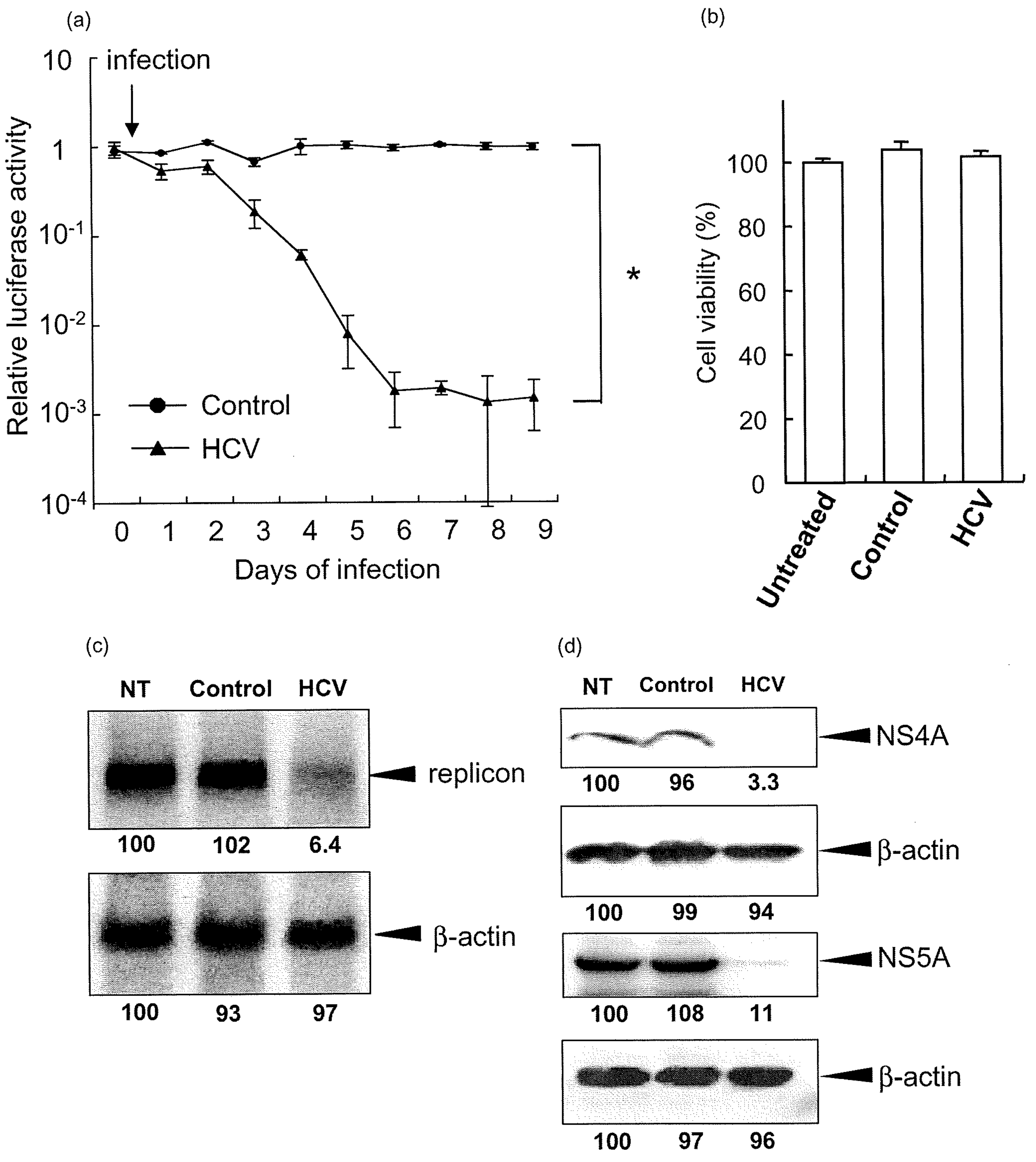

death.[18,30,31] Therefore, we examined the effects of the shRNA-expressing plasmids and adenoviruses on the activation of ISG expression in cells. The ISRE-reporter plasmid, pISRE-TA-Luc, and a control plasmid, peGFPneo, were transfected into Huh7 cells with plasmid pUC19-shRNA-HCV or pUC19-shRNA-Control, or adenovirus, AxshRNA-HCV or AxshRNA-Control, and the ISRE-mediated luciferase activities were measured. On day 2, the ISRE-luciferase activities did not significantly change in cells in which

Figure 4 Effect of a recombinant adenovirus expressing shRNA on HCV replicon. (a) Huh7/pRep-Feo cells were infected with AxshRNA-HCV or shRNA-Control at a multiplicity of infection (MOI) of 1. The cells were harvested, and internal luciferase activities were measured on day 0 though day 9 after adenovirus infection. Each assay was done in triplicate, and the value is displayed as a percentage of no treatment and as mean ± SD. An asterisk indicates a *P*-value of less than 0.05. (b) Dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay of Huh7/pRep-Feo cells. Cells were infected with indicated recombinant adenoviruses at an MOI of 1. The assay was done at day 6 of infection. Error bars indicate mean + SD. (c) Northern blotting. The upper panel shows replicon RNA, and the lower panel shows beta-actin mRNA. (d) Western blotting. Total cell lysates were separated on NuPAGE gel, blotted and incubated with monoclonal anti-NS4A or anti-NS5A antibodies. The membrane was re-blotted with antibeta-actin antibodies. NT, untreated Huh7/pRep-Feo cells; Control, cells infected with AxshRNA-Control; HCV, cells treated with AxshRNA-HCV. In panels (b) and (c), cells were harvested on day 6 after adenovirus infection at an MOI of 1.

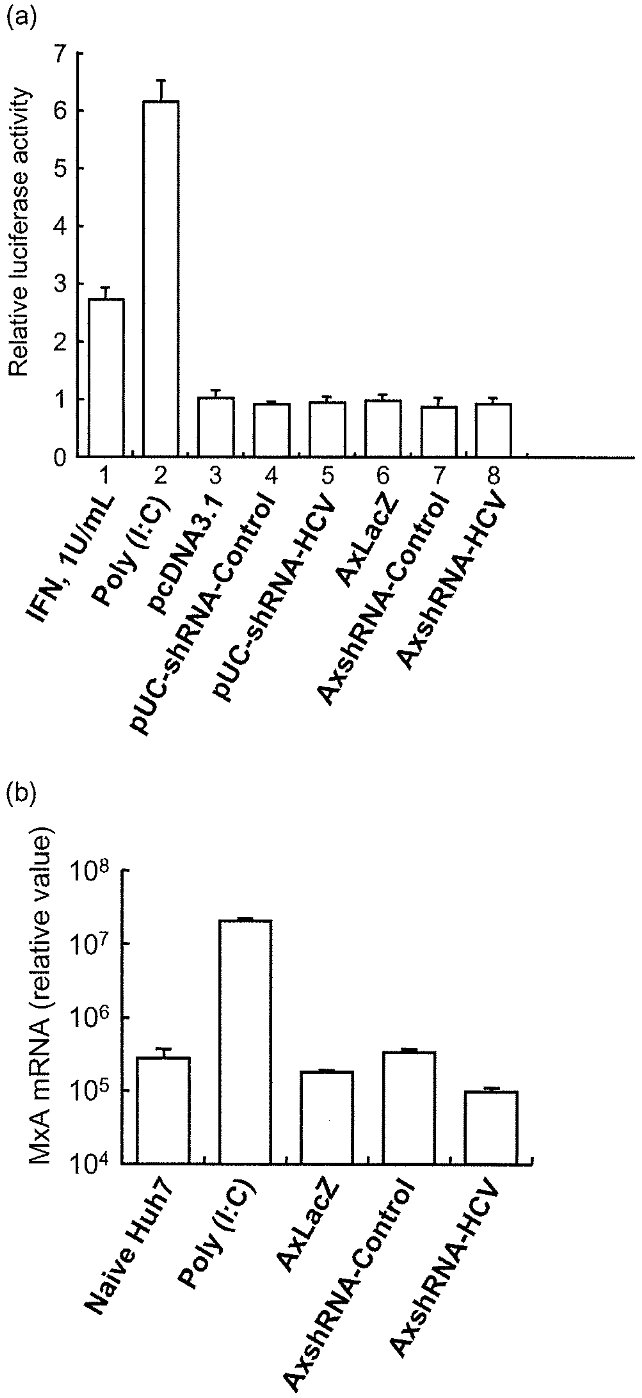

Figure 5 Interferon-stimulated gene responses by transfection of siRNA vectors. (a) Huh7 cells were seeded at 5 x 10^4 per well in 24-well plates on the day before transfection. As a positive control, 200 ng of pISRE-TA-Luc, or pTA-Luc, 1 ng of pRL-CMV, were transfected into a well using FuGENE-6 Transfection Reagent (Roche), and the cells were cultured with 1 U/mL of interferon (IFN) in the medium (lane 1). Lanes 3–5: 200 ng of pISRE-TA-Luc or pTA-Luc, and 1 ng of pRL-CMV were cotransfected with (lane2) 300 ng of poly (I : C), or 200 ng of plasmids (lane 3) pcDNA3.1, (lane 4) pUC19-shRNA-Control or (lane 5) pUC19-shRNA-HCV. Lanes 6–8: 200 ng of pISRE-TA-Luc or pTA-Luc, and 1 ng of pRL-CMV were transfected, and MOI = 1 of adenoviruses, (lane 6) AxLacZ, which expressed the beta-galactosidase (LacZ) gene under control of the chicken beta-actin (CAG) promoter as a control, (lane 7) AxshRNA-Control or (lane 8) AxshRNA-HCV were infected. Dual luciferase assays were performed at 48 h after transfection. The Fluc activity of each sample was normalized by the respective Rluc activity, and the respective pTA luciferase activity was subtracted from the pISRE luciferase activity. The experiment was done in triplicate, and the data are displayed as means + SD. (b) Huh7 cells were infected with indicated recombinant adenoviruses, AxLacZ, AxshRNA-Control and AxshRNA-HCV. RNA was extracted from each sample at day 6, and mRNA expression levels of an interferon-inducible MxA protein were quantified by the real-time RT-PCR analysis. Primers used were as follows: human MxA sense, 5′-CGA GGG AGA CAG GAC CAT CG-3′; human MxA antisense, 5′-TCT ATC AGG AAG AAC ATT TT-3′; human beta-actin sense, 5′-ACA ATG AAG ATC AAG ATC ATT GCT CCT CCT-3′; and human beta-actin antisense, 5′-TTT GCG GTG GAC GAT GGA GGG GCC GGA CTC-3′.

negative- or positive-control shRNA plasmids was transfected. (Fig. 5a). Similarly, the expression levels of an interferon-inducible MxA protein did not significantly change by transfection of shRNA-expression vectors (Fig. 5b). These results demonstrate that the shRNA used in the present study lack induction of the ISG responses both in the form of the expression plasmids and the adenovirus vectors.

Effect of siRNA and shRNA adenoviruses on HCV-JFH1 cell culture

The effects of HCV-targeted siRNA- and shRNA-expressing adenoviruses were confirmed by using HCV-JFH1 virus cell culture system. Transfection of the siRNA #331[14] into HCV-infected Huh7.5.1 cells resulted in substantial decrease of intracellular HCV RNA, while a control siRNA showed no effect (Fig. 6a). Similarly, infection of AxshRNA-HCV into Huh7.5.1/HCV-JFH1 cells specifically suppressed expression of HCV RNA (Fig. 6b).

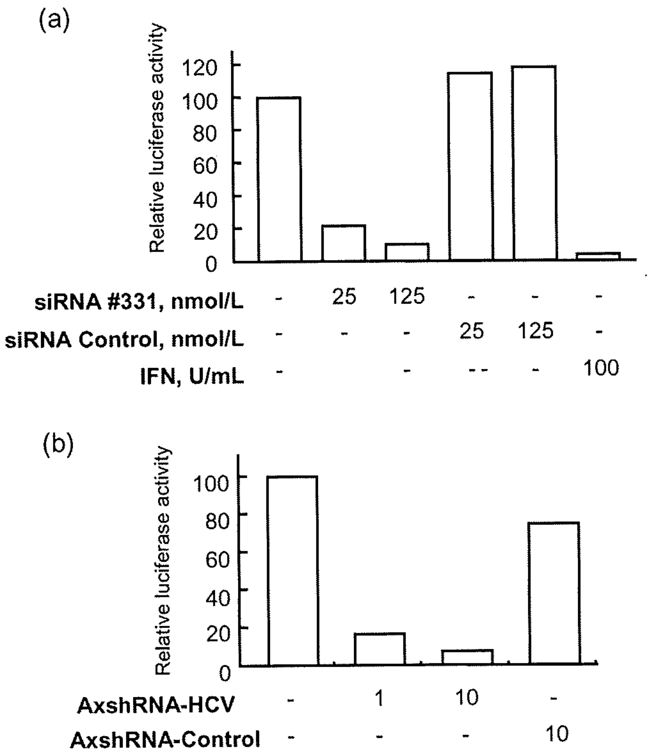

Figure 6 Effects of an siRNA and adenovirus expressing shRNA on HCV-JFH1 cell culture. (a) The siRNA #331, the siRNA-Control[14], (b) AxshRNA-HCV or AxshRNA-Control were, respectively, transfected or infected onto HV-JFH1-infected Huh7.5.1 cells. Seventy-two hours of the transfection or infection, expression level of HCV-RNA was quantified by real-time RT-PCR. The assays were repeated twice, and consistent results were obtained. IFN, recombinant interferon-alpha 2b.

Suppression of HCV-IRES-mediated translation *in vivo* by adenovirus expressing shRNA

The effects of the shRNA expression on the expression of the viral structural proteins *in vivo* were investigated using conditional HCV cDNA-transgenic mice, CN2-29.[28] Adenoviruses, AxshRNA-HCV, AxshRNA-Control or AxCAw1 were injected into CN2-29 mice in combination with AxCANCre, an adenovirus expressing *Cre* DNA recombinase. The mice were killed on the fourth day after the injection, and the hepatic expression of the HCV core protein was measured. The expressed amounts of the core protein were 143.0 ± 56.2 pg/mg and 108.5 ± 42.4 pg/mg in AxCAw1 and AxshRNA-Control-infected mice, respectively, and the expressed amount was significantly lower in mice injected with AxshRNA-HCV (28.7 ± 7.0 pg/mg, $P < 0.05$, Fig. 7a). Similarly, the induced expression of HCV core protein was not detectable by immunohistochemistry in AxshRNA-HCV infected liver tissue (Fig. 7c). Staining of a host cellular protein, albumin, was not obviously different between the liver infected with AxCAw1, AxshRNA-HCV and AxshRNA-Control (Fig. 7d). The expression levels of two ISG, IFN-beta and Mx1, in the liver tissue were not significantly different between individuals with and without injection of the adenovirus vectors (Fig. 7b). These results indicate specific shRNA silencing of HCV structural protein expression in the liver.

Discussion

The requirements to achieve a high efficiency using RNAi are: (i) selection of target sequences that are the most susceptible to RNAi; (ii) persistence of siRNA activity; and (iii) efficient *in vivo* delivery of siRNA to cells. We have used an shRNA sequence that was derived from a highly efficient siRNA (siRNA331), and constructed a DNA-based shRNA expression cassette that showed competitive effects with the synthetic siRNA (Fig. 2).[14] The shRNA-expression cassette does not only allow extended half-life of the RNAi, but also enables use of gene-delivery vectors, such as virus vectors. As shown in the results, a retrovirus vector expressing shRNA-HCV could stably transduce cells to express HCV-directed shRNA, and the cells acquired protection against HCV subgenomic replication (Fig. 3). An adenovirus vector expressing shRNA-HCV resulted in suppression of HCV subgenomic and protein expression by around three logs to almost background levels (Fig. 4). Consistent results were obtained by using an HCV cell culture (Fig. 6). More importantly, we have demonstrated *in-vivo* effects on viral protein expression in the liver using a conditional transgenic mouse model (Fig. 7). These results suggest that efficient delivery of siRNA could be effective against HCV infection *in vivo*.

An obstacle to applying siRNA technology to treat virus infections is that viruses are prone to mutate during their replication.[32] HCV continuously produces mutated viral strains to escape immune defense mechanisms. Even in a single patient, the circulating HCV population comprises a large number of closely related HCV sequence variants called quasispecies. Therefore, siRNA targeting the protein-coding sequence of the HCV genome, which have been reported by others,[15–19] may vary considerably among different HCV genotypes, and even among strains of the same genotype.[33] Our shRNA sequence targeted the 5′-UTR of HCV RNA, which is the most conserved region among various HCV isolates.[33] In addition, the structural constraints on the 5′-UTR, in terms of its requirement to direct internal ribosome entry and translation of viral proteins, might not permit the evolution of escape mutations. Our preliminary results have shown that the siRNA-HCV suppressed replication of an HCV genotype 2a replicon[34] to the same extent as the HCV 1b replicon.

Although the siRNA techniques rely on a high degree of specificity, several studies report siRNA-induced non-specific effect that may result from induction of ISG responses.[18,31] These effects may be mediated by activation of double-strand RNA-dependent protein kinase, toll-like receptor 3,[35] or possibly by a recently identified RNA helicase, RIG-I.[36] It remains to be determined whether these effects are generally induced by every siRNA construct. Sledz *et al.* have reported that transfection of two siRNA induced cellular interferon responses,[37] while Bridge *et al.* report that shRNA-expressing plasmids induced an interferon response but transfection of synthetic siRNA did not.[31] Speculatively, these effects on the interferon system might be construct dependent. Our shRNA-expression plasmids and adenoviruses did not activate ISG responses *in vitro* (Fig. 5a,b) or *in vivo* (Fig. 7b). We have preliminarily detected phosphorylated PKR (P-PKR) by western

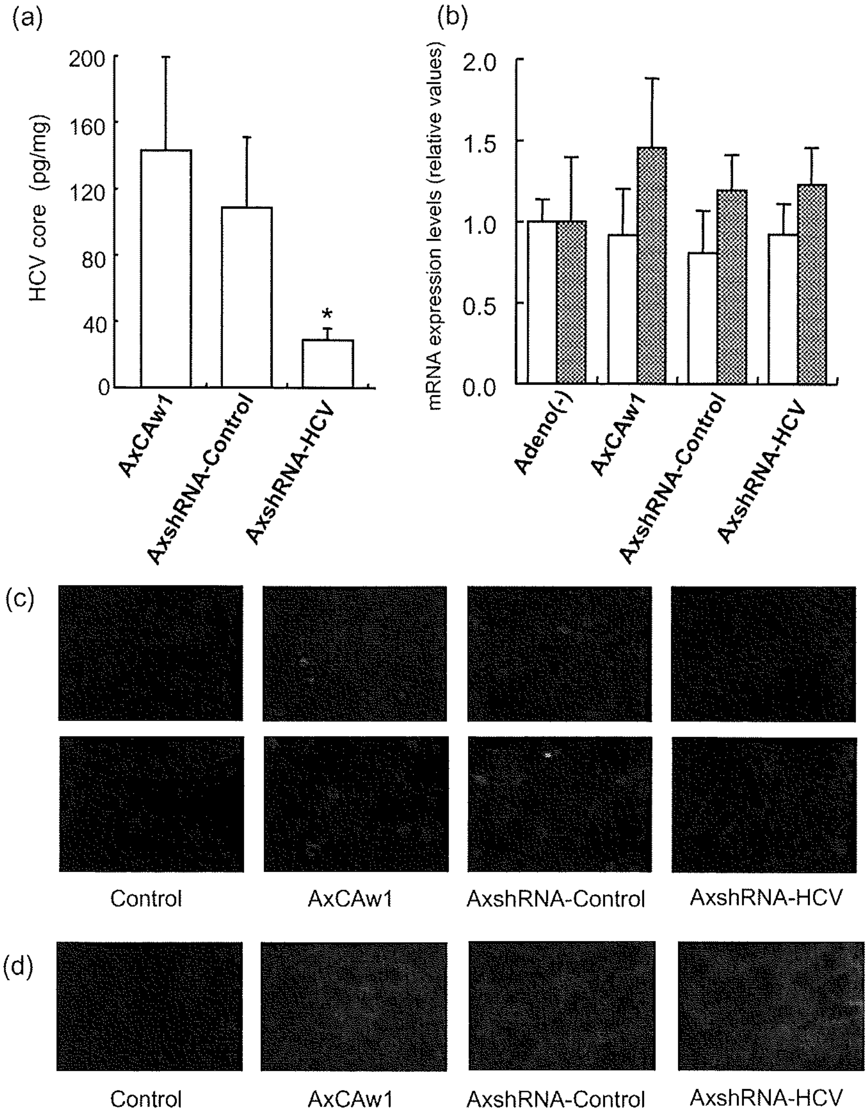

Figure 7 Effects of a recombinant adenovirus expressing shRNA on HCV core protein expression in CN2-29 transgenic mice. CN2-29 transgenic mice were administered with 1 x 10^9 PFU of AxCANCre combined with 6.7 x 10^8 PFU of AxshRNA-HCV, AxshRNA or AxCAw1. The mice were killed on day 4 after injection. (a) Quantification of HCV core protein in liver. Liver tissues were homogenized and used to determine the amount of HCV core protein. Each assay was done in triplicate, and the values are displayed as mean ± SD. Asterisk indicates P-value of less than 0.05. (b) Expression levels of mouse interferon-beta (white bars) and Mx1 (shaded bars) mRNA in the mouse liver tissue were quantified by the real-time RT-PCR analyses. Primers used were as follows: mouse interferon-beta sense, 5′-ACA GCC CTC TCC ATC AAC TA-3′; mouse interferon-beta antisense, 5′-CCC TCC AGT AAT AGC TCT TC-3′; mouse Mx1 sense, 5′-AGG AGT GGA GAG GCA AAG TC-3′; mouse Mx1 antisense, 5′-CAC ATT GCT GGG GAC TAC CA-3′; mouse beta-actin sense, 5′-ACT CCT ATG TGG GTG ACG AG-3′; mouse beta-actin antisense, 5′-ATA GCC CTC GTA GAT GGG CA-3′. Adeno (-) denotes mice without adenovirus administration. (c) Immunofluorescence microscopy of HCV core protein in the liver tissue. Liver sections of mice were stained using rabbit anticore polyclonal antibody and normal rabbit IgG as a negative control. The upper photographs were obtained at 400x magnification, and the lower photographs were at 1000x. (d) Immunofluorescence microscopy of albumin in liver. Liver sections from the mice were fixed and stained using rabbit antialbumin antibody and normal rabbit IgG as a negative control.

blotting, and found no apparent increase of P-PKR (data not shown). These results indicate that these target sequences and structures are of sufficient specificity to silence the target gene without eliciting non-specific interferon responses.

Beside the canonical action of siRNA, a sequence-specific cleavage of target mRNA, the siRNA could act as a micro-RNA that suppresses translational initiation of mRNA,[38] or it could mediate transcriptional gene silencing.[39] Regarding our *in-vivo* experiments, it was difficult to differentially analyze the effect of siRNA at individual sites of action because post-translational effect of siRNA concomitantly destabilizes target mRNA, which leads to apparent decrease of mRNA transcripts.

Efficiency and safety of gene transfer methods are the key determinants of the clinical success of gene therapy and an unresolved problem. There are several reports of delivery of siRNA or siRNA-expression vectors to cells *in vivo*;[12,40,41] however, gene delivery methods that are safe enough to apply to clinical therapeutics are currently under development. Adenovirus vectors are one of the most commonly used carriers for human gene therapies.[42–44] Our present results demonstrate that the adenoviral delivery of shRNA is effective in blocking HCV replication *in vitro* and virus protein expression *in vivo*. Adenovirus vectors have several advantages of efficient delivery of transgene both *in vitro* and *in vivo* and natural hepatotropism when administered *in vivo*. The AxshRNA-HCV specifically blocked expression of HCV structural proteins in a conditional transgenic mouse expressing those proteins. The current adenovirus vectors may cause inflammatory reactions in the target organ,[45] however, and produce neutralizing antibodies which make repeated administration difficult. These problems may be overcome by the improved constructs of virus vectors with attenuated immunogenicity or by the development of non-viral carriers for gene delivery.[46]

In conclusion, our results demonstrate the effectiveness and feasibility of the siRNA expression system. The efficiency of adenovirus expressing shRNA that target HCV suggests that delivery and expression of siRNA in hepatocytes may eliminate the virus and that this RNA-targeting approach might provide a potentially effective future therapeutic option for HCV infection.

Acknowledgments

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MAJOR ARTICLE

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)CACCCTATCAGGCAGTACCACAAGGCC(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'-AACAACAUCGUUUCAGGUCCUTT-3'; antisense, 5'-AGGGCCUGAAACGAUGUUGTT-3'). siRNA targeting enhanced green fluorescent protein (GFP) was used as the negative control (sense, 5'-CUUACGCUGAGUACUUCGATT-3'; antisense, 5'-UCGAAGUACUCAGCGUAATT-3'). (Underlined letters indicate deoxyribonucleotides.)

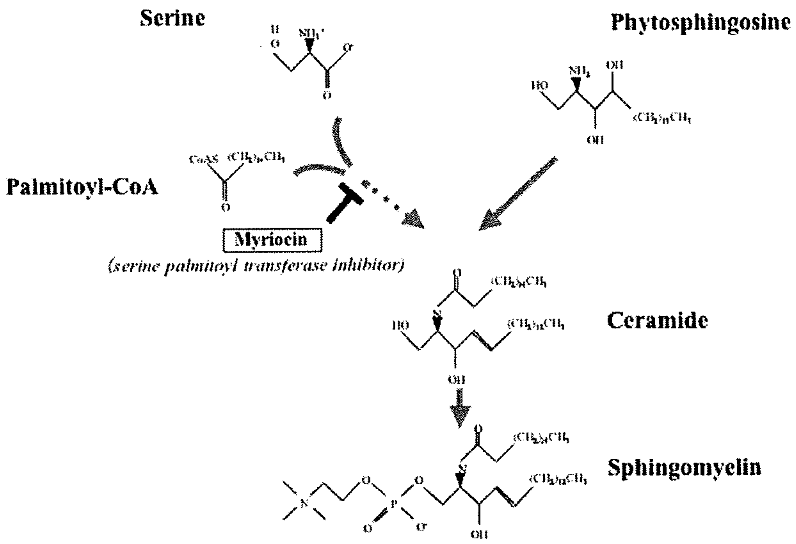


Figure 1. The sphingomyelin synthesis pathway. Serine palmitoyltransferase catalyzes the first committed step of sphingomyelin biosynthesis from serine and palmitoyl-coenzyme A (CoA). Myriocin inhibits the catalyzing activity of serine palmitoyltransferase. Phytosphingosine is known to work as a precursor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

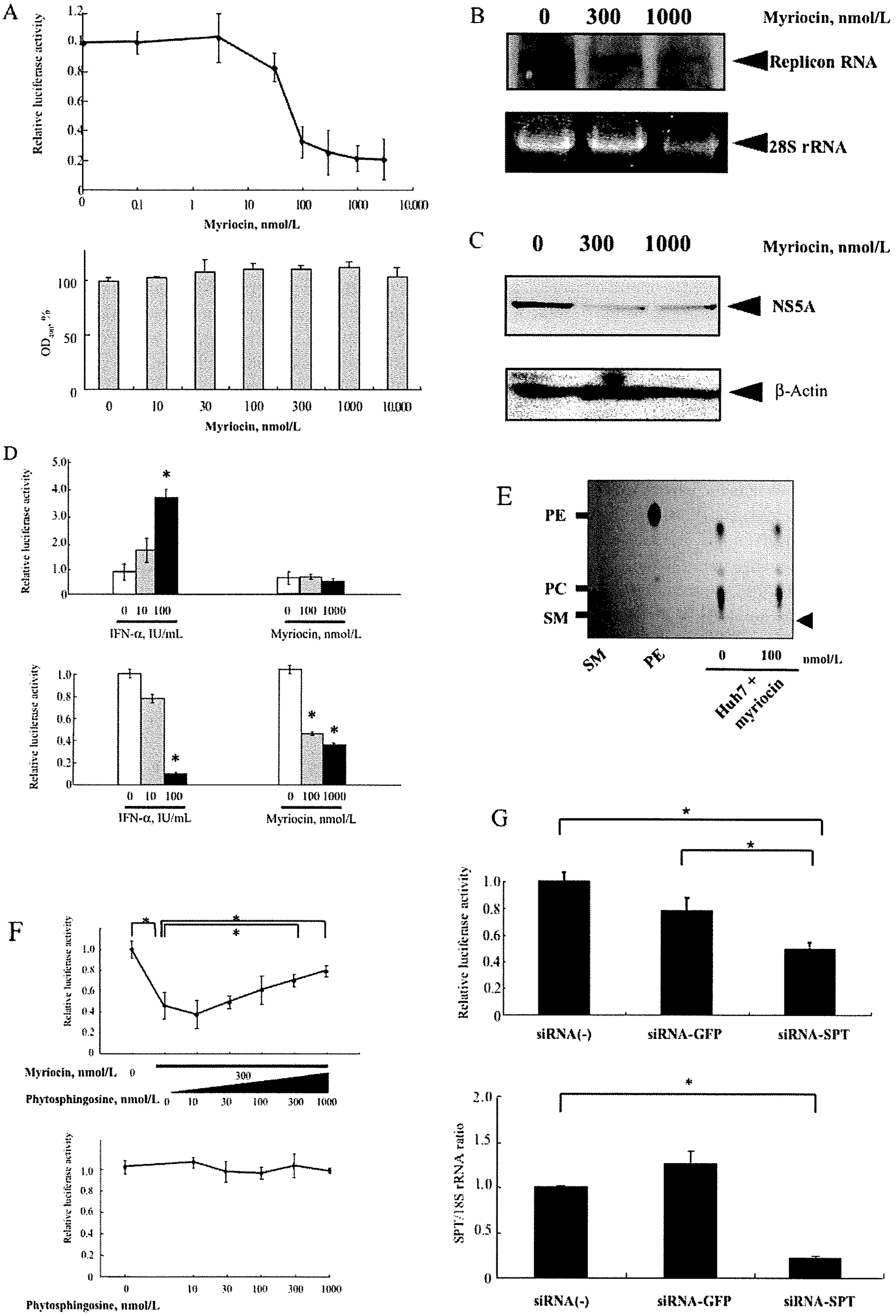
Specific suppression of the replication of a subgenomic HCV-1b replicon by an inhibitor of sphingomyelin synthesis. To clarify the role played by the sphingomyelin synthesis pathway in HCV replication, we added myriocin, a specific inhibitor of SPT that catalyzes the first committed step of sphingomyelin 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
1.2
1.0
0.8
0.6
0.4
0.2
0
0 0.1 1 10 100 1000 10,000
Myriocin, nmol/L
OD$_{590}$, %
100
50
0
0 10 30 100 300 1000 10,000
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
5.0
4.0
3.0
2.0
1.0
0
*
0 10 100
IFN-α, IU/mL
0 100 1000
Myriocin, nmol/L
1.0
0.8
0.6
0.4
0.2
0
*
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0 10 100
IFN-α, IU/mL
0 100 1000
Myriocin, nmol/L
E
PE
PC
SM
SM
PE
0
100
nmol/L
Huh7 + myriocin
F
*
*
*
Relative luciferase activity
1.0
0.8
0.6
0.4
0.2
0
Myriocin, nmol/L 0
300
Phytosphingosine, nmol/L 0 10 30 100 300 1000
Relative luciferase activity
1.0
0.8
0.6
0.4
0.2
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Phytosphingosine, nmol/L
G
*
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Relative luciferase activity
1.0
0.8
0.6
0.4
0.2
0
siRNA(-)
siRNA-GFP
siRNA-SPT
*
SPT/18S rRNA ratio
2.0
1.5
1.0
0.5
0
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 upregulation 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. $^*P < .05$.

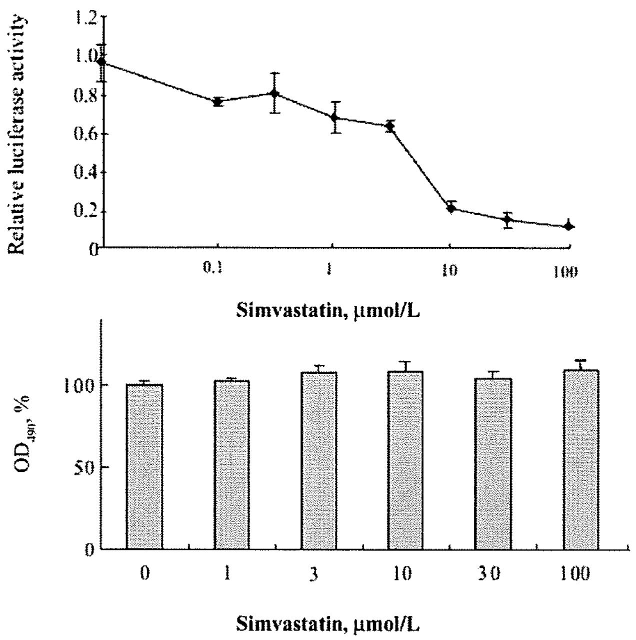

Figure 3. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by simvastatin. Huh7/Rep-Feo cells were cultured with various concentrations of simvastatin, and the luciferase assay was performed after 48 h of treatment *(upper panel)*. The dimethylthiazol carboxymethoxyphenyl 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

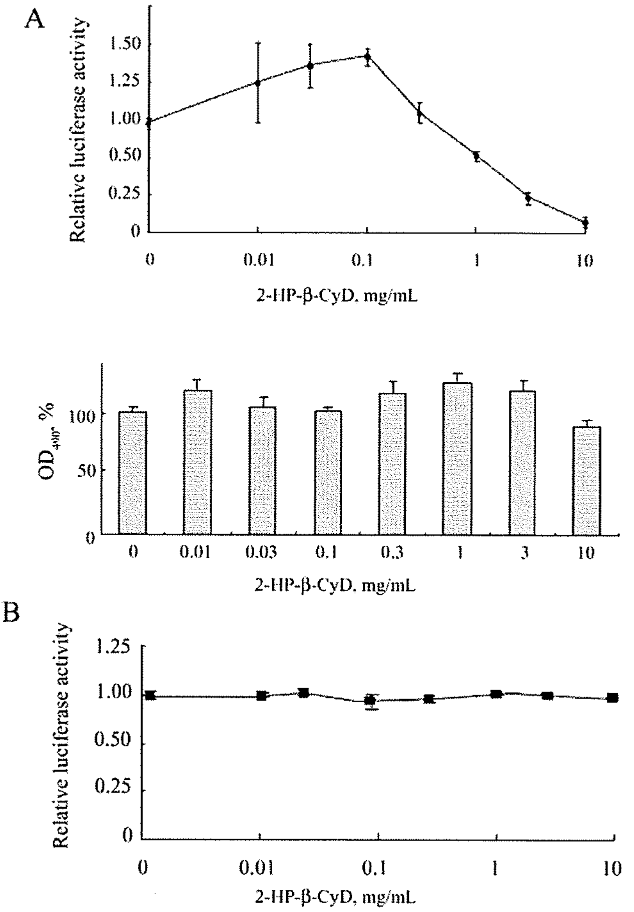

Figure 4. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD). *A*, Huh7/Rep-Feo cells cultured with various concentrations of 2-HP-β-CyD for 48 h. The luciferase assay was performed after 48 h of treatment *(upper panel)*. The 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.

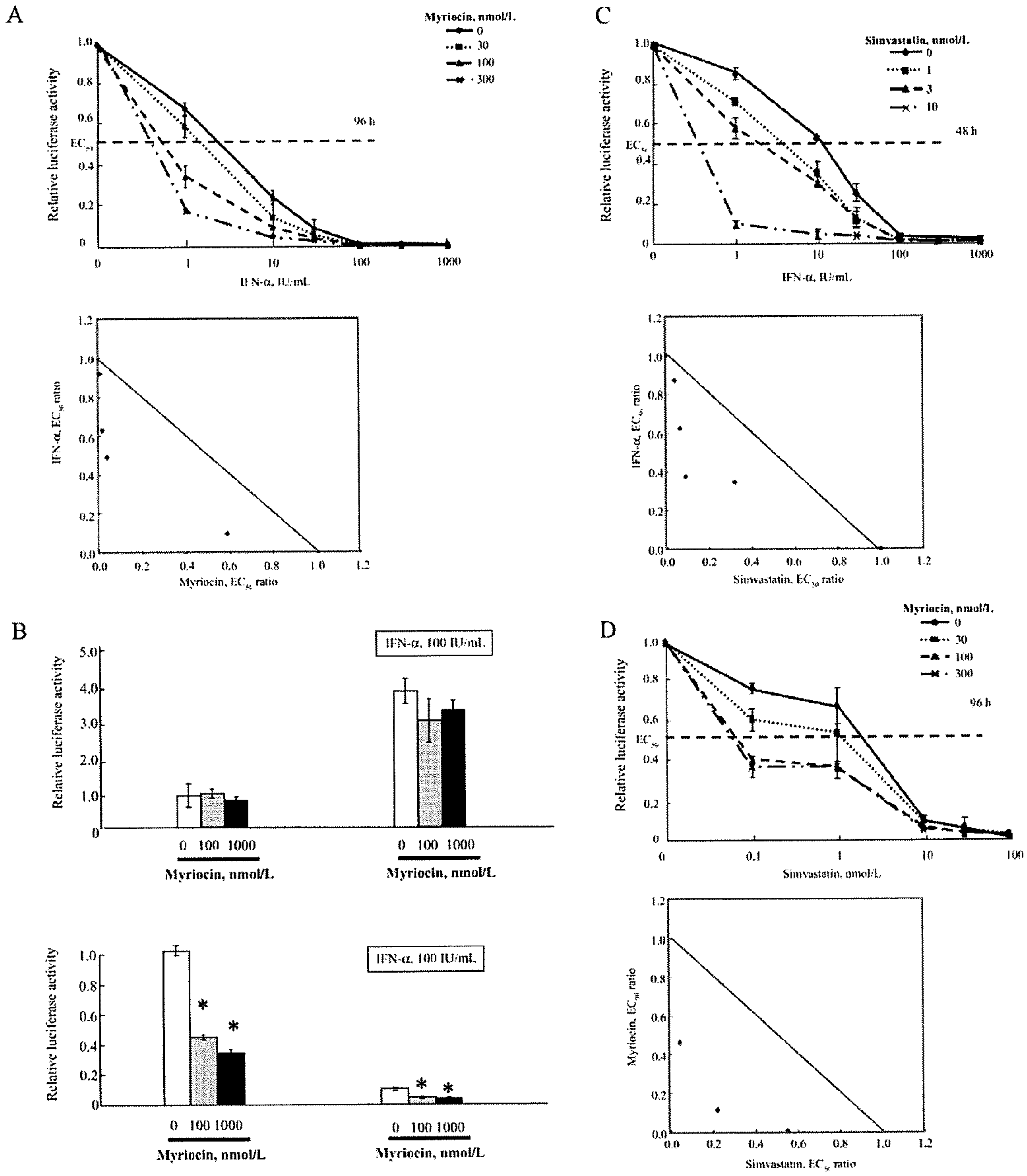

Figure 5. Synergistic inhibitory effects of myriocin with interferon (IFN), simvastatin with IFN, and myriocin with simvastatin. *A,* Synergistic inhibitory effect of myriocin with IFN on hepatitis C virus replication. Huh7/Rep-Feo cells were treated with combinations of myriocin and IFN at various concentrations. 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*).

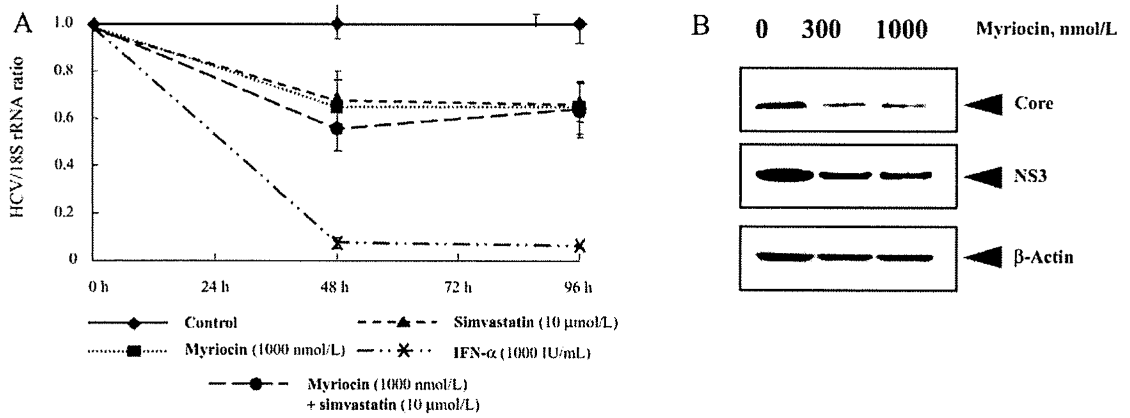

Figure 6. Suppression of JFH-1 hepatitis C virus (HCV) replication by myriocin and simvastatin. *A,* Cells containing JFH-1 HCV treated for 96 h with 1000 nmol/L myriocin, 10 μmol/L simvastatin, 1000 IU/mL IFN, or a combination of 1000 nmol/L myriocin and 10 μmol/L simvastatin. The cells were collected 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 inhibition 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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Original Article

Griseofulvin, an oral antifungal agent, suppresses hepatitis C virus replication *in vitro*

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Aim: Hepatitis C virus (HCV), which infects an estimated 170 million people worldwide, is a major cause of chronic liver disease. The current standard therapy for chronic hepatitis C is based on pegylated interferon (IFN)α in combination with ribavirin. However, the success rate remains at approximately 50%. Therefore, alternative agents are needed for the treatment of HCV infection.

Methods: Using an HCV-1b subgenomic replicon cell culture system (Huh7/Rep-Feo), we found that griseofulvin, an oral antifungal agent, suppressed HCV-RNA replication and protein expression in a dose-dependent manner. We also found that griseofulvin suppressed the replication of infectious HCV JFH-1. A combination of IFNα and griseofulvin exhibited a synergistic inhibitory effect in Huh7/Rep-Feo cells.

Results: We found that griseofulvin blocked the cell cycle at the G_2/M phase in the HCV subgenomic replicon cells, but did not inhibit HCV internal ribosome entry site-dependent translation.

Conclusion: Our results suggest that griseofulvin may represent a new approach to the development of a novel therapy for HCV infection.

Key words: cell cycle, griseofulvin, hepatitis C virus internal ribosome entry site, hepatitis C virus replicon, JFH-1

INTRODUCTION

HEPATITIS C VIRUS (HCV) is an etiologic agent of chronic liver disease,[1,2] and it is estimated that approximately 170 million people worldwide are infected with the virus. Chronic hepatitis C can lead to severe liver diseases, including fibrosis, cirrhosis, and hepatocellular carcinoma.[3] With advancements in HCV therapy, including the most recent combination of pegylated interferon (IFN)α and ribavirin, up to one-half of patients achieve a sustained virological response. However, the remainder cannot clear the virus, demonstrating a great need for more powerful therapeutic modalities.[4]

Investigations have been hampered by the lack of an efficient HCV cell culture system. In 1999, the establishment of an HCV subgenomic replicon cell culture system improved the situation. The subgenomic replicon RNA is composed of the HCV 5′ untranslated region (UTR) containing the internal ribosomal entry site (IRES), a neomycin phosphotransferase (neo) gene and the HCV non-structural (NS) proteins through 3–5B under the control of an encephalomyocarditis virus (EMCV) IRES, followed by the HCV 3′ UTR.[5] A HCV replicon carrying, in addition to the selectable marker, a gene encoding luciferase, can be used to screen a large number of compounds for antiviral activity.[6–8] The recent development of an *in vitro* HCV infection system provides an opportunity to evaluate inhibitors of all stages of the HCV life cycle.[9–11]

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Currently, proof of concept has been obtained in clinical trials of three different HCV NS3 protease inhibitors, BILN 2061,[12,13] telaprevir (VX-950),[14] and SCH 503034.[15] However, because of many factors, including possible side-effects and the emergence of drug-resistant mutants, there is still great need for improved therapies. We focused therefore on screening a set of licensed drugs which have not been recommended previously for antiviral use. Here, we found that the oral antifungal agent, griseofulvin, had a suppressive effect on HCV replication, assessed using the HCV-1b subgenomic replicon system and the particle-producing cell culture HCV-2a model of JFH-1. The mechanism of the anti-HCV activity of griseofulvin also was studied.

METHODS

Cell cultures and HCV replicon

THE HUMAN HEPATOMA cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin. For subgenomic replicon Huh7/Rep-Feo (HCV 1b replicon that expresses a chimeric protein consisting of neomycin phosphotransferase and firefly luciferase) cells,[7,8] the culture medium was supplemented with 250 g/mL G418. Huh 7.5.1/JFH-1 cells (Huh 7.5.1 chronically infected HCV JFH-1) were maintained in DMEM supplemented with 10% (v/v) fetal bovine serum, 100 IU/mL penicillin, and 100 µg/mL streptomycin.[16]

Reagents

Griseofulvin and fluconazole were purchased from Wako Pure Chemical (Tokyo, Japan). Itraconazole was purchased from LKT Laboratories (St Paul, MN, USA). Recombinant human IFNα-2b was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

Cell viability assays

For griseofulvin and fluconazole, viable cell growth was determined by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt (MTS) reduction assay using the Cell Titer 96 aqueous one solution cell proliferation assay (Promega, Madison, WI, USA), according to the manufacturer's protocol.

For itraconazole, viable cell growth was determined using the CellTiter-Glo luminescent cell viability assay (Promega, USA), according to the manufacturer's protocol.

Luciferase activity assays

Typically, Huh7/Rep-Feo cells were seeded in a 48-well plate at a density of 2×10^4 cells per well. Compounds were added to the culture medium at various concentrations. After 72 h of culture, the expression levels of the HCV replicon were measured by luciferase assay using the luciferase assay system (Promega, USA) and the Luminescencer-JNR AB-2100 (Atto, Tokyo, Japan).

The Huh7 cells stably transfected with the pEF Fluc IN vector were mock treated (control) or treated with 20 µM or 40 µM griseofulvin. After 72 h of culture, luciferase assays were performed using the luciferase assay system and the Luminescencer-JNR AB-2100. Luciferase activity was normalized by the protein concentration, measured using a BCA protein assay kit (Pierce, Rockford, IL, USA).

The Huh7 cells stably transfected with the pEF Rluc-HCV IRES Feo vector were mock treated (control) or treated with 20 µM griseofulvin. Dual luciferase activities were carried out at 8, 16, 24, and 32 h after exposure to griseofulvin using the dual luciferase reporter assay system and the Luminescencer-JNR AB-2100.

All assays were performed in triplicate, and the results were expressed as mean ± SD relative light units.

RNA analysis

Total cellular RNA was extracted from the Huh7/Rep-Feo cells using the RNAqueous-4PCR kit (Ambion, Austin, TX, USA). RNA was reverse transcribed with a Thermoscript reverse transcriptase kit (Invitrogen, Carlsbad, CA, USA).

Quantitative real-time polymerase chain reaction (PCR) was carried out using ABI Prism 7500 (Applied Biosystems, Foster City, CA, USA). The forward and reverse primers for the 5′ UTR of HCV-RNA were 5′-TGCGGAACCGGTGAGTACA-3′ and 5′-CTTAAGG TTTAGGATTCGTGCTCAT-3′, respectively. The fluorogenic probe used for the quantification of HCV-RNA was 5′-(FAM)-CACCCTATCAGGCAGTA-CCACAAGG CC-(TAMRA)-3′. Human 18S ribosomal RNA levels in the samples were analyzed by quantitative real-time PCR to normalize the RNA content. The forward and reverse primers for human 18S ribosomal RNA were 5′-ACTCTAGATAACCTCGGGCCGA-3′ and 5′-GATGT GGTAGCCGTTTCTCAGG-3′, respectively. The fluorogenic probe used for quantification of human 18S ribosomal RNA was 5′-(FAM)-CCATTCGAACGTCTGCCC TATCAACTTT-(TAMRA)-3′. The method has been described elsewhere.[17]

The primers used for reverse transcription (RT)–PCR were as follows: human 2′,5′-oligoadenylate synthetase (2′,5′-OAS): forward primer, 5′-CAATCAGCGAGGCC AGTAATC-3′ and reverse primer, 5′-TGGTGAGAAGTGC TGGGGTC-3′; human myxovirus resistance protein A (MxA): forward primer, 5′-GTCAGGAGT-TGCCCTT CCCA-3′ and reverse primer, 5′-GGCCCCTTCCTT ACCCTTA-3′; and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH): forward primer, 5′-GAAG GTGAAGGTCGGAGTC-3′ and reverse primer, 5′-CTT TAGGGTAGTGGTAGAAG-3′, respectively. Each reaction mixture contained cDNA (3 µL), 1.5 mM $MgCl_2$, 200 µM dNTP, 1 µM each primer, and 1.25 U AmpliTaq Gold (Applied Biosystems, USA) with 1× supplied reaction buffer. After activation of AmpliTaq Gold activity at 95 °C for 10 min, the temperature cycling conditions for MxA were 29 cycles consisting of denaturation at 95 °C for 30 s, annealing at 56 °C for 1 min, and extension at 72 °C for 1 min. For 2′,5′-OAS, the conditions were 32 cycles consisting of denaturation at 95 °C for 30 s, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min. For GAPDH, the conditions were 30 cycles consisting of denaturation at 95 °C for 30 s, annealing at 53 °C for 1 min, and extension at 72 °C for 1 min. PCR products were subjected to electrophoresis in a 3% agarose gel.

Western blotting

Preparation of cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis, and immunoblotting were performed as described previously.[18] The antibodies used in this study were the anti-NS3 antibody (Santa Cruz Biotechnology, USA) anti-NS5A antibody (Virogen, Watertown, MA, USA) and anti-β-actin antibody (Cell Signaling, Danvers, MA, USA). Alkaline phosphatase-conjugated secondary antibodies and CDP-Star chemiluminescent substrate (New England Biolabs, Beverly, MA, USA) were used for detection.

Cell cycle analysis

Harvested cells were washed once with phosphate-buffered saline (PBS) and fixed with 70% ethanol at 4 °C for 1 h. After an additional wash, the cells were treated with 250 µg/mL RNase A at 37 °C for 1 h and subsequently stained with 50 µg/mL propidium iodide at 4 °C for 1 h. The DNA content was then analyzed by FACSCalibur (BD Biosciences, Franklin Lakes, NJ, USA) with ModFit LT software (Verity Software House, Topsham, ME, USA).

Analyses of drug synergy

The effects of the treatment of Huh7/Rep-Feo cells with griseofulvin and IFNα, alone and in combination, were analyzed with CalcuSyn, a computer program based on the method of Chou and Talalaly.[19] After converting the dose-effect curves for each drug or drug combination to median-effect plots, the program calculated a combination index (CI). The CI of <1, 1, and >1 indicate synergy, an additive effect, and antagonism, respectively.

Plasmids and stable transfection

The plasmid pEF-Fluc-IN was constructed as follows. The fragment carrying the firefly luciferase was amplified from the pGL3 control vector (Promega, USA) by PCR using a pair of primers (5′-GAATTCATGGAAGAC GCCAAAAACATAAA-3′ [*Eco*RI site] and 5′-GCGGC CGCTTACACGGCGATCTTTCCGCCC-3′ [*Not*I site]). The PCR product was cloned into the pGEM-T Easy vector (Promega, USA). The EMCV IRES Neo fragment was excised from the pMXs-IN vector by *Not*I and *Sal*I digestion.[20] The *Eco*RI–*Sal*I fragment of the pCHO vector was excised from the pGag-pol-IRES-bs^r vector by *Eco*RI and *Sal*I digestion.[21] To construct pEF-Fluc-IB, the *Eco*RI–*Not*I fragment of firefly luciferase, and the *Not*I–*Sal*I fragment of the EMCV IRES Neo were inserted into the *Eco*RI and the *Sal*I site of pCHO by triple ligation.

The plasmid pEF Rluc-HCV IRES Feo was constructed as follows. The fragment carrying the Renilla luciferase was amplified from the phRL-TK vector (Promega, USA) by PCR using a pair of primers (5′-GAATTCATGGCT TCCAAGGTGTACGACCC-3′ [*Eco*RI site] and 5′-GGAT CCTTACTGCTCGTTCTTCAGCACGC-3′ [*Bam*HI site]). The fragment carrying the HCV IRES Feo was amplified from the pRep-Feo vector[7] by PCR using a pair of primers (5′-GGATCCGCCAGCCCCCGATTGGGGGCG AC-3′ [*Bam*HI site] and 5′-GTCGACTCAGAAGAAC TCGTCAAGAAGGC-3′ [*Sal*I site]). Each PCR product was cloned into the pGEM-T Easy vector. To construct pEF Rluc-HCV IRES Feo, the *Eco*RI–*Bam*HI fragment of Renilla luciferase, and the *Bam*HI–*Sal*I fragment of HCV IRES Feo were inserted into the *Eco*RI and *Sal*I site of pCHO by triple ligation.

The pEF-Fluc-IB and pEF Rluc-HCV IRES Feo was transfected into Huh7 cells using Effectene transfection reagent (QIAGEN, Hilden, Germany), according to the manufacturer's recommendation. Two days after transfection, the Huh7 cells were selected in a medium containing 250 µg/mL G418.

Immunofluorescent staining

After treatment with griseofulvin for 72 h, HCV JFH-1-infected cells were fixed with cold methanol and blocked using Blocking One (Nacalai Tesque, Kyoto, Japan). For the detection of the NS3 protein, the cells were incubated with the anti-NS3 antibody (Virogen, USA) for 1 h at room temperature. After washing with PBS, the cells were incubated with an Alexa Fluor 488 goat antimouse immunoglobulin G antibody (Molecular Probes, Eugene, OR, USA) for 1 h at room temperature. After washing with PBS, the cells were stained with 7-aminoactinomycin D for nuclear counterstaining, and analyzed using fluorescence microscopy.

RESULTS

Replication of a subgenomic HCV-1b replicon is suppressed by griseofulvin

WE INVESTIGATED THE anti-HCV effect and cell toxicity of griseofulvin in the HCV subgenomic replicon cells, Huh7/Rep-Feo. The luciferase activities of the Huh7/Rep-Feo cells showed that replication of the HCV replicon was suppressed by griseofulvin in a dose-dependent manner (Fig. 1a). Next, we performed a time-course experiment in which the luciferase activities of Huh7/Rep-Feo cells were measured at various time points after treatment with griseofulvin. As shown in Figure 1b, griseofulvin induced a decrease in the luciferase activities of Huh7/Rep-Feo cells over time. The treatment with griseofulvin had little effect on cellular viability at this range of concentration, as revealed by the MTS assay (Fig. 1c). The 50% effective concentration (EC_{50}) of griseofulvin was 6.13 ± 0.17 µM. The 50% cytotoxic concentration of this compound (CC_{50}) was 217.93 ± 3.49 µM. Thus the selectivity index (ratio of CC_{50} to EC_{50}) was 35.5 (Table 1). Furthermore, we examined the effect of other antifungal agents, fluconazole and itraconazole, on HCV-RNA replication. In contrast, fluconazole and itraconazole had little effect on HCV-RNA replication (Table 1).

We analyzed HCV-RNA levels in Huh7/Rep-Feo cells treated or not treated with griseofulvin using real-time RT–PCR. As shown in Figure 2a, treatment with griseofulvin decreased the replicon RNA titer in a dose-dependent manner. Similar results were seen at the protein level by monitoring the HCV non-structural proteins NS3 and NS5A. The Western blot analysis demon-

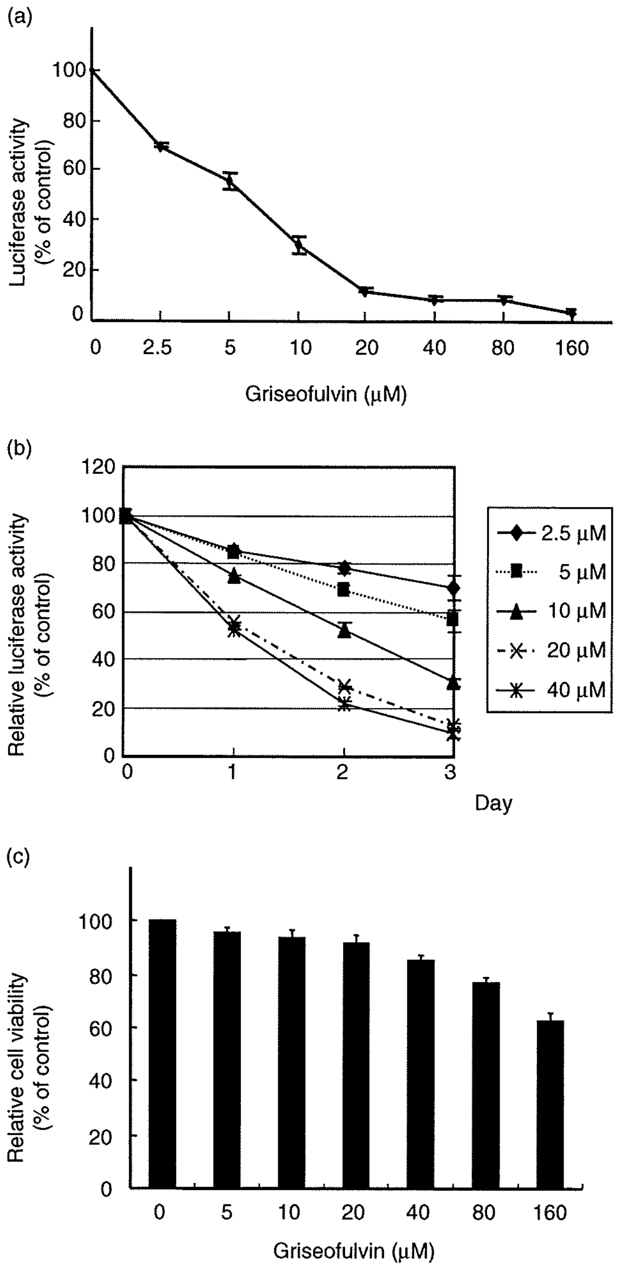

Figure 1 Inhibition of hepatitis C virus replication in Huh7/Rep-Feo cells by griseofulvin. (a) Huh7/Rep-Feo cells were cultured with various concentrations of griseofulvin in the medium and luciferase assays were performed after 72 h of culture. Luciferase assays were performed in triplicate. Error bars indicate mean ± standard deviation. (b) Huh7/Rep-Feo cells were treated with various concentrations of griseofulvin (2.5–40.0 µM). Luciferase activity was measured at the time points indicated after exposure to griseofulvin. (C) 5-(3-Carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt of Huh7/Rep-Feo cells cultured with the concentration of griseofulvin indicated.