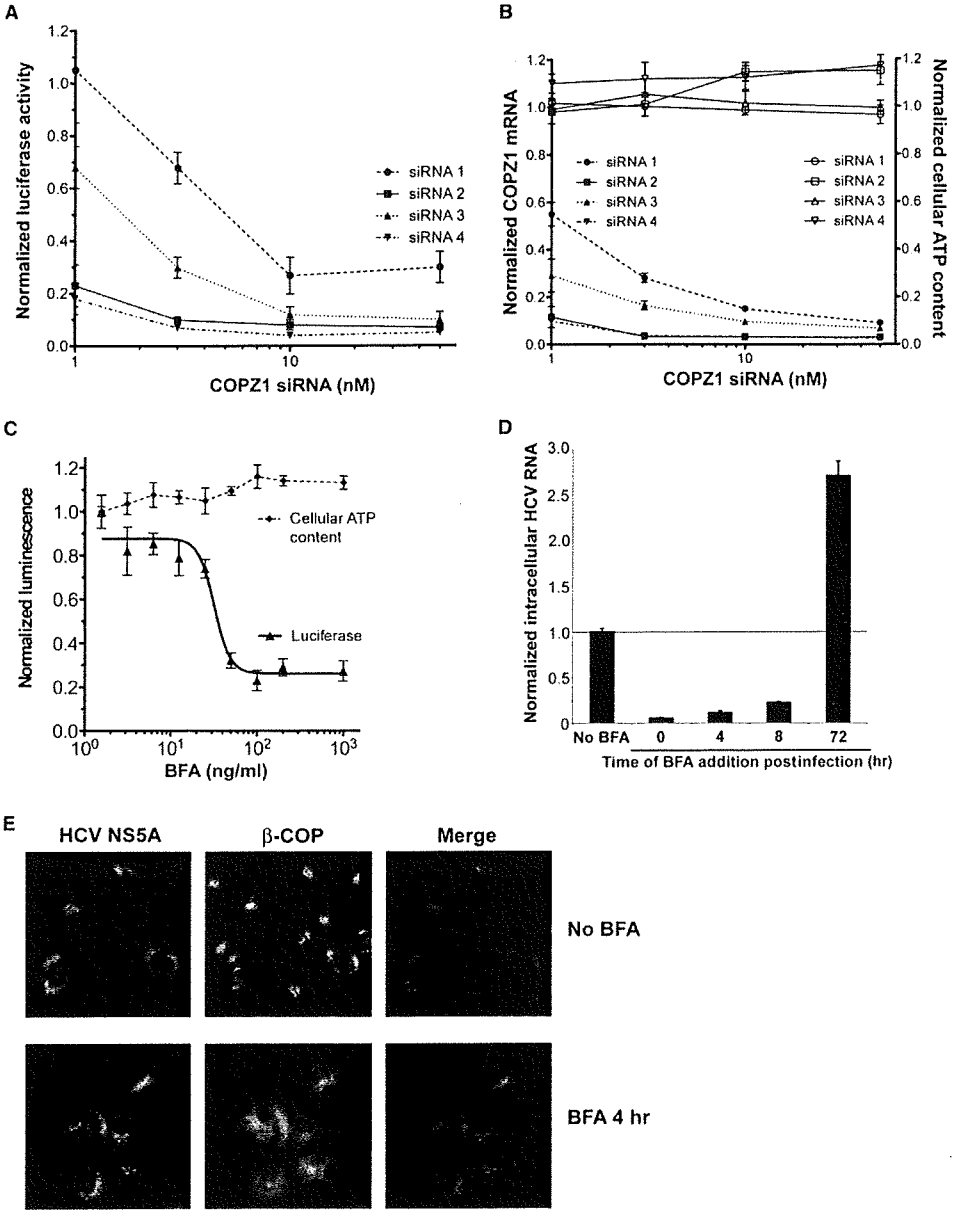

Figure 3. The COPI Complex Supports HCV Replication Early in the Viral Lifecycle

(A) Individual siRNA duplexes against *COPZ1* block replication of the full-length HCV replicon OR6 in a concentration-dependent manner. OR6 cells were transfected with the indicated siRNA duplexes for 64 hr and then assayed for luciferase activity. Values were obtained from quadruplicate wells in two independent experiments and are mean ± SD.

(B) Individual siRNA duplexes against *COPZ1* deplete *COPZ1* transcripts in a dose-dependent manner without detectable cytotoxicity. OR6 cells transfected with the indicated siRNA duplexes as in (A) were assayed for cellular ATP content (open symbols) or for *COPZ1* transcript levels by quantitative real-time PCR (closed symbols). Values were obtained from

triplicate qPCR measurements from duplicate wells in two independent experiments and are mean ± SD.

(C) Brefeldin A (BFA), a pharmacologic inhibitor of COPI function, blocks replication of the OR6 full-length replicon. OR6 cells were treated with the indicated concentrations of BFA for 24 hr and then assayed for luciferase activity (triangles) or cellular ATP content (diamonds). Values were obtained in quadruplicate and are mean ± SD.

(D) BFA blocks replication of the infectious JFH1 isolate early in viral infection and blocks HCV secretion late in infection. BFA was added at 100 ng/mL to Huh7.5.1 cells at the indicated times relative to the time of JFH1 infection. Intracellular HCV RNA was quantified by qPCR at 20 hr of BFA treatment and normalized to HCV RNA from mock treated cells. Values were obtained from triplicate qPCR replicates from duplicate wells and are mean ± SD.

(E) Preformed NS5A-positive structures are resistant to BFA treatment. OR6 cells were treated with 100 μg/mL BFA (lower panels) or 1% ethanol (upper panels) for 4 hr and then processed for immunofluorescence for HCV NS5A (left panels) and HCV NS5A (middle panels). Nuclei were counterstained with DAPI (blue). BFA causes dissociation of β-COP from the Golgi apparatus within minutes of treatment.

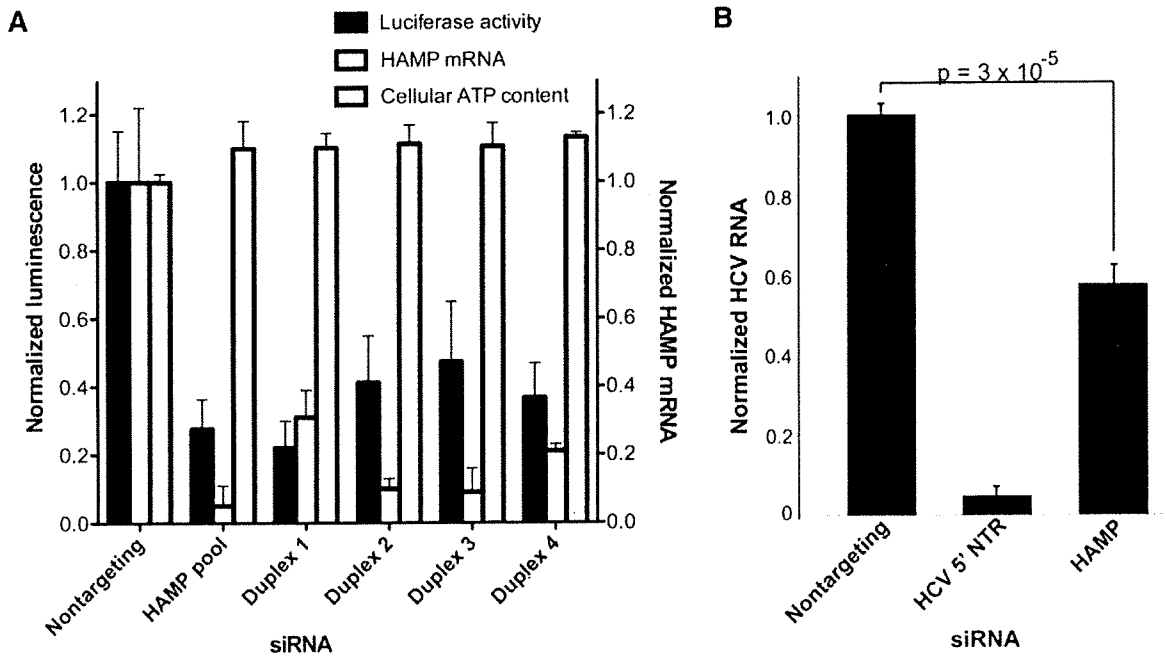

Figure 4. The Iron Regulatory Hormone Hepcidin Supports HCV Replication

(A) *HAMP* silencing blocks replication of the full-length HCV replicon OR6. OR6 cells were transfected with the indicated siRNAs for 72 hr and then assayed for luciferase activity (black bars), *HAMP* transcript levels (white bars), and cellular ATP content (gray bars). Values were obtained from quadruplicate wells in three independent experiments and are mean ± SD.

(B) *HAMP* silencing blocks replication of the infectious JFH1 HCV strain in Huh7 cells. Two-tailed p value was calculated by indepdendent two-sample t test. Values were obtained from triplicate qPCR replicates from duplicate wells in two independent experiments and are mean ± SD.

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Original Article

Two flavonoids extracts from *Glycyrrhizae radix* inhibit *in vitro* hepatitis C virus replication

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Aim: Traditional herbal medicines have been used for several thousand years in China and other Asian countries. In this study we screened herbal drugs and their purified compounds, using the Feo replicon system, to determine their effects on *in vitro* HCV replication.

Methods: We screened herbal drugs and their purified extracts for the activities to suppress hepatitis C virus (HCV) replication using an HCV replicon system that expressed chimeric firefly luciferase reporter and neomycin phosphotransferase (Feo) genes. We tested extracts and 13 purified compounds from the following herbs: *Glycyrrhizae radix*; *Rehmanniae radix*; *Paeoniae radix*; *Artemisiae capillari spica*; and *Rhei rhizoma*.

Results: The HCV replication was significantly and dose-dependently suppressed by two purified compounds, isoliquiritigenin and glycycoumarin, which were from *Glycyrrhizae radix*. Dose-effect analyses showed that 50% effective concentrations were 6.2 ± 1.0 μg/mL and 15.5 ± 0.8 μg/mL for isoliquiritigenin and glycycoumarin, respectively. The MTS assay did not show any effect on cell growth and viability at these effective concentrations, indicating that the effects of the two compounds were specific to HCV replication. These two compounds did not affect the HCV IRES-dependent translation nor did they show synergistic action with interferon-alpha.

Conclusion: Two purified herbal extracts, isoliquiritigenin and glycycoumarin, specifically suppressed *in vitro* HCV replication. Further elucidation of their mechanisms of action and evaluation of *in vivo* effects and safety might constitute a new anti-HCV therapeutics.

Key words: hepatitis C virus, herbal drugs, replicon

INTRODUCTION

HEPATITIS C VIRUS (HCV) infects 170 million people worldwide and is characterized by chronic liver inflammation and fibrogenesis leading to end-stage liver failure and hepatocellular malignancy.[1,2] The difficulty in eradicating HCV is attributable, in part, to limited treatment options against the virus. Currently, combination therapy using pegylated interferon-alpha (IFN) and ribavirin has been used worldwide.[3–5] The success rates, however, are almost half of patients treated. Furthermore, these therapies carry a significant risk of serious side effects. Thus, the development of alternative therapeutic agents against HCV is our high priority goal.

We have reported an HCV subgenomic replicon that expresses chimeric luciferase reporter "Feo" protein.[6] This Feo replicon supports stable and high levels of autonomous HCV RNA replication in transfected cells. Furthermore, the level of luciferase correlates well with levels of HCV RNA production, so that luciferase can be used as a reliable surrogate marker for HCV replication. This chimeric reporter replicon system has contributed the discovery of novel anti-HCV substances such as cyclosporins,[7–9] short interfering RNA,[10,11] interferon-gamma[12] and HMG-CoA reductase inhibitors.[13,14]

Traditional herbal drugs have been used for several thousand years in China and other Asian countries. Although these pharmacological activities are not fully

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Table 1 List of herbal drugs and their purified extracts

Herbal drug	Purified compound
Glycyrrhizae radix	Isoliquiritigenin
	Glycycoumarin
	Isoliquiritin
	Licuroside
Paeoniae radix	Paeoniflorin
	1,2,3,6-tetra-O-galloyl-β-D-glucose
Rhei Rhizoma	Rhein 8-O-β-glucoside
Rehmanniae radix	Acteoside
	Martynoside
	Isoacteoside
Artemisiae capillari spica	Demethoxycapillarisin
	3,4-di-o-galloylquinic acid
	Acteosyringone

characterized, they also have been safely used for many clinical conditions in Japan. For example, Sho-saiko-to (TJ-9; Xiao-Chae-Hu-Tang in Chinese), an oral medicine, which consists of seven herbal components (*Bupleuri radix, Pinelliae tuber, Scutellariae radix, Ginseng radix, Grycyrrhhizae radix, and Zingiberis rhizoma*),[15] has been clinically used for the treatment of chronic viral liver disease. It has been reported to regulate the cytokine production system in patients with hepatitis C[16] and to prevent the development of HCC in patients with non-B cirrhosis.[17] *Glycyrrhizin*, the major component of *Glycyrrhizae radix* (licorice), has also been used for the treatment of chronic hepatitis in Japan, known to have an alanine transaminase-lowering effect.[18,19] Despite the clinical effects of these herbal drugs, they did not suppress the HCV replication *in vitro*.[15]

In the present study, we applied the Feo replicon system to screen the herbal drugs and their purified compounds for their effects on *in vitro* HCV replication. Here, we show that two purified compounds from the herbal extracts specifically and substantially suppressed HCV replication.

MATERIALS AND METHODS

Purified compounds (Table 1)

THIRTEEN COMPOUNDS WERE purified from five herbal drugs: *Glycyrrhizae radaix; Rhemanniae radix; Paeoniae radix; Artemisiae Capillari Spica;* and *Rhei Rhizoma* (Table 1; Tsumura, Tokyo, Japan). These extracts were prepared at concentrations of 5 mg/mL in dimethyl sulfoxide (DMSO), then stored at −20°C until use. Recombinant human interferon (IFN) alpha-2b was obtained from Schering-Plough (NJ, USA).

Cell culture

A human hepatoma cell line, Huh7, was maintained in Dulbecco's modified Eagle's medium (Sigma, MO, USA) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. Huh7 cells expressing the HCV replicon were cultured in a medium containing 200 μg/mL G418 (Wako, Osaka, Japan).

HCV subgenomic replicon construct

An HCV subgenomic replicon plasmid, pHCV1bneodelS,[20] was reconstructed by substituting the neomycin phosphotransferase gene with a fusion gene comprising the firefly luciferase and neomycin phosphotrasnferase (pRep-Feo) (Fig. 1a). RNA was synthesized from pRep-

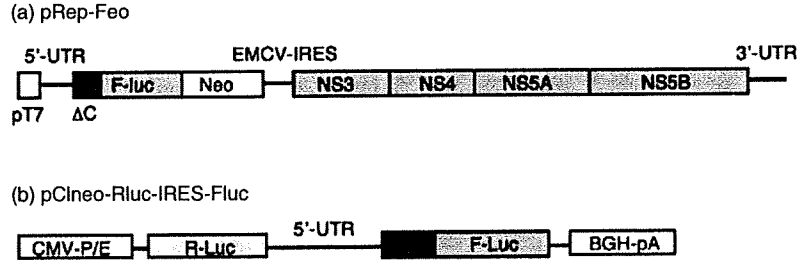

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "Feo"[6]. NS, nonstructural region; pT7, T7 promoter; 3′ UTR, 3′ untranslated region. (b) A plasmid, pCIneo-Rluc-IRES-Fluc, was constructed to analyze HCV-IRES-mediated translation efficiency . The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-internal ribosome entry site (IRES)-mediated initiation, was stably transfected into Huh7 cells.

Feo and transfected into Huh7 cells. After culture in the presence of G418, cell lines stably expressing the replicon were established.[10,21]

HCV-IRES reporter construct

A plasmid, pCIneo-Rluc-IRES-Fluc, was used to analyze HCV internal ribosome entry site (IRES)-mediated translation efficiency (Fig. 1b).[22] The plasmid, expressing a bicistronic RNA, in which *Renilla* luciferase (Rluc) was translated in a cap-dependent manner and firefly luciferase (Fluc) was translated by HCV-IRES-mediated initiation, was stably transfected into Huh7 cells. After culture in the presence of G418, Huh7/CRIF cells were established.[9] Activities of the HCV-IRES-mediated translation were measured by culture of Huh7/CRIF cells in the presence of drugs and by dual luciferase assays after 48 h.

Luciferase assays and measurements of antiviral activity

Huh7/Rep-Feo cells were cultured with various concentrations of herbal extracts or compounds. Levels of HCV replication were quantified by internal luciferase assay after 48 h of culture. Luciferase activities were quantified using a luminometer (Promega, WI, USA) and the Bright-Glo Luciferase Assay System (Promega). Assays were performed in triplicate and the results were expressed as means ± SD as percentages of the controls. The 50% effective concentrations (EC50) were calculated using probit method. The determination of EC50 was performed three times, and presented as mean ± SD in each compound.

Realtime RT-PCR analysis

Total cellular RNA was extracted from cultured cells or liver tissue using ISOGEN (Nippon Gene, Tokyo, Japan). Two μg of total cellular RNA was used to generate cDNA from each sample using the SuperScript II reverse-transcriptase (Invitrogen, CA, USA). The replicon RNA expression levels were measured using the Applied Biosystems 7500 Fast Realtime PCR System (Applied Biosystems, CA, USA) and QuantiTect SYBR Green PCR Kit (QIAGEN, CA, USA). Sequences of a pair of primers has been described elsewhere.[23]

Northern blottings

Expression of HCV subgenomic RNA was detected as previously reported.[24] Total cellular RNA was extracted from cells using ISOGEN (Nippon Gene, Tokyo, Japan). Fifteen micrograms of the total cellular RNA was electrophoresed on a 1.0% denaturing agarose-formaldehyde gel and was transferred to a Hybond-N+ nylon membrane (Amersham-Pharmacia Biotech, Sweden). The upper part of the membrane, which contained the HCV replicon RNA, was hybridized with a digoxigenin-labeled probe that was specific for the full-length replicon sequence, and the lower part of the membrane was hybridized with a probe specific for beta-actin. The signals were detected in a chemiluminescence reaction using a Digoxigenin Luminescent Detection Kit (Roche, Germany) and visualized using a Fluoro-Imager (Roche).

Western blottings

Western blotting was done as reported previously.[24] Thirty micrograms of total cell lysate was separated using NuPAGE 4–12% Bis-Tris gels (Invitrogen, CA, USA) and blotted onto an Immobilon polyvinylidene difluoride membrane (Roche). The membrane was incubated with the primary antibodies followed by a peroxidase-labeled anti IgG antibody, and visualized by chemiluminescence reaction (BM Chemiluminescence Blotting Substrate; Roche). The antibodies used were anti-NS5A (BioDesign, ME, USA), anti-core (provided by Dr. Wakita), and anti-beta-actin antibodies (Sigma).

MTS assays

To evaluate cell viability, MTS (dimethylthiazol carboxymethoxyphenyl sulfophenyl tetrazolium) assays were performed using a Cell Titer 96 Aqueous One Solution Cell Proliferation Assay (Promega) according to manufacturer's directions.

HCV-JFH1 virus cell culture

An *in vitro* transcribed HCV-JFH1 RNA[25] was transfected into Huh7.5.1 cells.[26] Naïve Huh7.5.1 cells were subsequently infected by culture supernatant of the JFH1-RNA transfected Huh-7.5.1 cells, and subjected to culture in the presence of drugs. Culture medium was collected serially and HCV core antigen was measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen; Ortho-Clinical Diagnostics, Tokyo, Japan). Cellular virus expression was measured by the Western blotting using anti-core antibodies.[27]

Statistical analyses

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

RESULTS

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

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

Realtime-RT-PCR and Western blotting analyses

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

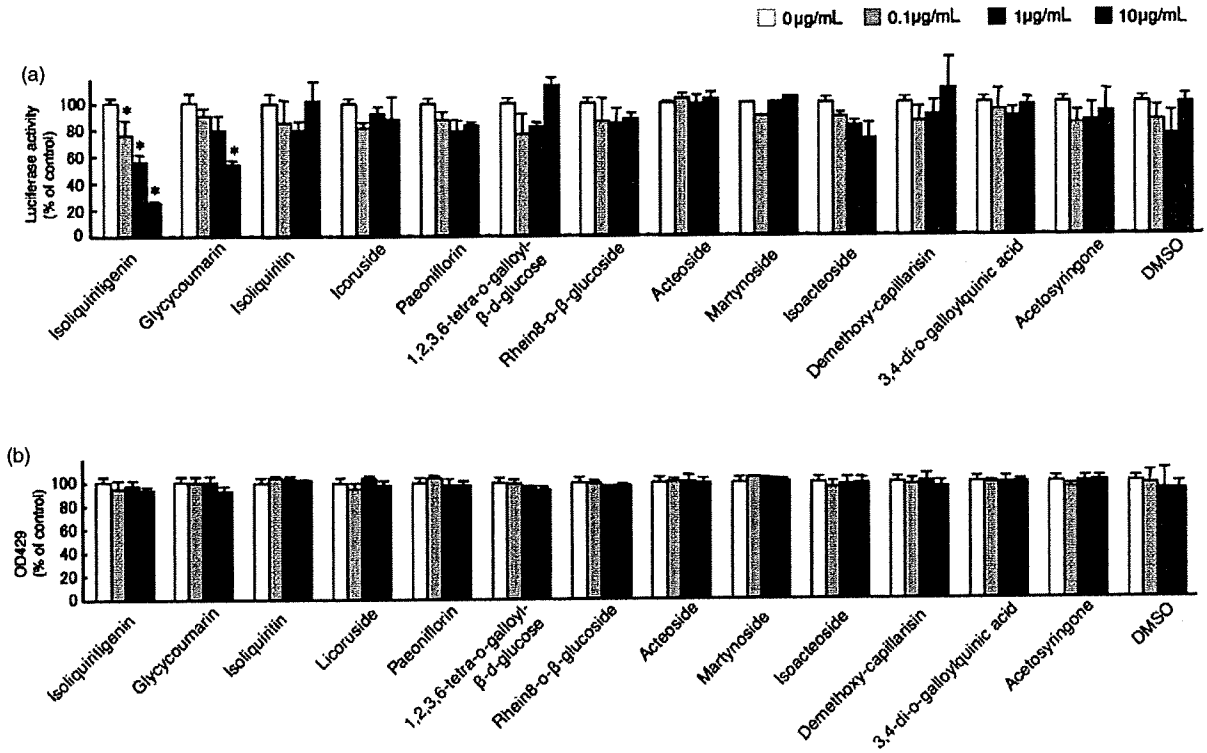

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured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

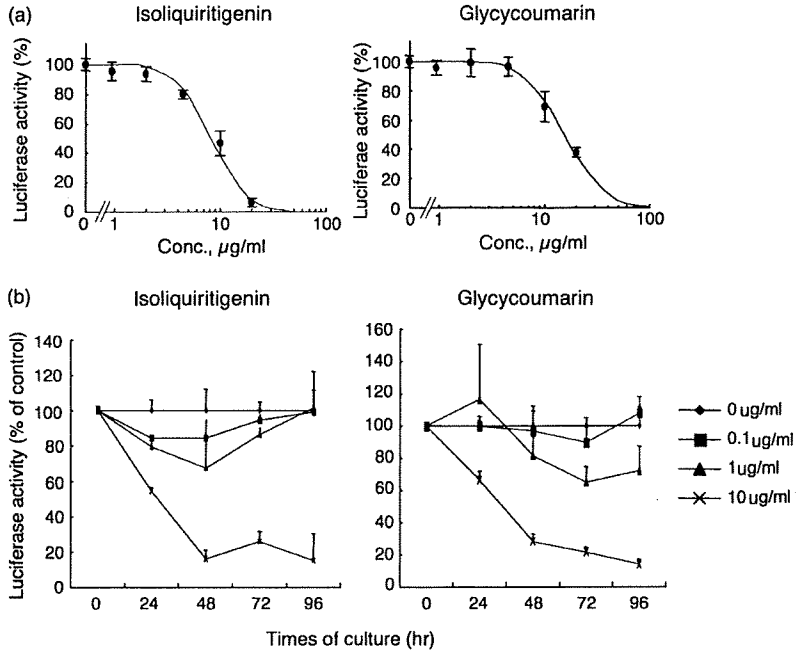

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo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

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

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

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

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

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

Isoliquiritigenin and glycycoumarin suppress HCV-JFH1 virus cell culture

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

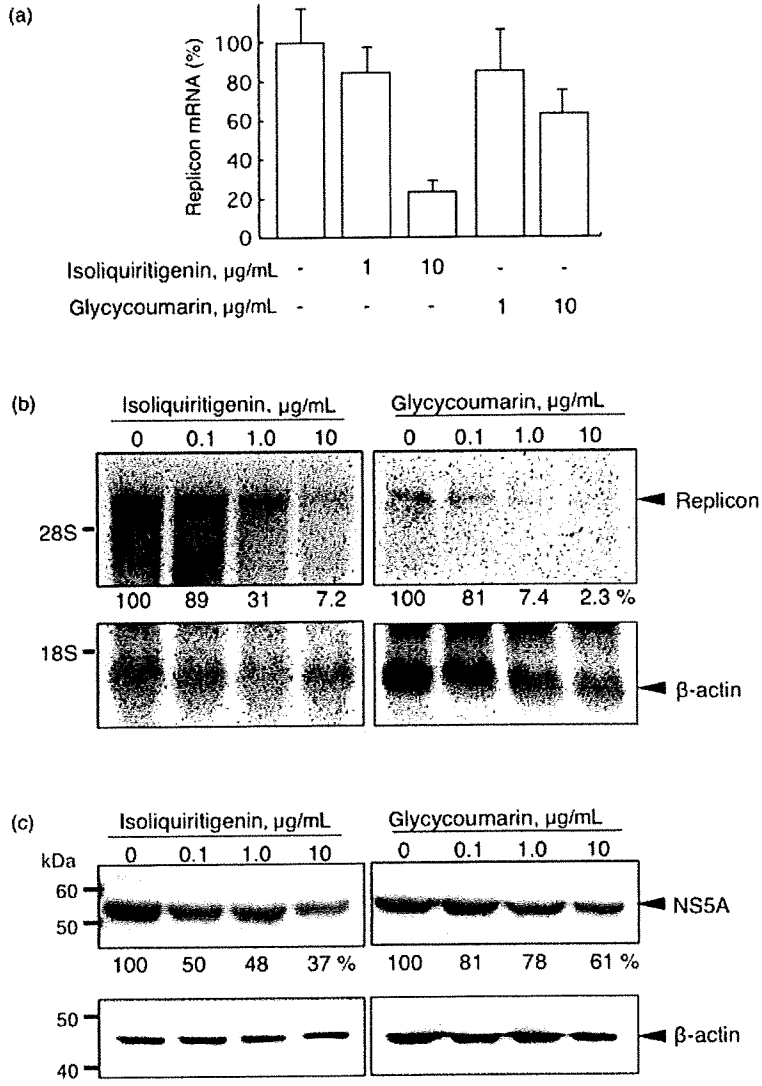

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot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

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

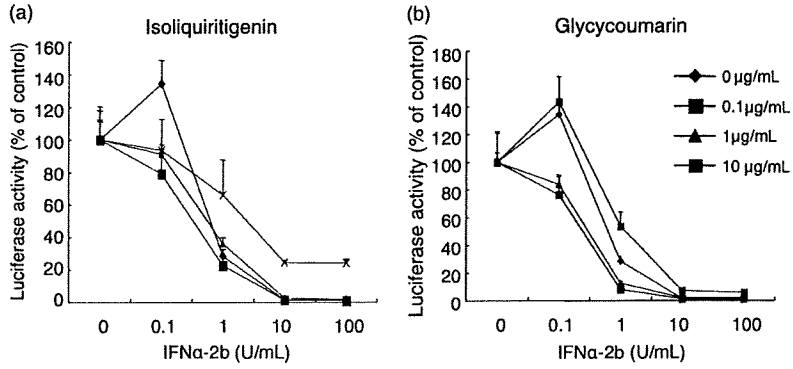

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured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

DISCUSSION

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

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

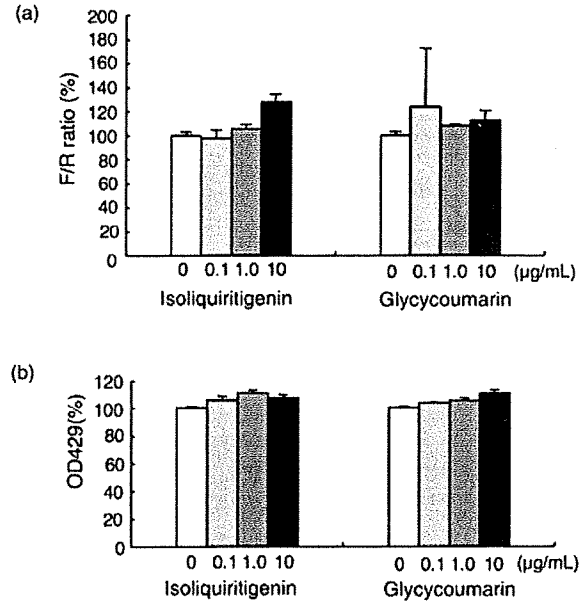

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin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

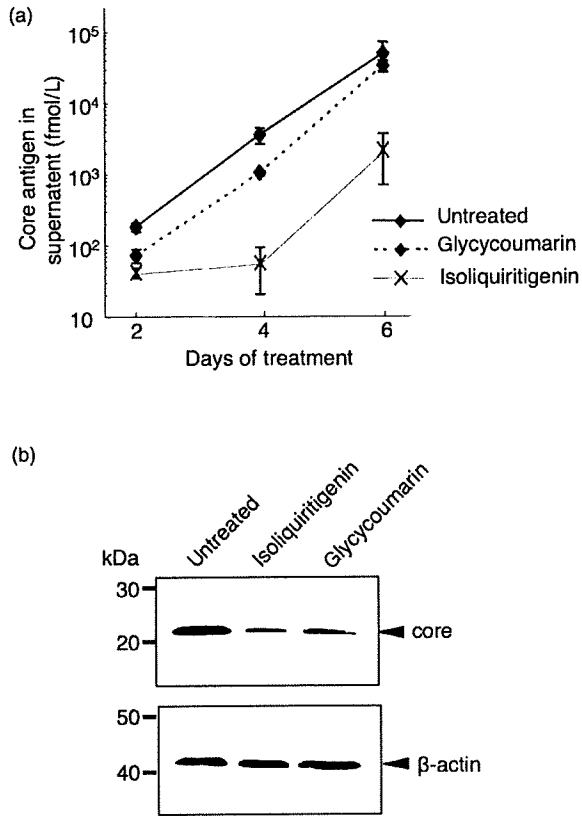

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin.* (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days,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

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

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

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

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

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ABSTRACT

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

1. Introduction

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

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

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

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constructed using HCV-positive sera from healthy carriers (1B-4 and 1B-5) and one replicon was constructed using serum sampled from a case of acute hepatitis C (KAH5). These replicons contained neomycin phosphotransferase (Neo) and *Renilla* luciferase (RL) genes at the first cistron of the replicon with the aim of conducting a stable and highly sensitive reporter assay. In this study of four replicons, we examined the anti-HCV reagents IFN-α, IFN-γ, IFN-λ, CsA, and various statins (pitavastatin (PTV), fluvastatin (FLV), and rosvastatin (RSV)), and we found diverse sensitivities among the replicons. Newly developed replicons will be useful tools for the present study regarding the diverse sensitivities of genotype 1b HCVs to anti-HCV reagents, including IFNs.

2. Materials and methods

2.1. HCV-positive sera and GeneBank accession numbers

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

2.2. Cell cultures

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

2.3. RT-nested PCR

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

2.4. Plasmid construction

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

2.5. RNA transcription

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

2.6. RNA transfection and G418-resistant cells

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

2.7. Western blot analysis

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

2.8. Quantification of HCV RNA

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

2.9. Northern blot analysis

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

2.10. Reagents

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

2.11. Luciferase reporter assay

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

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

2.12. Statistical analysis

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

3. Results

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

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

3.2. Diverse activity of various IFN types on HCV replicons

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

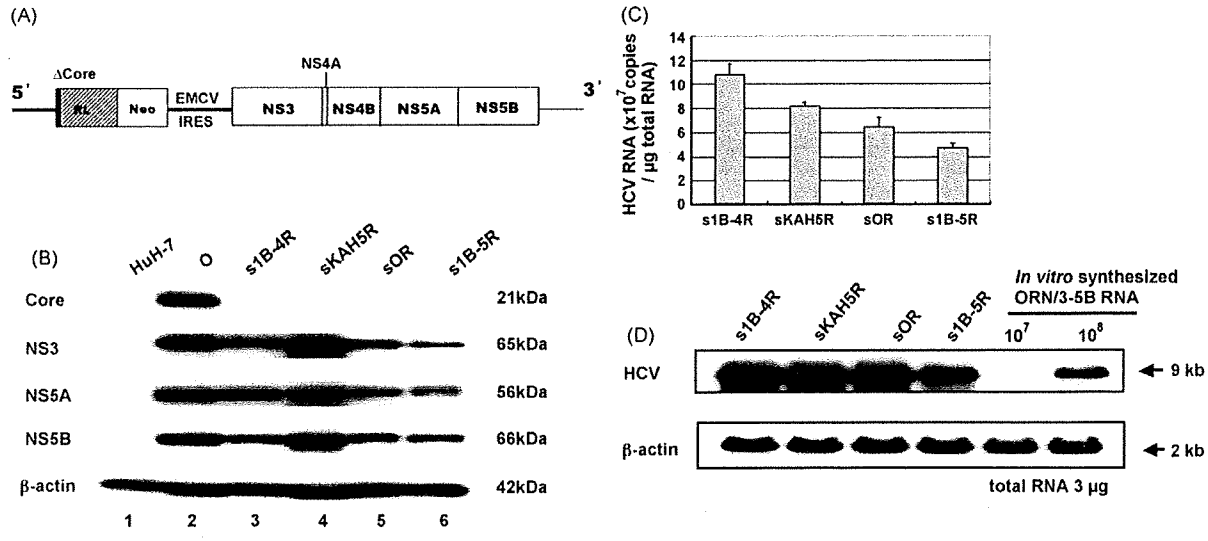

Fig. 1. The expression of HCV proteins and HCV RNAs in four replicon-harboring cell lines. (A) Schematic gene organization of subgenomic replicon RNA. The NS3 to NS5B region and 12 N-terminal amino acid residues of the Core (ΔC) are depicted in closed boxes. Untranslated regions, EMCV IRES, RL, and Neo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

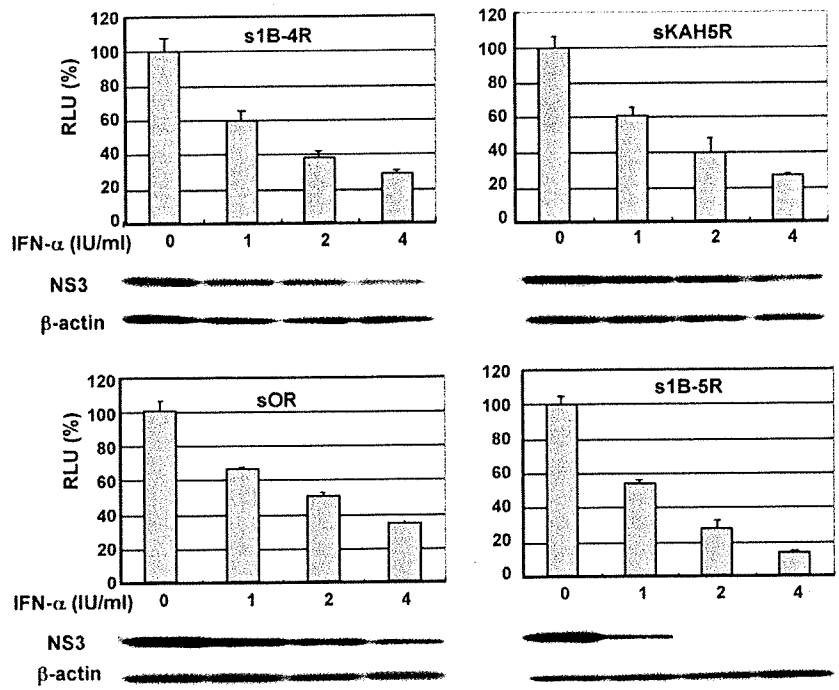

Fig. 2. The activity of IFN-α on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-α treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-α (0, 1, 2, and 4 IU/ml) for 72 h. Then, the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels). The percent relative luciferase unit (RLU (%)) was calculated with the RL activity of untreated cells assigned at a value of 100%. The data indicate means ± S.D.s of triplicate samples. All of the luciferase assays were repeated at least three times. β-Actin was used as a control for the amount of proteins loaded per lane.

coincided with the results of the reporter assay. Thus, these results indicated that genotype 1b replicons possess different sensitivities to IFN-α.

Next, we examined the sensitivity of four replicons to type II IFN, IFN-γ, because in our previous study, HCV (genotype 1b, AH1 strain) from a patient with AH C was found to be more resistant to IFN-γ than was HCV-O (Mori et al., 2008). In this study, sKAH5R was also derived from the serum of a patient with AH C. The reporter assay revealed that sKAH5R has the lowest sensitivity to IFN-γ (EC_{50}: 2.26 IU/ml) among the replicons tested (Fig. 3). To calculate the EC_{50} of IFN-γ to sKAH5R, we also treated sKAH5R with IFN-γ at 2 and 4 IU/ml for 72 h (data not shown). The EC_{50} of IFN-γ to s1B-4R, sOR,

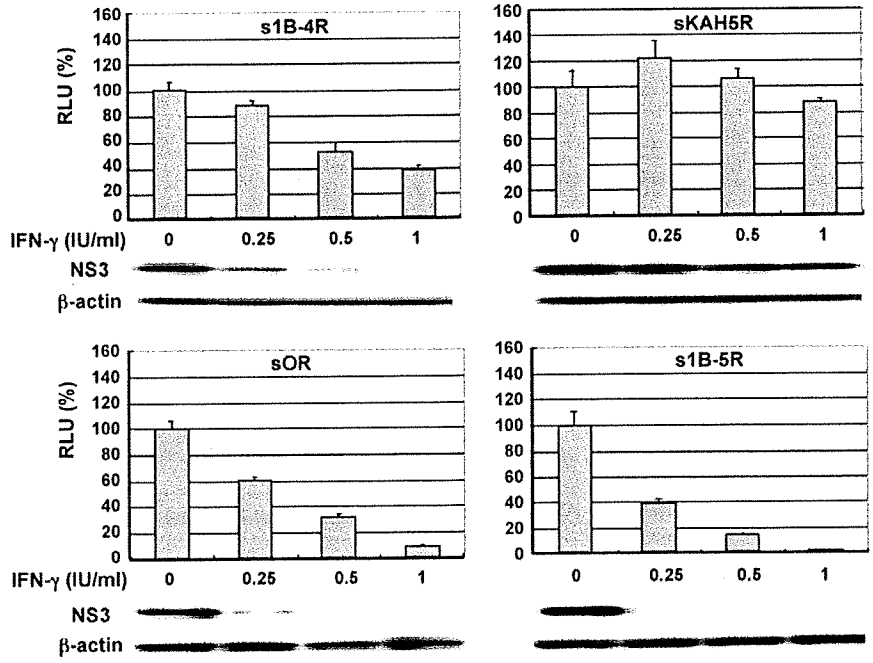

Fig. 3. The activity of IFN-γ on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in IFN-γ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-γ (0, 0.25, 0.5, and 1 IU/ml) for 72 h and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels). All of the luciferase assays were repeated at least three times.

and s1B-5R was 0.54, 0.33, and 0.21 IU/ml, respectively. The results of Western blot analyses of sensitivity to IFN-γ coincided with those of the reporter assay. Interestingly, again in this study, the HCV RNA derived from the patient with AH C was resistant to IFN-γ, as was the AH1 strain. These results may suggest that AH C in pathologic states of HCV infection may be involved in the IFN-γ resistance feature of the replicon. Further studies will be needed to clarify this issue.

We analyzed a recently identified type III IFN, IFN-λ, in terms of its anti-HCV activity against four HCV replicons. IFN-λ shares the same Jak/Stat signaling pathway with type I IFNs, which express a common set of IFN-stimulating genes (ISGs). However, IFN-λ uses distinct receptors composed of IFNLR1 and IL10R2. Here, sKAH5R and s1B-4R, respectively, exhibited the lowest and highest sensitivities to IFN-λ (EC_{50}: 8.25 and 1.50 ng/ml) (Fig. 4A). Additionally, sOR and s1B-5R exhibited moderate sensitivity to IFN-λ (EC_{50}: 4.48 and 4.82 ng/ml, respectively) (Fig. 4A). These diverse inhibitory activities of IFN-λ were also confirmed by Western blot analysis (Fig. 4B). Moreover, s1B-4R and sKAH5R showed similar sensitivities to IFN-α. However, it was of note that these replicons exhibited different degrees of sensitivity to IFN-λ, which uses a common Jak/Stat signaling pathway. These results suggest the presence of a complicated antiviral mechanism in type I and III IFNs. Recently, it was reported that IFN-λ in combination with IFN-α or IFN-γ enhanced anti-HCV activity (Pagliaccetti et al., 2008). Therefore, s1B-4R and sKAH5R are useful for the study in combination treatment of IFNs.

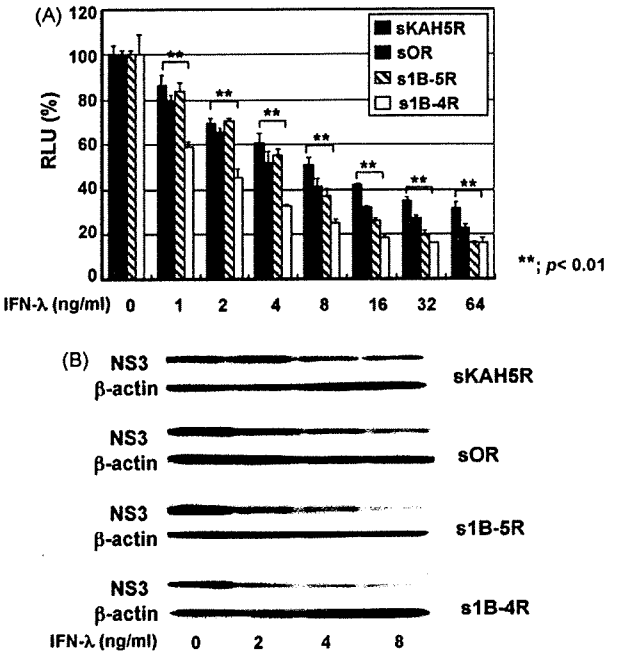

Fig. 4. Effects of IFN-λ on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in IFN-λ treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with IFN-λ (0, 2, 4, 8, 16, 32, and 64 ng/ml) for 72 h, and then the cells were subjected to RL assay (B) and Western blot analysis. Four replicon-harboring cell types were treated with IFN-λ (0, 2, 4, and 8 ng/ml) for 72 h and were subjected to Western blot analysis of NS3. All of the luciferase assays were repeated at least three times.

3.3. Diverse effects of PTV but not CsA on HCV replicons

Anti-HCV reagents other than IFNs were examined in terms of their effectiveness in the presence of various replicons. As CsA is a well-characterized anti-HCV reagent, we examined the sensitivities of the replicons to CsA by reporter assay. There were no significant differences in sensitivity to CsA among the replicons (Fig. 5). The EC_{50} of CsA to s1B-4R, sKAH5R, sOR, and s1B-5R was 0.71, 0.96, 1.10, and 0.85 μg/ml, respectively. We also obtained similar results by Western blot analysis. In contrast to the findings of the IFN study, there were no significant differences in sensitivity to CsA among the genotype 1b replicons tested.

Statins, which are HMG-CoA reductase inhibitors, are yet another well-characterized anti-HCV reagent. Therefore, we

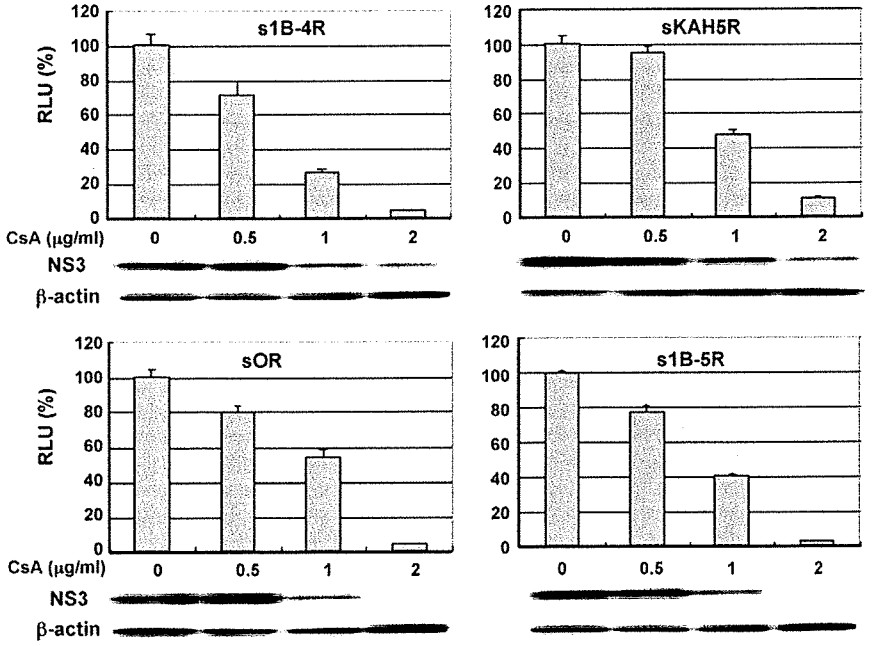

Fig. 5. The activity of CsA on HCV replicon RNA replication. Reporter assay and Western blot analysis for HCV replicons in CsA treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with CsA (0, 0.5, 1, and 2 μg/ml) for 72 h, and then the cells were subjected to RL assay (upper panels) and Western blot analysis for NS3 (lower panels), as described in Fig. 2. All of the luciferase assays were repeated at least three times.

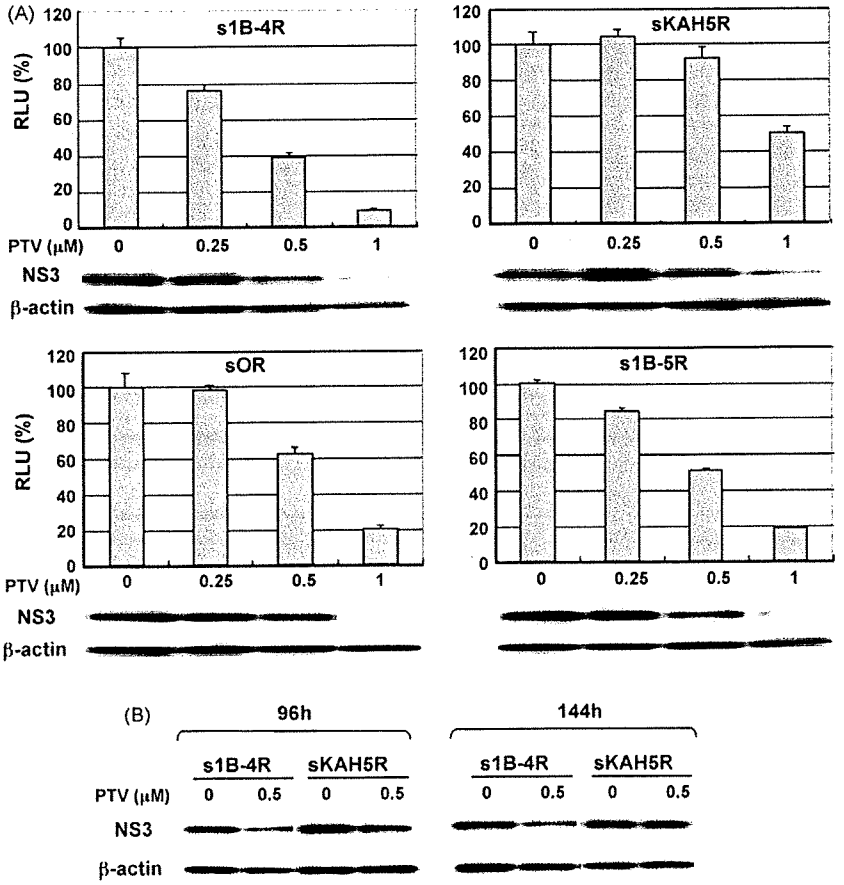

Fig. 6. The activity of PTV on HCV replicon RNA replication. (A) Reporter assay and Western blot analysis for HCV replicons in PTV treatment. s1B-4R cells, sKAH5R cells, sOR cells, and s1B-5R cells were treated with PTV (0, 0.25, 0.5, and 1 μM) for 72 h, and then the cells were subjected to RL assay and Western blot analysis, as described in Fig. 2. All of the luciferase assays were repeated at least three times. (B) s1B-4R cells and sKAH5R cells were treated with PTV (0 and 0.5 μM) for 96 and 144 h and were subjected to Western blot analysis.

examined the sensitivity of the replicons to PTV. The reporter assay revealed that sKAH5R has the lowest sensitivity to PTV (EC_{50}: 1.00 μM) among the replicons tested (Fig. 6A). The sensitivities of the other replicons to PTV were almost identical, and the EC_{50} values of s1B-4R, sOR, and s1B-5R were 0.40, 0.64, and 0.51 μM, respectively (Fig. 6A). We also obtained similar results by Western blot analysis using cell lysates at 72 h after treatment. The inhibition of HCV protein in s1B-4R persisted until 96 and 144 h after treatment with PTV (0.5 μM) (Fig. 6B). FBL2 is identified as a geranylgeranylated cellular protein required for HCV RNA replication (Wang et al., 2005). Therefore, we examined the expression levels of FBL2 in s1B-4R and sKAH5R. The expression levels of FBL2 mRNA were almost equal between both cells (Supplemental Fig. 2). This result indicates that low sensitivity of sKAH5R to statins is not due to the low expression of FBL2. Previously, we used an OR6 assay system to demonstrate that PTV inhibited genome-length HCV RNA replication, and the EC_{50} of PTV was found to be 0.45 μM (Ikeda et al., 2006; Ikeda and Kato, 2007). The EC_{50} values of PTV in three replicons other than sKAH5R were almost equal to that of PTV in OR6. These results, taken together, suggest that sKAH5R is resistant to PTV as well as to IFN-γ and IFN-λ.

3.4. Resistance to statins in a replicon from a patient with AH C

To further confirm that sKAH5R is resistant to statins, we examined the sensitivity of the replicons to FLV and RSV using a reporter assay. Here, sKAH5R exhibited the lowest sensitivity to FLV and RSV (Fig. 7). In the case of sKAH5R, the EC_{50} of FLV was 7.87 μM, and the EC_{50} of RSV exceeded 20 μM, because RSV was toxic to cells at concentrations of more than 20 μM. Moreover, sOR and s1B-5R showed almost equal and moderate sensitivities to both FLV and RSV. It was of note that these results were in agreement with those regarding PTV sensitivity, i.e., s1B-4R exhibited the highest sensitivity to both FLV and RSV. The EC_{50} values of FLV and RSV to s1B-4R were 2.82 and 10.12 μM, respectively. These results suggest that sKAH5R exhibits some resistance, and s1B-4R some sensitivity, to statins. Therefore, these replicons may serve as useful tools for investigating the mechanism of the anti-HCV activity of statins.

3.5. Polyclonal KAH5 replicon with a statin-resistant phenotype

sKAH5R replicon cells were found to possess the least sensitivity to statins among the replicon-harboring cells tested. However, the statin-resistant phenotype may be due to cell clonality rather than HCV strain, because the sKAH5R replicon cells used here were a cloned cell line selected from numerous G418-resistant colonies. We thus examined the sensitivity of polyclonal sKAH5R cells to statins, and then compared the results with those obtained using polyclonal s1B-4R cells in order to rule out this possibility. In polyclonal sKAH5R, the EC_{50} values of PTV and FLV were 0.88 and 6.56 μM, respectively (Fig. 8), and the EC_{50} of RSV exceeded 20 μM (Fig. 8), because RSV is toxic to these cells at concentrations of more than 20 μM. In polyclonal s1B-4R, the EC_{50} values of PTV, FLV, and RSV were 0.47, 3.41, and 10.00 μM, respectively (Fig. 8).

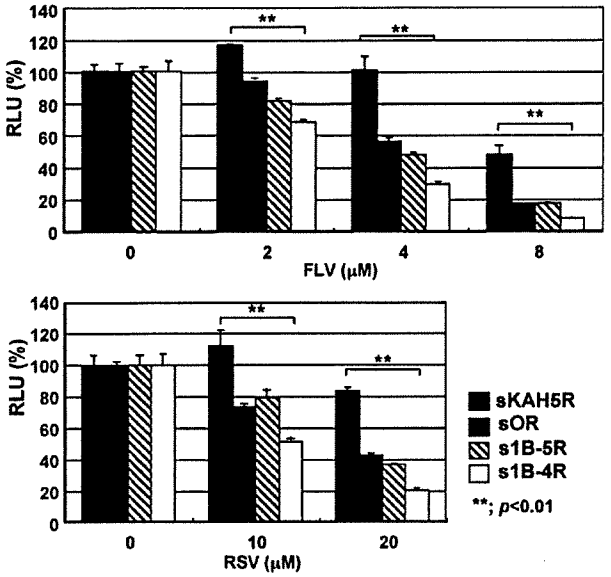

Fig. 7. HCV replicons exhibit diverse sensitivities to statins. Reporter assay of the sensitivity of HCV replicons to FLV. sKAH5R cells (light column), sOR cells (dark column) s1B-5R cells (shaded column), and s1B-4R cells (open column) were treated with FLV (0, 2, 4, and 8 μM) for 72 h (upper panel), and then the cells were subjected to an RL assay. A reporter assay of RSV sensitivity to HCV replicons was performed using RSV (0, 10, and 20 μM) (lower panel). All of the luciferase assays were repeated at least three times.

The polyclonal sKAH5R cells exhibited less sensitivity to PTV, FLV, and RSV than did polyclonal s1B-4R cells. These results suggest that the statin-resistant phenotype of sKAH5R is due to the KAH5 strain-specific viral factors rather than to the cell clonality of sKAH5R cells.

3.6. Second generation of sKAH5R possessed less sensitive phenotype to PTV than that of s1B-4R

To further demonstrate that the statin-resistant phenotype of sKAH5R is not due to the clonal specificity of the cells, we developed the second generation of sKAH5R and s1B-4R. Total RNAs from sKAH5R and s1B-4R were introduced into naïve OR6c cells. The second generation of sKAH5R and s1B-4R, designated as ssKAH5R and ss1B-4R, respectively, were selected as the polyclonal cells after 3 weeks G418 selection. ssKAH5R revealed less sensitive to PTV than ss1B-4R (EC_{50}: 0.76 μM vs. 0.43 μM) (Fig. 9A). These results further support that the viral factor plays the major role in the statin-resistant phenotype of sKAH5R.

On the contrary, there was no significant difference between ssKAH5R and ss1B-4R in the sensitivity to IFN-λ (EC_{50}: 4.1 ng/ml vs. 3.5 ng/ml) (Fig. 9B). These results suggest that cellular factors are dominant in the sensitivity to IFN-λ.

Table 1
EC_{50} of anti-HCV reagents to HCV replicons.

	s1B-4R	sKAH5R	sOR	s1B-5R
IFN-α (IU/ml)	1.44	1.37	2.35	1.10
IFN-γ (IU/ml)	0.54	2.26	0.33	0.21
IFN-λ (ng/ml)	1.50	8.25	4.48	4.82
CsA (μg/ml)	0.71	0.96	1.10	0.85
PTV (μM)	0.40	1.00	0.64	0.51
FLV (μM)	2.82	7.87	4.53	3.81
RSV (μM)	10.12	ND	17.52	17.10

ND: not determined.

4. Discussion

In the present study, we established an HCV replicon reporter assay system using four genotype 1b HCV strains (1B-4, KAH5, O, and 1B-5). Genotype 1 HCV infection accounts for most cases of resistance to current PEG-IFN-α and ribavirin therapy. However, in most previous reports, anti-HCV reagents have been assessed in terms of their effects using replicon(s) derived from only one or two HCV strain(s). Therefore, in order to further evaluate the anti-HCV activity of various reagents among the genotype 1b HCVs, we performed a comparative study using the present replicon reporter assay system, which was found to a precise, highly sensitive, and time-sparing assay compared to assays involving the quantification of HCV RNA. The EC_{50} values of anti-HCV reagents in four genotype 1b replicons are summarized in Table 1.

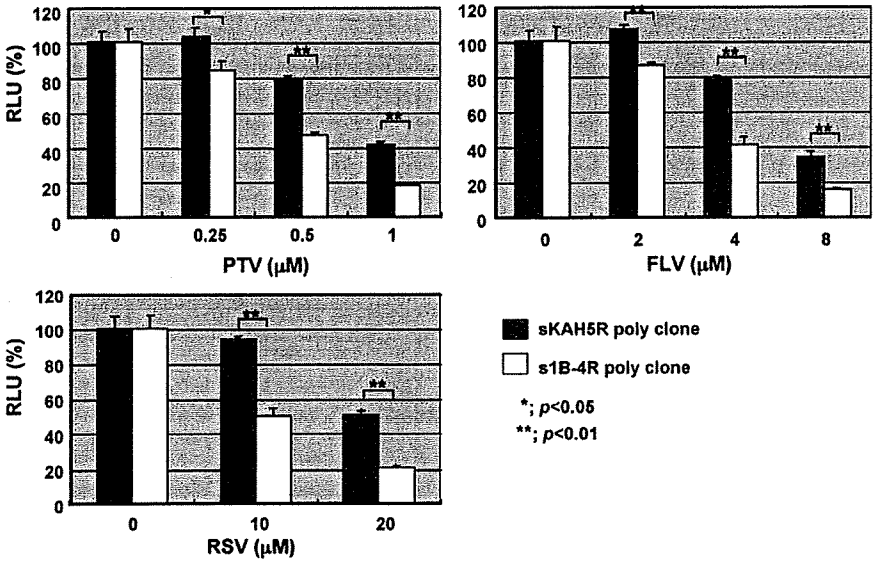

Fig. 8. Diverse sensitivities of polyclonal replicons to statins. Reporter assay of the sensitivity of polyclonal sKAH5R and s1B-4R replicons to PTV, FLV, and RSV. Polyclonal sKAH5R cells and polyclonal s1B-4R cells were treated with PTV (0, 0.25, 0.5, and 1 μM), FLV (0, 2, 4, and 8 μM), and RSV (0, 10, and 20 μM) for 72 h and then were subjected to RL assay. All the luciferase assays were repeated at least three times.