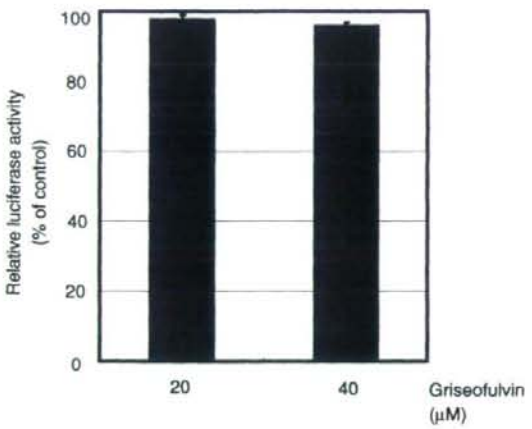

Figure 3 No inhibition of firefly luciferase activity by griseofulvin. pEF Fluc IN vector was stably transfected into Huh7 cells. Cells were cultured without (control) and with 20 μM or 40 μM griseofulvin for 72 h. Firefly luciferase assay was performed. Luciferase activity was normalized by the protein concentration. Error bars indicate mean ± SD.

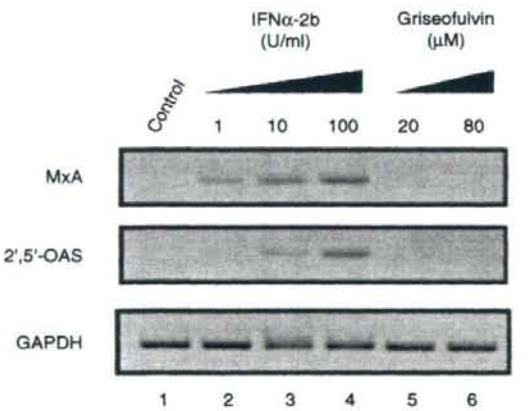

Figure 4 Griseofulvin elicited an interferon (IFN) response. Huh7/Rep-Feo cells were treated without (lane 1) or with 1, 10, or 100 U/mL IFNα-2b (lanes 2–4), and 20 (lane 5) or 80 μM griseofulvin (lane 6) for 72 h. Messenger RNA of human myxovirus resistance protein A (MxA), 2',5'-oligoadenylate synthetase (2',5'-OAS), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal control were detected by reverse transcription–polymerase chain reaction analysis.

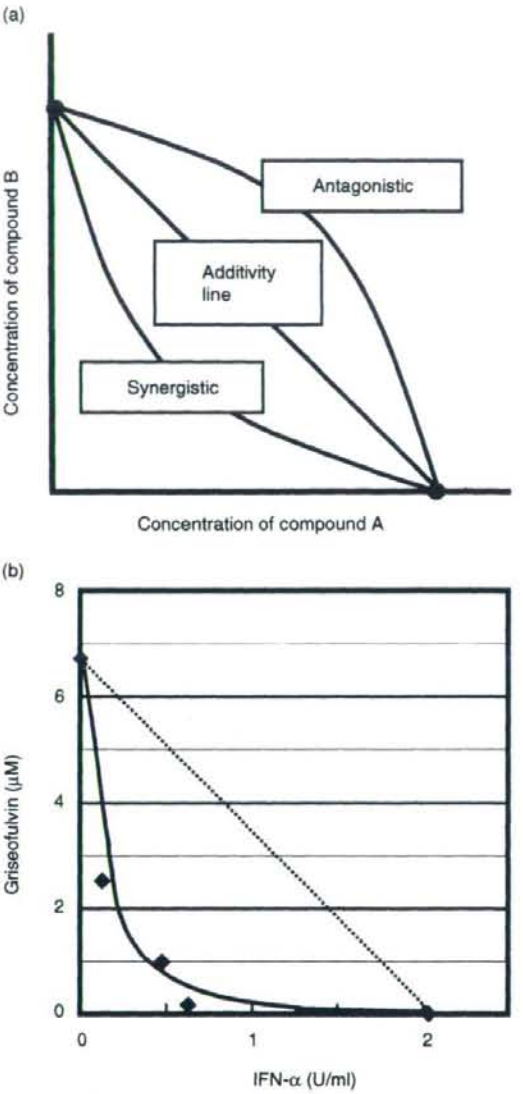

Figure 5 Effect of a combination of griseofulvin and interferon (IFN)α on intracellular hepatitis C virus (HCV)-RNA replication. (a) Representative isobologram for analyzing the interaction between two drugs. (b) Isobole plot of 50% inhibition of HCV-RNA replication. Huh7/Rep-Feo cells were treated with griseofulvin in combination with IFNα, and a luciferase assay was performed after 72 h of culture to obtain each isobole plot. Dotted line indicates an additive effect in the isobologram method used.

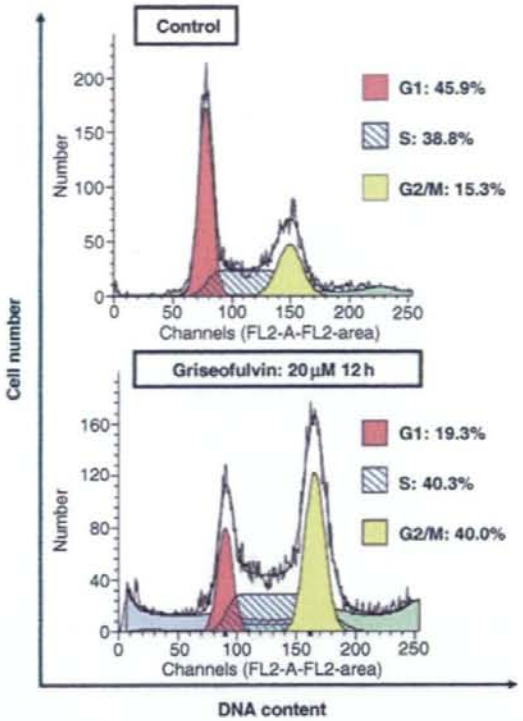

Figure 6 Griseofulvin induced G_2/M phase arrest in Huh7/Rep-Feo cells. Flow cytometry analysis of DNA content of untreated Huh7/Rep-Feo cells (control) and cells treated for 12 h with 20 μM of griseofulvin. [■ G_1: 45.9%, □ S: 38.8%, G_2/M: 15.3%; ■ G_1: 19.3%, □ S: 40.3%, G_2/M: 40.0%.]

(data not shown). The ratios of griseofulvin and IFNα were 9:1, 1:1, and 1:9. Each concentration of griseofulvin and IFNα at 50% inhibition was plotted on the X- and Y-axes, respectively, to generate an isobologram (Fig. 5b). As shown in Figure 5b, each plot fell far below the line showing additivity, indicating that the effect of the griseofulvin and IFNα combination on HCV-RNA replication is strongly synergistic.

Griseofulvin induces G_2/M cell cycle arrest in HCV replicon cells

As described previously, griseofulvin blocks cell cycle progression at the G_2/M phase in several human cell lines.[24] Here, we examined the effect of griseofulvin on cell cycle progression in Huh7/Rep-Feo cells. As shown in Figure 6, the population of griseofulvin-treated Huh7/Rep-Feo cells in the G_2/M phase at 12 h was 40%, compared to 15.3% for the control cell populations. These data imply that griseofulvin might have the potential to arrest Huh7/Rep-Feo cells in the G_2/M phase.

As described earlier, the treatment of Huh7/Rep-Feo cells with 20 μM griseofulvin for 12 h results in G_2/M arrest (Fig. 6), while treatment for 72 h had no effect on cell growth (Fig. 1c). To explain this discrepancy, we examined the growth kinetics of griseofulvin-treated Huh7/Rep-Feo cells. The cells were cultured with 20 μM griseofulvin, and cell growth was monitored by MTS assay. The cell viability declined gradually until 48 h after treatment with 20 μM griseofulvin, but increased from 48 h to 72 h (Fig. 7). These data indicate that treatment with 20 μM griseofulvin arrests Huh7/Rep-Feo cells in the G_2/M phase, but does not inhibit cell growth completely.

Griseofulvin does not inhibit HCV IRES-dependent translation

Previous studies have shown that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization and the cell cycle in G_2/M, inhibit

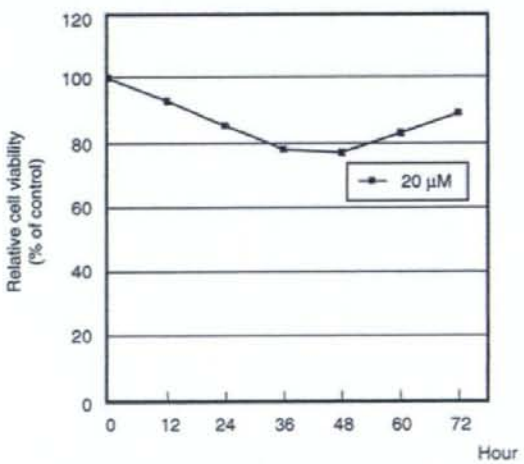

Figure 7 Growth kinetics of griseofulvin treatment of Huh7/Rep-Feo cells. Cells were cultured with [–•–] 20 μM griseofulvin, and cell viability was monitored by a 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl) tetrazolium inner salt assay at the times indicated. Error bars indicate mean ± SD.

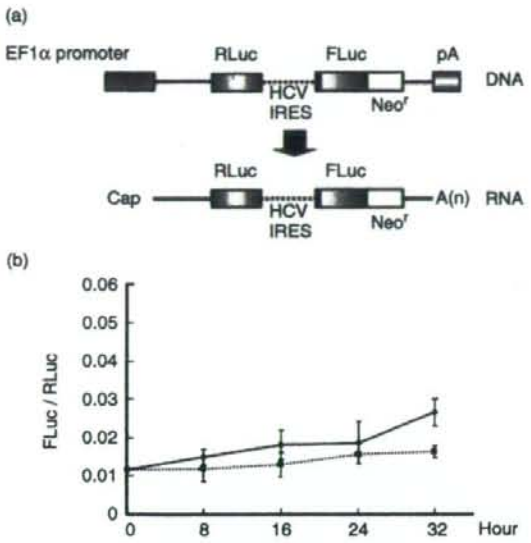

Figure 8 Griseofulvin does not influence hepatitis C virus (HCV) internal ribosomal entry site (IRES)-mediated translation. (a) Structure of the plasmid, pEF-Rluc-HCV IRES Feo. Transcription is initiated under the control of a composite elongation factor 1α (EF1α) promoter. Upstream cistron encodes Renilla luciferase (RLuc) and is translated by a cap-dependent mechanism in transfected cells, while the downstream cistron encodes a fusion (Feo) of the firefly luciferase (Fluc) and neomycin phosphotransferase (Neo^r) genes, translated under the control of the HCV IRES. (b) pEF-Rluc-HCV IRES Feo was stably transfected into Huh7 cells. Cells were treated without (control –◆–) and with 20 μM griseofulvin –●–. Dual luciferase activities were measured at the indicated time points after exposure to griseofulvin. Values are displayed as ratios of Fluc to Rluc. Error bars indicate mean ± SD.

HCV replication, but not HCV IRES-dependent translation.[25] Therefore, we determined whether G_2/M cell cycle arrest by griseofulvin affects HCV IRES-dependent translation using Huh7 cells transfected with pEF Rluc-HCV IRES Feo (Fig. 8a). The treatment of these cells with 20 μM griseofulvin resulted in no significant change of the internal luciferase activities, a concentration that suppressed the expression of the HCV replicon and arrested the HCV replicon cells in the G_2/M phase (Fig. 8b). These results suggested that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation, as shown previously for vinblastine sulfate and nocodazole.

Griseofulvin suppressed JFH-1 HCV replication

The studies described thus far were carried out using the subgenomic HCV-1b replicon system. Recently, Wakita *et al.* established a cell culture model for HCV. This system, known as the JFH-1 system and based on genotype 2a HCV, allows the production of a virus that can be efficiently propagated in cell culture (HCVcc).[10] Therefore, we examined the effect of griseofulvin using the JFH-1 system. The Huh7.5.1/JFH-1 cells (cells persistently infected with HCV JFH-1) were cultured with 10 μM or 20 μM griseofulvin for 72 h. We detected the HCV NS3 protein in Huh7.5.1/JFH-1 HCV cells by immunostaining. As shown in Figure 9, in the absence of griseofulvin treatment, the NS3 protein was localized predominantly in the perinuclear region. After treatment of griseofulvin, the NS3 protein expression level was reduced substantially (Fig. 9). This result indicates that griseofulvin also suppresses HCV replication in the JFH-1 HCVcc system.

DISCUSSION

WE HAVE SHOWN here that griseofulvin inhibits the replication of HCV in the HCV subgenomic replicon cells, Huh7/Rep-Feo. In this reporter-based subgenomic replicon system, the EC_{50} of griseofulvin for the inhibition of HCV replication, determined by measurement of the luciferase activity, was approximately 6.13 μM. The real-time RT–PCR and Western blot analyses revealed that both RNA synthesis and its translation were inhibited by griseofulvin in a dose-dependent manner. The treatment of Huh7/Rep-Feo cells with griseofulvin did not activate the IFN inducible gene responses, suggesting that the inhibitory mechanism of griseofulvin in HCV replication is independent of the IFN signaling pathway. Moreover, we demonstrated that the combination treatment of griseofulvin and IFNα had a synergistic inhibitory effect in Huh7/Rep-Feo cells. We also demonstrated that griseofulvin suppressed replication of JFH-1 HCV.

A previous study demonstrated that griseofulvin induces G_2/M arrest in several human cell lines.[24] Here, we show that griseofulvin arrested the Huh7/Rep-Feo cells in the G_2/M phase. Recently, several studies have shown a correlation between HCV IRES-mediated translation and the cell cycle. Honda *et al.* reported that the HCV IRES activity was highest in the G_2/M phase.[26] In contrast, Venkatesan *et al.* reported that the HCV IRES activity was lowest in the G_2/M,[27] while other studies

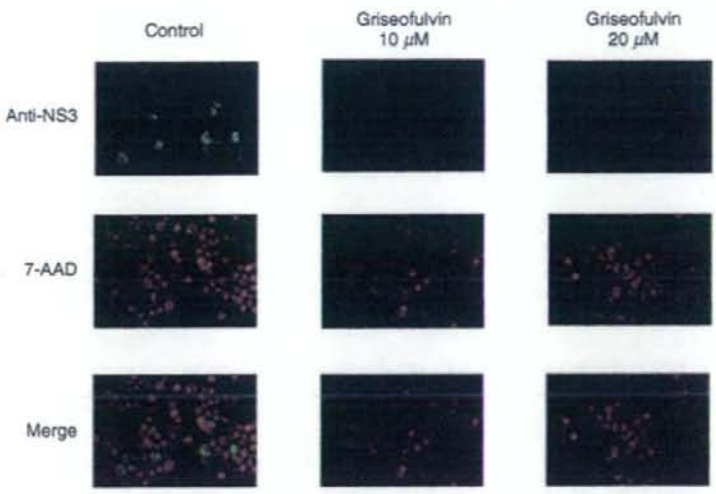

Figure 9 Griseofulvin suppresses JFH-1 replication. Immunofluorescent staining of Huh7.5.1/JFH-1 cells treated with various concentrations of griseofulvin. Hepatitis C virus NS3 protein is stained green and nuclei are stained with 7-aminoactinomycin D (7-AAD; red).

reported that the HCV IRES activity was independent of the stage of the cell cycle.[28,29] In addition, Bost *et al.* reported that several cell cycle inhibitors (vinblastine sulfate, colchicine, nocodazole, and cytochalasin D) did not affect HCV IRES-dependent translation.[25] We also have shown that cell cycle arrest by griseofulvin did not affect HCV IRES-dependent translation. Accordingly, our results support the hypothesis that the HCV IRES activity is independent of the cell cycle.

Previous studies have demonstrated that vinblastine sulfate and nocodazole, well-characterized inhibitors of microtubule polymerization, are able to inhibit HCV-RNA replication in HCV subgenomic replicon cells.[25] These findings indicate that microtubule polymerization is required for the formation of the HCV replication complex. Griseofulvin has been shown to arrest human cells in the G_2/M phase by acting on microtubule polymerization.[30] Thus it is speculated that the inhibition of microtubule polymerization by griseofulvin may influence the formation of the HCV-RNA replication complex. Further, defining the mechanism of action of griseofulvin against HCV replication may be important for defining a novel target for anti-HCV therapy.

Griseofulvin has been used for many years for the treatment of ringworm and other dermatophyte infections. Moreover, griseofulvin does not have significant toxicity for humans. Consequently, the development of derivatives of this compound may be a useful strategy for future therapeutic intervention in chronic hepatitis C.

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REFERENCES

1 Alter HJ, Purcell RH, Shih JW *et al.* Detection of antibody to hepatitis C virus in prospectively followed transfusion recipients with acute and chronic non-A, non-B hepatitis. *N Engl J Med* 1989; **321**: 1494–500.
2 Choo QL, Kuo G, Weiner AJ *et al.* Isolation of a cDNA clone derived from a blood-borne non-A, non-B viral hepatitis genome. *Science* 1989; **244**: 359–62.
3 Alter MJ. Epidemiology of hepatitis C. *Hepatology* 1997; **26**: 62S–5S.
4 Fried MW, Shiffman ML, Reddy KR *et al.* Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. *N Engl J Med* 2002; **347**: 975–82.
5 Lohmann V, Korner F, Koch J *et al.* Replication of subgenomic hepatitis C virus RNAs in a hepatoma cell line. *Science* 1999; **285**: 110–13.

6 Kim SS, Peng LF, Lin W *et al.* A cell-based, high-throughput screen for small molecule regulators of hepatitis C virus replication. *Gastroenterology* 2007; **132**: 311–20.

7 Tanabe Y, Sakamoto N, Enomoto N *et al.* Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon- alpha. *J Infect Dis* 2004; **189**: 1129–39.

8 Yokota T, Sakamoto N, Enomoto N *et al.* Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. *EMBO Rep* 2003; **4**: 602–8.

9 Lindenbach BD, Evans MJ, Syder AJ *et al.* Complete replication of hepatitis C virus in cell culture. *Science* 2005; **309**: 623–6.

10 Wakita T, Pietschmann T, Kato T *et al.* Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. *Nat Med* 2005; **11**: 791–6.

11 Zhong J, Gastaminza P, Cheng G *et al.* Robust hepatitis C virus infection in vitro. *Proc Natl Acad Sci USA* 2005; **102**: 9294–9.

12 Hinrichsen H, Benhamou Y, Wedemeyer H *et al.* Short-term antiviral efficacy of BILN 2061a hepatitis C virus serine protease inhibitor, in hepatitis C genotype 1 patients. *Gastroenterology* 2004; **127**: 1347–55.

13 Lamarre D, Anderson PC, Bailey M *et al.* An NS3 protease inhibitor with antiviral effects in humans infected with hepatitis C virus. *Nature* 2003; **426**: 186–9.

14 Reesink HW, Zeuzem S, Weegink CJ *et al.* Rapid decline of viral RNA in hepatitis C patients treated with VX-950: a phase Ib, placebo-controlled, randomized study. *Gastroenterology* 2006; **131**: 997–1002.

15 Sarrazin C, Rouzier R, Wagner F *et al.* SCH 503034, a novel hepatitis C virus protease inhibitor, plus pegylated interferon alpha-2b for genotype 1 nonresponders. *Gastroenterology* 2007; **132**: 1270–8.

16 Amemiya F, Maekawa S, Itakura Y *et al.* Targeting lipid metabolism in the treatment of hepatitis C. *J Infect Dis* 2008; **197**: 361–70.

17 Martell M, Gomez J, Esteban JI *et al.* High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. *J Clin Microbiol* 1999; **37**: 327–32.

18 Okada Y, Osada M, Kurata S *et al.* p53 gene family p51 (p63) -encoded, secondary transactivator p51B (TAp63alpha) occurs without forming an immunoprecipitable complex with MDM2, but responds to genotoxic stress by accumulation. *Exp Cell Res* 2002; **276**: 194–200.

19 Chou TC, Talaly P. A simple generalized equation for the analysis of multiple inhibitions of Michaelis–Menten kinetic systems. *J Biol Chem* 1977; **252**: 6438–42.

20 Kitamura T, Koshino Y, Shibata F *et al.* Retrovirus-mediated gene transfer and expression cloning: powerful tools in functional genomics. *Exp Hematol* 2003; **31**: 1007–14.

21 Morita S, Kojima T, Kitamura T. Plat-E: an efficient and stable system for transient packaging of retroviruses. *Gene Ther* 2000; **7**: 1063–6.

22 Blight KJ, Kolykhalov AA, Rice CM. Efficient initiation of HCV RNA replication in cell culture. *Science* 2000; **290**: 1972–4.

23 Frese M, Pietschmann T, Moradpour D *et al.* Interferon-alpha inhibits hepatitis C virus subgenomic RNA replication by an MxA-independent pathway. *J Gen Virol* 2001; **82**: 723–3.

24 Ho YS, Duh JS, Jeng JH *et al.* Griseofulvin potentiates antitumorigenesis effects of nocodazole through induction of apoptosis and G2/M cell cycle arrest in human colorectal cancer cells. *Int J Cancer* 2001; **91**: 393–401.

25 Bost AG, Venable D, Liu L *et al.* Cytoskeletal requirements for hepatitis C virus (HCV) RNA synthesis in the HCV replicon cell culture system. *J Virol* 2003; **77**: 4401–8.

26 Honda M, Kaneko S, Matsushita E *et al.* Cell cycle regulation of hepatitis C virus internal ribosomal entry site-directed translation. *Gastroenterology* 2000; **118**: 152–62.

27 Venkatesan A, Sharma R, Dasgupta A. Cell cycle regulation of hepatitis C and encephalomyocarditis virus internal ribosome entry site-mediated translation in human embryonic kidney 293 cells. *Virus Res* 2003; **94**: 85–95.

28 Nelson HB, Tang H. Effect of cell growth on hepatitis C virus (HCV) replication and a mechanism of cell confluence-based inhibition of HCV RNA and protein expression. *J Virol* 2006; **80**: 1181–90.

29 Scholle F, Li K, Bodola F, Ikeda M *et al.* Virus–host cell interactions during hepatitis C virus RNA replication: impact of polyprotein expression on the cellular transcriptome and cell cycle association with viral RNA synthesis. *J Virol* 2004; **78**: 1513–24.

30 Panda D, Rathinasamy K, Santra MK *et al.* Kinetic suppression of microtubule dynamic instability by griseofulvin: implications for its possible use in the treatment of cancer. *Proc Natl Acad Sci USA* 2005; **102**: 9878–83.

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.)

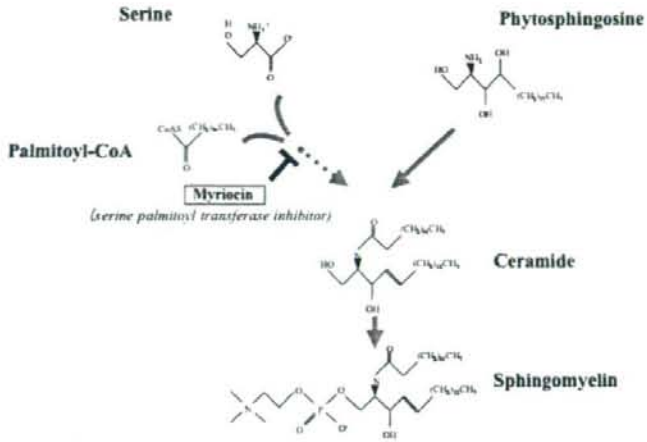

Figure 1. The sphingomyelin synthesis pathway. Serine palmitoyltransferase catalyzes the first committed step of sphingomyelin biosynthesis from serine and palmitoyl-coenzyme A (CoA). Myriocin inhibits the catalyzing activity of serine palmitoyltransferase. Phytosphingosine is known to work as a precursor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

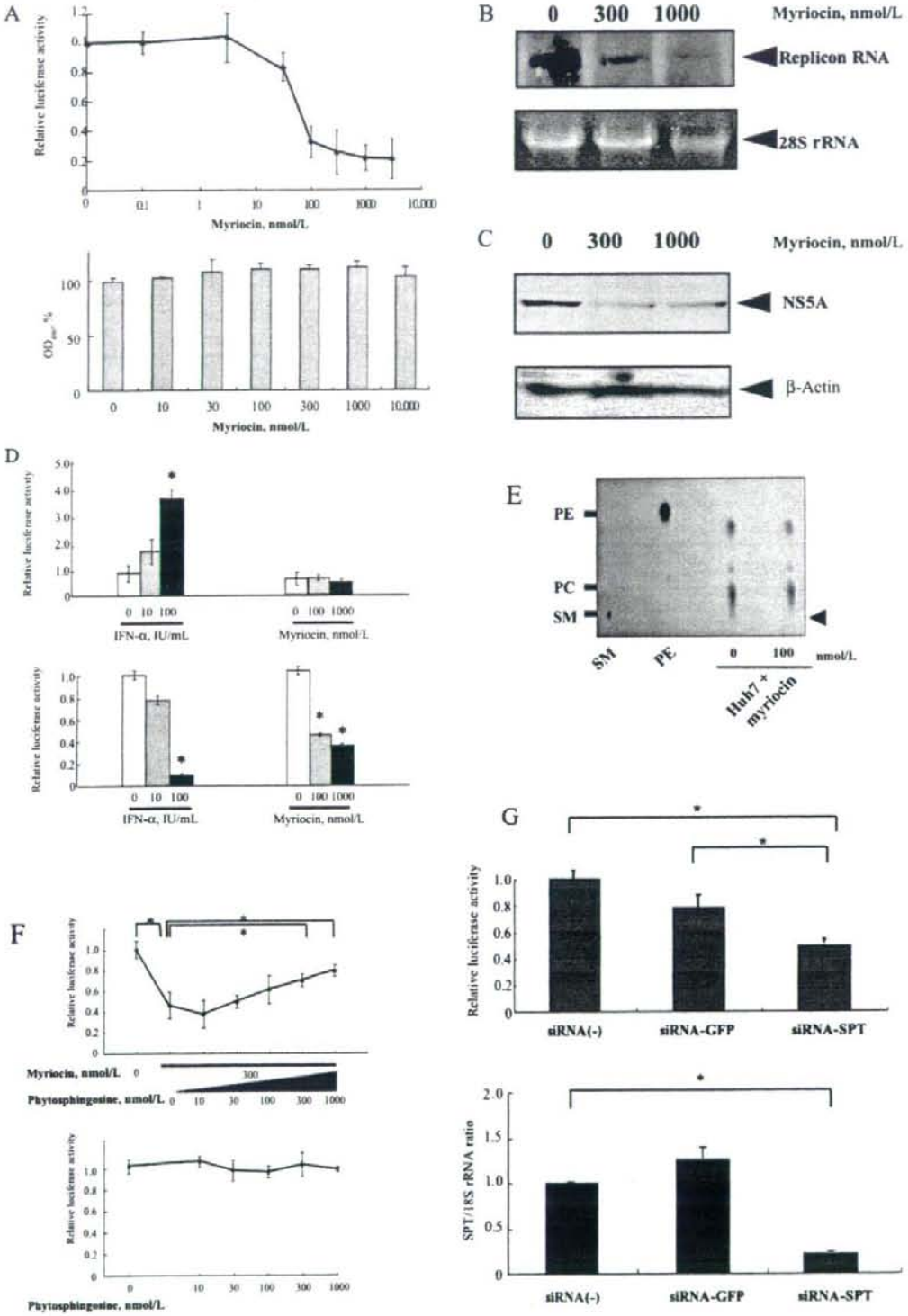
A
Relative luciferase activity
Myriocin, nmol/L
B
0 300 1000
Myriocin, nmol/L
Replicon RNA
28S rRNA
C
0 300 1000
Myriocin, nmol/L
NS5A
β-Actin
D
Relative luciferase activity
IFN-α, IU/mL
Myriocin, nmol/L
E
PE
PC
SM
SM PE 0 100 nmol/L
Huh7 + myriocin
F
Myriocin, nmol/L
Phytosphingosine, nmol/L
Phytosphingosine, 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. $^*P < .05$.

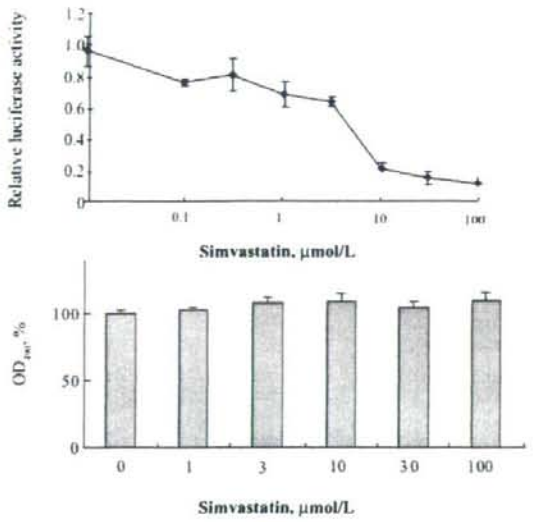

Figure 3. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by simvastatin. Huh7/Rep-Feo cells were cultured with various concentrations of simvastatin, and the luciferase assay was performed after 48 h of treatment *(upper panel)*. The dimethylthiazol carboxymethoxyphenyl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

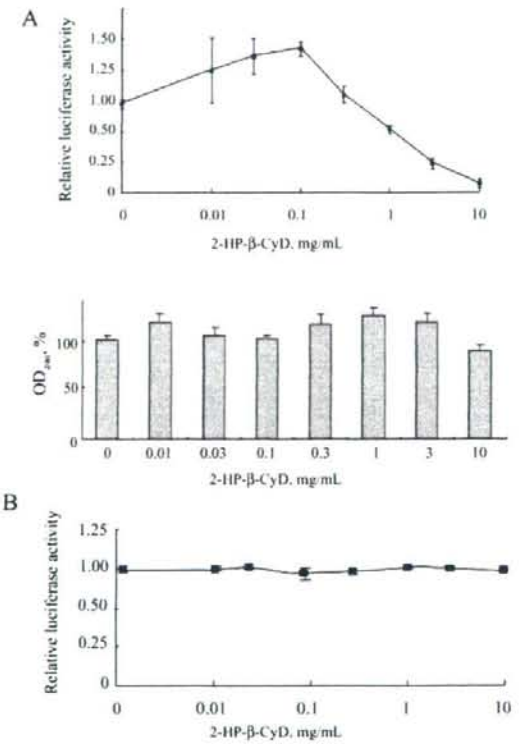

Figure 4. Inhibition of replication of a subgenomic hepatitis C virus genotype 1b replicon by 2-hydroxypropyl-β-cyclodextrin (2-HP-β-CyD). *A*, Huh7/Rep-Feo cells cultured with various concentrations of 2-HP-β-CyD for 48 h. The luciferase assay was performed after 48 h of treatment *(upper panel)*. The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

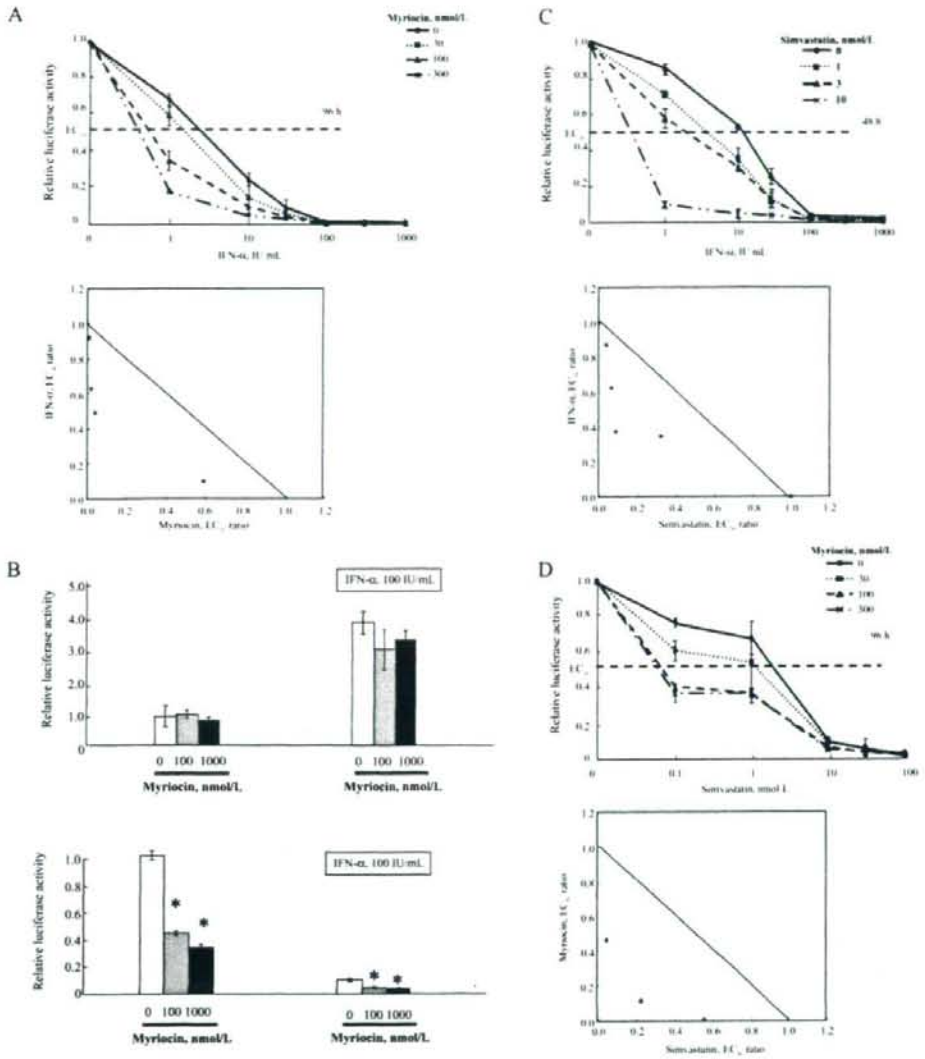

Figure 5. Synergistic inhibitory effects of myriocin with interferon (IFN), simvastatin with IFN, and myriocin with simvastatin. *A*, Synergistic inhibitory effect of myriocin with IFN on hepatitis C virus replication. Huh7/Rep-Feo cells were treated with combinations of myriocin and IFN at various concentrations.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

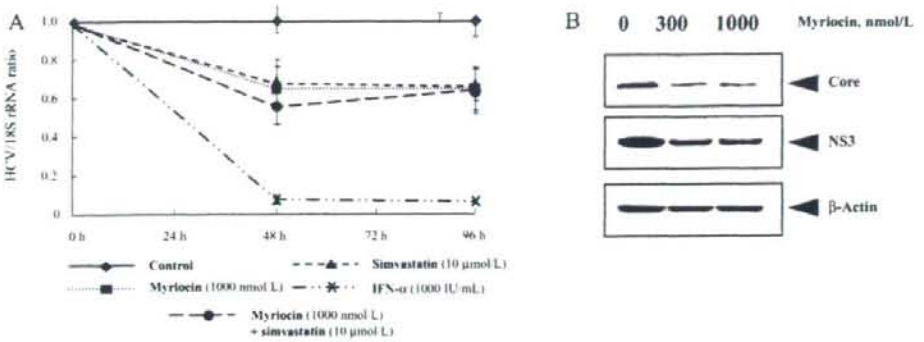

Figure 6. Suppression of JFH-1 hepatitis C virus (HCV) replication by myriocin and simvastatin. *A*, Cells containing JFH-1 HCV treated for 96 h with 1000 nmol/L myriocin, 10 μmol/L simvastatin, 1000 IU/mL IFN, or a combination of 1000 nmol/L myriocin and 10 μmol/L simvastatin. The cells were collected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

References

1. Global surveillance and control of hepatitis C. Report of a WHO consultation organized in collaboration with the Viral Hepatitis Prevention Board, Antwerp, Belgium. J Viral Hepat **1999**; 6:35–47.
2. Hoofnagle JH. Hepatitis C: the clinical spectrum of disease. Hepatology **1997**; 26:15S–20S.
3. Seeff LB. Natural history of chronic hepatitis C. Hepatology **2002**; 36: S35–46.
4. Fried MW, Shiffman ML, Reddy KR, et al. Peginterferon alfa-2a plus ribavirin for chronic hepatitis C virus infection. N Engl J Med **2002**; 347: 975–82.
5. Wengler G, Nowak T, Castle E. Description of a procedure which allows isolation of viral nonstructural proteins from BHK vertebrate cells infected with the West Nile flavivirus in a state which allows their direct chemical characterization. Virology **1990**; 177:795–801.
6. Shi ST, Schiller JJ, Kanjanahaluethai A, Baker SC, Oh JW, Lai MM. Colocalization and membrane association of murine hepatitis virus gene 1 products and de novo-synthesized viral RNA in infected cells. J Virol **1999**; 73:5957–69.
7. van der Meer Y, Snijder EJ, Dobbe JC, et al. Localization of mouse hepatitis virus nonstructural proteins and RNA synthesis indicates a role for late endosomes in viral replication. J Virol **1999**; 73:7641–57.
8. Ahlquist P, Noueiry AO, Lee WM, Kushner DB, Dye BT. Host factors in positive-strand RNA virus genome replication. J Virol **2003**; 77:8181–6.
9. Egger D, Wolk B, Gosert R, et al. Expression of hepatitis C virus proteins induces distinct membrane alterations including a candidate viral replication complex. J Virol **2002**; 76:5974–84.
10. Moradpour D, Gosert R, Egger D, Penin F, Blum HE, Bienz K. Membrane association of hepatitis C virus nonstructural proteins and identification of the membrane alteration that harbors the viral replication complex. Antiviral Res **2003**; 60:103–9.
11. Gao L, Aizaki H, He JW, Lai MM. Interactions between viral nonstructural proteins and host protein hVAP-33 mediate the formation of hepatitis C virus RNA replication complex on lipid raft. J Virol **2004**; 78: 3480–8.
12. Aizaki H, Lee KJ, Sung VM, Ishiko H, Lai MM. Characterization of the hepatitis C virus RNA replication complex associated with lipid rafts. Virology **2004**; 324:450–61.
13. Mannova P, Fang R, Wang H, et al. Modification of host lipid raft proteome upon hepatitis C virus replication. Mol Cell Proteomics **2006**; 5: 2319–25.
14. Leu GZ, Lin TY, Hsu JT. Anti-HCV activities of selective polyunsaturated fatty acids. Biochem Biophys Res Commun **2004**; 318:275–80.
15. Kapadia SB, Chisari FV. Hepatitis C virus RNA replication is regulated by host geranylgeranylation and fatty acids. Proc Natl Acad Sci USA **2005**; 102:2561–6.
16. Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N. Different anti-HCV profiles of statins and their potential for combination therapy with interferon. Hepatology **2006**; 44:117–25.
17. Sakamoto H, Okamoto K, Aoki M, et al. Host sphingolipid biosynthesis as a target for hepatitis C virus therapy. Nat Chem Biol **2005**; 1:333–7.
18. Shi ST, Lee KJ, Aizaki H, Hwang SB, Lai MM. Hepatitis C virus RNA replication occurs on a detergent-resistant membrane that cofractionates with caveolin-2. J Virol **2003**; 77:4160–8.
19. Wakita T, Pietschmann T, Kato T, et al. Production of infectious hepatitis C virus in tissue culture from a cloned viral genome. Nat Med **2005**; 11:791–6.
20. Zhong J, Gastaminza P, Cheng G, et al. Robust hepatitis C virus infection in vitro. Proc Natl Acad Sci USA **2005**; 102:9294–9.

21. Nakagawa M, Sakamoto N, Enomoto N, et al. Specific inhibition of hepatitis C virus replication by cyclosporin A. Biochem Biophys Res Commun **2004**; 313:42–7.
22. Tanabe Y, Sakamoto N, Enomoto N, et al. Synergistic inhibition of intracellular hepatitis C virus replication by combination of ribavirin and interferon- alpha. J Infect Dis **2004**; 189:1129–39.
23. Bligh EG, Dyer WJ. A rapid method of total lipid extraction and purification. Can J Biochem Physiol **1959**; 37:911–7.
24. Martell M, Gomez J, Esteban JI, et al. High-throughput real-time reverse transcription-PCR quantitation of hepatitis C virus RNA. J Clin Microbiol **1999**; 37:327–32.
25. Yokota T, Sakamoto N, Enomoto N, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep **2003**; 4:602–8.
26. Schmidt-Mende J, Bieck E, Hugle T, et al. Determinants for membrane association of the hepatitis C virus RNA-dependent RNA polymerase. J Biol Chem **2001**; 276:44052–63.
27. Pietschmann T, Lohmann V, Rutter G, Kurpanek K, Bartenschlager R. Characterization of cell lines carrying self-replicating hepatitis C virus RNAs. J Virol **2001**; 75:1252–64.
28. Lai VC, Dempsey S, Lau JY, Hong Z, Zhong W. In vitro RNA replication directed by replicase complexes isolated from the subgenomic replicon cells of hepatitis C virus. J Virol **2003**; 77:2295–300.
29. O'Leary JG, Chan JL, McMahon CM, Chung RT. Atorvastatin does not exhibit antiviral activity against HCV at conventional doses: a pilot clinical trial. Hepatology **2007**; 45:895–8.
30. Umehara T, Sudoh M, Yasui F, et al. Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model. Biochem Biophys Res Commun **2006**; 346:67–73.

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HEPATOLOGY

Inhibition of hepatitis C virus infection and expression *in vitro* and *in vivo* by recombinant adenovirus expressing short hairpin RNA

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Abstract

Background and Aim: We have reported previously that synthetic small interfering RNA (siRNA) and DNA-based siRNA expression vectors efficiently and specifically suppress hepatitis C virus (HCV) replication *in vitro*. In this study, we investigated the effects of the siRNA targeting HCV-RNA *in vivo*.

Methods: We constructed recombinant retrovirus and adenovirus expressing short hairpin RNA (shRNA), and transfected into replicon-expressing cells *in vitro* and transgenic mice *in vivo*.

Results: Retroviral transduction of Huh7 cells to express shRNA and subsequent transfection of an HCV replicon into the cells showed that the cells had acquired resistance to HCV replication. Infection of cells expressing the HCV replicon with an adenovirus expressing shRNA resulted in efficient vector delivery and expression of shRNA, leading to suppression of the replicon in the cells by ~10^{-3}. Intravenous delivery of the adenovirus expressing shRNA into transgenic mice that can be induced to express HCV structural proteins by the Cre/*lox*P switching system resulted in specific suppression of virus protein synthesis in the liver.

Conclusion: Taken together, our results support the feasibility of utilizing gene targeting therapy based on siRNA and/or shRNA expression to counteract HCV replication, which might prove valuable in the treatment of hepatitis C.

Introduction

Hepatitis C virus (HCV), which affects 170 million people worldwide, is one of the most important pathogens causing liver-related morbidity and mortality.[1] The difficulty in eradicating HCV is attributable to limited treatment options against the virus and their unsatisfactory efficacies. Even with the most effective regimen with pegylated interferon (IFN) and ribavirin in combination, the efficacies are limited to less than half of the patients treated.[2] Given this situation, the development of safe and effective anti-HCV therapies is one of our high-priority goals.

RNA interference (RNAi) is a process of sequence-specific, post-transcriptional gene silencing that is initiated by double-stranded RNA.[3,4] Because of its potency and specificity, RNAi rapidly has become a powerful tool for basic research to analyze gene functions and for potential therapeutic applications. Recently, successful suppression of various human pathogens by RNAi have been reported, including human immunodeficiency viruses,[5,6] poliovirus,[7] influenza virus,[8] severe acute respiratory syndrome (SARS) virus[9] and hepatitis B virus (HBV).[10–13]

We and other researchers have reported that appropriately designed small interfering RNA (siRNA) targeting HCV genomic RNA can efficiently and specifically suppress HCV replication *in vitro*.[14–19] We have tested siRNA designed to target the well-conserved 5′-untranslated region (5′-UTR) of HCV-RNA, and identified the most effective target, just upstream of the translation initiation codon. Furthermore, transfection of DNA-based vectors expressing siRNA was as effective as that of synthetic siRNA in suppressing HCV replication.[14]

In this study, we explored the further possibility that efficient delivery and expression of siRNA may be effective in suppression and elimination of HCV replication and that delivery of such

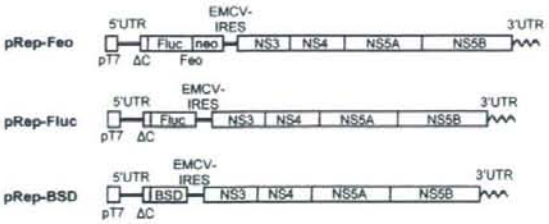

Figure 1 Structures of HCV replicon plasmids. The pRep-Feo expressed a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase (GenBank accession No. AB119282).[14,20] The pRep-Fluc expressed the Fluc protein. The pRep-BSD expressed the blasticidin S (BSD) resistance gene. pT7, T7 promoter; 5'UTR, HCV 5'-untranslated region; ΔC, truncated HCV core region (nt. 342–377); neo, neomycin phosphotransferase gene; EMCV, encephalomyocarditis virus; NS3, NS4, NS5A and NS5B, genes that encode HCV non-structural proteins; 3'UTR, HCV 3'-untranslated region.

HCV-directed siRNA *in vivo* may be effective in silencing viral protein expression in the liver. Here, we report that HCV replication was suppressed *in vitro* by recombinant retrovirus and adenovirus vectors expressing short hairpin RNA (shRNA) and that the delivery of the adenovirus vector to mice *in vivo* specifically inhibited viral protein synthesis in the liver.

Methods

Cells and cell culture

Huh7 and Retro Pack PT67 cells (Clontech, Palo Alto, CA, USA) were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon, G418 (Wako, Osaka, Japan) was added to the culture medium to a final concentration of 500 μg/mL.

HCV replicon constructs and transfection

HCV replicon plasmids, pRep-Feo, pRep-Fluc and pRep-BSD were constructed from were constructed from a virus, HCV-N strain, genotype 1b.[21] The pRep-Feo expressed a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase.[14,20] The pRep-Fluc and the pRep-BSD expressed the Fluc and blasticidin S (BSD) resistance genes, respectively (Fig. 1). The replicon RNA synthesis and the transfection protocol have been described previously.[22]

Synthetic siRNA and siRNA-expression plasmid

The design and construction of HCV-directed siRNA vectors have been described.[14] Briefly, five siRNA targeting the 5'-UTR of HCV RNA were tested for their efficiency to inhibit HCV replication, and the most effective sequence, which targeted nucleotide position of 331 though 351, was used in the present study. To construct shRNA-expressing DNA cassettes, oligonucleotide inserts were synthesized that contained the loop sequence (5'-TTC AAG AGA-3') flanked by sense and antisense siRNA sequences (Fig. 2a). These were inserted immediately downstream of the human U6 promoter. To avoid a problem in transcribing shRNA because of instability of the DNA strands arising from the tight palindrome structure, several C-to-T point mutations, which retained completely the silencing activity of the shRNA, were introduced into the sense strand of the shRNA sequences (referred to as 'm').[23] A control plasmid, pUC19-shRNA-Control, expressed shRNA directed towards the Machado–Joseph disease gene, which is a mutant of ataxin-3 gene and is not normally expressed. We have previously described the sequence specific activity of the shRNA-Control.[24]

Prior to construction of the virus vectors, we tested silencing efficiency of five shRNA constructs of different lengths that covered the target sequence (Fig. 2a). The shRNA-HCV-19, shRNA-HCV-21 and shRNA-HCV-27 had target sequences of 19, 21 and 27 nucleotides, respectively. Transfection of these shRNA constructs into Huh7/pRep-Feo showed that shRNA with longer target sequences had better suppressive effects (Fig. 2b). Therefore, we used shRNA-HCV-27m (abbreviated as shRNA-HCV) in the following study.

Recombinant retrovirus vectors

The U6-shRNA expression cassettes were inserted into the *StuI*/*Hin*dIII site of a retrovirus vector, pLNCX2 (Clontech) to construct pLNCshRNA-HCV and pLNCshRNA-Control (Fig. 2c). The plasmids were transfected into the packaging cells, Retro Pack PT67. The culture supernatant was filtered and added onto Huh7 cells with 4 μg/mL of polybrene. Huh7 cell lines stably expressing shRNA were established by culture in the presence of 500 μg/mL of G418.

Recombinant adenovirus

Recombinant adenoviruses expressing shRNA were constructed using an Adenovirus Expression Vector Kit (Takara, Otsu, Japan). The U6-shRNA expression DNA cassette was inserted into the *SwaI* site of pAxcw to construct pAxshRNA-HCV and pAxshRNA-Control. The adenoviruses were propagated according to the manufacturer's protocol (AxshRNA-HCV and AxshRNA-Control; Fig. 2c). A 'multiplicity of infection' (MOI) was used to standardize infecting doses of adenovirus. The MOI stands for the ratio of infectious virus particles to the number of cells being infected. An MOI = 1 represents equivalent dose to introduce one infectious virus particle to every host cell that is present in the culture.

Plasmids for assays of interferon responses

pISRE-TA-Luc (Invitrogen, Carlsbad, CA, USA) contained five copies of the consensus interferon stimulated response element (ISRE) motifs upstream of the Fluc gene. pTA-Luc (Invitrogen), which lacks the enhancer element, was used for background determination. The pcDNA3.1 (Invitrogen) was used as an empty vector for mock transfection. pRL-CMV (Promega, Madison, WI, USA), which expresses the *Renilla* luciferase protein, was used for normalization of transfection efficiency.[25] A plasmid, peGFPneo (Invitrogen), was used to monitor percentages of transduced cells.

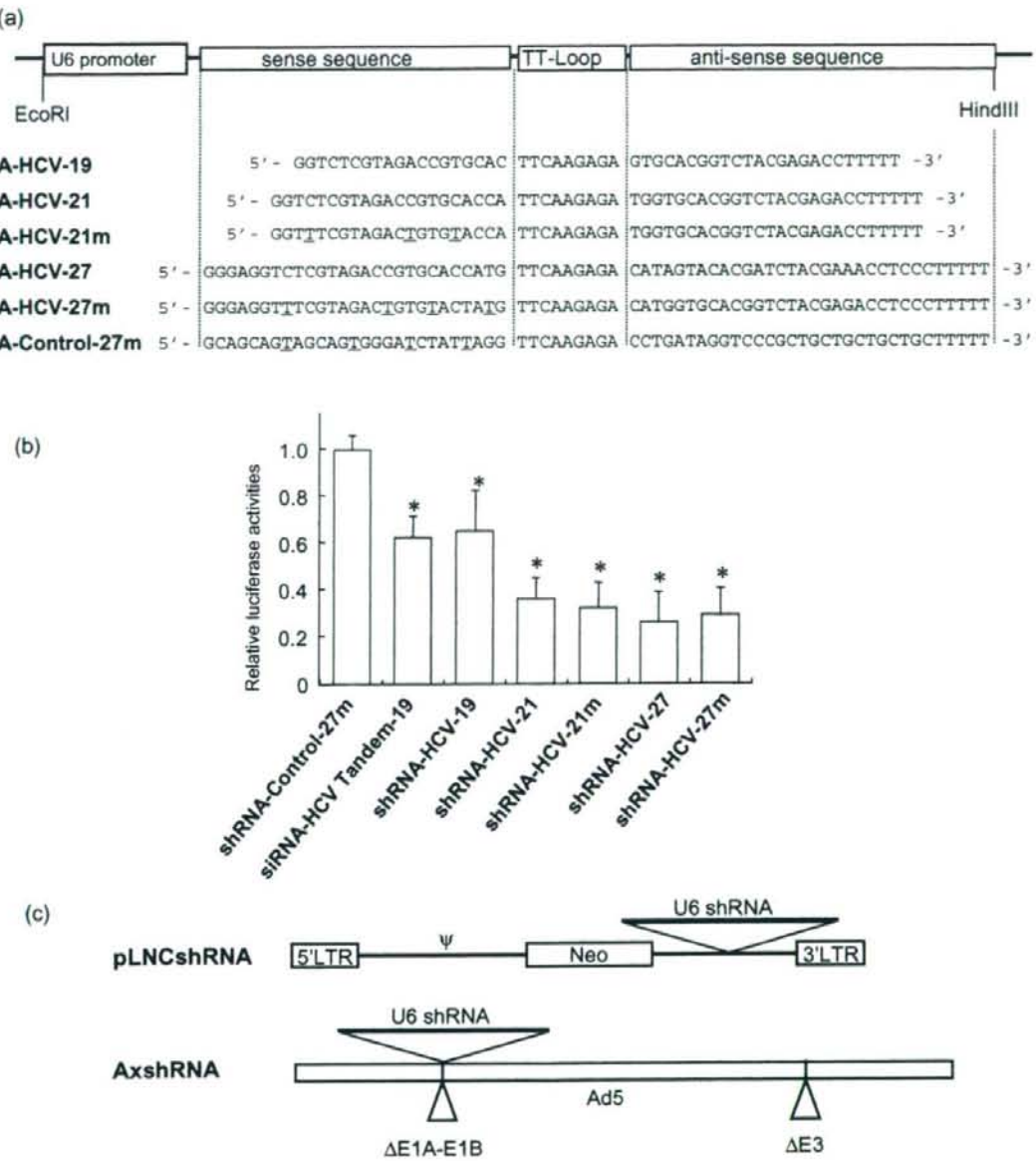

Figure 2 Structure of shRNA-expression constructs and shRNA sequences. (a) Structure of shRNA-expression cassette and shRNA sequences. TT-Loop, the loop sequence. The shRNA-Control was directed toward an unrelated target, Machado–Joseph disease gene. Underlined letters indicate C-to-T point mutations in the sense strand. (b) The shRNA-expression plasmids were transfected into Huh7/pRep-Feo cells, and internal luciferase activities were measured at 48 h of transfection. Each assay was done in triplicate, and the values are displayed as mean + SD. $^*P < 0.05$. (c) pLNCshRNA, structure of a recombinant retrovirus expressing shRNA. Ψ, the retroviral packaging signal sequence. AxshRNA, structure of a recombinant adenovirus expressing shRNA.

Real-time RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Total cellular RNA (2 μg) was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen). The mRNA expression levels were measured using the Light Cycler PCR and detection system (Roche, Mannheim, Germany) and Light Cycler Fast Start DNA Master SYBR Green 1 mix (Roche).

Luciferase assays

Luciferase activity was measured using a luminometer, Lumat LB9501 (Promega) and the Bright-Glo Luciferase Assay System (Promega) or the Dual-Luciferase Reporter Assay System (Promega).

Northern and western hybridization

Total cellular RNA was separated by denaturing agarose-formaldehyde gel electrophoresis, and transferred to a nylon membrane. The membrane was hybridized with a digoxigenin-labeled probe specific for the full-length replicon sequence, and subsequently with a probe specific for beta-actin. The signals were detected by chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche), and visualized by Fluoro-Imager (Roche). For the western blotting, 10 μg of total cell lysate was separated on NuPAGE 4.12% Bis-TrisGel (Invitrogen), and blotted onto an Immobilon PVDF Membrane (Roche). The membrane was incubated with monoclonal antibodies specific for HCV-NS5A (BioDesign, Saco, ME, USA), NS4A (Virogen, Watertown, MA, USA), or beta-actin (Sigma), and detected by a chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; POD, Roche).

Transient-replication assays

A replicon, pRep-Fluc, was transfected into cells and the luciferase activities of the cell lysates were measured serially. To correct the transfection efficiency, each value was divided by the luciferase activity at 4 h after the transfection.

Stable colony formation assays

Cells were transfected with a replicon, pRep-BSD, and were cultured in the presence of 150 μg/mL of BSD (Invitrogen). BSD-resistant cell colonies appeared after ~3 weeks of culture, and were counted.

HCV-JFH1 virus cell culture

An *in-vitro* transcribed HCV-JFH1 RNA[26] was transfected into Huh7.5.1 cells.[27] Naive Huh7.5.1 cells were subsequently infected by the culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to siRNA or drug treatments. Replication levels of HCV-RNA were quantified by the realtime RT-PCR by using primers that targeted HCV-NS5B region, HCV-JFH1 sense: 5′-TCA GAC AGA GCC TGA GTC CA-3′, and HCV-JFH1 antisense: 5′-AGT TGC TGG AGG GCT TCT GA-3′.

Mice and adenovirus infection

Transgenic mice, CN2-29, inducibly express mRNA for the HCV structural proteins (genotype1b, nucleotides 294–3435) by the Cre/*lox*P switching system.[28] The transgene does not contain full-length HCV 5′-UTR, but shares the target sequence of the shRNA-HCV. Although the transgenic mouse CN2 has been previously reported as expressing higher levels of the viral proteins, the expression levels of the viral core protein in the CN2-29 mice are modest and similar to that in the liver of HCV patients. Thus, we chose CN2-29 mice in the present study.

The mice were infected with AxshRNA-HCV or controls (AxshRNA-Control or AxCAw1) in combination with AxCAN-Cre, which expressed Cre recombinase. Three days after the infection, the mice were killed and HCV core protein in the liver was measured as described below. The BALB/c mice were maintained in the Animal Care Facility of Tokyo Medial and Dental University, and transgenic mice were in the Tokyo Metropolitan Institute of Medical Science. Animal care was in accordance with institutional guidelines. The review board of the university approved our experimental animal studies and all experiments were approved by the institutional animal study committees.

Measurement of HCV core protein in mouse liver

The amounts of HCV core protein in the liver tissue from the mice was measured by a fluorescence enzyme immunoassay (FEIA)[29] with a slight modification. Briefly, the 5F11 monoclonal anti-HCV-core antibody was used as the first antibody on the solid phase, and the 5E3 antibody conjugated with horseradish peroxidase was the second antibody. This FEIA can detect as little as 4 pg/mL of recombinant HCV-core protein. Contents of the HCV core protein in the liver samples were normalized by the total protein contents and expressed as pg/mg total protein.

Immunohistochemical staining

Liver tissue was frozen with optimal cutting temperature (OTC) compound (Tissue Tek; Sakura Finetechnical, Tokyo, Japan). The sections (8 μm thick) were fixed with a 1:1 solution of acetone : methanol at −20°C for 10 min and then washed with phosphate-buffered saline (PBS). Subsequently, the sections were incubated with the IgG fraction of an anti-HCV core rabbit polyclonal antibody (RR8)[28] in blocking buffer or antialbumin rabbit polyclonal antibody (Dako Cytomation, Glostrup, Denmark) in PBS overnight at 4°C. The sections were incubated with secondary antibody, Alexa-antirabbit IgG (Invitrogen) or TRITIC-antirabbit IgG (Sigma), for 2 h at room temperature. Fluorescence was observed using a fluorescence microscope.

Statistical analyses

Statistical analyses were performed using Student's *t*-test; *P*-values of less than 0.05 were considered to be statistically significant.

Results

Retrovirus transduction of shRNA can protect from HCV replication

Retrovirus vectors propagated from pLNCshRNA-HCV and pLNCshRNA-Control were used to infect Huh7 cells, and cell lines were established that constitutively express shRNA-HCV and shRNA-Control (Huh7/shRNA-HCV and Huh7/shRNA-Control, respectively). There were no differences in the cell morphology or growth rate between shRNA-transduced and non-transduced Huh7 cells (data not shown). The HCV replicon, pRep-Fluc, was transfected into Huh7/shRNA-HCV, Huh7/shRNA-Control and naive Huh7 cells by electroporation. In Huh7/shRNA-Control and naive Huh7 cells, the initial luciferase activity at 4 h decreased temporarily, which represents decay of the transfected replicon RNA, but increased again at 48 h and 72 h, which demonstrate de novo synthesis of the HCV replicon RNA. In contrast, transfection into Huh7/shRNA-HCV cells resulted in a decrease in the initial luciferase activity, reaching background by 72 h (Fig. 3a). Similarly, transfection of the replicon, pRep-BSD, into Huh7 cells and BSD selection yielded numerous BSD-resistant colonies in the naive Huh7 (832 colonies) and Huh7/shRNA-Control cell lines (740 colonies), while transfection of Huh7/shRNA-HCV, which expressed shRNA-HCV, yielded obviously fewer colonies (five colonies), indicating reduction of colony forming units by $\sim 10^2$ (Fig. 3b). There was no difference in shape, growth or viability between cells expressing the shRNA or not. These results indicated that cells expressing HCV-directed shRNA following retrovirus transduction acquired resistance to HCV replication.

Effect of recombinant adenoviruses expressing shRNA on *in vitro* HCV replication

We investigated subsequently the effects of recombinant adenovirus vectors expressing shRNA. AxshRNA-HCV and AxshRNA-Control were used separately to infect Huh7/pRep-Feo cells, and the internal luciferase activities were measured sequentially (Fig. 4a). AxshRNA-HCV caused continuous suppression of HCV RNA replication. Six days postinfection, the luciferase activities fell to background levels. In contrast, the luciferase activities of the Huh7/pRep-Feo cells infected with AxshRNA-Control did not show any significant changes compared with untreated Huh7/pRep-Feo cells (Fig. 4a). The dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium (MTS) assay showed no significant difference between cells that were infected by recombinant adenovirus and uninfected cells (Fig. 4b). In the northern blotting analysis, the cells were harvested 6 days after infection with the adenovirus at an MOI of 1. Feo-replicon RNA of 9.6 kb, which was detectable in the untreated Huh7/pRep-Feo cells and in the cells infected with AxshRNA-Control, diminished substantially following infection with the AxshRNA-HCV (Fig. 4c). Densitometries showed that the intracellular levels of the replicon RNA in the Huh7/pRep-Feo cells correlated well with the internal luciferase activities. Similarly in the western blotting, cells were harvested 6 days after infection with adenovirus. Levels of the HCV NS4A and NS5A proteins that were translated from the HCV replicon decreased following infection with the AxshRNA-HCV (Fig. 4d). These results indicated that the decrease in luciferase activities was due to specific suppressive effects of shRNA on expression of HCV genomic RNA and the viral proteins, and not due to non-specific effects caused by the delivery of shRNA or to toxicity of the adenovirus vectors.

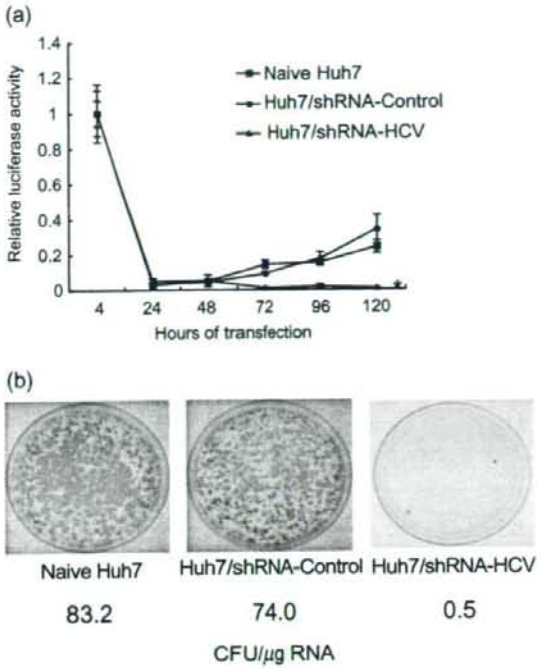

Figure 3 HCV replication can be inhibited by shRNA-HCV which was stably transfected into cells. Huh7/shRNA-HCV and Huh7/shRNA-Control stably express shRNA-HCV or shRNA-Control, respectively, following retroviral transduction. (a) Transient replication assay. An HCV replicon RNA, pRep-Fluc, was transfected into naive Huh7, Huh7/shRNA-HCV and Huh7/shRNA-Control cells. Luciferase activities of the cell lysates were measured serially at the times indicated, and the values were plotted as ratios relative to luciferase activities at 4 h. The luciferase activities at 4 h represent transfected replicon RNA. The data are mean ± SD. An asterisk denotes a *P*-value of less than 0.001 compared with the corresponding value of the naive Huh7 cells. (b) Stable colony formation assay. The HCV replicon, pRep-BSD, was transfected into naive Huh7, Huh7/shRNA-HCV and Huh7/shRNA-Control cells. The cells were cultured in the presence of blasticidin S (BSD) in the medium for ~3 weeks, and the BSD-resistant colonies were counted. These assays were repeated twice. The colony-forming units per microgram RNA (CFU/μg RNA) are shown at the bottom.

Absence of interferon-stimulated gene responses by siRNA delivery

It has been reported that double-stranded RNA may induce interferon-stimulated gene (ISG) responses which cause instability of mRNA, translational suppression of proteins and apoptotic cell