5 HuS-E/2 cells were cultured in 1 ml Mebiol gel (Mebiol Inc., Kanagawa, Japan)/well in 12-well plates. Five hundred microliters of fresh medium was overlaid on the solidified gel, and was changed every 2 days. Cell

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extraction from the gel was done at the designated time points according to the manufacturer's protocol.

RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR (Q-PCR). At the designated time points, total cellular RNA was extracted and 1 μg of total RNA was used as a template for RT-PCR and for the quantitative detection of HCV-RNA using real-time RT-PCR (Q-PCR) as previously described [10].

HCV infection experiment. HCV infection experiments were carried out using sera from patients infected with HCV. Infection in 2D culture was undertaken as previously described [5]. For 3D/TGP cultured cells, the gel was solidified, and 50 μl HCV-containing patient serum with a titer of 1×10^6 HCV-RNA/ml was added to the culture and mixed. The culture was continued until the cells were extracted. Following extraction from 3D-TGP, cells were centrifuged and washed three times thoroughly with PBS. RNA was then extracted from the cells as described above. HCV infection into HuS-E/2 cells was also examined in the presence of anti-E2 mouse monoclonal antibody (917) as outlined previously [8].

Treatment of cells with PPARα signaling agonists and antagonists. Fenofibrate or MK886 (Sigma–Aldrich, USA) were added to the culture medium of HuS-E/2 (2D-HuS-E/2) cells from day 0 of HCV infection; or the culture medium of LucNeo#2 replicon harboring cells. The cells were then cultured to the designated time point.

Microarray analysis. Gene expression profiles of 3D/TGP cultured HuS-E/2 cells were obtained by microarray analysis (3D-Genes Human 25, Toray, Tokyo, Japan) and compared to those of cells cultured in 2D.

Results

3D/TGP cultures enhance HCV proliferation in HuS-E/2 cells

Infection and proliferation of the HCV genotype 1b (HCV-RC5) derived from the serum of patient RC5 in HuS-E/2 cells cultured in 3D/TGP (3D/TGP-HuS-E/2 cells) was investigated and compared with that of HuS-E/2 cells cultured in 2D (2D-Hus-E/2). As outlined in Fig. 1A, the HCV-RNA levels in the 3D/TGP-HuS-E/2 cells were significantly higher at all of the time points examined following infection than in the 2D-HuS/E2 cells, suggesting that the 3D/TGP system greatly enhances the proliferation of naturally occurring HCV in HuS-E/2 cells. Similar results were also obtained for sera from additional patients (data not shown). To examine whether the infection is viral envelope-receptor mediated, the infection experiments using serum treated with anti-HCV-E2 antibody (α-E2) or with anti-tubulin (negative control) was also performed. Pre-incubation of the serum with α-E2 significantly reduced the total amount of HCV-RNA in the cells upon infection (Fig. 1B). This result suggested that the infection of natural HCV into 3D/TGP-HuS-E/2 cells was HCV-E2-dependent.

Inhibition of natural HCV replication in HuS-E/2 cells by Interferon

In order to test the effects of anti-viral agents on natural HCV replication in 3D/TGP HuS-E/2 cells, 50–100 U/ml of IFNα was added to the medium overlaying the HCV-RC5 infected 3D/TGP-HuS-E/2 cells. The two treatment concentrations resulted in the inhibition of HCV-RNA replication in 3D-HuS-E/2 cells by

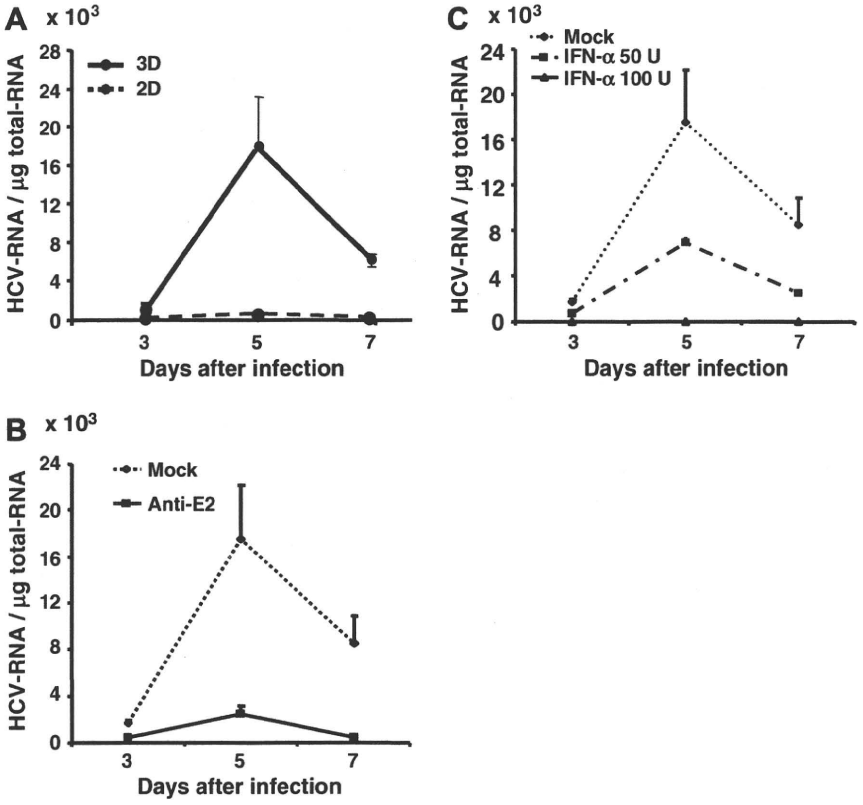

Fig. 1. HCV infection into 3D/TGP-HuS-E/2 cells. (A) 3D/TGP significantly enhanced HCV proliferation in HuS-E/2 cells. HCV patient serum was used to infect a similar number of HuS-E/2 cells cultured in 2D (hashed line) or 3D/TGP (solid line) culture for 24 h. Cells were then harvested and lysed at the indicated time points (3–7 days). The quantity of genomic HCV-RNA per 1 μg total RNA was determined by Q-PCR analysis. (B) Anti-E2 antibodies blocked HCV infection. HCV infection was performed as described in panel A in the presence of Anti-E2 specific or anti-tubulin (control) antibodies. (C) IFNα inhibits HCV replication in 3D/TGP-HuS-E/2 cells. HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock), 50 U/ml, or 100 U/ml IFNα overlaid on the gel containing the cells and HCV proliferation measured as described above.

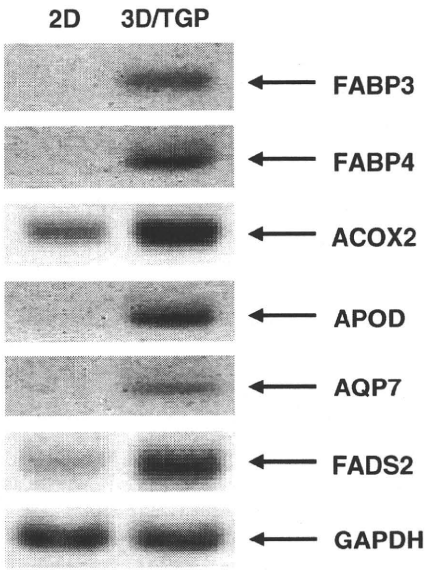

Fig. 2. RT-PCR analysis of the expression of genes identified by microarray. The PPARα regulated genes were increased in 3D/TGP-HuS-E/2 cells (3D-TGP) and their expression levels measured by RT-PCR. 2D represents RNA samples from 2D-HuS-E/2 cells. Twenty cycles of amplification were undertaken for the RT-PCR analysis. GAPDH expression served as an internal control. *Abbreviations:* FABP3, fatty acid binding proteins 3; FABP4, fatty acid binding proteins 4; ACOX2, acyl-coenzyme A oxidase 2; APOD, apolipoprotein D; AQP7, aquaporin 7; FADS2, fatty acid desaturase 2; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

approximately 50–60% and almost completely, respectively, when compared to the replication in cells receiving mock treatment (Fig. 1C). These results demonstrate that the IFNα treatment was effective on HCV derived from RC5 and that 3D/TGP-HuS-E/2 cells may be useful for the screening of anti-HCV drugs for the treatment of natural HCV.

Increased activation of the PPARα signaling pathway in 3D cultured HuS-E/2 cells

Given that 3D/TGP-HuS-E/2 cells demonstrated enhanced proliferation of natural HCV, the gene expression profiles of these cells was compared with that of cells cultured under normal 2D conditions using microarray analysis in order to identify the factors required for the enhanced proliferation. Among the 24,268 genes compared in this analysis, 212 genes demonstrated a greater than four folds index increase in expression in 3D/TGP than standard cultured cells. Cell signaling pathway analysis of these 212 genes showed that six genes, including fatty acid binding proteins 4 and 3 (FABP4 and 3), apolipoprotein D (APOD), aquaporin 7 (AQP7), acyl-coenzyme A oxidase 2 (ACOX2), and fatty acid desaturase 2 (FADS2), were targets of PPARα signaling [9–12]. The increased expression of these genes in the 3D/TGP-HuS-E/2 cells was further confirmed by RT-PCR analysis (Fig. 2). Given that PPARα is an essential factor for normal hepatocyte function [13], these results indicate that 3D/TGP culture enhances the hepatocyte-specific characteristics of HuS-E/2 cells.

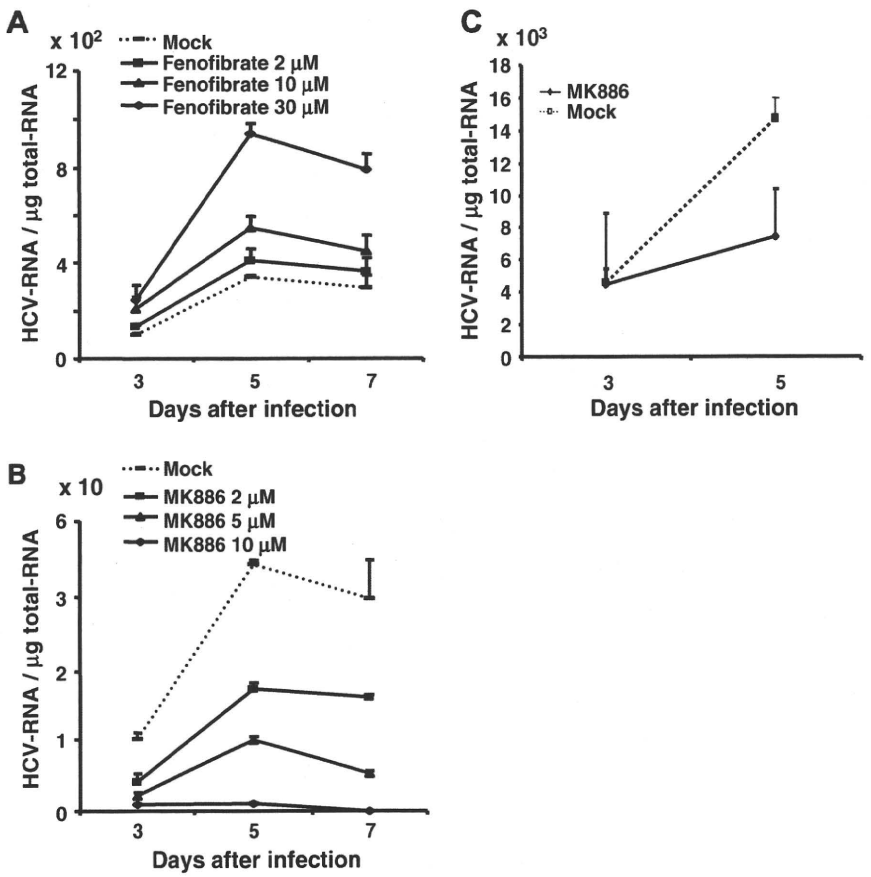

Fig. 3. The effects of PPARα agonists and antagonists on natural HCV proliferation. (A) HuS-E/2 cells were infected with HCV and fresh medium supplemented with or without (Mock) 2, 10, or 30 μM of fenofibrate overlaid on the cells. (B) Medium supplemented with or without (Mock), 2, 5, or 10 μM of MK886 was overlaid on 2D-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR. (C) Medium supplemented with or without (Mock), 10 μM of MK886 was overlaid on 3D/TGP-HuS-E/2 cells infected with HCV. HCV proliferation following treatment was measured by Q-PCR.

PPARα signaling affects HCV replication

We next examined the potential role of PPARα signaling on HCV proliferation by monitoring HCV replication in 2D-HuS-E/2 cells that had been infected with HCV-RC5 and subsequently treated with the PPARα agonist fenofibrate [14] or the PPARα antagonist MK886 [14] (Fig. 3B). As outlined in Fig. 3A, a dose-dependent increase in HCV replication was observed in fenofibrate-treated cells. In contrast, a dose-dependent decrease in HCV proliferation was observed in the presence of MK886. Similarly, treatment with MK886 reduced HCV proliferation in 3D/TGP-HuS-E/2 cells (Fig. 3C). The response of HCV proliferation in response to fenofibrate and MK886 treatment was also analyzed in LucNeo#2 cells that contained HCV replicon RNA (LNMH14) derived from the HCV-1b genome (Fig. 4A). Luciferase expression in these cells represented replication of the HCV replicon [6] and, as shown in Fig. 4A, luciferase activity in the cells treated with fenofibrate or MK886 also showed either enhancement or suppression of replicon proliferation, respectively. In addition, the increased HCV replication following fenofibrate treatment was completely abolished when treated with MK886 simultaneously. As MK886 is known to induce apoptosis when administered in high doses [15], the cell viability was examined using the XTT assay. There were no significant effects on cell viability after treatment with fenofibrate. Although MK886 resulted in a minor reduction in XTT values when high doses (10–15 μM) were administered, this reduction was not statistically significant when compared to its effect on HCV replication (Fig. 4B). This result suggests that PPARα signaling is required for HCV replication and that suppression of PPARα signaling has an anti-HCV effect.

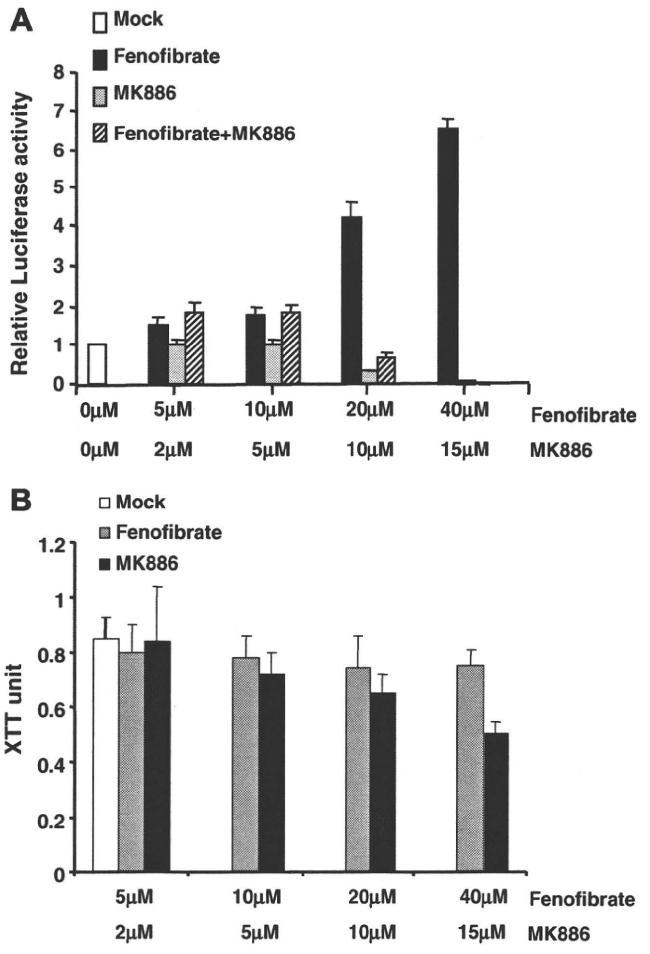

Fig. 4. The effects of PPARα agonists and antagonists on the replication of HCV subgenomic replicons. (A) LucNeo#2 cells containing a HCV subgenomic replicon termed LNMH14, were mock treated or treated with fenofibrate, MK886, or a combination of both fenofibrate and MK886 at the indicated concentrations for 2 days. Luciferase activity derived from the replicon was then measured as an indicator of HCV replication [7]. (B) Following treatment with fenofibrate and Mk886, LucNeo#2 cells were cultured for 2 days and cell viability measured using the XTT assay (Roche, Mannheim, Germany).

Discussion

In the current study, we demonstrated that immortalized hepatocyte HuS-E/2 cells cultured in 3D/TGP support the infection and replication of natural HCV derived from patient sera. Unlike recombinant HCVs, which have been required to adapt to sublines of HuH-7 cells [16], the population of the natural HCV is fairly polymorphic, demonstrating different responses to a variety of anti-viral agents [17,18]. The 3D/TGP-HuS-E/2 cells have the advantage of being a small-scale 3D cultured cells, which are cultured in 12-well plates at a density of 1×10^5/well, that allow the study of both viral and cellular events. In the current study, it demonstrated a 2 log increase in susceptibility to natural HCV infection and replication when compared to conventional 2D culture systems. Thus it offers an important advantage in the study of natural HCV infection and replication, and the response of natural HCV to anti-HCV drugs.

As the ability of HuS-E/2 cells to support infection and replication of natural HCV was greatly altered by the culture conditions, it is likely that the culture system described in our study will provide important information in regards to the cellular factors that support the HCV life cycle. The microarray study showed that the expression of some genes related to the PPARα signaling pathway were upregulated in the 3D cultured HuS-E/2 cells. Using both PPARα signaling agonists and antagonists, PPARα signaling was shown to affect infection and proliferation of natural HCV. PPARα is a ligand-activated transcription factor that is primarily expressed in tissues with high lipid metabolism including the liver, where it functions as one of three major nuclear receptors and is essential for its normal function [19]. Similar to a part of our data, a negative effect on HCV replication was previously observed in the replicon-bearing cells treated with siRNA for PPARα, with only 50% reduction of HCV-RNA [20]. In this study, even a large dose of PPARα agonist enhanced natural HCV replication in the 2D-HuS-E/2 cells for three times, despite the 2 logs enhancement of HCV proliferation in 3D/TGP culture. This implies that additional factors activated in 3D/TGP-HuS-E/2 cells may be required for the efficient HCV proliferation. Further analysis of the microarray data may provide us with further information on factors that may prove useful in the development of anti-HCV drugs.

In conclusion, the novel *in vitro* culture system combining TGP and immortalized hepatocytes described in this study demonstrated efficient support of natural HCV infection and replication. This system may be used in future virological studies to define new anti-HCV strategies. It may also prove useful for the specific design of effective individual therapy according to patient-specific strains.

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Isolation and gene analysis of interferon α-resistant cell clones of the hepatitis C virus subgenome

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Abstract

Hepatitis C virus (HCV) proteins appear to play an important role in IFN-resistance, but the molecular mechanism remains unclear. To clarify the mechanism in HCV replicon RNA harboring Huh-7 cells (Huh-9-13), we isolated cellular clones with impaired IFNα-sensitivity. Huh-9-13 was cultured for approximately 2 months in the presence of IFNα, and 4 IFNα-resistant cell clones showing significant resistances were obtained. When total RNA from clones was introduced into Huh-7 cells, the transfected cells also exhibited IFNα-resistance. Although no common mutations were present, mutations in NS3 and NS5A regions were accumulated. Transactivation of IFNα and IFNα-stimulated Stat-1 phosphorylation were reduced, and the elimination of HCV replicon RNA from the clones restored the IFNα signaling. These results suggest that the mutations in the HCV replicon RNA, at least in part, cause an inhibition of IFN signaling and are important for acquisition of IFNα resistance in Huh-9-13.

Keywords: Hepatitis C virus; Replicon; Interferon resistance; Stat-1; Nonstructural protein NS5A

Introduction

Hepatitis C virus (HCV) is the major cause of post-transfusion non-A non-B hepatitis. Approximately 170 million individuals worldwide were estimated to be infected with HCV (Alter, 1997). It has been suggested that the development of liver cirrhosis and hepatocellular carcinoma are consequences of chronic infection with HCV (Hijikata et al., 1993b; Tong et al., 1995).

HCV, a member of the *Flaviviridae* family, has a single-stranded positive-sense linear RNA genome of about 9.5 kb (Hijikata et al., 1991; Kato et al., 1990; Takamizawa et al., 1991). The RNA encodes a single precursor polyprotein of approximately 3010 amino acids (Choo et al., 1991; Okamoto et al., 1991, 1992) that is co- and post-translationally cleaved to produce individual structural (Core, E1, E2) and nonstructural proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) by both host and viral proteases (Hijikata et al., 1993a,b; Houghton, 1996).

The cell line Huh-9-13, in which the HCV subgenome can self-replicate, was established by R. Bartenschlager's group (Lohmann et al., 1999). The HCV subgenomic RNA consists of the entire nonstructural coding region of the Con1 strain of the HCV genome, except for the neomycin-resistant gene. This cell line provides significant information for understanding the replication of the HCV genome and is useful as a powerful screening tool for developing anti-HCV drugs (Bartenschlager et al., 2000, 2001).

Interferon alpha (IFNα) is widely used for the treatment of patients with chronic HCV infection; however, the effectiveness of IFNα, especially in genotype 1b, is low at only about 20–30% (Lindsay, 1997), although combination therapy with Ribavirin improves treatment outcomes (up to 50–60%) (McHutchison et al., 1998). According to reports of epidemiologic analysis conducted in Japan, IFN treatment outcomes are related with mutations within a 40 amino acid sequence in NS5A (amino acid

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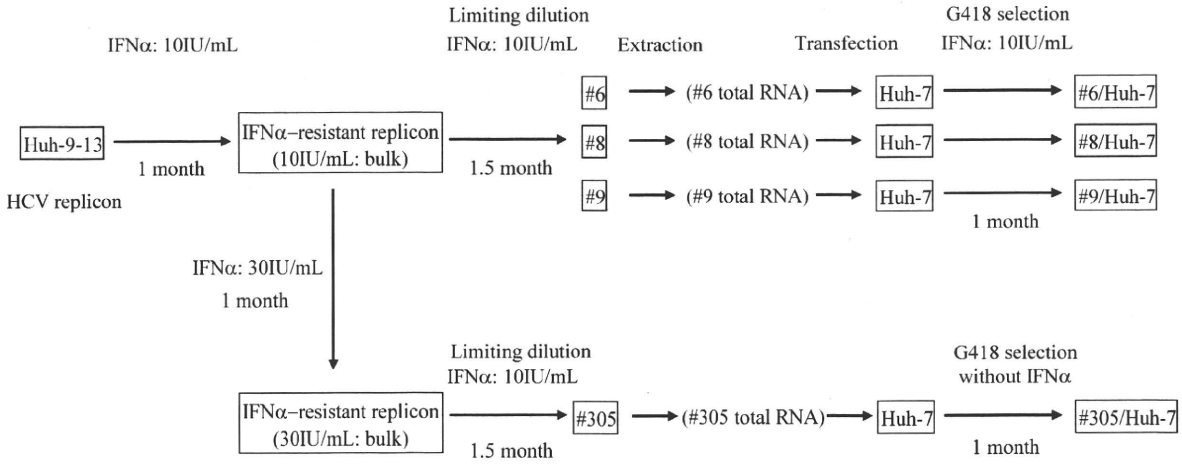

Fig. 1. An outline of the process used for isolation of replicon cells showing IFNα-resistance. Total RNA transfection derived from replicons to naive Huh-7 cells was performed using DMRIE-C reagent (Invitrogen).

(A)

	Cell	EC50(IU/mL)	Fold reduction
Original	Huh-9-13	0.7	1.0
IFNα–resistant	#6	6.9	9.5
	#8	6.7	9.2
	#9	10.2	13.9
	#305	99.2	135.6

(B)

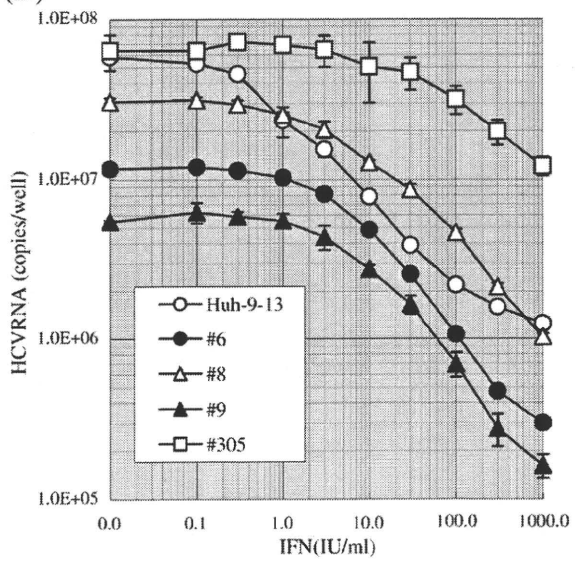

Fig. 2. Reactivity for IFNα in established IFNα-resistant replicon cells (#6, #8, #9, and #305) and original replicon cells (Huh-9-13). The cells were treated with IFNα for 48 h, and the amount of HCV RNA was measured by quantitative RT-PCR. (A) EC_{50} value (IU/mL) of IFNα in each replicon and fold reduction of the value compared to original replicon (Huh-9-13). (B) Change in copy number of HCV RNA in original and IFNα-resistant replicons by IFNα treatment. These experiments were performed in triplicate and mean values are shown.

numbers 2209–2248, based on the sequence of the prototype for HCV-J polyprotein) called the interferon sensitivity determining region (ISDR) (Enomoto et al., 1996). However, it is not clear how NS5A functionally interacts with IFN signals. Alternatively, NS5A is shown to inhibit the activity of double-stranded RNA (dsRNA)-activated protein kinase (PKR) and 2′-5′-oligoadenylate synthetase (2′-5′-OAS) induced by IFNα (Gale et al., 1997; Noguchi et al., 2001; Taguchi et al., 2004).

Recently, Meylan et al. and other groups reported that HCV-NS3-4A protease cleaved Cardif (Meylan et al., 2005) (also designated as VISA (Xu et al., 2005), MAVS (Seth et al., 2005), IPS-1 (Kawai et al., 2005)) and suppressed IFN production through RIG-I signaling. Cardif interacts with RIG-I (Yoneyama et al., 2004) mediated through CARD domains in both molecules

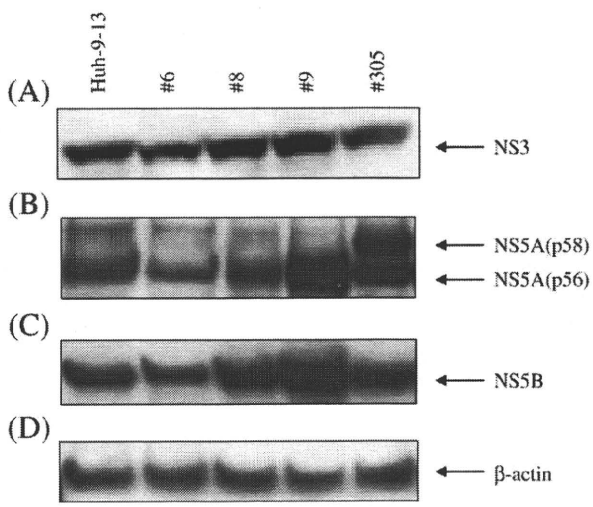

Fig. 3. Western blot analysis of the established IFNα-resistant replicon cells (#6, #8, #9, and #305) and original replicon cells (Huh-9-13). Expression of β-actin was used as an internal control of cellular protein in the replicon cells. Each cell line was inoculated on a 60-mm plate at 3×10^5 cells/well. Twenty-four hours after inoculation, the cells were lysed with SDS sample buffer. Total proteins were subjected to a 2/15% SDS gradient gel, and were subsequently immunoblotted by NS3 (A), NS5A (B), NS5B (C), and β-actin (D) antibody.

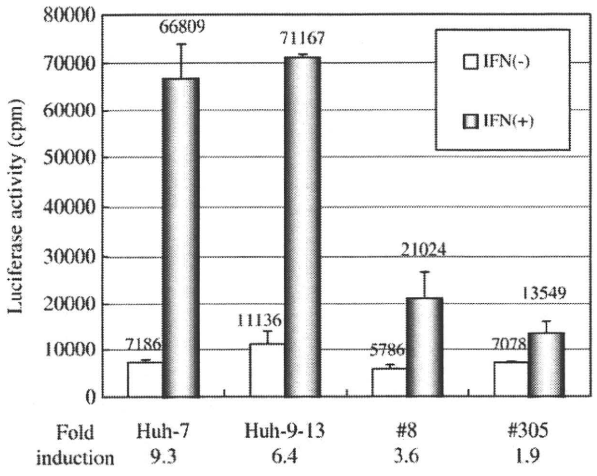

Fig. 4. Transactivation of ISRE in IFNα-resistant replicon cell lines (#8 and #305), original replicon (Huh-9-13), and parental Huh-7 cells by reporter gene (pISRE/Luc) analysis. The cells were stimulated with 1000 IU/mL of IFNα for 24 h after transfection of reporter plasmid DNA. White bars show control (no addition of IFNα) luciferase activity, and black bars show the activity under IFNα stimulation. Values of luciferase activity by IFNα stimulation relative to those of untreated cells are shown below the panel as 'fold induction'.

in a dsRNA-dependent manner, and transduce IFN production signals through the activation of nuclear factor κB (NFκB) and interferon regulatory factor 3 (IRF-3).

Despite bearing an HCV-1b genotype-derived replicon with mutations in ISDR, the replicon cells do not show resistances to IFN (Frese et al., 2002; Guo et al., 2001, 2004). Concerning this point, some reports regarding IFN-resistance acquisition and analysis of this property in the replicon cells (Namba et al., 2004; Sumpter et al., 2004; Zhu et al., 2005) showed involvement of various factors such as viral and/or host gene alterations participating in IFNα-resistance in replicon cells.

Here, we isolated IFNα-resistant clones of the HCV subgenome with accumulated mutations, especially in NS3 and NS5A regions. We observed impairment of phosphorylation of Stat-1 in cells bearing the IFNα-resistant HCV replicon. Our findings suggest that NS5A contributes to the acquisition of IFNα-resistant phenotype in HCV replicon cells.

Results

Establishment of IFNα-resistant replicon cell lines

HCV replicon cells were cultured for approximately 1 month in the presence of 10 IU/mL IFNα. HCV RNA titer decreased during the culture; however, the appearance of cells less sensitive to IFNα during prolonged culture was observed by quantitative RT-PCR. The resistant cells were then cloned by limiting dilution. Three clones (Fig. 1: #6, #8, and #9) were obtained, and mixed pools of these resistant cells were further selected in the presence of 30 IU/mL IFNα for another 4 weeks. After confirming decreased sensitivity to IFNα at this dose, the clone

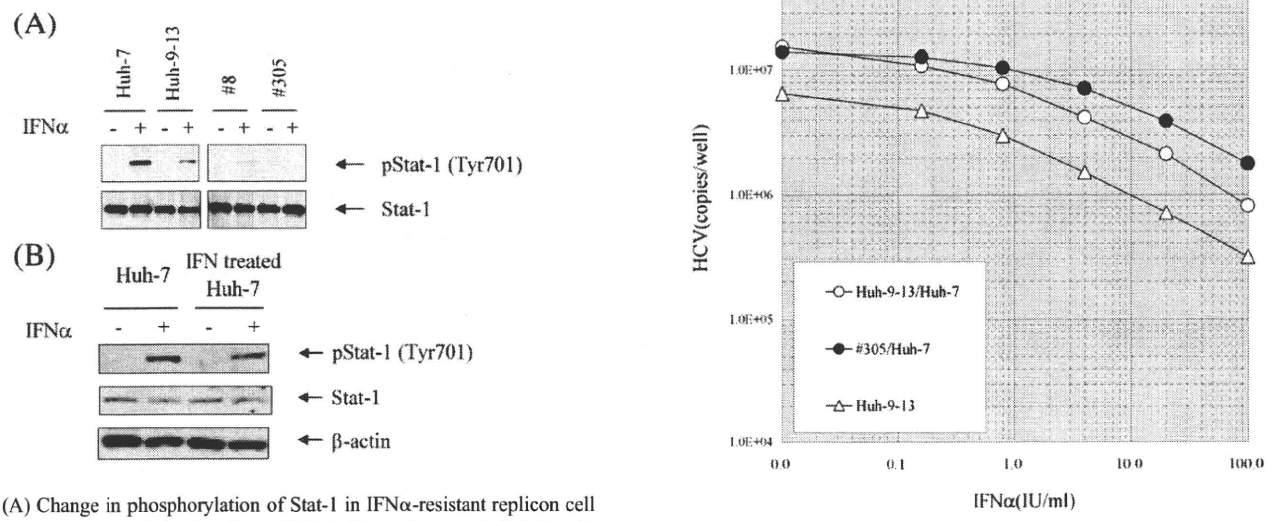

Fig. 5. (A) Change in phosphorylation of Stat-1 in IFNα-resistant replicon cell lines (#8 and #305), original replicon (Huh-9-13) and parental Huh-7 cell. Phosphorylation of Stat-1 was analyzed by western blot analysis using anti-phospho-Stat-1 (Tyr701) antibody. The cells were cultured in medium with or without 500 IU/mL of IFNα for 30 min. Upper panel represents a phospho-Stat-1 (Tyr701) and lower panel shows a Stat-1. Western blot analysis was performed as described in Materials and methods. (B) Change in phosphorylation of Stat-1 in Huh-7 cells maintained in the presence or absence of IFNα (10 IU/mL) for 4 weeks. Upper panel represents a phospho-Stat-1 (Tyr701), middle panel shows a Stat-1 and lower panel shows a β-actin. Phosphorylation of Stat-1 in these cells was examined as described above.

(A)

	Cell	IFN selection	EC50 (IU/mL)	Fold Reduction
Total RNA transfection	Huh-9-13/Huh-7	(-)	0.7	1.0
	#305/Huh-7	(-)	4.1	5.9
original	Huh-9-13	(-)	0.6	1.2

(B)

Fig. 6. Reactivity for IFNα in the Huh-7 cells, #305/Huh-7, transfected with total RNA of #305 replicon cells and in the Huh-7 cells, Huh-9-13/Huh-7, transfected with total RNA of original replicon cells (Huh-9-13). These transfected cells were selected with G418 in the absence of IFNα. The amount of HCV RNA was analyzed by quantitative RT-PCR, as described in Fig. 2. (A) EC_{50} value (IU/mL) of IFNα in Huh-9-13/Huh-7 and #305/Huh-7. (B) Change in copy number of HCV RNA in Huh-9-13/Huh-7 and #305/Huh-7 by IFNα treatment. These experiments were performed in triplicate and mean values are shown.

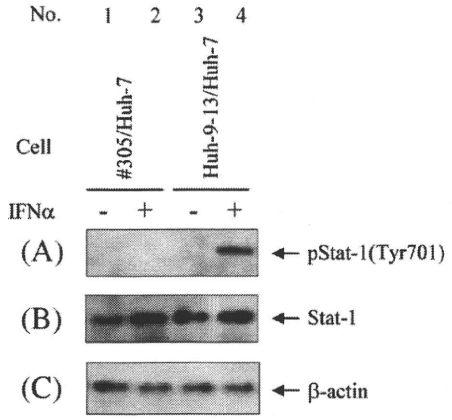

Fig. 7. Phosphorylation of Stat-1 in #305/Huh-7 and Huh-9-13/Huh-7 described in Fig. 6. The experiment was performed as described in Fig. 5. Each panel shows (A) phospho-Stat-1 (Tyr701), (B) Stat-1, and (C) β-actin. (Lanes 1 and 2) Huh-7 cells transfected with IFNα-resistant replicon (#305) total RNA (#305/Huh-7). (Lanes 3 and 4) Huh-7 cells transfected with original replicon (Huh-9-13) total RNA (Huh-9-13/Huh-7).

(Fig. 1; #305) showing highest resistance to IFNα was obtained. Sensitivities of these clones to IFNα are shown in Fig. 2. The basal HCV RNA levels in these cells (#6, #8, #9, and #305) were almost equal to that in the original replicon cells (Huh-9-13). The EC_{50} value of IFNα for the original replicon (Huh-9-13) was 0.7 IU/mL, compared to 6.9 IU/mL, 6.7 IU/mL, 10.2 IU/mL, and 99.2 IU/mL for resistant clones #6, #8, #9, and #305, respectively. These results demonstrate that sensitivity to IFNα based on EC_{50} value decreased 9 to 135-fold in the IFNα-resistant clones.

Characterization of IFNα-resistant replicon cell lines

First, expression of HCV NS proteins (NS3, NS5A, and NS5B) in IFNα-resistant replicon cell lines (#6, #8, #9, and #305) was analyzed by western blot. We detected expression of all the NS proteins in these cell lines as well as in original replicon cell (Huh-9-13) at almost at the same levels, although the levels of NS5A and NS5B in clone #6 were slightly low (Fig. 3). Interestingly, only clone #305 exhibited a different migration of NS5A, corresponding to the size of hyper-phosphorylated form (p58) in addition to the size of basal phosphorylated form (p56).

To analyze the change in IFNα signal transduction in two representative IFNα-resistant replicon cell lines (#8 and #305), we carried out a reporter gene assay using a firefly luciferase gene fused with three repeats of an ISG15-type IFN-stimulated responsive element (ISRE) as a reporter construct (pISRE/Luc). After transfection of pISRE/Luc to these replicon cells, the cells were stimulated with 1000 IU/mL of IFNα for 24 h. As shown in Fig. 4, the transactivation by IFNα in original replicon cells (Huh-9-13) was slightly reduced compared with that of parental cell line Huh-7 (Huh-7, 9.3-fold; Huh-9-13, 6.4-fold). Luciferase activity of #8 and #305 was more diminished than that of Huh-9-13 (#8, 3.6-fold; #305, 1.9-fold). The extent of decline of transactivation by IFNα treatment in these resistant replicon cell lines was dependent on the extent of IFNα-resistance, as quantified by RT-PCR (Fig. 2). It is suggested that the genetic alteration in HCV replicon RNA confers on IFNα-resistance in these cell lines.

In relation to the reporter gene analysis, JAK-STAT pathway activated by type I IFN was analyzed in IFNα-resistant replicons containing cells (#8 and #305). Phosphorylation of Stat-1, one of the important molecules in the JAK-STAT signal transduction pathway, was lowered in original replicon cells (Huh-9-13) compared with that in parental Huh-7 (Fig. 5A). However, severely impaired phosphorylation of Stat-1 was observed in the IFNα-resistant replicons containing cells (#8 and #305) compared with original replicon cells (Huh-9-13) (Fig. 5A). Furthermore, phosphorylation of Stat-1 was also decreased in #305 containing cells maintained in the absence of IFNα for 4 weeks, and the degree of decrease of Stat-1 phosphorylation was almost equal to that maintained in the presence of IFNα (data not shown). In contrast to these observations, Huh-7 cells, the parental cell of Huh-9-13 that was maintained in the presence of IFNα for 4 weeks did not show the significant alteration of Stat-1 phosphorylation compared with that maintained in the absence of IFNα (Fig. 5B). These results suggest that reduction of phosphorylation of Stat-1 in these IFNα-resistant replicon cell lines is caused by alteration of HCV replicon RNA and it may correlate with suppression of transcription from the reporter gene (Fig. 4).

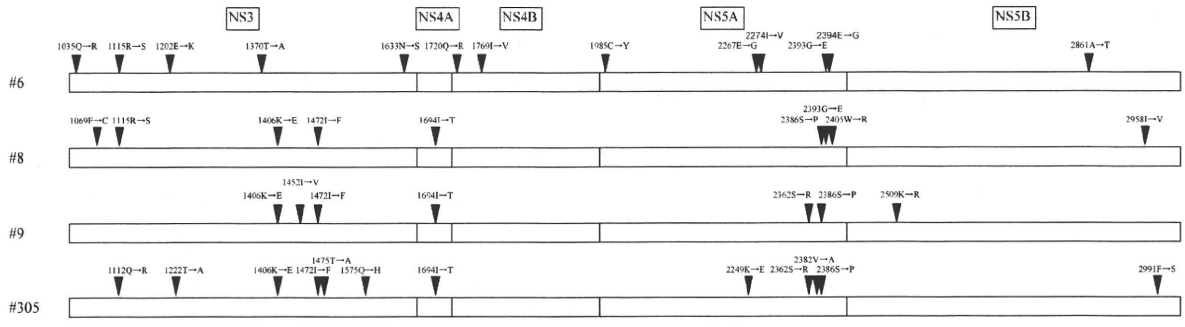

Fig. 8. The amino acid sequence deduced from nucleotide sequence in IFNα-resistant replicon cells. The nucleotide sequence was determined by an RT-PCR direct sequencing method. Arrows indicate the amino acid substitutions that were detected only in IFNα-resistant replicons compared with original replicon (Huh-9-13). The numbering of amino acids was referred to that of complete polyprotein of the isolate.

HCV replicon RNA confers IFNα-resistance

To confirm the role of HCV subgenomic RNA from clone #305 for acquisition of IFNα-resistance, total RNA was extracted from the cells and transfected to naive Huh-7 cells. The transfected cells were selected with G418 in the absence of IFNα. HCV negative-stranded replicon RNA, replication intermediate, and HCV NS proteins (NS3, NS5A and NS5B) were detected in the cells (data not shown).

Concerning the cells transfected with total RNA from IFNα-resistant #305 cell (#305/Huh-7) or the cells transfected with total RNA from original Huh-9-13 replicon cell (Huh-9-13/Huh-7), IFNα-sensitivity (EC_{50}) was analyzed (Fig. 6). IFNα-sensitivity (EC_{50}) of the Huh-9-13/Huh-7 showed 0.7 IU/mL, whereas the #305/Huh-7 showed 4.1 IU/mL. EC_{50} values of the Huh-7 cells bearing IFNα-resistant replicon derived from clone #305 were approximately 6-fold higher than that of Huh-7 cells bearing the original replicon. Although IFNα-resistance (EC_{50}) of the cells bearing #305 RNA was not as high as that of original #305, this finding suggests that acquisition of IFNα-resistance of these cells was due to genetic alteration of the replicon RNA.

We investigated the phosphorylation status of Stat-1 by stimulation of IFNα in these cells. As shown in Fig. 7, phosphorylation of Stat-1 in #305/Huh-7 (lane 2) was suppressed compared with that in Huh-9-13/Huh-7 (lane 4), suggesting that the IFNα-resistant HCV replicon derived from #305 is responsible for acquisition of the decreasing response to Stat-1 phosphorylation stimulated by IFNα.

Direct sequencing analysis of IFNα-resistant replicons

Nucleotide sequences in the NS region of each resistant clone were determined by RT-PCR direct sequencing. Sites of mutation that were detected only in IFNα-resistant replicons are shown by arrowheads and numbers (N-terminus of NS3 was denoted as 1027 based on the numbering of the complete polyprotein of the isolate), together with conversion of amino acids by arrows (Fig. 8). Although synonymous mutations are clustered in NS3 and the C-terminal region of NS5A, there were no common mutations among these resistant clones. Moreover, no mutations located at the positions as in IFNα-resistant replicons established by Namba et al. (2004) and Sumpter et al. (2004) were found in the present study. Mutations in the ISDR of NS5A were reported

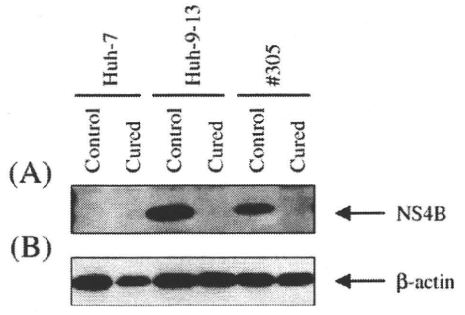

Fig. 9. Expression of NS protein (NS4B) (A) and β-actin (B) was confirmed in 'cured cells' by western blot analysis. Huh-7 cells with JTP-71892 as well as replicon cells (Huh-9-13 and #305) were analyzed likewise.

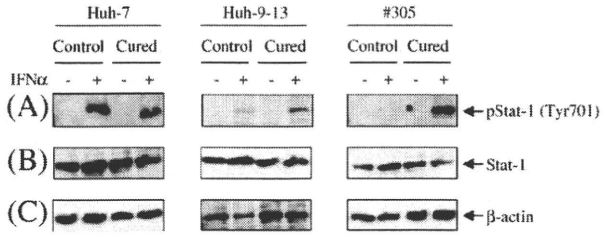

Fig. 10. Phosphorylation of Stat-1 (Tyr701) in 'cured cells'. Phosphorylation of Stat-1 (Tyr701) (A) by IFNα stimulation was investigated by western blot analysis. Stat-1 (B) and β-actin (C) were also analyzed. IFNα stimulation and western blot analysis were performed as described in Fig. 5.

to play an important role in outcome of IFN treatment to patients with genotype 1b of HCV in Japan (Enomoto et al., 1996); however, the amino acid sequence of ISDR was preserved among these replicon cell lines in our experiments.

Characterization of 'cured cells' obtained by IFNα-resistant HCV replicon cells

To clarify the role of HCV replicon RNA in resistance to IFNα, the replicon cells (Huh-9-13 and #305) were treated with JTP-71892 (1 μM) for more than 1 month to establish 'cured cells', as described in Materials and methods. JTP-71892 is a JTK-109-derivative synthesized in our laboratory, which has a potent inhibitory effect on HCV replication (Hirashima et al., 2006). The amounts of HCV replicon RNA in both replicon-bearing cell types were decreased less than what could be detected by quantitative RT-PCR, while the amounts of GAPDH mRNA used as a control did not show any difference (data not shown). The representative HCV NS protein, NS4B, was not detected in the 'cured cells' (Fig. 9).

The phosphorylation status of Stat-1 was then analyzed in these cells. The Stat-1 phosphorylation (Tyr701) by IFNα stimulation has restored remarkably in 'cured cells' (derived from both Huh-9-13 and #305) (Fig. 10). There was no obvious difference in the extent of Stat-1 phosphorylation by JTP-71892 treatment in Huh-7, indicating that restoration of Stat-1 phosphorylation was not due to JTP-71892. There was no clear difference in the amount of non-phosphorylated Stat-1 and β-actin expression by the IFNα stimulation or JTP-71892 treatment among these cell clones. These results suggest that HCV replicon RNA contributes to IFNα-resistance through impairment of phosphorylation of Stat-1, at least in part.

Discussion

We cultured HCV replicon cells in the presence of 10 and 30 IU/mL IFNα to isolate IFNα-resistant clones. Four different resistant clones with differing sensitivities to IFNα were isolated. The sensitivity for IFNα attenuated more than 100-fold in the #305 replicon, which was isolated in the presence of 30 IU/mL of IFNα and showed the most remarkable resistance in our study.

We analyzed the appearance of G418-resistant cells, #305/Huh-7, obtained by transfection of total RNA from the IFNα-

resistant replicon-bearing cells to Huh-7 by culturing them in the absence of IFNα, as shown in Fig. 1. IFNα sensitivities of the Huh-7 cells transduced with HCV replicon RNA of #305 cells were about 6-fold lower than those transfected with total RNA of original replicon cells (Huh-9-13), in coincident with a reduction of Stat-1 phosphorylation. However, #305/Huh-7 conferred a lesser extent of IFNα-resistance compared with that of parental #305 (Figs. 2 and 6). Although some factors other than HCV replicon RNA itself may participate in acquisition of IFNα-resistance in #305 cells, these results suggest that replicon RNA derived from #305 was significantly involved in regulation of IFNα signaling. The 'cured cells', from which HCV genomic RNA was removed from IFNα-resistant replicon cell line (#305) after treatment with 1 μM of JTP-71892, a potent HCV replication inhibitor, resulted in restoration of IFNα signaling to parental Huh-7. This finding suggests that HCV replicon RNA plays important roles in suppression of Stat-1 function. Moreover, this effect is dependent on mutation of HCV replicon RNA.

Mutations of amino acids were clustered throughout the whole region of NS3 and the C-terminus of NS5A in the IFNα-resistant replicon RNAs; however, there were no common amino acid mutations among the clones. This result may suggest the possibility that a change of plural functions participates in the acquisition of resistance. Whereas we did not identify common mutations, four amino acid mutations, K1406E, I1472F, I1694T, and S2386P, in NS3/4A and NS5A were shown to be common in #8, #9, and #305. In particular, the mutation at S2386P in NS5A located near region V3, one of the important prediction factors of the outcome in clinical IFN therapy (Nousbaum et al., 2000; Puig-Basagoiti et al., 2005), is found in #9 and #305. The nucleotide sequence of ISDR region was preserved between original replicon and IFNα-resistant replicons.

Concerning the mutations in NS5A region of #305, we established 3 chimeric replicon cell clones harboring Huh-9-13 replicon that was substituted with NS5A coding region derived from #305, which was selected by G418 in the absence of IFNα. These cell clones showed reduction of IFNα sensitivity (EC_{50}) as 20 to 30 times as those of normal replicon cell (Huh-9-13). Although chimeric replicons harboring #305 NS5A showed lesser extent of IFNα-resistance than that of #305 replicon cell, NS5A of #305 plays an important role in acquisition of IFNα-resistance in the replicon cell (data not shown).

Naka et al. (2005) reported that nonsense mutations and deletions of type I IFN receptor genes (IFNAR1, IFNAR2c) were found in certain clones of replicon cells that gained IFNα-resistance. However, we did not detect any such mutation or deletion in either of these genes in this work. Furthermore, we were not able to obtain resistant phenotype by IFN treatment at high concentrations of more than 1000 IU/mL.

In #305, among other IFNα-resistant clones, substantial amount of slow migrating form of NS5A was observed. From previous reports (Asabe et al., 1997, Ide et al., 1997; Kaneko et al., 1994; Kim et al., 1999; Reed et al., 1997, 1998; Tanji et al., 1995), it is supposed that this form is hyper-phosphorylated NS5A with 58 KD. Hyper-phosphorylated form of NS5A (p58) negatively participates in replication of HCV RNA in replicon cells (Appel et al., 2005; Evans et al., 2004; Huang et al., 2006; Neddermann et al., 2004). However, the quantity of basal HCV replication in #305 was almost the same as in other replicon cells, including Huh-9-13. Thus, it is likely that the hyper-phosphorylation of NS5A does not contribute to suppression of replication of HCV replicon. Rather, it may be related to a potent IFNα-resistance in #305 via un-identified mechanisms. Further studies are needed to clarify the role of hyper-phosphorylated NS5A in IFNα-resistance.

Concerning effects of NS5A on IFN signaling, it was reported that transiently- or stably-transfected NS5A inhibits IFN-stimulated Stat-1 phosphorylation and transactivation of ISRE in hepatocyte-derived cell lines, including Huh-7 cell (Gong et al., 2007; Lan et al., 2007). These authors also suggested the interaction of NS5A with Stat-1. Although these evaluation methods were different from that of our replicon system, they lend additional credibility to the suggestion that NS5A plays an important role in regulation of IFN signaling via inhibition of Stat-1 phosphorylation.

Stat-1 phosphorylation by IFNα stimulation was suppressed in IFNα-resistant replicon cells. The degree of suppression of Stat-1 phosphorylation was related to the sensitivity of IFNα in IFNα-resistant replicons (Fig. 5A). Moreover, the decrease of Stat-1 phosphorylation in #305 cells maintained in the absence of IFNα for 4 weeks was almost same level as that maintained in the presence of IFNα, suggesting that IFNα pressure did not induce a negative feedback (i.e. leading to the degradation of IFN receptor) loop in our experimental system. In contrast, Stat-1 phosphorylation was not changed significantly in parental Huh-7 cells that were maintained in the presence of IFNα compared with that maintained in the absence of IFNα (Fig. 5B), suggesting that Stat-1 phosphorylation in the parental Huh-7 cells was not affected with IFNα pressure and that the alteration of HCV replicon confers the IFNα-resistance. Stat-1 phosphorylation was also suppressed in the Huh-7 cells transfected with total RNA from IFNα-resistant replicon (Fig. 7). Moreover, the 'cured cells' showed a restoration of Stat-1 phosphorylation (Fig. 10). These observations suggest that IFNα-resistance in IFNα-resistant replicon cells depends on a change in Stat-1 phosphorylation, at least in part. For unknown reasons, we could not detect phosphorylation of Stat-2 (Tyr689), Stat-3 (Tyr705) (Sarcar et al., 2004; Zhu et al., 2005), JAK-1 (Tyr1022), or Tyk-2 (Tyr1054) in these cells. Concerning these proteins in the replicon cells, further investigation is needed to understand their roles in acquisition of IFNα-resistance.

Although the underlying mechanism of acquisition of IFNα-resistance gained by HCV replicon RNA remains unclear, clarification of detailed analysis of the role of Stat-1 in regard to IFN signaling in HCV replicon cells may contribute to the development therapeutic agents.

Materials and methods

Cell culture

Huh-9-13 cells harboring HCV subgenomic (NS3-3′X) replicon and parental Huh-7 cells were purchased from ReBLikon GmbH. Cells were cultured in Dulbecco's modified Eagle's

medium (DMEM) supplemented with 10% fetal bovine serum. To Huh-9-13 cells, 1 mg/mL of G418 (Geneticin; Invitrogen), a selective marker for replicated HCV subgenome was added.

IFN treatment

Huh-9-13 cells were seeded in a 75-cm^2 flask at a density of 3×10^5 cells/flask. Twenty-four hours after cell seeding, human IFNα (Sumiferon®300; Dainippon Sumitomo Pharma) was added so that the final concentration in medium was 10 IU/mL. Control cells were cultured in medium with no other additional substances. Cell passages were performed approximately every 7 days and the cells were cultured for approximately 1 month in the presence of IFNα (10 IU/mL). After decreases in sensitivity to IFNα were confirmed in the IFNα-treated groups by quantitative RT-PCR, IFNα-resistant cell phenotypes were further cultured for about 1 month in the presence of 30 IU/mL IFNα, and sensitivity to IFNα was then also measured in these cells. The cells cultured in the presence of 10 or 30 IU/mL of IFNα were cloned by a limiting dilution method using 96-well plates: cells were seeded at 1 cell/well and cultured in medium containing 10 IU/mL IFNα. After culture for about two to three weeks, survival and growth of cloned cells were confirmed, and then colonies were isolated and added to 48-well plates containing the test substance in 500 μL of culture medium per well. The proliferated cells in the 48-well plates were transferred to 6-well plates, and these were further put into 75-cm^2 cell culture flasks for subculture. Thereafter, subculture passage was performed approximately every 7 days. Cloning and subculture were performed in the presence of IFNα.

Measurement of IFN-sensitivity (quantitative analysis of HCV replicon and GAPDH mRNA)

IFN-sensitivity of IFNα-treated replicon cells was measured by quantitative RT-PCR. Cells (1×10^4 cells/well) were seeded in 96-well plates in the presence of 0, 0.1, 0.3, 1, 3, 10, 30, 100, 300, or 1000 IU/mL of IFNα. Forty-eight hours after cultivation with IFNα, the cells were harvested to extract total RNA using a total RNA extraction kit (RNeasy® 96; Qiagen) in accordance with the instruction manual. Quantification of HCV replicon RNA in the prepared RNA was performed using TaqMan® EZ RT-PCR Core Reagent (ABI) using a sequence detector under the following conditions: sense-primer: 5′-CGGGAGAGCCATAGTGG-3′ (130-S17; Greiner), antisense-primer: 5′-AGTACCACAAGGCCTTTCG-3′ (290-R19; Greiner), probe: 5′(FAM)-CTGCGGAACCGGTGAGTACAC (TAMRA)-3′ (148-S21FT; TaKaRa) (Takeuchi et al., 1999), RT-PCR reaction conditions: 50 °C, 2 min→60 °C, 30 min→95 °C, 5 min→45 cycles×(95 °C, 20 s→62 °C, 1 min). The number of copies in the samples was determined using a standard curve calibrated with 10^4 to 10^8 copies of synthesized HCV RNA standards encoding from 5′ terminus to E2 region, and recorded as amount of HCV RNA.

Direct sequencing analysis of HCV replicon RNA

Nucleotide sequences of HCV replicon RNA were analyzed by direct sequencing method. The NS region of total RNA extracted from IFNα-resistant replicon clones was divided into four fragments and amplified using an RT-PCR kit (ReverTra Dash®; TOYOBO). Four primers (HCV-NS-1RV: 5′-ATAGCACTCGCACAGAACCGA-3′; Greiner, HCV-NS-2RV: 5′-GGAACCGTTTTTCACATGTCC-3′; Greiner, HCV-NS-3RV: 5′-ATGTGGTTAACGGCCTTGCT-3′; Greiner, HCV-NS-4RV: 5′-TCATCGGTTGGGGAGTAGATAGA-3′; Greiner) were used for reverse transcription (RT). For polymerase chain reaction (PCR), another four primers (HCV-NS-1FW: 5′-ATGGCGCCTATTACGGCCTA-3′; Greiner, HCV-NS-2FW: 5′-TGTTCGATTCCTCGGTTCTGT-3′; Greiner, HCV-NS-3FW: 5′-CCCCTTCTTCTCATGTCAACG-3′; Greiner, HCV-NS-4 FW: 5′-GGAACCTATCCAGCAAGCCC-3′; Greiner) were used in addition to the primers for RT.

RT and PCR reactions were conducted in accordance with the instruction manual provided with the kit. RT reaction was conducted at 42 °C, 20 min, and the reaction mixtures were then heated to 99 °C, 5 min. The PCR reaction was performed for 30 cycles under the following conditions: 98 °C, 10 s; 60 °C, 2 s; then 74 °C, 90 s.

Sequencing was performed using a BigDye Terminator Cycle Sequencing Ready Reaction Kit (ABI). One μL of amplified RT-PCR product for each clone was purified using QIAquick Gel Extraction kit (Qiagen) and the sequence primers were used to prepare each of the reaction solutions in accordance with the manufacturer's procedure. Twenty μL of each solution was allowed to react for 25 cycles under the conditions: 96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min; then 72 °C, 7 min. The solutions were then purified by Dye EX 2.0 (Qiagen) in accordance with the instruction manual. After that, the samples were applied for sequencing analysis using an ABI PRISM 3100 genetic analyzer (ABI).

The NS region (5952 bp, 1984 amino acids) in sequenced samples underwent gene analysis using Vector NTI analysis software (Invitrogen). In a comparison of deduced amino acid sequences based on nucleotide sequences among the four IFNα-resistant replicon clones and original replicons, the NS regions were compared to that of the original replicon clone to identify mutations. The amino acid sequence of the original replicon cells was included among the materials provided with the Huh-9-13 cell line product from ReBLikon GmbH.

Reporter gene analysis

We attempted to clarify IFNα transactivation in IFNα-resistant replicons. Firefly luciferase fused gene with three repeats of an ISG15-type IFN-stimulated responsive element (ISRE) was used as a reporter construct (pISRE/Luc). HCV replicon cells or Huh-7 cells (3×10^5 cells/well) were seeded on a 60-mm plate in the absence of IFNα. Eight hours after cell seeding, the reporter construct (3 μg) was transfected using FuGENE6 (Roche) as a transfection reagent, following the instruction manual. The transfected cells were cultured further 12 to 14 h, and then the cells (1×10^4 cells) were inoculated on a 96-well plate and cultured for 24 h with or without 1000 IU/mL of IFNα. The luciferase activity was measured by adding Steady Glo® to the cells using TopCount (Packard).

Western blot analysis

The cell lysates were prepared in Laemmli buffer (BIO-RAD) and subjected to SDS-2/15% gradient PAGE and transferred onto nitrocellulose membranes. To detect expression of HCV NS proteins, antibodies against NS3, NS4B, NS5A, and NS5B were used. Anti-β-actin antibody (Sigma) was also used for detection of β-actin as an internal control.

To investigate the phosphorylation of Stat-1 at Tyr701 in HCV replicon cells and its parental Huh-7 cells, the cells were cultured in the medium containing 500 IU/mL of IFNα for 30 min. After cell lysates were prepared as previously described, western blot analysis was performed using an anti-phospho-Stat-1 (Tyr701) antibody (Cell Signaling Technology) or an anti-Stat-1 antibody (BD Transduction Laboratories). Immunocomplexes were detected by visualization using enhanced chemiluminescence (Amersham Biosciences).

Transfection of total RNA derived from replicon cells to naive Huh-7

Total RNA (5 μg) extracted from HCV replicon cells was transfected to Huh-7 cells using DMRIE-C transfection reagents, in accordance with the instruction manuals provided with the reagents. The transfected cells were cultured in the absence of IFNα and selected with 1000 μg/mL of G418 for 4 weeks. Drug-resistant cells were collected and reactivity to IFNα was measured as described in previous section.

Elimination of HCV replicon RNA from replicon cells (Isolation of 'cured' replicon)

To remove HCV replicon RNA from replicon cells, HCV replicon cells were treated ('cured') with HCV RNA-dependent RNA polymerase NS5B inhibitor, JTP-71892, JTK-109-derivatives synthesized in our laboratory (Hirashima et al., 2006; Ishida et al., 2006). The replicon cells (5×10^4 cells) were inoculated on a 60-mm plate and further cultured in the presence of the compound (1 μM) for about 4 weeks. The cell culture was performed in the absence of G418, to prevent survival of the compound-resistant clones. Medium was exchanged with fresh medium containing the compound twice per week. The finding that 1 μM of JTP-71892 does not exhibit any toxicity or growth inhibition in long-term culture had been previously confirmed.

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Variants in *IL28B* in Liver Recipients and Donors Correlate With Response to Peg-Interferon and Ribavirin Therapy for Recurrent Hepatitis C

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BACKGROUND & AIMS: Patients with hepatitis C virus (HCV)–related liver disease frequently undergo orthotopic liver transplantation, but recurrent hepatitis C is still a major cause of morbidity. Patients are treated with peg-interferon and ribavirin (PEG-IFN/RBV), which has substantial side effects and is costly. We investigated genetic factors of host, liver donor, and virus that might predict sensitivity of patients with recurrent hepatitis C to PEG-IFN/RBV. **METHODS:** Liver samples were analyzed from 67 HCV-infected recipients and 41 liver donors. Liver recipient and donor DNA samples were screened for single nucleotide polymorphisms near the *IL28B* genes (rs12980275 and rs8099917) that affect sensitivity to PEG-IFN/RBV. HCV RNA was isolated from patients and analyzed for mutations in the core, the IFN sensitivity-determining region, and IFN/RBV resistance-determining regions in nonstructural protein 5A. **RESULTS:** In liver recipients and donors, the *IL28B* single nucleotide polymorphism rs8099917 was significantly associated with a sustained viral response (SVR; $P = 0.003$ and $P = .025$, respectively). Intrahepatic expression of *IL28* messenger RNA was significantly lower in recipients and donors that carried the minor alleles (T/G or T/T) in rs8099917 ($P = .010$ and .009, respectively). Genetic analyses of *IL28B* in patients and donors and of the core and nonstructural protein 5A regions encoded by HCV RNA predicted an SVR with 83% sensitivity and 82% specificity; this was more effective than analysis of any single genetic feature. **CONCLUSIONS: In patients with recurrent HCV infection after orthotopic liver transplantation, combination analyses of single nucleotide polymorphisms of *IL28B* in recipient and donor tissues and mutations in HCV RNA allow prediction of SVR to PEG-IFN/RBV therapy.**

Keywords: ISDR; IRRDR; Genetic Analysis; Genetic Variations.

Hepatitis C virus (HCV) infection affects 170 million people worldwide and can lead to decompensated cirrhosis and hepatocellular carcinoma.[1,2] As a result, HCV-related liver disease is the leading indication for orthotopic liver transplantation (OLT) worldwide.[3,4] However, several reports have shown that post-OLT patient and graft survival are significantly negatively affected by HCV recurrence after OLT.[5,6] This can be mitigated by achievement of a sustained virological response (SVR) with pegylated interferon and ribavirin (PEG-IFN/RBV) therapy.[7] However, many patients cannot tolerate curative doses or do not respond to therapy with PEG-IFN/RBV.[6,8] Because of the substantial cost of therapy, both financial and with regard to side effects, it would be ideal to be able to predict which patients would benefit from PEG-IFN/RBV therapy for recurrent HCV.[9,10]

Many reports have demonstrated that HCV-RNA mutations, including those of amino acid residues 70 and 91 in the Core region,[11] and those in the interferon sensitivity determining region (ISDR)[12] and variable region 3 domain[13] in the nonstructural protein 5A (NS5A), were significantly associated with IFN sensitivity in patients infected with genotype 1 HCV. We previously reported that these genetic mutations have a significant impact on patients' responsiveness to PEG-IFN/RBV therapy for recurrent hepatitis C after OLT.[14] However, in addition to viral factors, host factors can also be used to predict IFN sensitivity. For example, Asahina et al demonstrated that the pretreatment induction level of IFN-stimulated genes (ISGs) was significantly associated with SVR to PEG-IFN/RBV therapy.[15] In addition, it was recently reported that single nucleotide polymorphisms near the IL28B gene on chromosome 19q13, rs12980275, or rs8099917 are significantly associated with the sensitivity of IFN/RBV combination therapy for chronic hepatitis

Abbreviations used in this paper: DW, double-wild; ETR, end of treatment response; HCV, hepatitis C virus; IRRDR, interferon/ribavirin resistance-determining region; ISDR, interferon sensitivity-determining region; ISG, interferon-stimulated gene; mRNA, messenger RNA; NR, nonresponse; NS5A, nonstructural protein 5A; OLT, orthotopic liver transplantation; PCR, polymerase chain reaction; PEG-IFN, pegylated interferon; RBV, ribavirin; SVR, sustained viral response.

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