RESULTS

Suppression of HCV replication by purified herbal extracts, isoliquiritigenin and glycycoumarin

TO SCREEN THE herbal drugs and these purified extracts (Table 1) for their antiviral effects against HCV replication, Huh7/Rep-Feo cells were cultured with various concentrations of 5 herbal extracts; *Glycyrrhizae radaix, Rhemanniae radix, Paeoniae radix, Artemisiae capillari spica, and Rhei rhizoma,* and 13 compounds purified from these herbal extracts. Levels of HCV replication were quantified by internal luciferase assay after 48 h. None of the herbal extracts showed any effects on HCV replication (data not shown). On the other hand, among the 13 purified compounds, isoliquiritigenin and glycycoumarin, which were purified from *Glycyrrhizae radix,* suppressed replication of HCV replicon in a dose-dependent manner. The EC50s were 6.2 ± 1.0 and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively (Figs 2a,3a). The MTS assay did not show any effect on cell growth and viability (Fig. 2b), indicating that the antiviral action of the two compounds is not due to cytotoxic or antiproliferative effects. Huh7/Rep-Feo cells were cultured with various concentrations of isoliquiritigenin and glycycoumarin, and the dose-effect correlation and time courses of replicon expression were measured by luciferase assay. After addition of each compounds, suppressive effect of the HCV replicon lasted for 48 h in a dose and time-dependent manner (Fig. 3b).

Realtime-RT-PCR and Western blotting analyses

In the realtime RT-PCR analysis and Northern blot analyses, levels of the replicon RNA decreased in a dose-dependent manner following treatment with isoliquir-

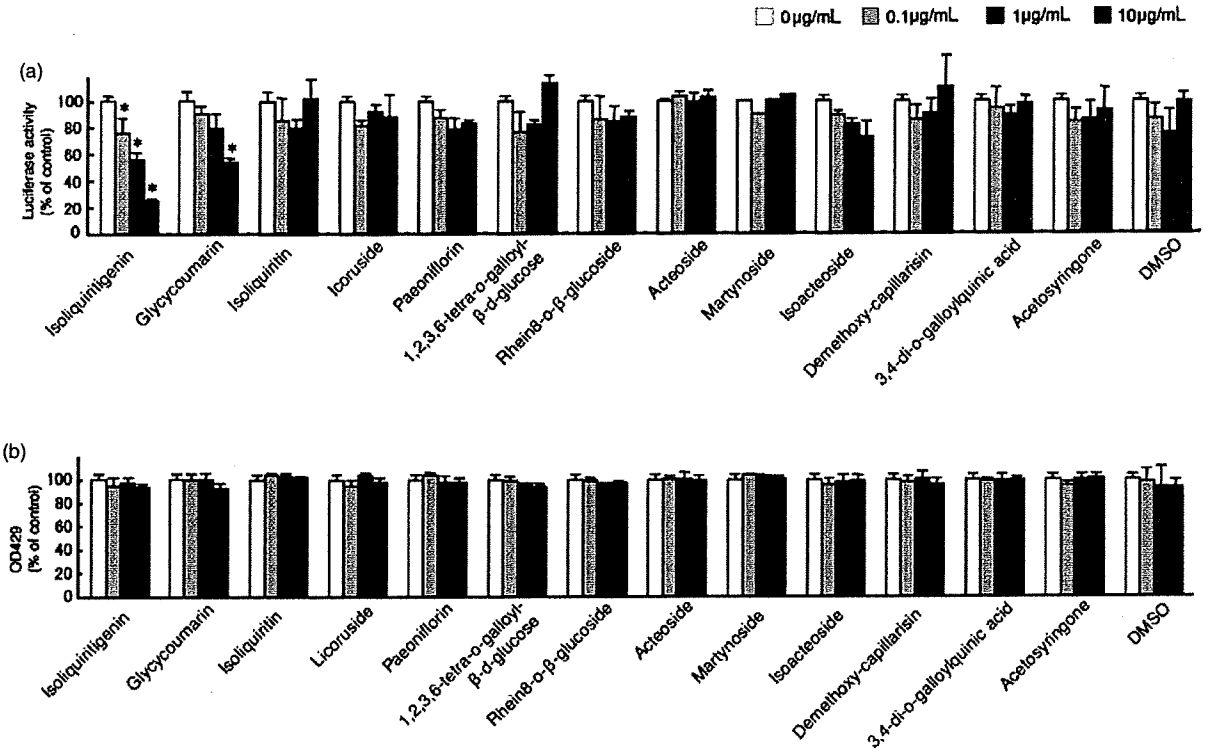

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Asterisks indicate p-values of less than 0.05. (b) MTS assay of Huh7/Rep-Feo cells cultured with the concentrations of 13 compounds indicated. Error bars indicate mean ± SD.

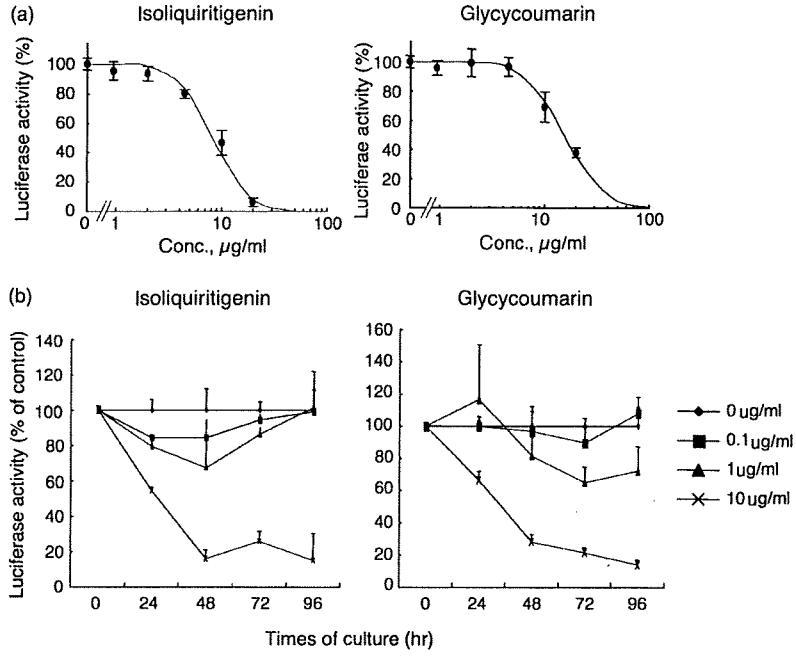

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo cells were cultured with the concentrations of isoliquiritigenin and glycycoumarin indicated. The internal luciferase activities were measured at times of culture indicated. Assays were performed in triplicate. Error bars indicate mean ± SD.

itigenin and glycycoumarin (Fig. 4a,b). Similarly, in Western blot analysis, the HCV non-structural protein, NS5A, which was translated from the HCV replicon, decreased by corresponding amounts in response to treatment with isoliquiritigenin and glycycoumarin (Fig. 4c). Densitometric analysis of NS5A protein showed that the intracellular levels of the virus protein in Huh7/Rep-Feo cells correlated well with the luciferase activities.

Absence of synergistic anti-HCV effects of interferon-alpha with isoliquiritigenin or glycycoumarin

To determine whether IFN and these two compounds have a synergistic inhibitory effect on the replicon, Huh7/Rep-Feo cells were cultured with combinations of IFNα-2b and isoliquiritigenin or glycycoumarin at various concentrations. The relative dose-inhibition curves of IFN were plotted under each fixed concentrations of isoliquiritigenin or glycycoumarin of 0, 0.1, 1, 10 μg/mL, respectively (Fig. 5). The curves did not show synergy of the two compounds and IFN against the HCV replicon. To see whether the action of isoliquiritigenin and glycycoumarin involve interferon-Jak/STAT-ISRE pathway, we conducted ISRE reporter assays. We transfected the p-55C1BLuc plasmid in Huh7 cells and cultured the cells in the presence of isoliquiritigenin or glycycoumarin. After 12 h of incubation, those drugs did not activate ISRE-promoter activities (data not shown). These results suggested that the action of the compounds on the intracellular replication of HCV replicon was independent of the IFN-ISRE pathway.

Isoliquiritigenin and glycycoumarin do not suppress the HCV IRES-dependent translation

We next determined whether these two compounds suppress HCV IRES-dependent translation, we used Huh7 cell line that had been stably transfected with pCIneo-Rluc IRES-Fluc (Huh7/CRIF; Fig. 1b). Treatment of these cells with isoliquiritigenin or glycycoumarin resulted in no significant change of the internal luciferase activities at concentrations of these two compounds that suppressed expression of the HCV replicon (Fig. 6a). The MTS assay did not show any effect on cell growth and viability at concentrations used in this assay (Fig. 6b).

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

The demonstrated inhibitory effects isoliquiritigenin and glycycoumarin on HCV subgenomic replication were validated further by using HCV-JFH1 cell culture system.[25] As shown in Figure 7a, treatment of the cells with the two compounds suppressed time-dependent

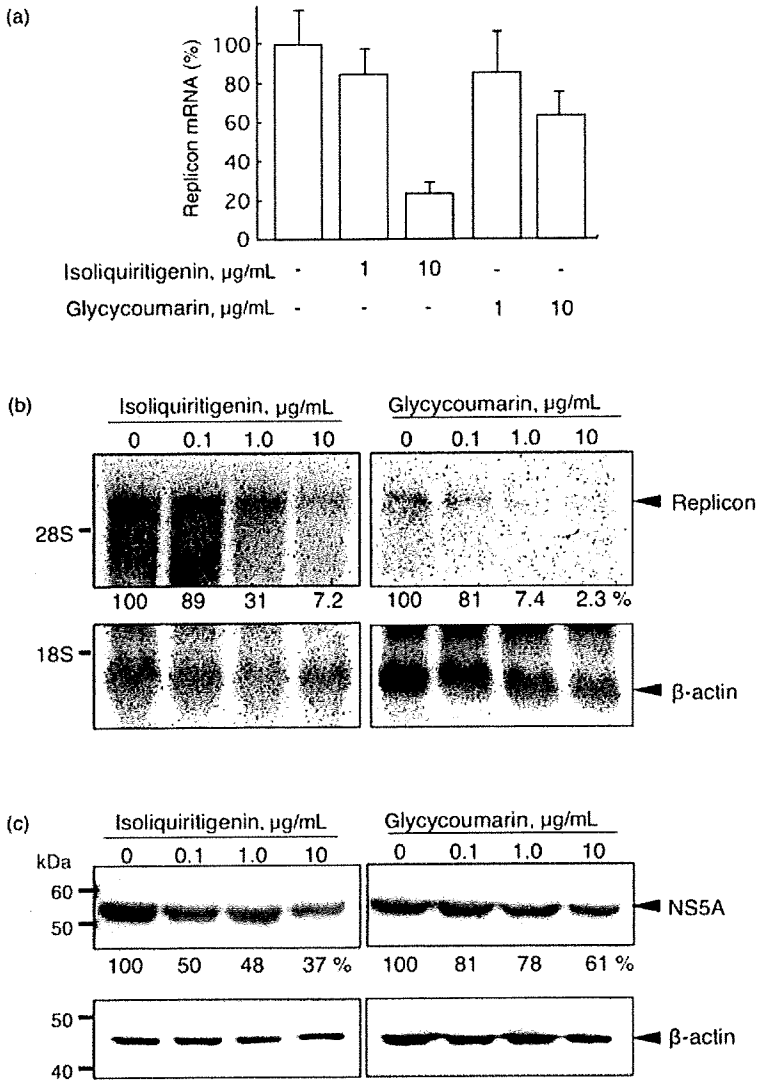

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot hybridization. Fifteen micrograms of total cellular RNA was electrophoresed in each lane. The upper part of the membrane containing the hepatitis C virus replicon RNA was hybridized with a digoxigenin-labeled probe specific for the replicon sequence, and the lower part was hybridized with beta-actin probe. Densitometry for replicon RNA was performed and indicated as percents of drug-negative control. (c) Western blotting. Thirty micrograms of total cellular protein was electrophoresed in each lane. Densitometry of NS5A protein was performed and indicated as percents of drug-negative control.

increase of HCV core antigen in the medium. In all time points, core antigen levels were significantly lower in culture that were treated with isoliquiritigenin and glycycoumarin than the untreated culture. The effect of glycycoumarin was partly reversed on day six probably due to chemical instability of the compound. Consistently, the Western blot showed that the cellular HCV core protein expression was substantially suppressed by treatment with isoliquiritigenin and glycycoumarin (Fig. 7b).

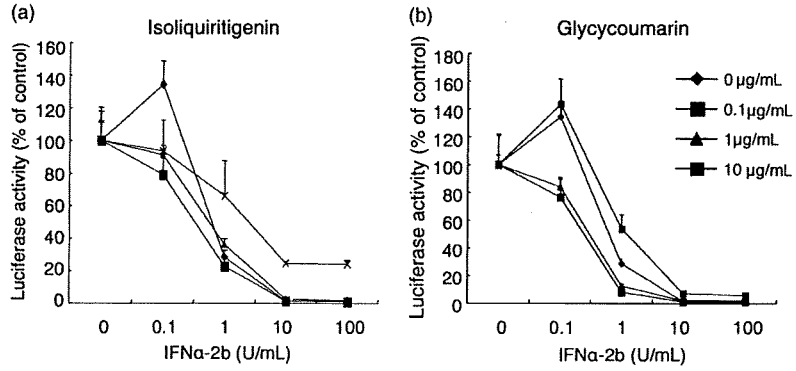

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured after 48 h of culture. Assays were performed in triplicate. Error bars indicate mean ± SD. Plots of 100% in each curves represent replicon expression levels that were treated with indicated amounts of isoliquiritigenin or glycycoumarin and without IFN.

DISCUSSION

THE PRESENT STUDY demonstrates that two purified herbal extracts, isoliquiritigenin and glycycoumarin, isolated from *Glycyrrhizae radix*, suppress replication of an HCV replicon (Fig. 2). Northern and Western blot analyses reveal that both RNA synthesis and its translation were reduced by the two compounds in dose- and time-dependent manners (Figs 3,4). The two drugs did not show activation of type-I interferon-dependent, ISRE-mediated transcription or synergistic action with interferon-alpha on HCV replication (Fig. 5,6), which suggests that the anti-HCV effects of the compounds are independent of interferon-antiviral mechanisms. Finally, we have demonstrated that the two compounds show inhibitory effects on HCV virus cell cultures (Fig. 7).

Flavonoid is a class of plant pigment, found in wide range of green vegetables and fruits. They are classified into flavon, flavonol, flavanone, flavanol, isoflavone, chalcone, anthocyanin and catechin, according to their molecular structures. Many flavonoids have various biological functions such as antibacterial,[28] antioxidative and anticarcinogeninc activities.[29] Isoliquiritigenin is a simple chalcon derivative and found in licorice and vegetables including shallots and bean sprouts. Isoliquiritigenin has several biochemical activities similar to other flavonoids. It has various biochemical activities such as antioxidative and superoxide scavenging activities,[30] an antiplatelet aggregation effect,[31] an inhibitory effect on aldose reductase activity,[32] estrogenic properties[33] and selective inhibition of H2 receptor-mediated signaling.[34]

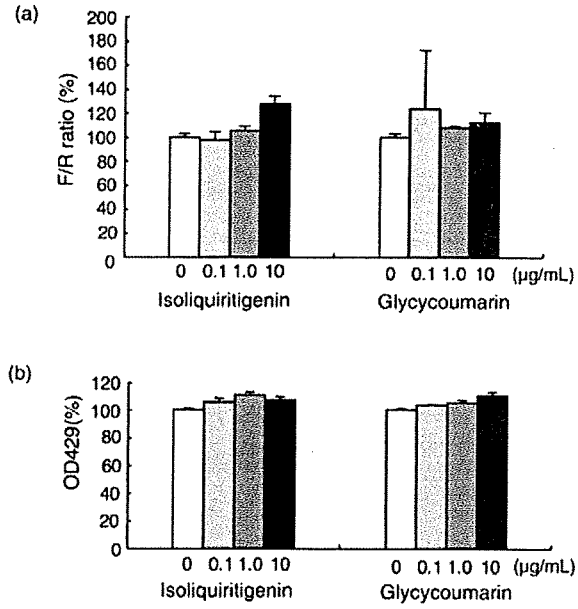

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated, and dual luciferase activities were measured after 48 h of treatment. Values are displayed as ratios of Fluc to Rluc. Error bars indicated mean ± SD. (b) MTS assay of Huh7/neo-Rluc-IRES-Fluc cells cultured with isoliquiritigenin or glycycoumarin at the concentrations indicated. MTS assays at 48 h after treatment with each drug were performed in triplicate. Error bars indicate mean + SD.

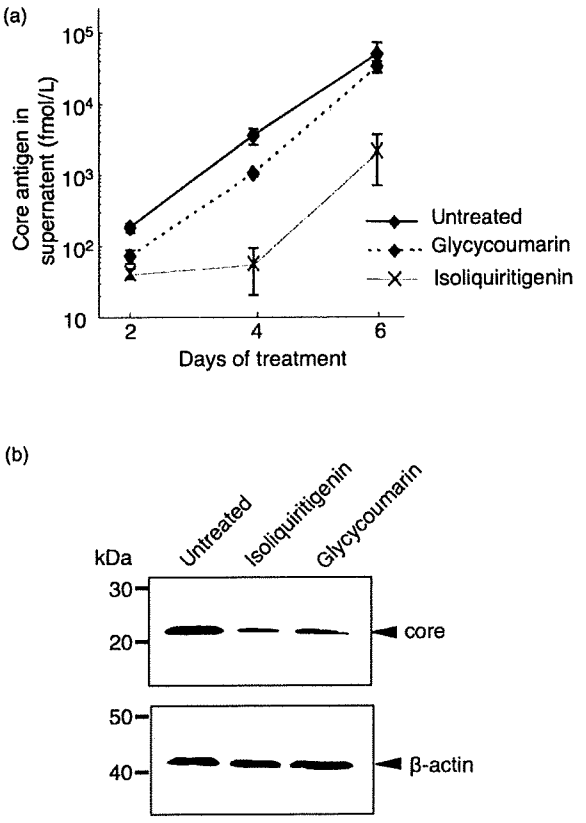

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, and HCV core antigen was measured. Assays were done in triplicate and indicated as mean ± SD. (b) Cells were harvested at day 6, and Western blotting was performed using anti-core and anti-beta-actin antibodies.

Extracts of a licorice root, *Glycyrrhizae radix*, show anti-inflammatory properties in chronic and acute liver inflammation,[35] and are widely and extensively prescribed in Japan as Strong Neominophagen C (SNMC). A major ingredients of *Glyzyrrizae radix* are glycyrrhizin and liquiritin. However, glycyrrhizin and liquiritin did not suppress HCV replication, suggesting that the commercially available SNMC will not elicit antiviral effects against HCV. On the other hand, there have been reports on the pharmacological action of glycycoumarin. Glycycoumarin displays antibacterial properties in the upper respiratory tract in infections such as *Streptococcus pyogenes, Haemophilus infuenzae* and *Moraxella catarrhalis*,[36] and methicillin-resistant *Staphylococcus aureus*,[37] but the mechanisms of action is unclear.

To our knowledge, there have been no reports on the serum concentration of glycycoumarin and isoliquiritigenin in patients taking medicines or dietary supplements containing *Glyzyrrizae radix*. However, therapeutic doses of 3–12 g per day of powdered root have been suggested for pathological conditions including chronic hepatitis, muscle cramp, acute gastritis, and urolithiasis. Thus, further studies are required to assess the human exposure to these flavonoids, the pharmacological dose-dependent properties and the tissue distribution and drug kinetics.

Considering the current status of limited therapy options for HCV infection and their unsatisfactory outcomes, large scale screening of anti-HCV molecules for the development of novel antiviral therapies is called for. In the present study, we have screened Chinese herbal extracts for the ability to suppress HCV replication, and identified two extracts, isoliquiritigenin and glycycoumarin, which specifically suppressed HCV replication. These results suggest that these agents will be a promising for use in the stabilization of HCV replication and active liver inflammation. In addition, further investigations of the action of these drugs on the expression, processing or maturation of HCV proteins may elucidate new aspects of the viral infection and replication and may constitute novel molecular targets for anti-HCV chemotherapeutics.

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Targeting Lipid Metabolism in the Treatment of Hepatitis C Virus Infection

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Recently, microdomains of organelle membranes rich in sphingomyelin and cholesterol (called "lipid rafts") have been considered to act as a scaffold for the hepatitis C virus (HCV) replication complex. Using the HCV cell culture system, we investigated the effect of myriocin, a sphingomyelin synthesis inhibitor, on HCV replication. We also investigated the combined effect of myriocin with interferon (IFN) and myriocin with simvastatin. Myriocin suppressed replication of both a genotype 1b subgenomic HCV replicon (Huh7/Rep-Feo) and genotype 2a infectious HCV (JFH-1 HCV) in a dose-dependent manner (for subgenomic HCV-1b, maximum of 79% at 1000 nmol/L; for genomic HCV-2a, maximum of 40% at 1000 nmol/L). Combination treatment with myriocin and IFN or myriocin and simvastatin attenuated HCV RNA replication synergistically in Huh7/Rep-Feo cells. Our data demonstrate that the sphingomyelin synthesis inhibitor strongly suppresses replication of both the subgenomic HCV-1b replicon and the JFH-1 strain of genotype 2a infectious HCV, indicating that lipid metabolism could be a novel target for HCV therapy.

Hepatitis C virus (HCV) is a major etiologic agent of liver diseases, affecting 170 million people worldwide [1]. Fifty-five percent to 85% of acute infections become persistent [2], and at least 20% of patients with chronic HCV infection progress to cirrhosis within 20 years [3]. With therapeutic advances, including the recent combination of pegylated interferon (IFN) plus ribavirin, half of patients can achieve a sustained virologic response [4]. However, the remaining half cannot clear the virus, demonstrating a strong need for HCV-specific therapies.

Positive-strand RNA viruses replicate intracellularly on certain membrane structures, including the endoplasmic reticulum [5], the Golgi apparatus [6], endosomes, and lysosomes [7]. During replication, RNA viruses form distinct replication complexes made of several membrane compartments and viral proteins [8]. In HCV, the membranous web (consisting of vesicles in a membranous matrix) has been described in the cellular matrix of HCV replicon–harboring cells [9, 10]. This membranous web is considered to be the HCV replication complex, consisting of viral and host proteins.

Recent studies suggest that the HCV replication complexes are formed on lipid rafts (which are detergent-insoluble microdomains of intracellular vesicular membranes rich in cholesterol and sphingolipid) [11–13]. It has been reported that viral nonstructural proteins and both positive- and negative-sense HCV RNAs were localized distinctively in a fraction of lipid rafts when subgenomic HCV replicon cells were subjected to membrane flotation analysis [12]. On the other hand, recent studies have demonstrated that agents related to lipid metabolism affect the replication of genotype 1 HCV. Leu et al. [14] reported that polyunsaturated fatty acids exerted strong anti-HCV activity on a subgenomic HCV-1b replicon. Moreover, 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitors (statins), which prevent cholesterol synthesis, have been shown to suppress replication of ge-

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nomic and subgenomic HCV-1b replicons [15, 16]. Even though the precise mechanism has not been defined, these agents may attenuate HCV replication through the destruction of lipid rafts, according to their pharmacological actions. If this is the mechanism, sphingomyelin, the remaining and essential component of lipid rafts, might play a role in HCV replication. With this in view, recent studies have demonstrated that a sphingomyelin synthesis inhibitor attenuated the replication of a subgenomic HCV-1b replicon in cultured cells [17] and the replication of genomic HCV-1 in a chimeric mouse model [18]. However, investigation of anti-HCV activity in these agents has been limited to genotype 1 HCV, and the combined effect of these agents has not been determined. If they do not target the HCV structure itself but exert their antiviral activity through destruction of the host's lipid raft, it would be plausible to speculate that they might be effective irrespective of the viral isolate, and the combined effect of these agents might be additive or synergistic.

In the present study, we investigated the role played by the sphingomyelin synthesis pathway and the mevalonate pathway in HCV replication, using a subgenomic HCV-1b replicon and the particle-producing cell culture HCV 2a model of JFH-1 HCV [19].

MATERIALS AND METHODS

Cell culture and HCV replicon. The human hepatoma cell lines Huh7 and Huh7.5.1 [20] were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal calf serum at 37°C in 5% CO_2. The subgenomic HCV replicon used was derived from Rep-Feo (genotype 1b) [21, 22], and a full-length genomic HCV RNA was derived from genotype 2a JFH-1 HCV [19]. Subgenomic or genomic HCV RNA was synthesized from replicon cDNA–harboring plasmids (pRep-Feo and pJFH-1) by means of T7 polymerase (RiboMax Large Scale RNA Production System; Promega) and transfected into these cells. For the subgenomic replicon, cell lines stably expressing the replicon were established (Huh7/Rep-Feo) in the presence of 500 μg/mL G418.

Reporter plasmids and luciferase assay. pISRE-TA-Rluc expressing the *Renilla* luciferase reporter gene under control of the IFN-stimulated response element (ISRE) was constructed by replacing the firefly luciferase gene with the *Renilla* luciferase gene of pISRE-TA-Luc, purchased from Invitrogen. Luciferase activity was quantified using the Bright-Glo or Dual-Luciferase assay system (both from Promega) and a luminometer (AB-2250; ATTO). Assays were performed in triplicate, and the results were expressed as mean ± SD percentages of the control values. QuantiLum recombinant luciferase (Promega) was used as the positive control for the analysis.

Reagents. The reagents used included myriocin (Biomol), IFN-α 2b (Santa Cruz Biotechnology), phytosphingosine hydrochloride (Sigma), 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD; Sigma), and simvastatin (Cosmobio).

Northern blotting. Total cellular RNA was extracted from cells by means of Isogen (Wako). The RNA was separated by denaturing agarose-formaldehyde gel electrophoresis and transferred to a membrane from a NorthernMax kit (Ambion). The membrane was hybridized with a digoxigenin-labeled probe that was specific for the nonstructural replicon sequence. The signals were detected in a chemiluminescence reaction by using a digoxigenin detection kit (Roche) and were visualized by using an LAS-1000 imaging system (Fuji Film).

Western blotting. Ten micrograms of total cell lysate was separated using NuPAGE 4%–12% Bis-Tris gel (Invitrogen) and was blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with an anti-core monoclonal antibody (MAb; Affinity Bioreagents), an anti-NS3 MAb (Virogen), an anti-NS5A MAb (gift from Burckstummer, Robert Koch Institute), or a anti–β-catenin MAb (Sigma). Detection was done in a chemiluminescence reaction (ECL; Amersham).

Dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assays. To evaluate cytotoxicity, MTS assays were performed using a CellTiter 96 Aqueous One Solution Cell Proliferation Assay (Promega), in accordance with the manufacturer's instructions.

Thin-layer chromatography (TLC). The lipid fraction of cells treated with myriocin was extracted using the method of Bligh and Dyer [23], and total lipids from the cells treated with myriocin were extracted with 3 mL of chloroform. The extracts were spotted onto silica gel TLC plates (Merck) and were chromatographed with chloroform-methanol-water (65:25:4 [vol/vol/vol]). The plate was visualized with a molybdenum spray.

Real-time reverse-transcription polymerase chain reaction (RT-PCR). TaqMan RT-PCR targeting the 5' untranslated region was used for the quantitation of intracellular genomic JFH-1 HCV RNA. The sequences of the sense and antisense primers and the TaqMan probe were 5'-TGCGGAACCGGTGAGTACA-3', 5'-CTTAAGGTTTAGGATTCGTGCTCAT-3', and 5'-(FAM)CAC-CCTATCAGGCAGTACCACAAGGCC(TAMRA)-3', respectively. The method has been described elsewhere [24].

Short interfering RNA (siRNA) analysis. The sequence encoding the LCB1 subunit of serine palmitoyltransferase (SPT) was selected as the target for siRNA (sense, 5'-AACAA-CAUCGUUUCAGGUCCUTT-3'; antisense, 5'-AGGGCCUG-AAACGAUGUUGTT-3'). siRNA targeting enhanced green fluorescent protein (GFP) was used as the negative control (sense, 5'-CUUACGCUGAGUACUUCGATT-3'; antisense, 5'-UCG-AAGUACUCAGCGUAATT-3'). (Underlined letters indicate deoxyribonucleotides.)

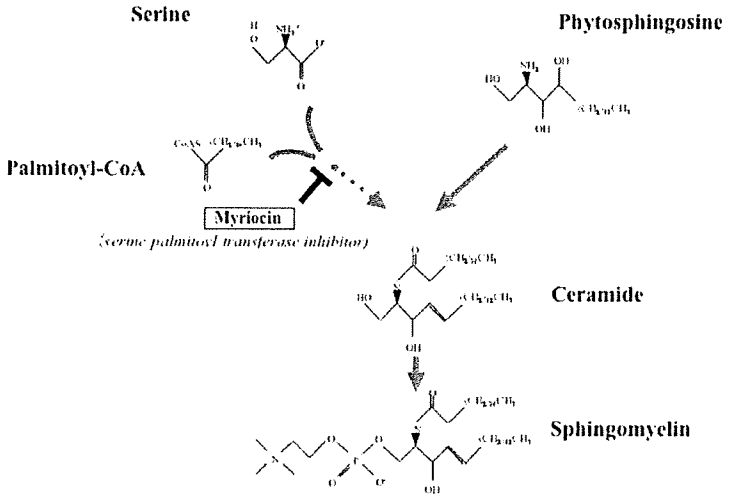

Figure 1. The sphingomyelin synthesis pathway. Serine palmitoyltransferase catalyzes the first committed step of sphingomyelin biosynthesis from serine and palmitoyl-coenzyme A (CoA). Myriocin inhibits the catalyzing activity of serine palmitoyltransferase. Phytosphingosine is known to work as a precursor of ceramide in both mammalian and fungal cells.

Statistical analyses. Statistical analyses were performed using Student's *t* test; statistically significant differences were defined as those for which $P < .05$.

RESULTS

Specific suppression of the replication of a subgenomic HCV-1b replicon by an inhibitor of sphingomyelin synthesis. To clarify the role played by the sphingomyelin synthesis pathway in HCV replication, we added myriocin, a specific inhibitor of SPT that catalyzes the first committed step of sphingomyelin biosynthesis (figure 1), to the medium of Huh7/Rep-Feo cells. The luciferase activity, reflecting replication of the subgenomic HCV-1b replicon, dropped to 37% and 21% of the control at myriocin concentrations of 100 and 1000 nmol/L, respectively (figure 2*A*, *upper panel*), but myriocin did not cause toxicity to the cultured cells (figure 2*A*, *lower panel*). The result indicates that the decrease in HCV replication is due to a specific suppressive effect of myriocin and not to the cytotoxicity of myriocin. Northern hybridization analysis also demonstrated a substantial reduction of the subgenomic HCV replicon RNA in Huh7/Rep-Feo cells treated with myriocin in a dose-dependent manner (figure 2*B*). Similarly, Western blot analysis demonstrated a decrease in HCV NS5A after treatment with myriocin (figure 2*C*).

No enhancement of ISRE promoter activity after myriocin treatment. To determine whether the effect of myriocin in suppressing the subgenomic HCV replicon was associated with the activation of IFN-stimulated genes, the ISRE–*Renilla* luciferase plasmid was transfected into Huh7/Rep-Feo cells, and these cells were cultured with various concentrations of myriocin. As a positive control for the enhancement of ISRE reporter activity, the ISRE–*Renilla* luciferase–transfected cells were cultured with IFN. Myriocin had no significant effect on ISRE promoter activity, whereas IFN significantly up-regulated ISRE activity (figure 2*D*, *upper panel*). In contrast, firefly luciferase activity in the Huh7/Rep-Feo cells, reflecting HCV replication, was inhibited by both IFN and myriocin in a dose-dependent manner (figure 2*D*, *lower panel*). These results demonstrate that the action of myriocin on HCV replication is independent of the IFN pathway.

Decrease in the sphingomyelin content of Huh7 cells after myriocin treatment. To clarify whether myriocin really inhibits the biosynthesis of sphingomyelin in Huh7 cells, we treated Huh7 cells with 100 nmol/L myriocin and analyzed the change in the cellular phospholipid composition by TLC. As demonstrated in figure 2*E*, the cellular sphingomyelin content decreased after myriocin treatment, but no significant change was observed in other cellular phospholipids.

Restoration of HCV replication by addition of phytosphingosine. To confirm that suppression of HCV RNA replication was due to depletion of sphingomyelin, we incubated replicon cells with phytosphingosine, a precursor of ceramide in mammalian and fungal cells, in the presence of myriocin. Treatment with phytosphingosine restored HCV replication in a dose-dependent manner (figure 2*F*, *upper panel*). On the other hand, phytosphingosine by itself did not have any effect on HCV replication (figure 2*F*, *lower panel*). This result indicates that inhibition of HCV replication was the direct result of depletion of sphingomyelin.

Suppression of HCV replication by knocking down SPT with siRNA. Next, we determined whether inhibition of SPT expression suppresses HCV replication by knocking down SPT with siRNA. As demonstrated in the upper panel of

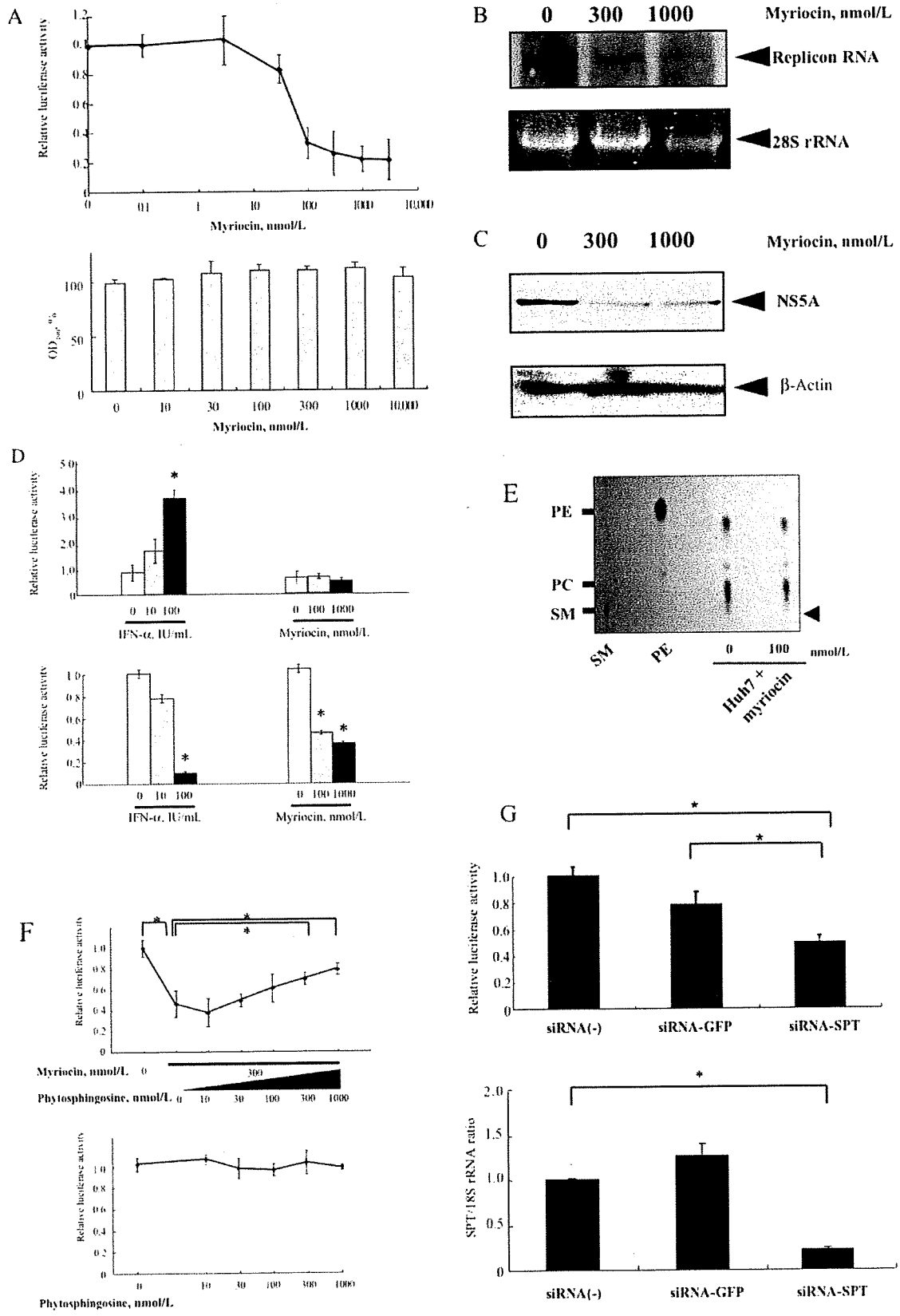
A
Relative luciferase activity
Myriocin, nmol/L
B
0 300 1000
Myriocin, nmol/L
Replicon RNA
28S rRNA
C
0 300 1000
Myriocin, nmol/L
NS5A
β-Actin
D
Relative luciferase activity
IFN-α, IU/mL
Myriocin, nmol/L
E
PE
PC
SM
SM PE 0 100 nmol/L
Huh7 + myriocin
F
Relative luciferase activity
Myriocin, nmol/L
Phytosphingosine, nmol/L
Phytosphingosine, nmol/L
G
Relative luciferase activity
siRNA(-)
siRNA-GFP
siRNA-SPT
SPT/18S rRNA ratio
siRNA(-)
siRNA-GFP
siRNA-SPT

figure 2*G*, HCV replication was suppressed significantly by siRNA targeting SPT compared with no siRNA or siRNA targeting GFP (negative control). We confirmed with real-time PCR that the siRNA targeting SPT significantly decreased expression of SPT mRNA (figure 2*G*, *lower panel*). This result indicates that the SPT enzyme plays an important role in HCV replication.

Inhibition of the replication of a subgenomic HCV-1b replicon by an HMG-CoA reductase inhibitor (simvastatin). HMG-CoA reductase inhibitors have been reported to suppress replication of subgenomic and genomic HCV-1b replicons [15, 16]. Because cholesterol is another important component of lipid rafts, it may be speculated that depletion of cholesterol by HMG-CoA reductase inhibitors disrupts the lipid raft, affecting the ability of the HCV replicon to replicate in Huh7 cells. To confirm the effect of HMG-CoA reductase inhibitors on the subgenomic HCV-1b replicon, we examined the effect of simvastatin by means of Huh7/Rep-Feo cells. Cultures of Huh7/Rep-Feo cells with simvastatin at concentrations of 0–100 μmol/L showed a dose-dependent reduction of the subgenomic HCV-1b replicon (figure 3, *upper panel*). The MTS assay showed that treatment with simvastatin had no toxic effect on Huh7/Rep-Feo cells in the dose range used (figure 3, *lower panel*). These results demonstrated that simvastatin specifically suppressed replication of a subgenomic HCV-1b replicon. However, because recent studies showed that statins suppress HCV replication through inhibition of geranylgeranylation of certain proteins rather than inhibition of cholesterol synthesis [15], we also examined the effect on HCV replication of 2-HP-β-CyD, an agent known to deplete cholesterol directly from membranes. As demonstrated in figure 4*A*, 2-HP-β-CyD also suppressed HCV replication without cytotoxicity. To confirm that 2-HP-β-CyD did not inhibit firefly luciferase activity nonspecifically rather than by suppressing HCV RNA, we incubated recombinant firefly luciferase with various concentrations of 2-HP-β-CyD in the culture medium, and the medium was subjected to luciferase analysis. As demonstrated in figure 4*B*, 2-HP-β-CyD did not affect luciferase activity. These results indicate that cholesterol itself plays an important role in HCV replication.

Synergistic inhibitory effects of myriocin with IFN, simvastatin with IFN, and myriocin with simvastatin. We carried out the following assay to determine whether myriocin and IFN have a synergistic inhibitory effect on HCV replication. Huh7/Rep-Feo cells were treated with combinations of myriocin and IFN at various concentrations. The relative dose-inhibition curves of IFN were plotted for each fixed concentration of myriocin (0, 30, 100, and 300 nmol/L). As demonstrated in the upper panel of figure 5*A*, the curves shifted to the left with increasing concentrations of myriocin, demonstrating the synergy of the 2 drugs against the subgenomic HCV-1b replicon. Isobologram analysis also confirmed the synergy (figure 5*A*, *lower panel*). To determine whether this synergistic effect was associated with up-regulation of the IFN-stimulated gene responses, we investigated the combined effect of myriocin and IFN on ISRE activity. As demonstrated in figure 5*B* *(upper panel, right)*, myriocin did not enhance the ISRE–*Renilla* luciferase activity induced by IFN, but

Figure 2. Specific inhibition of the replication of a subgenomic hepatitis C virus (HCV) genotype 1b replicon by myriocin. *A*, Inhibition of HCV replicon replication by myriocin. By use of Huh7/Rep-Feo cells expressing a selectable chimeric luciferase reporter Feo gene, the intracellular replication level of an HCV replicon was quantified on the basis of luciferase activity [22, 25]. Huh7/Rep-Feo cells were cultured with various concentrations of myriocin. After 96 h of treatment, the luciferase assay was performed, as described in Materials and Methods *(upper panel)*. In the dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay, Huh7/Rep-Feo cells were cultured with various concentrations of myriocin for 96 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments. *B*, Northern hybridization. Huh7/Rep-Feo cells were cultured with various concentrations of myriocin and harvested at 96 h after administration. Ten micrograms of total cellular RNA was electrophoresed in each lane. The membrane containing the HCV replicon RNA was hybridized using a digoxigenin-labeled probe specific for the replicon sequence *(upper panel)*, and 28S human ribosomal RNA (rRNA) was used as an internal control *(lower panel)*. Lane 1, no myriocin; lane 2, 300 nmol/L myriocin; lane 3, 1000 nmol/L myriocin. *C*, Western blotting. Ten micrograms of total cellular protein was electrophoresed in each lane. Anti-NS5A monoclonal antibody was used as the primary antibody to detect HCV proteins *(upper panel)*, and β-actin was used as an internal control *(lower panel)*. Lane 1, no myriocin; lane 2, 300 nmol/L myriocin; and lane 3, 1000 nmol/L myriocin. *D*, No enhancement of interferon (IFN)–stimulated response element (ISRE) promoter activity by myriocin. To investigate whether the effect of myriocin was associated with the activation of IFN-stimulated genes, the ISRE–*Renilla* luciferase plasmid was transfected into Huh7/Rep-Feo cells in the presence of myriocin. The upper panel demonstrates the ISRE–*Renilla* luciferase activity at 48 h after transfection. The lower panel demonstrates the firefly luciferase activity of the Huh7/Rep-Feo cells, reflecting HCV replication. Data are means ± SDs of triplicates from 2 independent experiments. $^*P < .05$. *E*, Decrease in the sphingomyelin (SM) content of Huh7 cells after myriocin treatment. The change in the cellular phospholipid content was analyzed by thin-layer chromatography. Huh7 cells were cultured alone or with 100 nmol/L myriocin for 96 h. PC, phosphatidylcholine; PE, phosphatidylethanolamine. *F*, Restoration of the HCV replication that was suppressed by myriocin after the addition of phytosphingosine. Huh7/Rep-Feo cells were cultured with myriocin alone or with various concentrations of phytosphingosine. The luciferase assay was performed after 72 h of treatment *(upper panel)*. Huh7/Rep-Feo cells were also cultured with phytosphingosine alone as indicated for 72 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments. $^*P < .05$. *G*, Suppression of HCV replication by knocking down of serine palmitoyltransferase (SPT) with short interfering RNA (siRNA). Huh7/Rep-Feo cells were transfected with 10 nmol/L siRNA oligonucleotides targeting the LCB1 subunit of SPT or control siRNA targeting green fluorescent protein (GFP). The luciferase activity of the HCV replicon was measured 72 h after transfection *(upper panel)*. SPT mRNA expression at 72 h after siRNA transfection was analyzed by real-time polymerase chain reaction. The SPT mRNA level was measured relative to 18S rRNA *(lower panel)*. Values are shown as ratios to negative control levels and as the means ± SDs of triplicates from 2 independent experiments. siRNA(−), no siRNA. $^*P < .05$.

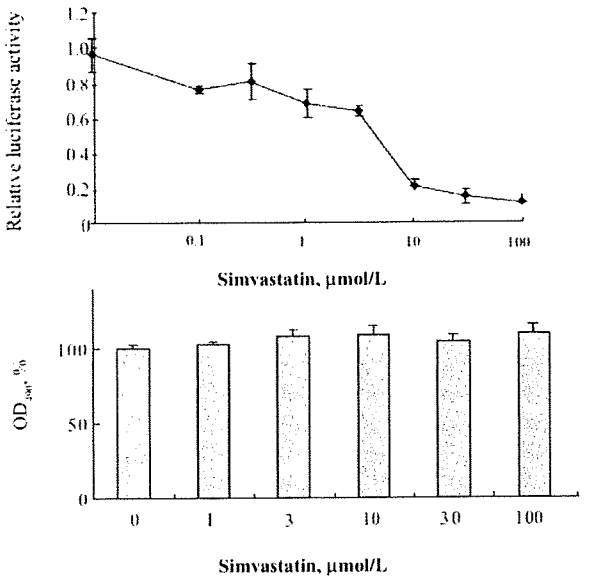

Figure 3. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by simvastatin. Huh7/Rep-Feo cells were cultured with various concentrations of simvastatin, and the luciferase assay was performed after 48 h of treatment *(upper panel)*. The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7/Rep-Feo cells were cultured with various concentrations of simvastatin for 48 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments.

it significantly enhanced IFN-induced suppression of the firefly luciferase activity reflecting HCV replication *(lower panel, right)*. This demonstrated that the synergistic effect was not caused by up-regulation of the IFN-stimulated genes. We also assessed the synergy of simvastatin with IFN and of myriocin with simvastatin. In each case, the 2 drugs showed synergistic effects at the concentrations indicated (figure 5*C* and 5*D*). In all cases, the MTS reduction values at the drug concentrations used in this assay did not show any significant decrease (data not shown). These results indicate that the synergistic effects on HCV replication of IFN with myriocin, IFN with simvastatin, and myriocin with simvastatin were exerted through their pharmacological effects and were not due to the augmentation of cytotoxicity.

Suppression of JFH-1 HCV replication by myriocin and simavastatin. The experiments described thus far were done using the subgenomic HCV-1b replicon system. Recently, Wakita et al. [19] established an infectious HCV model in cultured cells. This system, known as the JFH-1 system and based on genotype 2a HCV, secretes viral particles into the medium, and the medium is infectious for chimpanzees. This JFH-1 system completely mimics HCV infection in vivo and is considered more suitable for analyzing the effect of drugs. Therefore, we examined the effect of myriocin and simvastatin using the JFH-1 system. Huh7.5.1/JFH-1 HCV cells were cultured for 96 h with 1000 nmol/L myriocin, 10 μmol/L simvastatin, 1000 IU/mL IFN, and a combination of 1000 nmol/L myriocin and 10 μmol/L simvastatin. The intracellular JFH-1 HCV RNA titer was analyzed using real-time RT-PCR. As demonstrated in figure 6*A*, intracellular JFH-1 HCV RNA treated with myriocin or simvastatin decreased to 60% of control in 96 h, demonstrating that the inhibitory effect of myriocin and simvastatin on replication was not restricted to the subgenomic HCV-1b replicon. When both agents were used in combination, JFH-1 HCV RNA also

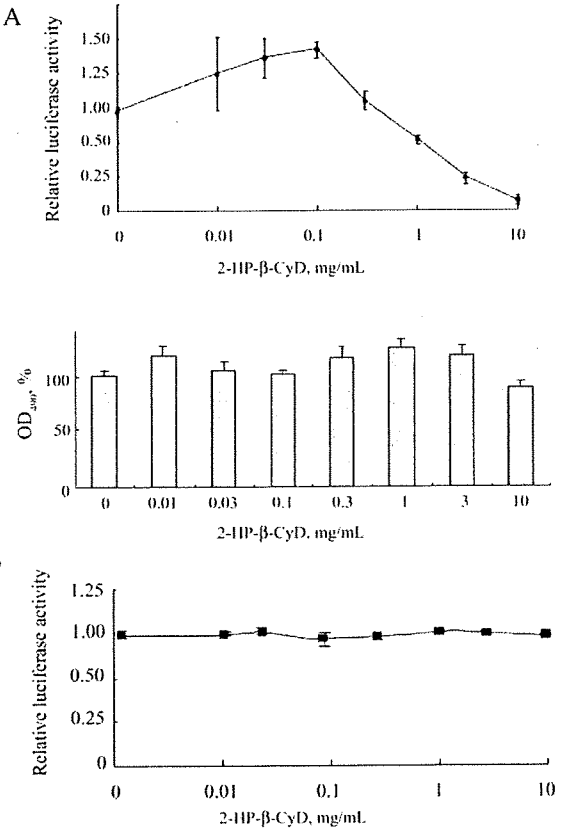

Figure 4. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD). *A*, Huh7/Rep-Feo cells cultured with various concentrations of 2-HP-β-CyD for 48 h. The luciferase assay was performed after 48 h of treatment *(upper panel)*. The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium assay was performed after Huh7/Rep-Feo cells were cultured with various concentrations of 2-HP-β-CyD for 48 h *(lower panel)*. Data are means ± SDs of triplicates from 2 independent experiments. *B*, Recombinant firefly luciferase incubated with various concentrations of 2-HP-β-CyD in the culture medium at 37°C for 48 h. The medium was collected and subjected to luciferase analysis. Data are means ± SDs of triplicates from 2 independent experiments.

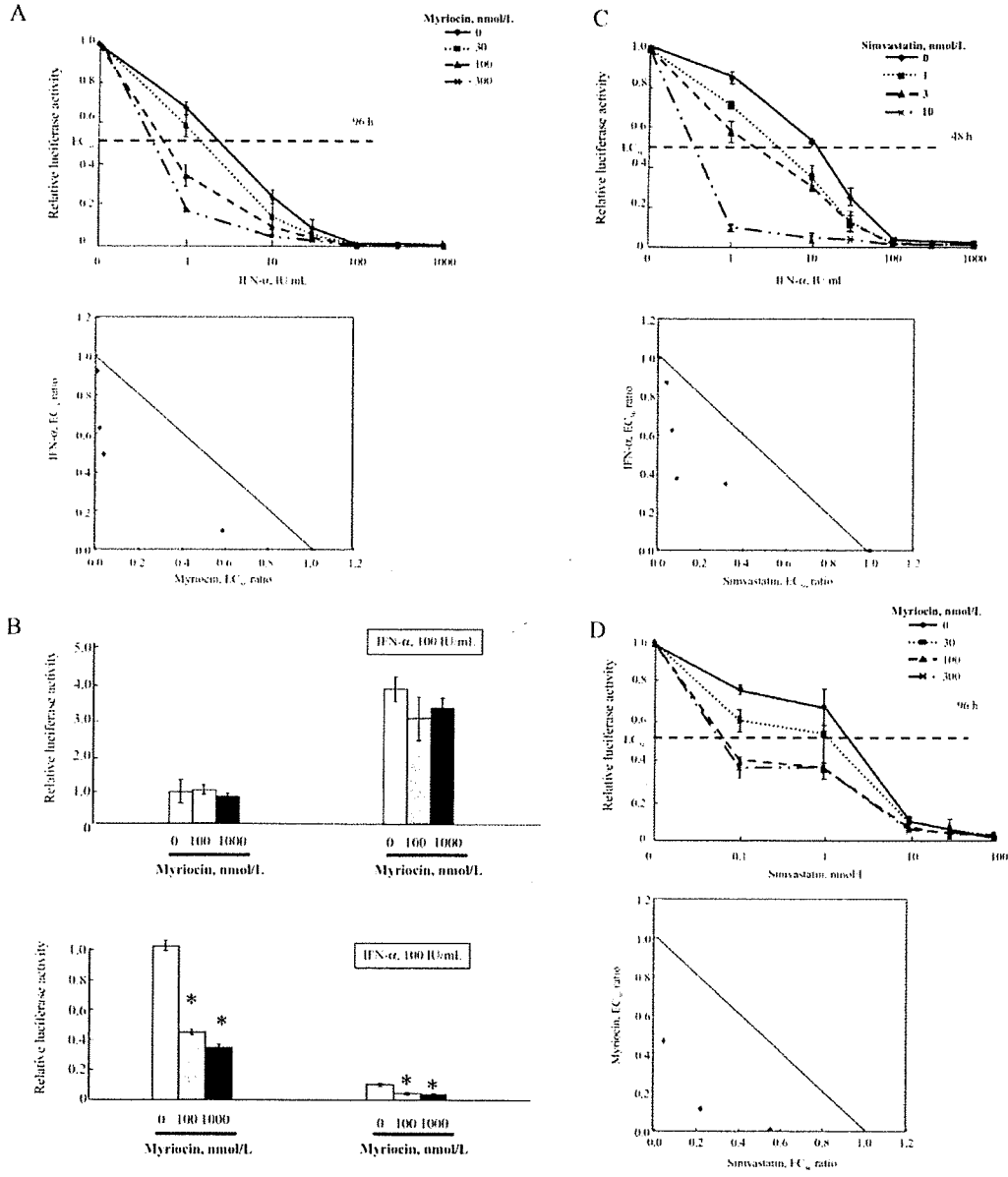

Figure 5. Synergistic inhibitory effects of myriocin with interferon (IFN), simvastatin with IFN, and myriocin with simvastatin. *A*, Synergistic inhibitory effect of myriocin with IFN on hepatitis C virus replication. Huh7/Rep-Feo cells were treated with combinations of myriocin and IFN at various concentrations. The upper panel shows the relative dose-inhibition curves of IFN plotted for each fixed concentration of myriocin (0, 30, 100, and 300 nmol/L). The lower panel shows the isobologram analysis for the combination of myriocin with IFN. *B*, IFN-stimulated response element (ISRE) promoter activity induced by a combination of myriocin with IFN. Huh7/Rep-Feo cells transfected with ISRE–*Renilla* luciferase were cultured with various concentrations of myriocin alone *(left)* or with 100 IU/mL IFN *(right)*. The upper panel demonstrates the ISRE–*Renilla* luciferase activity at 48 h after transfection. The lower panel demonstrates the firefly luciferase activity of the Huh7/Rep-Feo cells, reflecting hepatitis C virus (HCV). Data are means ± SDs of triplicates from 2 independent experiments. $^*P < .05$. *C*, Synergistic inhibitory effect of simvastatin with IFN on HCV replication. *D*, Synergistic inhibitory effect of simvastatin and myriocin on HCV replication.

decreased to almost 60% of the control at 48 and 96 h after treatment. However, no evident synergistic inhibitory effect was observed (figure 6*A*). To clarify the inhibitory effect of myriocin on JFH-1 HCV, we performed Western blot analysis for JFH-1 HCV proteins. As demonstrated in figure 6*B*, a substantial decrease in the core and NS3 proteins of JFH-1 HCV was observed 96 h after treatment with myriocin, confirming the RT-PCR results (figure 6*B*).

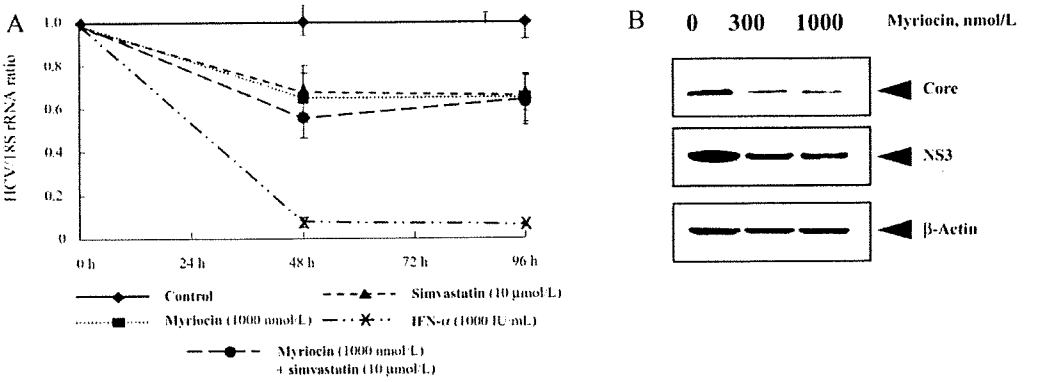

Figure 6. Suppression of JFH-1 hepatitis C virus (HCV) replication by myriocin and simvastatin. *A*, Cells containing JFH-1 HCV treated for 96 h with 1000 nmol/L myriocin, 10 μmol/L simvastatin, 1000 IU/mL IFN, or a combination of 1000 nmol/L myriocin and 10 μmol/L simvastatin. The cells were collected at 48 and 96 h, and the JFH-1 HCV RNA level relative to 18S rRNA was analyzed by real-time polymerase chain reaction. Values are shown as the ratios to negative control values (cells receiving no treatment) and as means ± SDs. *B*, Western blotting. Cells containing JFH-1 HCV were treated with 300 or 1000 nmol/L of myriocin and harvested at 96 h after administration. Ten micrograms of total cellular protein was electrophoresed in each lane. Anti-core monoclonal antibody (MAb) and anti-NS3 MAb were used as the primary antibodies to detect JFH-1 HCV proteins. β-Actin was detected as an internal control. Lane 1, no myriocin; lane 2, 300 nmol/L myriocin; and lane 3, 1000 nmol/L myriocin.

DISCUSSION

In the present study, we demonstrated that the sphingomyelin synthesis inhibitor myriocin suppressed not only replication of a subgenomic HCV-1b replicon but also replication of the JFH-1 strain of infectious genotype 2a HCV. We also demonstrated that simvastatin suppressed replication of both a subgenomic HCV-1b replicon and JFH-1 HCV. When a subgenomic HCV-1b replicon was used, the anti-HCV activity of both myriocin and simvastatin was enhanced synergistically with IFN. Moreover, when myriocin and simvastatin were used together, their anti-HCV activity was enhanced synergistically.

What is the mechanism by which myriocin suppresses viral replication? Because myriocin is a specific inhibitor of SPT, which catalyzes the first committed step of sphingomyelin biosynthesis, we speculated that myriocin exerts its action by inhibiting production of downstream substrates, especially sphingomyelin. The findings that siRNA targeted against SPT decreased HCV replication and that HCV replication was restored by addition of phytosphingosine, a precursor of sphingomyelin, demonstrated that the effect was specific to SPT activity. Moreover, the fact that treatment of Huh7 cells with myriocin did not enhance the ISRE promoter activity indicated that the inhibitory effects of myriocin were independent of those of IFN. It is known that intracellular replication of most RNA viruses occurs on certain membrane structures—including the endoplasmic reticulum, the Golgi apparatus, endosomes, and lysosomes—by making replication complexes at these sites [5–7]. For HCV, it has been reported by several groups that in vitro replication activity is located in the membrane fractions of cultured cells [26–28]. In addition, newly synthesized HCV RNA and the nonstructural proteins in replicon cells were colocalized in detergent-resistant membrane structures, most likely lipid rafts [18]. Caveolin-2, a lipid raft protein, was also shown to colocalize with the nonstructural proteins [18]. According to these findings, the HCV replication complex machinery is considered to form on a lipid raft. Therefore, because sphingomyelin is the major component of the lipid raft, it is plausible to speculate that myriocin disrupted lipid raft formation and inhibited HCV replication.

Cholesterol is another major component of lipid rafts and might also be targeted for anti-HCV therapy. Because cholesterol is synthesized in the mevalonate pathway, an inhibitor of the pathway might act to disrupt lipid rafts. In accordance with this concept, statins, which are HMG-CoA reductase inhibitors, already have been reported to suppress the replication of genomic and subgenomic HCV-1b replicons [15, 16]. In the present study, we also confirmed that simvastatin suppressed replication of a subgenomic HCV-1b replicon without toxicity. Moreover, we showed for the first time that the suppressive effect was also observed in an infectious HCV-2a model of JFH-1 HCV. Meanwhile, recent studies found that the effect of statins was attributable to inhibition of geranylgeranylation rather than depletion of cholesterol, because addition of geranylgeraniol rescued HCV suppression induced by statins [15]. However, although geranylgeranylation might play a role in HCV regulation, the importance of cholesterol itself has not yet been determined. To clarify further the role played by cholesterol in HCV replication, we investigated the effect of 2-HP-β-CyD, which is known to deplete cholesterol directly from cells. As demonstrated in figure 4, specific suppression of HCV replication by 2-HP-β-CyD indicated the importance of cholesterol itself for HCV replication. It is unlikely that these agents suppressed replication of the subgenomic replicon through inhibi-

tion of encephalomyocarditis virus internal ribosome entry site (EMCV-IRES) activity, because they also significantly suppressed replication of a full-length genomic HCV (JFH-1 HCV) that does not include EMCV-IRES (figure 6*A*; data for 2-HP-β-CyD not shown).

Although we observed an inhibitory effect of myriocin and simvastatin on both the subgenomic HCV-1b replicon and JFH-1 HCV, there was a difference in efficacy between the 2 HCV systems; the subgenomic HCV-1b replicon was more sensitive to and was more strongly inhibited by either agent alone or in combination, compared with JFH-1 HCV. This result was unexpected, because we had speculated that these agents might be effective irrespective of the viral isolate if these agents targeted not the virus itself but rather host factors, such as lipid rafts. However, there are several differences between these 2 systems, and we cannot directly compare the results. In particular, the subgenomic HCV replicon lacks viral structural proteins and has only an HCV RNA intracellular replication step, whereas JFH-1 HCV includes all steps of the HCV life cycle. We do not know the precise target of the agents, and further studies are still needed.

Is it really possible to use these agents in clinical HCV treatment? Especially because statins have been used in the treatment of hyperlipidemia for many years worldwide with proven safety, it would be ideal if we could use statins as one therapeutic application for anti-HCV therapy. Most recently, O'Leary et al. [29] undertook a human pilot study and treated 10 patients with atorvastatin for 12 weeks; they reported that there was no statistically significant change in HCV RNA levels compared with pretreatment levels. The reason for the discrepancy between in vitro and in vivo findings is unknown. However, as also discussed by O'Leary et al., the most plausible explanation for this discrepancy is that the plasma concentrations of atorvastatin after a conventionally approved dose were unlikely to reach those found to be effective in cell culture medium. According to their calculations, to inhibit HCV RNA replication the plasma atorvastatin concentration should be 3 logs higher than that achieved by a conventional dose. However, even though it would be difficult to inhibit HCV RNA replication with statins alone, a clinical antiviral effect might be still achieved if statins were used in combination with IFN (or myriocin), because a synergistic effect was observed in our in vitro study. To determine the synergistic effect in vivo, however, further clinical trials are needed. On the other hand, although promising in vitro, myriocin has not yet been used for human clinical diseases, and its safety has not been established. However, in chimeric mice, the plasma myriocin concentration equivalent to culture medium effectively inhibited HCV RNA replication, and drug toxicity was not observed at this concentration [30]. This finding suggested the possibility that myriocin could be used in vivo, although further studies are needed.

In conclusion, we have demonstrated that inhibition of the sphingomyelin synthesis pathway and the mevalonate pathway both effectively suppressed HCV replication in vitro, indicating that lipid metabolism could be an important target for new anti-HCV therapies.

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Replicons from genotype 1b HCV-positive sera exhibit diverse sensitivities to anti-HCV reagents

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ABSTRACT

Half of the population of genotype 1 HCV is resistant to current pegylated-interferon-α (PEG-IFN-α) and ribavirin therapy. The resistance to IFN therapy is an urgent problem, especially in patients with genotype 1 HCV infection. However, sensitivities among HCV strains to anti-HCV reagents including IFNs have not been thoroughly addressed. Here, we established three different subgenomic replicons (1B-4, 1B-5, and KAH5 strains) in addition to our previously established replicon (O strain). We comparatively examined the sensitivities of four replicons to IFN-α, IFN-γ, IFN-λ, cyclosporine A, and fluvastatin. Among the replicons, the 1B-4 and KAH5 replicons were the most sensitive and resistant, respectively to IFN-λ (EC_{50}: 1.50 μM vs. 8.50 μM) and fluvastatin (EC_{50}: 2.82 μM vs. 7.87 μM), although these replicons possessed similar features in terms of genetic distance from the O strain, HCV RNA expression levels, and sensitivity to IFN-α (EC_{50}: 1.44 IU/ml vs. 1.37 IU/ml) and cyclosporine A (EC_{50}: 0.71 μg/ml vs. 0.96 μg/ml). These replicons are thus useful tools for examining the mechanism of anti-HCV activity, especially in IFN-λ and statins.

1. Introduction

Hepatitis C virus (HCV) belongs to *Flaviviridae* family and contains a positive single-stranded RNA genome of 9.6 kb (Kato et al., 1990; Tanaka et al., 1996). The viral genome encodes a single polyprotein of approximately 3010 amino acid residues, which is proteolytically processed by host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (Kato, 2001). HCV infection frequently causes chronic hepatitis C (CH C) and progresses to fatal cirrhosis and hepatocellular carcinoma. The current standard therapy for CH C is pegylated-interferon-α (PEG-IFN-α) and ribavirin. However, the cure rate of the therapy for the treatment of CH C is limited to approximately 50% (Firpi and Nelson, 2007). The major cause of resistance to this therapeutic approach was observed in genotype 1 HCVs. However, the mechanisms of the diverse sensitivity to IFN therapy among genotype 1 HCVs have remained unclear. Therefore, the development of more effective anti-HCV reagents is an urgent issue.

Since the HCV replicon system was developed by Lohmann et al. (1999), several groups have reported candidate anti-HCV reagents. Statin, a 3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor, is one of the well-characterized anti-HCV reagents and its anti-HCV activity has been shown to be due to the inhibition of geranylgeranylation of host proteins (Ikeda et al., 2006; Kapadia and Chisari, 2005; Ye et al., 2003). Cyclosporine A (CsA), an immunosuppressant, is another well-characterized anti-HCV reagent that inhibits HCV RNA replication via its interaction with cyclophilins (CyPs) (Inoue et al., 2007; Nakagawa et al., 2005; Watashi et al., 2003). In addition to type I IFNs (α and β) and type II IFN (γ), recently identified type III IFN (λ) has been reported to possess anti-HCV activity in cell culture (Doyle et al., 2006; Marcello et al., 2006; Robek et al., 2005). Subgenomic HCV replicons have been reported since the breakthrough of the Con1(1b) replicon using different HCV strains: H77 (1a), N (1b), 1B-1 (1b), O (1b), JFH 1 (2a), and AH1 (1b) (Blight et al., 2003; Ikeda et al., 2002, 2005; Kato et al., 2003a,b; Kishine et al., 2002; Lohmann et al., 1999; Mori et al., 2008; Pietschmann et al., 2002). Moreover, a number of groups have examined anti-HCV reagents using the established replicon. However, such studies have been conducted using replicon(s) from only one or two HCV strain(s). To date, there has been no comprehensive study regarding the diverse sensitivities of anti-HCV reagents to genotype 1 HCV replicons from different strains.

To address this issue, we developed three HCV replicons from different genotype 1b HCV positive sera, in addition to our previously reported O strain (Ikeda et al., 2005). Two replicons were constructed using HCV-positive sera from healthy carriers (1B-4

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and 1B-5) and one replicon was constructed using serum sampled from a case of acute hepatitis C (KAH5). These replicons contained neomycin phosphotransferase (Neo) and *Renilla* luciferase (RL) genes at the first cistron of the replicon with the aim of conducting a stable and highly sensitive reporter assay. In this study of four replicons, we examined the anti-HCV reagents IFN-α, IFN-γ, IFN-λ, CsA, and various statins (pitavastatin (PTV), fluvastatin (FLV), and rosvastatin (RSV)), and we found diverse sensitivities among the replicons. Newly developed replicons will be useful tools for the present study regarding the diverse sensitivities of genotype 1b HCVs to anti-HCV reagents, including IFNs.

2. Materials and methods

2.1. HCV-positive sera and GeneBank accession numbers

Serum O (previously described as 1B-2), 1B-4, and 1B-5 were derived from an HCV-positive healthy carrier and have been described previously (Ikeda et al., 1997). Serum KAH5 was obtained from a patient with acute hepatitis C (AH C) who provided prior informed consent. The nucleotide sequence data for 1B-4, 1B-5, and KAH5 will appear in the DDBJ, EMBL, and GeneBank nucleotide sequence databases under accession nos. AB442219, AB442220, and AB442222, respectively.

2.2. Cell cultures

Three HCV-positive sera (KAH5, 1B-4, and 1B-5 strains) were used for the development of subgenomic replicons with reporter (RL). We first established 9, 4, and 6 replicon harboring clonal cell lines derived from KAH5, 1B-4, and 1B-5 strains, respectively. Then, after characterization for these cell lines, we selected the representative clonal cell lines and designated sKAH5R (clone 6), s1B-4R (clone 2), and s1B-5R (clone 4) as sKAH5R, s1B-4R, and s1B-5R, respectively (Supplemental Fig. 1A, B, and C). sO and O cells were used as subgenomic and genome-length HCV RNA-harboring cells with a Neo gene in the first cistron, as previously described (Kato et al., 2003a; Ikeda et al., 2005). These cells were derived from a hepatoma cell line, HuH-7, and were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, penicillin, streptomycin, and 0.3 mg/ml of G418 (Geneticin; Invitrogen, Carlsbad, CA). The cells were passaged twice weekly at a 5:1 split ratio. The sequences in the original subgenomic replicons were described above and appeared in the database with indicated accession numbers.

2.3. RT-nested PCR

HCV RNAs were prepared from HCV-positive sera (1B-4, 1B-5, and KAH5) using ISOGEN-LS (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's protocol. These RNA samples were used for RT-PCR in order to amplify the NS2 to NS5B region (6.0 kb) of the HCV genomes. RT was performed with the OligodA23 primer, 5′-AAAAAAAAAAAAAAAAAAAAAAA-3′. The primer pair 542: 5′-GTAGAGCCCGTCGTCTTCTCTGACATGGA-3′ and 9388R: 5′-ATGGCCTATTGGCCTGGAGTG-3′ was employed in the first-round PCR (35 cycles). The primer pair 3295X: 5′-ATTATTCTAGACTGACATGGAGACCAAGATCATCAC-3′ and 9357RX: 5′-ATTATTCTAGACCCGTTCACCGGTTGGGGAGCAG-3′, containing the XbaI site (underlined) was employed in the second-round PCR (35 cycles). SuperScript III reverse transcriptase (Invitrogen) and KOD-plus DNA polymerase (Toyobo, Osaka, Japan) were used for RT and PCR, respectively.

2.4. Plasmid construction

To construct an HCV replicon with RL and Neo genes, we used a previously described pRN/3-5B/KE plasmid as a cassette vector (Ikeda et al., 2005). Basically, the NS3 to NS5B region was replaced with RT-PCR products from sera with 1B-4, 1B-5, and KAH5 at SpeI (located in NS3) and BsiWI (located in NS5B) sites. The PCR products were further amplified with the primers NS3 SpeI: 5′-ATCATCACTAGTCTCACAGGCCGGGACAAGAAAC-3, containing the SpeI site (underlined); and NS5B BsiWI: 5′-CTTGGTCCGTACGGCCCAGTTGAAGAGGTACTTGC-3′, containing the BsiWI site (underlined). The amplified fragments were digested with SpeI and BsiWI, and were ligated into the pRN/3-5B/KE cassette vector, which was predigested with SpeI and BsiWI.

2.5. RNA transcription

Plasmid DNAs were linearized by XbaI digestion and were used for RNA synthesis with T7 MEGAscript (Ambion) as previously described (Kato et al., 2003a).

2.6. RNA transfection and G418-resistant cells

Ten micrograms of *in vitro* synthesized HCV replicon RNAs were introduced into HuH-7 derived cells (OR6c cells) by electroporation, and the cells were selected in the presence of G418 (0.3 mg/ml) for 3 weeks as described previously (Mori et al., 2008).

2.7. Western blot analysis

The preparation of cell lysates, sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and immunoblotting were performed as described previously (Kato et al., 2003a). The antibodies used in this study were those against Core, NS3, NS5A, and NS5B. β-actin antibody (AC-15, Sigma) was used as a control for the amount of protein loaded per lane. Immunocomplexes were detected with the Renaissance enhanced chemiluminescence assay (PerkinElmer Life Science, Boston, MA).

2.8. Quantification of HCV RNA

The RNAs were prepared from an HCV replicon RNA replicating cell line, and 2 μg of each total RNA was used for RT-qPCR with 5′-UTR of an HCV-specific primer pair, as described previously (Ikeda et al., 2005). Experiments were conducted in triplicate.

2.9. Northern blot analysis

Total RNA was extracted from the cultured cells using an RNeasy Mini Kit according to the manufacturer's protocol (QIAGEN). Three micrograms of total RNA were used for the analysis. HCV-specific RNA and β-actin were detected according to a previously described method (Ikeda et al., 2005).

2.10. Reagents

IFN-α and IFN-γ were purchased from Sigma, and CsA was obtained from Calbiochem (San Diego, CA). IFN-λ (IL-29) was purchased from WAKO. PTV was purchased from the Kowa Company, Ltd. (Tokyo, Japan). FLV was purchased from Calbiochem. RSV was obtained from AstraZeneca.

2.11. Luciferase reporter assay

For the luciferase assay, 1.0–1.5 × 10^4 HCV replicon-harboring cells were plated onto 24-well plates in triplicate and were cultured

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for 24 h. The cells were treated with each anti-HCV reagent for 72 h. Then the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol. All the luciferase assays were repeated at least three times.

2.12. Statistical analysis

Statistical comparison of the luciferase activity in various treatment groups was performed using Student's *t*-test. *P* values of less than 0.05 were considered statistically significant.

3. Results

3.1. Establishment of four subgenomic replicon-harboring cell lines using different genotype 1b HCV sera

We tried to establish replicon-harboring cells from different HCV-positive sera to assess the sensitivity of anti-HCV reagents among genotype 1b HCV strains. To this end, three sera (1B-4, KAH5, and 1B-5) were used to amplify the NS region of HCV genomes by reverse transcription-polymerase chain reaction (RT-PCR). The dicistronic replicons were designed as shown in Fig. 1A. RL and Neo genes were introduced into the first cistron and translation was driven by the HCV internal ribosomal entry site (IRES) leading to the expression of RL and Neo as a fusion protein. In the second cistron, NS3 to NS5B was translated via the encephalomyocarditis virus (EMCV) IRES (Fig. 1A). We introduced *in vitro*-synthesized HCV replicon RNAs (10 μg) into OR6c cells, in which HCV RNA was eliminated from OR6 cells by IFN-α treatment. After 3 weeks of G418 selection, we obtained HCV replicon-harboring cell colonies, i.e., more than 100 colonies from KAH5 and 20 colonies from 1B-4. However, no colony formation was observed among 1B-5 replicon-RNA-introduced cells. Therefore, we next attempted to perform the electroporation of a 1B-5 replicon with mutations derived from the HCV sequence in s1B-5 replicon-harboring cells, in which the replicating HCV replicon possessed only neomycin-resistant genes in the first cistron (data not shown). The mutations introduced into 1B-5 replicon were E1758D and I1851F in NS4B and R2192W and E2414Q in NS5A. Consequently, we established 9, 4, and 6 replicon-harboring cells from KAH5, 1B-4, and 1B-5, respectively, and confirmed the expression of HCV RNA and proteins. In addition to three replicon RNAs, the previously described ORN/3-5B/KE replicon RNA was also introduced into OR6c cells and selected as sOR in this study (Ikeda et al., 2005). The representative clonal cell lines, which grow healthy and stably expressed abundant HCV proteins, are used in the following experiments (Supplemental Fig. 1A, B, and C). These replicon-harboring cell lines were established from genotype 1b HCV strains: 1B-4, KAH5, O, and 1B-5 and were designated as s1B-4R, sKAH5R, sOR, and s1B-5R, respectively. We confirmed the expression of NS3, NS5A, and NS5B proteins in all replicon-harboring cells (Fig. 1B). The expression levels of HCV RNAs in the replicon-harboring cells were examined for the 5′-UTR by quantitative RT- PCR (RT-qPCR) (Fig. 1C). s1B-4R cells exhibited the highest levels of expression of HCV RNA (approximately 10^8 copies/μg total RNA), followed by sKAH5R, sOR, and s1B-5R cells (Fig. 1C). All of the replicon-harboring cells expressed HCV RNA at levels greater than at least 4×10^7 copies/μg total RNA. Northern blot analysis also demonstrated the presence of HCV-specific RNA with a length of approximately 9 kb in the total RNA extracts from four replicon-harboring cells (Fig. 1D). These four genotype 1b HCV replicon reporter systems were established and used for further analyses of sensitivity to anti-HCV reagents.

3.2. Diverse activity of various IFN types on HCV replicons

IFN-α belongs to the type I IFN group and is currently used as standard therapy for patients with CH C. Therefore, first we evaluated the activity of IFN-α using the four developed replicons and a reporter assay. The s1B-4R and sKAH5R replicons showed almost equal and moderate sensitivity to IFN-α (EC_{50}: 1.44 and 1.37 IU/ml, respectively) (Fig. 2). The s1B-5R and sOR replicons, respectively, exhibited the highest (EC_{50}: 1.10 IU/ml) and lowest (EC_{50}: 2.35 IU/ml) sensitivity to IFN-α among the replicons tested (Fig. 2). We also examined the activity of IFN-α on HCV protein expression levels in these four replicons. The findings from the Western blot analysis of the sensitivity to IFN-α coincided with

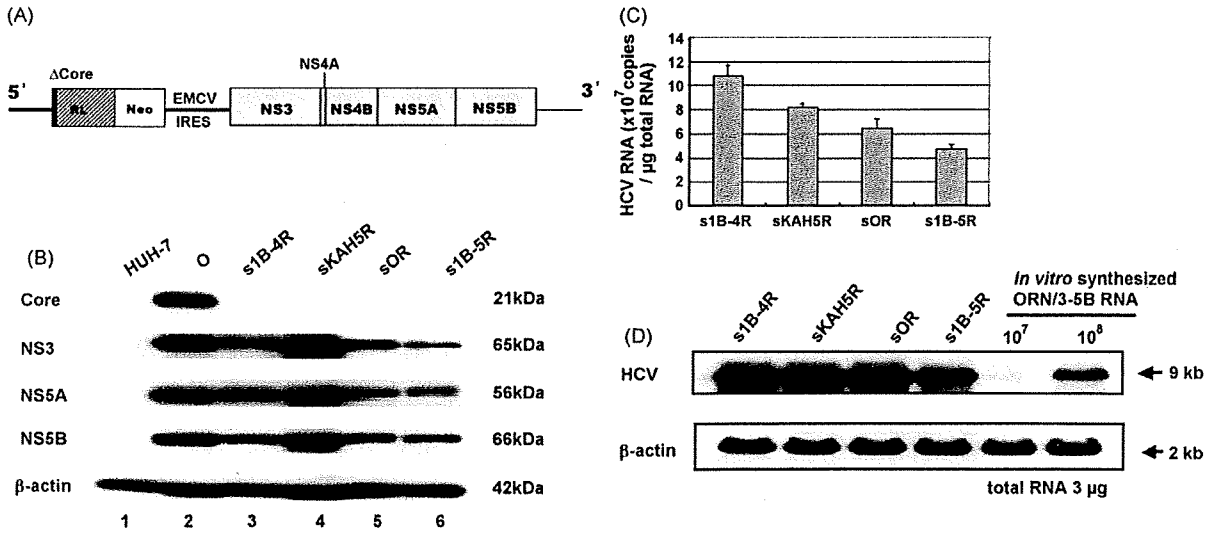

Fig. 1. The expression of HCV proteins and HCV RNAs in four replicon-harboring cell lines. (A) Schematic gene organization of subgenomic replicon RNA. The NS3 to NS5B region and 12 N-terminal amino acid residues of the Core (ΔC) are depicted in closed boxes. Untranslated regions, EMCV IRES, RL, and Neo genes are indicated by thin lines, thick line, shaded box, and open box. (B) Western blot analysis of HCV proteins. Production of Core, NS3, NS5A, and NS5B in HuH-7 cells (lane 1), O cells (lane 2), s1B-4R cells (lane 3), sKAH5R cells (lane 4), sOR cells (lane 5), and s1B-5R cells (lane 6) were analyzed by immunoblotting using anti-Core, anti-NS3, anti-NS5A, and anti-NS5B antibodies. (C) RT-qPCR analysis. The levels of HCV RNA in G418-resistant cells were quantified by LightCycler PCR. (D) Northern blot analysis. RNAs from s1B-4R, sKAH5R, sOR, and s1B-5R cells were used for comparison. *In vitro*-synthesized ORN/3-5B RNA was also used for comparative analyses.

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