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Hepatitis C virus NS5A protein increases hepatic lipid accumulation via induction of activation and expression of PPARgamma

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ABSTRACT

Steatosis is an established risk factor for disease progression in cases of chronic hepatitis C. Recently it was demonstrated that Hepatitis C virus (HCV) core and non-structural (NS) 2 proteins (NS2) induce lipid accumulation in hepatic cells. However, it has yet to be determined whether other HCV proteins are associated with lipid metabolism. The NS5A augmented the transcriptional activity and gene expression of PPARγ. Furthermore, NS5A increased the ability to recruit the transcriptional coactivator PGC-1s to the PPRE with PPARγ, as well as the interaction with PPARγ2 and PGC-1α. Our results indicate that NS5A may exploit multiple strategies that enhance PPARγ-induced lipid accumulation.

Structured summary:
MINT-7229685: *PPAR gamma 2* (uniprotkb:P37231-2) *physically interacts* (MI:0914) with *PGC1 alpha* (uniprotkb:Q9UBK2) by *pull down* (MI:0096)
MINT-7229712: *PPAR gamma 2* (uniprotkb:P37231-2) *physically interacts* (MI:0914) with *NS5A* (uniprotkb:P26662) by *pull down* (MI:0096)
MINT-7229698: *PPAR gamma 2* (uniprotkb:P37231-2) *physically interacts* (MI:0914) with *PGC1 alpha* (uniprotkb:Q9UBK2) by *anti tag coimmunoprecipitation* (MI:0007)
MINT-7229731: *PPAR gamma 2* (uniprotkb:P37231-2) *physically interacts* (MI:0914) with *NS5A* (uniprotkb:P26662) by *anti tag coimmunoprecipitation* (MI:0007)

1. Introduction

Hepatitis C virus (HCV) is classified into the genus *Hepacivirus* of the family *Flaviviridae* [1]. As the case with all the members of this family, HCV is an enveloped, single-stranded, and positive-sense RNA virus. Upon translation, HCV polyprotein is proteolytically processed by both cellular and viral proteases into at least 10 individual proteins, including four structural proteins (core, E1, E2 and p7) and six NS proteins (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [2]. As the penultimate protein processed from HCV polyprotein precursor, NS5A is a proline-rich hydrophilic phosphoprotein and may exist in dimeric form [3]. Although no intrinsic enzymatic activity has yet been ascribed to NS5A, it likely functions via interactions with other NS proteins and host cell factors [2].

Peroxisome proliferators-activated receptors (PPARs) are ligand-activated nuclear receptors belonging to the steroid/thyroid hormone receptor superfamily; 3 isoforms designated as α, β/δ, and γ exist, all of which are known to be involved in lipid homeostasis. Increased PPARγ expression has been reported in high-fat diet-induced liver steatosis [4].

Chronic HCV infection may result in a wide clinical and prognostic spectrum of severity and progression rates in cases of liver disease, ranging from chronic hepatitis to cirrhosis and hepatocellular carcinoma [5]. Hepatic steatosis is detected in almost 50% of HCV-infected patients, which suggests that it is a crucial contributor to nonalcoholic fatty liver disease (NAFLD) [6], a severe and progressive liver disease resulting in the development of cirrhosis [7]. Recently, it has been reported that HCV core proteins increase hepatic lipid accumulation via the activation of SREBP-1 and PPARγ [8], and that NS2 can upregulate the transcription of SREBP-1c and fatty acid synthase (FAS) [9]. However, the precise molecular mechanisms underlying HCV NS5A-associated steatosis have yet to be clearly characterized. In this study, we explored the possibility that NS5A induces lipid accumulation in hepatocytes and the activity and expression of PPARγ may be deregulated, therefore playing roles in NS5A-induced hepatic triglycerides deposits. Our

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results indicate that NS5A-mediated deregulation of PPARγ may be involved in HCV-induced fatty liver disease.

2. Materials and methods

2.1. Plasmids, reagents, and antibodies

pNK/Flag/NS5A, pcDNA4/myc/PGC-1α, and pcDNA3/Flag/PGC-1β were generously donated by Dr. Seishi Murakami, Dr. Toren Finkel, and Dr. Bruce M. Spiegelman [10–12]. pcDNA3/HA/PPARγ, pcDNA3/GFP/PPARγ, pcDNA3/GST/PPARγ, pcDNA3/HA/PGC-1α, pM/PGC-1α, pVP16/PGC-1α, and pcDNA3/HA/NS5A constructs were subcloned via the insertion of the PCR fragments of ORF into pcDNA3/HA, pM (Gal4), pVP16 (Clontech), or pcDNA3/GFP [13,14]. Other constructs were all described previously [8,13].

Rosiglitazone and Ciglitazone were obtained from Cayman. The transfection reagent PolyFect and JetPEI were purchased from QIAGEN and Polyplus-transfection. All other reagents were purchased from Sigma. The antibodies against PPARγ, GST, and GFP were purchased from Santa Cruz Biotechnology Inc. and Actin, Flag, and HA-antibody was obtained from Sigma, Cell Signaling, and Roche, respectively. The anti-NS5A polyclonal antibody was kindly gifted from Dr. Soon B. Hwang (Hallym University, Korea).

2.2. Cell culture and transient ransfection

Chang liver, HepG2, and Huh7 cell lines were maintained in DMEM–10% fetal bovine serum (FBS; Abclone). Transient transfections were conducted using PolyFect or JetPEI with the indicated reporter plasmids and mammalian expression vectors. Total amounts of expression vectors were maintained at constant levels via the addition of empty vectors. Relative luciferase activities were measured with luciferin (BD Biosciences).

2.3. Establishment of Chang liver cells expressing NS5A proteins

Chang liver cells were transfected with 2 μg of pcDNA3/HA or pcDNA3/HA/NS5A using JetPEI reagents in accordance with the manufacturer's instructions. After 48 h, the cells were grown in a medium containing 800 μg/ml G418. Following 2 weeks of selection, sorted single cells were grown under an additional 2 weeks of selection and expanded into stable cells. The candidate clones were analyzed via Western blotting using specific HA-antibody.

2.4. RNA isolation and RT-PCR

Total RNA was prepared using TRIzol (Invitrogen) in accordance with the manufacturer's recommendations. The cDNA was synthesized from 3 μg of total RNA with Moloney murine leukemia virus (MMLV) Reverse Transcriptase (Promega) using a random hexamer (Cosmo, Korea) for 1 h at 37 °C. A one-twenty fifth aliquot of the cDNA was subjected to PCR amplification using gene-specific primers. The PCR primers for PPARγ gene amplification were: 5′-GAAATGACCATGGTTGAC-3′ (sense), 5′-GATGCAGGCTCCACTTTG-3′ (antisense); for NS5A amplification: 5′-TAGCAGTGCTCACTTCCATGCTCA-3′ (sense), 5′-AGGATCTCCGCCGCAATGGATATT-3′ (antisense); for β-actin gene amplification: 5′-GACTACCTCATGAAGATC-3′ (sense), and 5′-GATCCACATCTGCTGGAA-3′ (antisense).

2.5. Establishment of Huh7 cells expressing HCV replicons and interferon-cured cells

An HCV subgenomic replicon plasmid, pRep-Feo, was derived from pRep-Neo (originally referred to as pHCVIbneo-delS) [15]. The pRep-Feo expressed a fusion gene comprised of *firefly* luciferase (Fluc) and neomycin phosphotransferase, as described

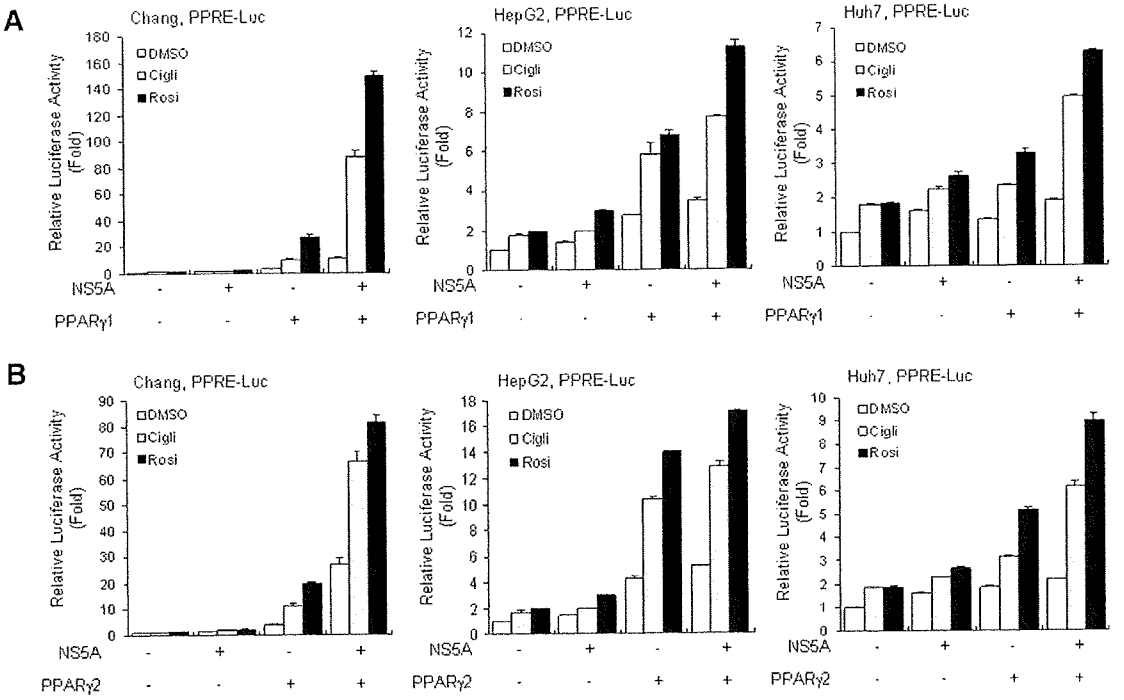

Fig. 1. NS5A induces the transcriptional activity of PPARγ in hepatocytes. (A) PPRE-tk-Luc was cotransfected with the expression vectors for PPARγ1 and NS5A into Chang liver, HepG2, and Huh7 cells. Transfected cells were incubated in the presence or absence of 10 μM Ciglitazone and Rosiglitazone. (B) Cells were cotransfected with a PPRE-tk-luciferase reporter, PPARγ2, and NS5A. Transfected cells were incubated for 24 h in the presence or absence of 10 μM Ciglitazone and Rosiglitazone. Luciferase activity was measured and values are expressed as means ± S.D. for at least two or more independent experiments.

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elsewhere. Replicon RNA was synthesized in vitro using T7-RNA polymerase and transfected into the Huh7 cells via electroporation. After culturing in the presence of G418, the cell lines stably expressing the replicons were established and designated Huh7/Rep-Feo. To establish interferon-alpha (IFN-α)-cured cells, HCV replicon cells were treated with 10^3 U/ml IFN-α (PBL Biomedical Laboratories) for 10 days. The expression of HCV replicon was confirmed by RT-PCR and Western blotting.

2.6. In vivo interaction assays

Forty-eight hours after transfection, the cells were solubilized with 300 μl of radioimmunoprecipitation assay (RIPA) buffer. The cleared lysates were mixed with 40 μl of glutathione-sepharose beads and rotated overnight at 4 °C. The bound proteins were eluted in 15 mM reduced glutathione, separated via SDS–PAGE, and transferred to polyvinylidene difluoride (PVDF) membranes for blotting.

2.7. Coimmunoprecipitation

For the immunoprecipitations, cells were plated and transfected as indicated plasmids. Cells were lysed via the addition of RIPA buffer and an aliquot of the lysate was removed for the input control, and the remainder was immunoprecipitated overnight with the specific antibody and protein G-Sepharose (50% suspension; Invitrogen). The lysates and immunoprecipitates were separated via SDS–PAGE and transferred onto PVDF membranes for blotting.

2.8. Oil Red O staining

ORO staining was conducted in accordance with the previously described procedure, with minor modifications [14]. Cells were stained overnight in freshly diluted ORO solution. The stain was then removed, and the cells were washed twice with water and absorbance of eluted ORO by adding 100% isopropanol at 500 nm was measured in a spectrophotometer.

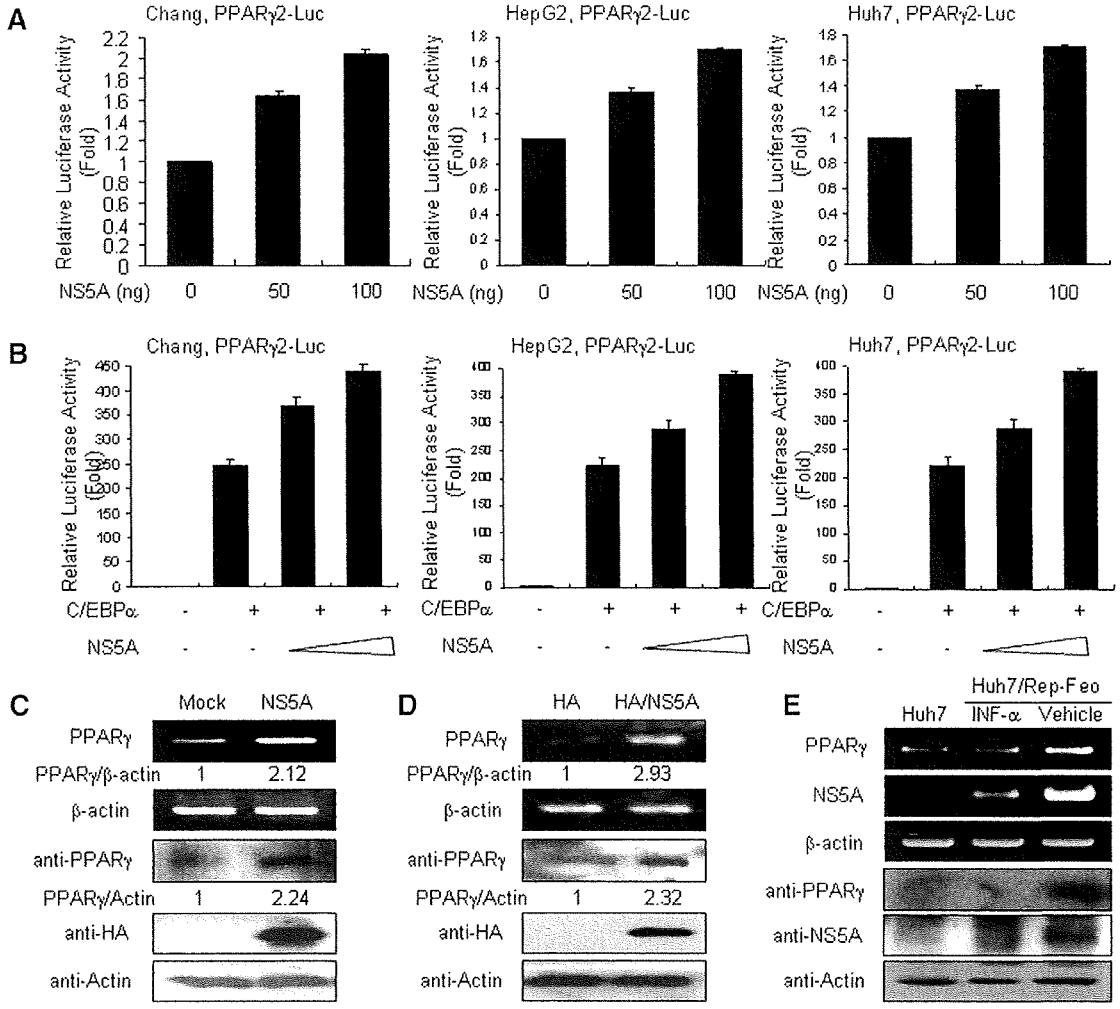

Fig. 2. NS5A promotes PPARγ mRNA and protein expression. (A) Chang liver, HepG2, and Huh7 cells expressing NS5A (50 and 100 ng) were assayed for their ability to transactivate a PPARγ2 promoter. (B) Cells were transfected with indicated expression plasmid. After 48 h of transfection, luciferase activity was determined. (C) The increase of PPARγ gene expression in transiently NS5A-transfected Chang liver cells. RT-PCR and Western blotting were performed as indicated. (D) The enhanced expression of PPARγ gene in Chang/HA/NS5A stable cells. Western blotting and RT-PCR were performed. (E) Total cell lysates or RNAs were harvested from Huh7, IFN-α-cured and HCV subgenomic replicon cells. RT-PCR and immunoblotting were performed as indicated.

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2.9. RNA interference and transfection

For the siRNA-mediated downregulation of PPARγ, PPARγ-specific siRNA and negative control siRNA were purchased from Bioneer (Daejeon, Korea). Chang liver cells were transfected with either the siRNA molecule specific for PPARγ or a negative control siRNA using HiPerFect reagent (QIAGEN).

2.10. Statistical analysis

Statistical analyses were carried out by unpaired or paired *t*-test as appropriate. All data are reported as means ± S.D. *P* value of <0.05 was considered significant.

3. Results

3.1. HCV NS5A induces the transcriptional activity of PPARγ

Chronic hepatitis C (CHC) is associated frequently with hepatic steatosis and PPARγ cultivates hepatic steatosis. We investigated the effect of nine individual proteins of HCV (core191, core-glycoprotein E1 fusion protein, E2, and six NS proteins, NS2, NS3, NS4A, NS4B, NS5A, and NS5B) on PPARγ transactivation. In order to determine whether nine proteins affect the transcriptional activity of PPARγ, we cotransfected a reporter plasmid harboring multimerized PPAR binding sites with the expression plasmid of PPARγ1, PPARγ2, and nine proteins. In particular, core191 and NA5A significantly affected the transcriptional activity of PPARγ1 and PPARγ2. Previously, we reported that core191 increases hepatic lipid accumulation by SREBP1 and PPARγ activation [8].

As shown in Fig. 1A and B, NS5A profoundly augmented the activation of reporter gene expression by PPARγ1 (Fig. 1A) and PPARγ2 (Fig. 1B) in the presence or absence of two synthetic PPARγ agonists, Ciglitazone and Rosiglitazone, in a variety of hepatic cell lines. These results indicate that NS5A protein is an important regulator in HCV-induced PPARγ activation.

3.2. HCV NS5A enhances the mRNA and protein expression of PPARγ

It might be expected that the increased PPARγ activity induced by NS5A is accompanied by increases in the levels of its gene expression. We found that NS5A augmented the promoter activity

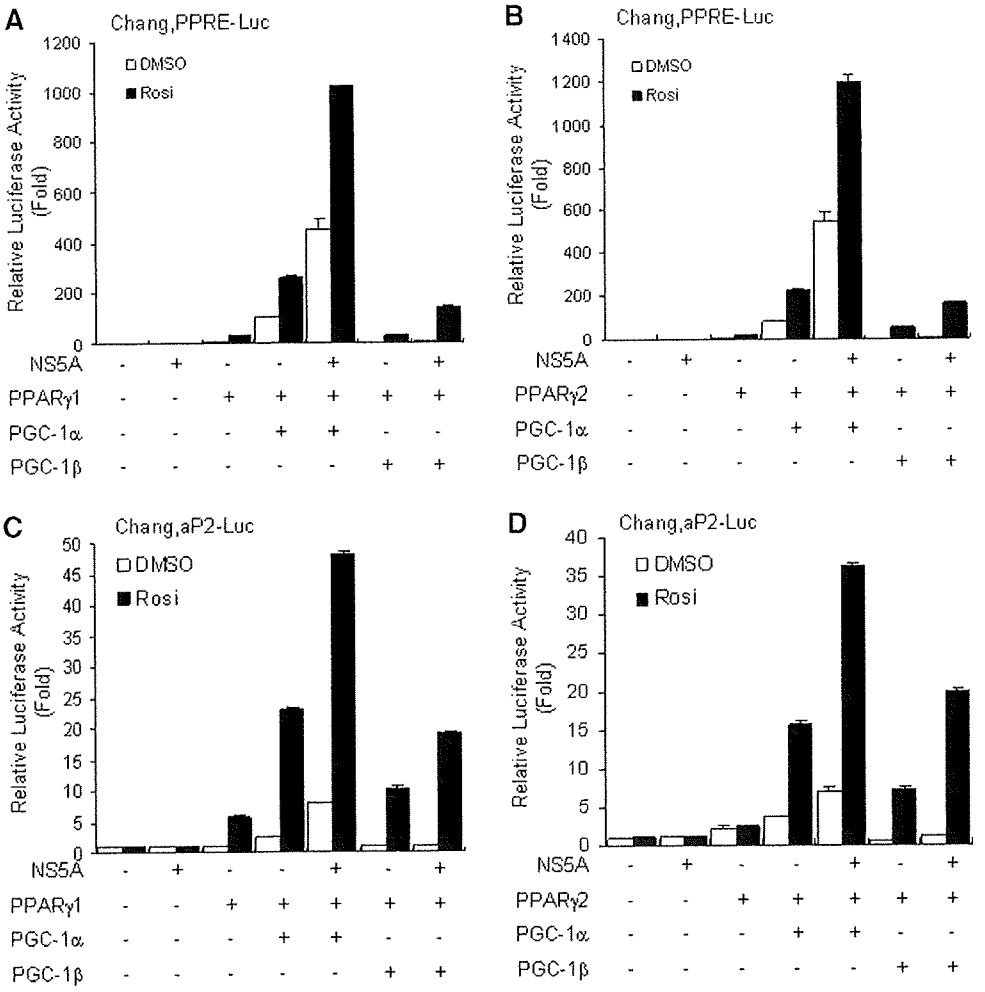

Fig. 3. PGC-1s potentiate the PPARγ-mediated transactivation with NS5A. (A and B) Chang liver cells were transiently transfected with PPRE-tk-Luc construct, in combination with PPARγ1 (A) or PPARγ2 (B) and NS5A in the presence or absence of PGC-1α and PGC-1β transfection. Cells were treated with 10 μM Rosiglitazone or vehicle (DMSO) for 24 h. (C and D) Chang liver cells expressing PPARγ1 (C), or PPARγ2 (D), NS5A, PGC-1α, and PGC-1β were assayed for their ability to transactivate the aP2 promoter. Cells were treated with 10 μM Rosiglitazone or vehicle for 24 h. Luciferase activity was measured.

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of PPARγ2 gene as a dose-dependent manner, as shown in comparisons of luciferase activity with basal levels (Fig. 2A). Since C/EBPα has been reported to upregulate PPARγ expression [16], we investigated the PPARγ2-luciferase reporter activity with cotransfection of C/EBPα and NS5A expression plasmids. A reporter assay indicated that NS5A increased the activity of PPARγ2 promoter in cooperation with C/EBPα as a dose-dependent manner (Fig. 2B).

Furthermore, as shown in Fig. 2C, the mRNA and protein induction in RT-PCR and Western blotting were confirmed for PPARγ expression by transiently transfected NS5A. In addition, to better assess the role of NS5A protein in PPARγ expression, we established Chang liver cells evidencing stable expression of HA-tagged NS5A proteins, and confirmed the expression by immunoblotting with the HA-antibody. We investigated that PPARγ protein and mRNA expression were significantly elevated in Chang/HA/NS5A stable cells (Fig. 2D).

To further investigate whether the expression of PPARγ was regulated by viral protein in the context of HCV RNA replication, Huh7/Rep-Feo cells were treated with IFN-α for 10 days. The mRNA and protein expression levels of PPARγ were significantly elevated in HCV subgenomic replicon cells as compared with Huh7 or the IFN-α-cured cells.

3.3. HCV NS5A increases the transactivation of PGC-1s

PGC-1 was initially identified as a PPARγ-interacting protein from a BAT library, and was subsequently shown to associate with an array of nuclear receptors (NRs) and transcription factors [17,18]. Initially, the cotransfection of PGC-1α and PGC-1β with PPARγ1 (Fig. 3A) or PPARγ2 (Fig. 3B) in the presence of NS5A expression further stimulated transactivity of the PPRE promoter. In particular, PGC-1α increased PPARγ1 and PPARγ2 activity more in the presence of NS5A as compared to PGC-1β. We additionally assessed the effects on the native promoter activity of adipocyte fatty-acid-binding protein, aP2 (encoded by *Ap2*), a member of the intracellular fatty-acid-binding protein (FABP) family, which is an intact target of PPARγ in adipose cells [19]. Although aP2 genes predominantly are not expressed in liver, we exploited the

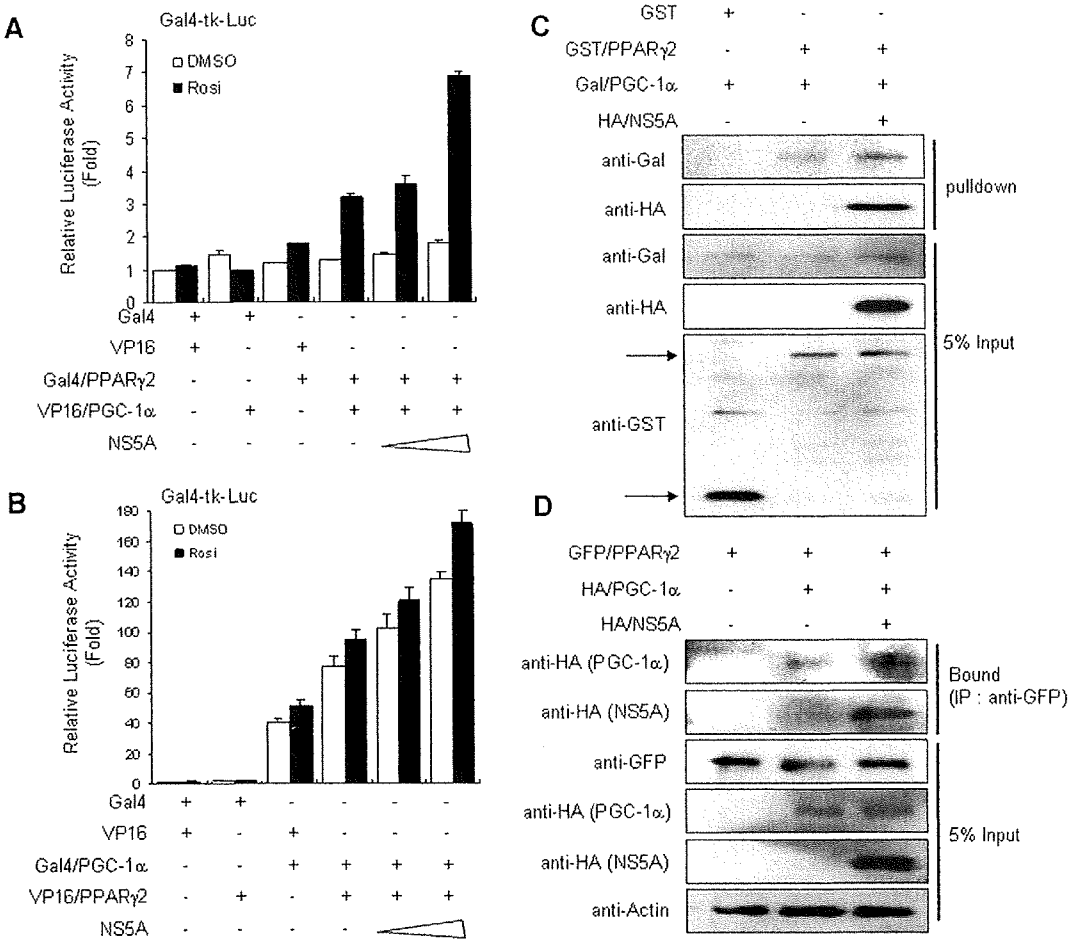

Fig. 4. NS5A enhances the interaction with PPARγ2 and PGC-1α. (A) Chang liver cells were cotransfected with expression vectors encoding Gal4/PPARγ2, VP16/PGC-1α, NS5A, and the reporter construct Gal4-tk-Luc. Cells were grown in the absence or presence of 10 μM Rosiglitazone. The histogram represents the transcriptional activity of the various Gal4/DBD/PPARγ fusion proteins. (B) Chang liver cells were cotransfected with expression vectors encoding Gal4/PGC-1α, VP16/PPARγ2, NS5A, and the reporter construct Gal4-tk-Luc. Cells were grown 24 h in the absence or presence of 10 μM Rosiglitazone. The luciferase assay was performed. (C) Chang liver cells were transfected with expression vectors for Gal/PGC-1α, HA/NS5A, GST/PPARγ2, and GST empty vector. Whole cell lysates were incubated with GST or GST/PPARγ2 fusion proteins bound to glutathione-sepharose 4B bead and bounded proteins were analyzed by Western blot. (D) Chang liver cells were transfected with plasmids as indicated. Total lysates from transfected cells were subjected to immunoprecipitation using antibodies specific for the GFP-epitope tag. Both lysates and precipitates were analyzed by immunoblotting with antibodies specific for HA.

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overexpression of the aP2 promoter. As shown in Fig. 3C and D, the cotransfection of PGC-1α and PGC-1β with PPARγ1 (Fig. 3C) or PPARγ2 (Fig. 3D) and NS5A further elevated transcription from the native promoters of aP2.

3.4. HCV NS5A induces the recruitments of PGC-1α

Above results prompted us to attempt to investigate using various constructs whether the interaction between the PPARγ and PGC-1α enhances by NS5A proteins. We exploited the mammalian two hybrid system. It was observed that the interaction between Gal4-fused PPARγ2 and VP16-fused PGC-1α was enhanced by NS5A protein as a dose-dependent manner (Fig. 4A). Furthermore, the reverse attempt using Gal4/PGC-1α and VP16/PPARγ2 showed effects similar to those observed in Fig. 5A (Fig. 4B).

In an effort to determine whether NS5A enhances the interaction between PPARγ2 and PGC-1α on the cellular level, we also employed other approaches. Chang liver cells were transfected with constructs encoding for the GST-fused PPARγ2, Gal-tagged PGC-1α, and HA-tagged NS5A as indicated. As shown in Fig. 4C, the results of in vivo GST pulldown indicated that PGC-1α only interacted with GST-PPARγ2 and not GST proteins. Also, NS5A significantly enhanced the interaction between two proteins. We observed similar results using coimmunoprecipitation using anti-GFP antibodies. The GFP-tagged PPARγ2 was bound to HA-tagged PGC-1α and expression of NS5A enhanced the interaction of two proteins (Fig. 4D). Collectively, these findings indicate that NS5A performs a critical function in the formation of an active transcriptional complex of PPARγ2 and PGC-1α for PPARγ activation.

3.5. PPARγ is necessary for HCV NS5A-induced hepatic lipid accumulation

In order to examine the effects of NS5A protein in the lipid accumulation, Chang/HA/NS5A stable cells and parent Chang/HA stable cells were stained with ORO. The percentage of ORO-positive cells of Chang liver/HA/NS5A stable clone were significantly higher than in Chang/HA stable cells (Fig. 5A). Also, in an effort to determine whether PPARγ performs a function in NS5A-induced lipid accumulation, we attempted to knockdown PPARγ expression using siRNA specific for PPARγ. Chang liver cells were cotransfected with the NS5A construct and/or siPPARγ. As shown in Fig. 5B, siPPARγ-transfected cells did not affect hepatic lipid accumulation, even in the presence of NS5A. These results clearly indicate that PPARγ is a crucial transcription factor which mediates NS5A-induced lipid accumulation.

4. Discussion

HCV infects >170 million individuals worldwide and causes liver disease, including hepatic steatosis, cirrhosis, and eventually hepatocellular carcinoma (HCC) [20]. The reported prevalence of steatosis in patients with CHC varies between 40% and 80%, depending on the features of the population studied with regard to alcohol consumption, the prevalence of overweight/obesity, diabetes, and other risk factors of fatty liver [21]. HCV core protein expression has been demonstrated to activate various pathways of lipid metabolism [8,22,23]. However, the precise functions of other proteins among HCV proteins in the development of fatty liver remain to be determined.

NS5A interferes with the response to interferon and appears to perform a crucial role in viral replication [24]. Also, NS5A augments the anti-apoptotic effect of bcl-2 and inhibits the pro-apoptotic action of bax [25]. However, other functions of NS5A have yet to be clearly defined. In this report, our results provide the first evidence suggesting that NS5A protein can increase hepatic lipid accumulation by inducing an increase in the expression and activity of PPARγ.

It is well established that modulation of PPAR activity maintains cellular and whole-body glucose and lipid homeostasis. Recent studies have established a role for hepatic PPARγ in the development of hepatic steatosis in mouse model. According to these studies, hepatic PPARγ is able to contribute to hepatic steatosis through increasing hepatic triglyceride production and hepatic lipid uptake [26,27].

In conclusion, we have determined that NS5A augments hepatic PPARγ activation and expression. It has been demonstrated that NS5A enhances the recruitment of PGC-1α to PPARγ via the

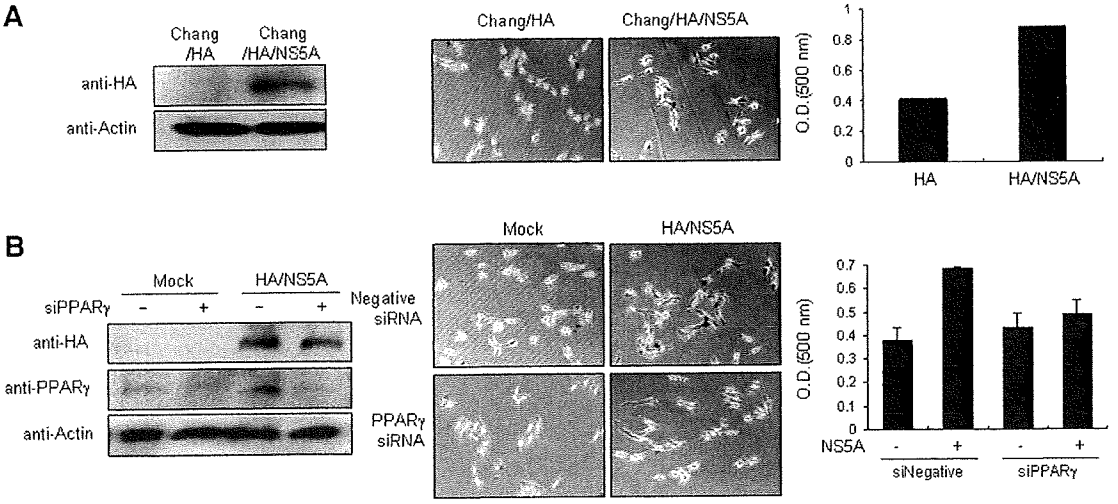

Fig. 5. HCV NS5A increases hepatic lipid accumulation. (A) The effect of expression of NS5A proteins in hepatic lipid accumulation. The expression levels of NS5A protein in Chang/HA/NS5A stable cells were analyzed by Western blotting using HA antibodies. ORO staining reveals the increased hepatic lipids in Chang/HA/NS5A stable cells compared with parent stable cells. (B) The effect of PPARγ in NS5A-induced lipid accumulation. For the siRNA-mediated downregulation of PPARγ, negative control siRNA or PPARγ-specific siRNA was transfected with or without NS5A proteins into Chang liver cells. The transfected cells were analyzed by Western blotting. The siPPARγ were transfected with or without NS5A into Chang liver cells. Cells were stained with ORO and photographed. The absorbance of eluted ORO was determined at 500 nm.

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enhancement of interaction between these proteins. Hence, the results regarding NS5A pave the way for an important function as a selective target of PPARγ coactivation circuitry. Notably, we determined that NS5A ameliorates hepatic lipid accumulation via the regulation of PPARγ and may contribute to HCV-induced hepatic steatosis.

Acknowledgments

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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, pHCV1bneo-delS,[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-

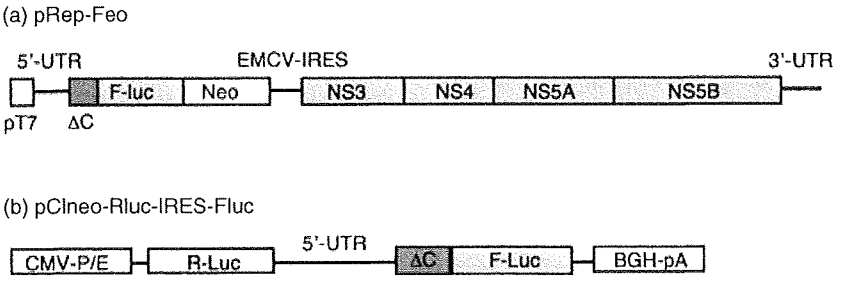

Figure 1 HCV subgenomic replicon and reporter plasmid constructs. (a) An HCV subgenomic replicon plasmid, pRep-Feo, was reconstructed from HCV1bneo-delS by replacing the neomycin phosphotransferase (Neo) gene with a fusion gene comprising the firefly luciferase (Fluc) and Neo, which we designated as "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-

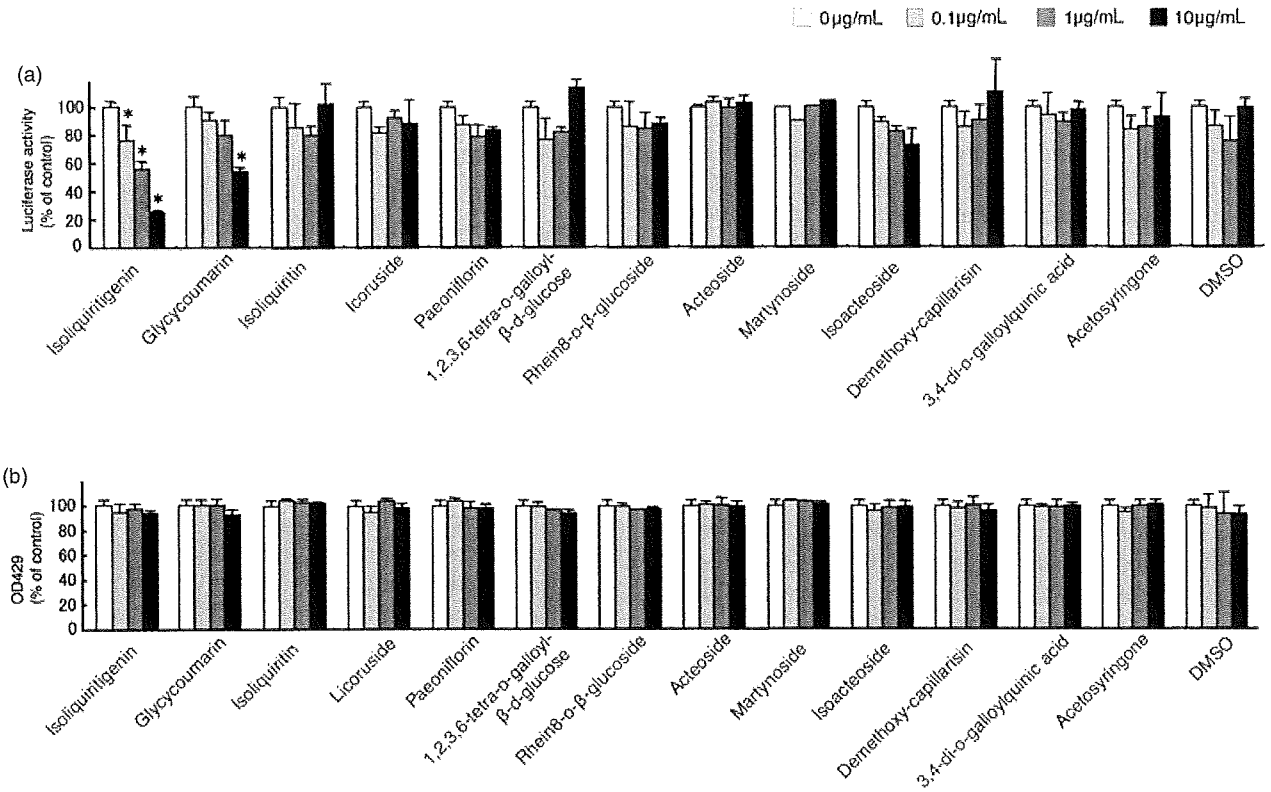

Figure 2 Effects of purified extracts from herbal drugs on expression of HCV replicon. (a) Huh7/Rep-Feo cells, which constitutively express the HCV Feo replicon, were cultured in the presence of 13 compounds at concentrations of 0, 0.1, 1, and 10 μg/mL. The internal luciferase activities were measured 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.

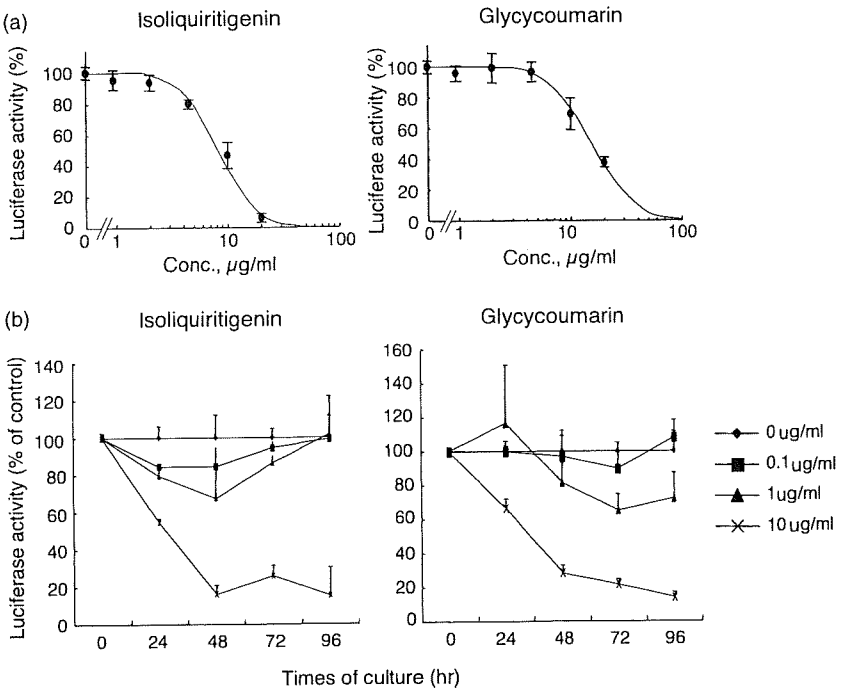

Figure 3 Dose- and time-dependent suppression of HCV replication by isoliquiritinenin and glycycoumarin. (a) Relative log (dose)-response plots for isoliquiritigenin or glycycoumarin. Error bars indicate mean ± SD of triplicate analyses. Calculated probit curves are overlaid in each plot. (b) Huh7/Rep-Feo 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

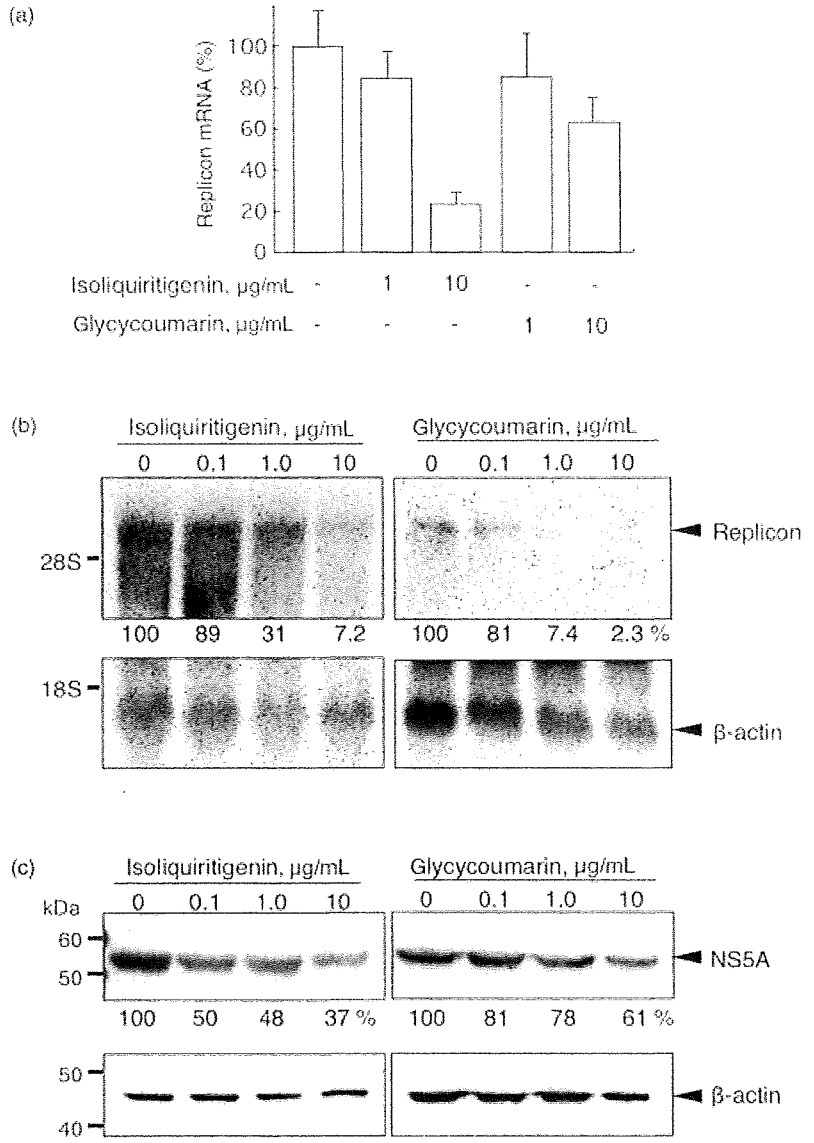

Figure 4 Suppression of replicon RNA and NS5A synthesis by isoliquiritigenin and glycycoumarin. Huh7/Rep-Feo cells were cultured with indicated concentrations of two compounds, isoliquiritigenin and glycycoumarin, and harvested at 48 hr after exposure. (a) Real-time RT-PCR analyses. (b) Northern-blot 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).

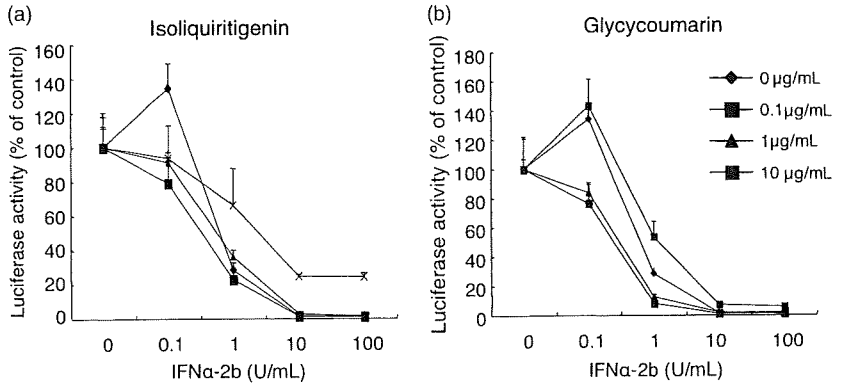

Figure 5 Effects of (a) isoliquiritigenin and (b) glycycoumarin used in combination with interferon(IFN)-α on HCV replication. Huh7/Rep-Feo cells were cultured with combinations of IFN-α-2b and isoliquiritigenin or glycycoumarin at concentrations indicated. The internal luciferase activities were measured 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]

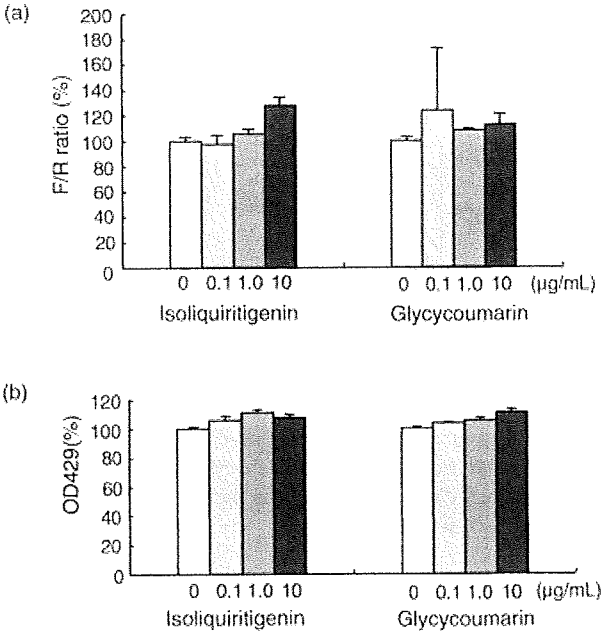

Figure 6 Isoliquiritigenin and glycycoumarin do not influence the HCV IRES-mediated translation. A bicistronic reporter gene plasmid, pCIneo-Rluc-IRES-Fluc, was stably trasfected into Huh7 cells (Huh7/CRIF, *see* the Methods). (a) Dual luciferase assay. The cells were cultured with isoliquiritigenin 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.

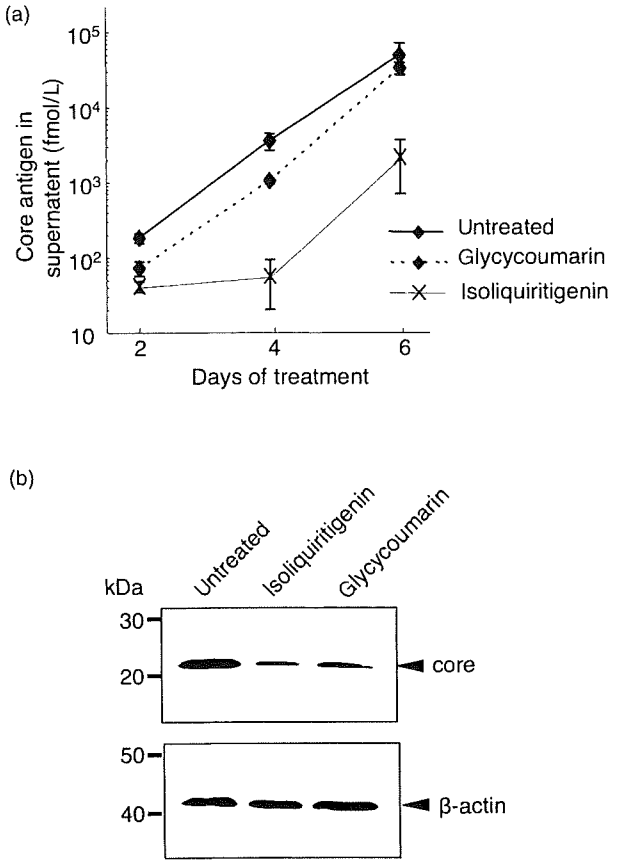

Figure 7 Suppresion of HCV-JFH1 virus expression by *isoliquiritigenin and glycycoumarin*. (a) Naïve Huh 7.5.1 cells were infected with culture supernatant of HCV-JFH1-infected cells and were subjected to culture in the presence of indicated drugs. Culture supernatants were collected at indicated days, 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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Antiviral Effects of the Interferon-Induced Protein Guanylate Binding Protein 1 and Its Interaction with the Hepatitis C Virus NS5B Protein

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Interferons (IFNs) and the interferon-stimulated genes (ISGs) play a central role in antiviral responses against hepatitis C virus (HCV) infection. We have reported previously that ISGs, including guanylate binding protein 1 (GBP-1), interferon alpha inducible protein (IFI)-6-16, and IFI-27, inhibit HCV subgenomic replication. In this study we investigated the effects of these ISGs against HCV in cell culture and their direct molecular interaction with viral proteins. HCV replication and virus production were suppressed significantly by overexpression of GBP-1, IFI-6-16, or IFI-27. Knockdown of the individual ISGs enhanced HCV RNA replication markedly. A two-hybrid panel of molecular interaction of the ISGs with HCV proteins showed that GBP-1 bound HCV-NS5B directly. A protein truncation assay showed that the guanine binding domain of GBP-1 and the finger domain of NS5B were involved in the interaction. Binding of NS5B with GBP-1 inhibited its guanosine triphosphatase GTPase activity, which is essential for its antiviral effect. Taken together, interferon-induced GBP-1 showed antiviral activity against HCV replication. *Conclusion:* Binding of the HCV-NS5B protein to GBP-1 countered the antiviral effect by inhibition of its GTPase activity. These mechanisms may contribute to resistance to innate, IFN-mediated antiviral defense and to the clinical persistence of HCV infection. (HEPATOLOGY 2009;50:1727-1737.)

Hepatitis C virus (HCV) is one of the most important pathogens causing liver-related morbidity and mortality.[1] Hepatitis C is characterized by persistent infection of the liver, leading to the development of chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma. Type-I interferon (IFN) plays a central role in eliminating viruses, not only by way of therapeutic applications[2] but also as a natural cellular antiviral mechanism.[3,4] Interferons are produced naturally in response to virus infection and to cellular exposure to IFN itself. Binding of the IFNs to their receptors activates the Jak-STAT pathway to form a complex with IFN-stimulated gene factor-3 (ISGF3), which translocates to the nucleus, binds the IFN-stimulated response element (ISRE) located in the promoter/enhancer region of the IFN-stimulated genes (ISGs), and activates expression of ISGs.

HCV subgenomic replicons constitute *in-vitro* models that simulate cellular autonomous replication of HCV

Abbreviations: CLEIA, chemiluminescence enzyme immunoassay; Fluc, firefly luciferase; GBP-1, guanylate binding protein 1; GTPase, guanosine triphosphatase; HCV, hepatitis C virus; IFN, interferon; IgG, immunoglobulin G; ISG, interferon-stimulated gene; ISGF3, IFN-stimulated gene factor-3; IRF-1, interferon regulatory factor 1; ISRE, IFN-stimulated response element; NF-κB, nuclear factor-kappaB; NS, nonstructural.

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genomic RNA. Replication of the HCV replicon can be abolished by treatment with small amounts of type-I and type-II IFNs.[5,6] These findings suggest that various molecules encoded by the ISGs have antiviral activities against HCV replication. We have reported previously that the baseline activities of ISG expression are substantially decreased in cells expressing HCV replicon and that this decrease is partly attributable to the transcriptional suppression of interferon regulatory factor 1 (IRF-1).[7] We performed expressional screening of ISGs to investigate their antiviral effects against HCV replication and showed that guanylate binding protein 1 (GBP-1), interferon alpha inducible protein (IFI)-6-16, and IFI-27 had novel activities against cells harboring an HCV replicon.[8] In this study we investigated the antiviral effects and molecular mechanism of GBP-1, IFI-6-16, and IFI-27 on HCV-JFH1-infected cells.

Materials and Methods

Cells and Cell Culture. Huh7, Huh7.5.1, and 293T cells were maintained in Dulbecco's modified minimal essential medium (Sigma, St. Louis, MO) supplemented with 10% fetal calf serum at 37°C under 5% CO_2. To maintain cell lines carrying the HCV replicon (Huh7/Rep-Feo cells), G418 (Nacalai Tesque, Kyoto, Japan) was added to the culture medium to a final concentration of 500 μg/mL.

HCV Replicon Constructs and Transfection. The HCV replicon plasmids, which contain Rep-Feo, were derived from the HCV-N strain, pHC1bneo/delS (1b-Feo) and HCV-JFH1 strain, pSGR-JFH1 (2a-Feo).[6,9] These constructs express a chimeric reporter protein of firefly luciferase (Fluc) and neomycin phosphotransferase. The replicon RNA synthesis and transfection have been described (Huh7/Rep-1bFeo, Huh7/Rep-2aFeo).[10,11]

HCV Cell Culture System. A plasmid, pJFH1-full,[12] which encodes the full-length HCV-JFH1 sequence, was linearized and used as a template for synthesis of HCV RNA using the RiboMax Large Scale RNA Production System (Promega, Madison, WI). After DNaseI (RQ-1, RNase-free DNase, Promega) treatment, the transcribed HCV RNA was purified using ISOGEN (Nippon Gene, Tokyo, Japan). For the RNA transfection, Huh-7.5.1 cells were washed twice, and 5×10^6 cells were suspended in Opti-MEM I (Invitrogen, Carlsbad, CA) containing 10 μg of HCV RNA, transferred into a 4-mm electroporation cuvette, and subjected to an electric pulse (1,050 μF and 270 V) using the Easy Ject system (EquiBio, Middlesex, UK). After electroporation, the cell suspension was left for 5 minutes at room temperature and then incubated under normal culture conditions in a 10-mm diameter cell culture dish. The transfected cells were split every 3 to 5 days. The culture supernatants were subsequently transferred onto uninfected Huh-7.5.1 cells.

Construction of Plasmids Expressing ISGs and Analysis of Their Effect on HCV Subgenomic and Genomic Replication. We constructed plasmids expressing GBP-1, IFI-6-16, IFI-27, and IRF-1. The full-length human ISGs were amplified by polymerase chain reaction (PCR) from Huh7 cells and cloned into pcDNA3.1D/V5-His-TOPO (pcDNA4/TO/myc-his for IRF-1) (Invitrogen) to yield the mammalian expression construct, pcDNA-ISG. The ISG-expression plasmid, pcDNA-ISG, was transfected into Huh7/Rep-1bFeo or Huh7/2aFeo cells, and the replication level of the HCV replicon was analyzed by luciferase assay. A plasmid, pcDNA3.1D/V5-His/lacZ (Invitrogen), was used as a control plasmid vector for mock transfection.

Another plasmid, pcDNA-ISG, was transfected into HCV-JFH1 cell culture systems. Forty-eight hours after transfection the culture supernatants, total cellular RNA, and protein, which were used for quantification of HCV core antigen, were harvested.

Luciferase Assays. Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo Luciferase Assay System (Promega) or a Dual-Luciferase Reporter Assay System (Promega).

Real-Time Reverse Transcription (RT)-PCR Analysis. Total cellular RNA was isolated using ISOGEN (Nippon Gene). Two micrograms of total cellular RNA were used to generate complementary DNA (cDNA) from each sample using SuperScript II (Invitrogen) reverse transcriptase. Expression of messenger RNA (mRNA) was quantified using the TaqMan Universal PCR Master Mix and the ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). The primers have been described.[13]

Quantification of HCV Core Antigen in Culture Supernatants. Culture supernatants of JFH1-RNA transfected Huh-7.5.1 cells were collected on the days indicated, passed through a 0.45-μm filter (MILLEX-HA, Millipore, Bedford, MA), and stored at −80°C. The concentrations of core antigen in the culture supernatants were measured using a chemiluminescence enzyme immunoassay (CLEIA) according to the manufacturer's protocol (Lumipulse Ortho HCV Antigen, Ortho-Clinical Diagnostics, Tokyo, Japan).

Synthetic Short Hairpin RNA (shRNA) and shRNA-Expression Plasmid. ISG-directed shRNA expression vectors (pUC19-shRNA-ISG) were designed and constructed as described.[11] Briefly, oligodeoxyribonucleotides encoding shRNA sequences were synthesized and cloned just downstream of the human U6 promoter in the

plasmid pUC19. To avoid problems of structural instability of DNA strands arising from the tight palindrome structure during transcription of shRNA, several point mutations were introduced into the sense strand of the shRNA sequences, which retained fully the silencing activity of the shRNA.[11] Sequences of the shRNAs are shown in Supporting Table 1.

Construction of Plasmids Expressing Full-Length and Truncated HCV-NS Proteins. Expression plasmids of HCV-NS3, NS4B, NS5A, and NS5B were constructed by inserting PCR-amplified fragments encoding each HCV-NS protein into pcDNA4/TO/myc-his (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, and pcDNA-NS5B, respectively). The plasmids, which expressed truncated HCV-NS5B proteins, were generated by insertion of various fragments amplified by PCR using pcDNA-NS5B into pcDNA4/TO/myc-his.

Immunoprecipitation Assay. Plasmids expressing HCV-NS protein (pcDNA-NS3, pcDNA-NS4B, pcDNA-NS5A, or pcDNA-NS5B) and plasmids expressing ISG (pcDNA-GBP-1, pcDNA-IFI-6-16, or pcDNA-IFI-27) were cotransfected into HEK-293 T cells. Forty-eight hours after transfection, cellular proteins were harvested and immunoprecipitation assay was performed using an Immunoprecipitation Kit according to the manufacturer's protocol (Roche Applied Science, Mannheim, Germany). The immunoprecipitated proteins were analyzed by western blotting.

Mammalian Two-Hybrid Assay. Mammalian two-hybrid assay (Stratagene, La Jolla, CA) is a method for detecting protein-protein interactions in vivo in mammalian cells. In this assay a gene encoding the HCV-NS protein (NS3, NS4B, NS5A, and NS5B) was fused to the DNA-binding domain of the yeast protein GAL4 (a bait plasmid, pcCMV-BD), whereas another gene (ISG; GBP-1, IFI-6-16, and IFI-27) was fused to the transcriptional activation domain of the mouse protein nuclear factor-kappaB (NF-κB) (an acceptor plasmid, pCMV-AD). These two-hybrid constructs are cotransfected into Huh-7 cells with a reporter plasmid encoding the firefly-luciferase gene. If the ISG protein and HCV-NS protein interact, they create a functional transcriptional activator by bringing the activation domain into close proximity with the DNA-binding domain; this can be detected by expression of the luciferase reporter gene. The ISG-encoding site was inserted into a plasmid, pCMV-AD, by cloning from a plasmid, pcDNA-ISG (pCMV-AD-ISG). The HCV-NS protein gene, which was subcloned from a plasmid, pcDNA-NS protein, was also inserted into a plasmid, pCMV-BD (pCMV-BD-NS protein). The plasmids pCMV-AD-ISG and pCMV-BD-NS protein were cotransfected with a reporter plasmid, pFR-luc encoding Fluc into Huh-7 cells. Cellular proteins were harvested after 48 hours and luciferase assays were performed.

Immunohistochemistry. Huh7.5.1 cells infected with HCV-JFH-1 were seeded onto 18-mm round microcover glasses (Matsunami, Tokyo, Japan). After transfection of plasmids expressing ISG, pcDNA-ISG, Huh7.5.1 cells were fixed with cold acetone. The cells were incubated with the primary antibodies for 1 hour at 37°C and with Alexa Flour 488 goat antimouse immunoglobulin G (IgG) antibody and Alexa 568 donkey antigoat IgG antibody (Molecular Probes, Eugene, OR) for 1 hour at room temperature. Cells were mounted with VectaShield Mounting Medium and DAPI (Vector Laboratories, Burlingame, CA) and visualized with fluorescence microscopy (BZ-8000, Keyence, Osaka, Japan).

The Establishment of a Mutant Form of GBP-1. In order to introduce mutations into GBP-1, the full-length human ISGs were amplified by PCR from a human liver cDNA library (Invitrogen) and cloned into pCMV-Tag Epitope Tagging Mammalian Expression Vectors; pCMV-GBP-1 (Stratagene). The following mutations were introduced into the DNA fragment in the subcloning vector by site-directed mutagenesis (Quick-Change II Site-Directed Mutagenesis Kit; Stratagene): R48P; pCMV-GBP1.R48P.

Guanosine Triphosphatase (GTPase) Assay. Cellular proteins were harvested from Huh7 cells 48 hours after transfection of a plasmid expressing HCV-NS5B, pcDNA-NS5B, or a plasmid for mock transfection, pcDNA3. A GTPase assay was performed to examine GTPase activity of cellular proteins using GTPase ELIPA kits (Cytoskelton, Denver, CO). The assay is based on an absorbance shift (340 to 360 nm) that occurs when 2-amino-6-mercapto-7-methylpurine ribonucleoside is catalytically converted to 2-amino-6-mercapto-7-methyl purine in the presence of inorganic phosphate (Pi). The reaction is catalyzed by purine nucleoside phosphorylase. One molecule of inorganic phosphate will yield one molecule of 2-amino-6-mercapto-7-methyl in an essentially irreversible reaction. Thus, the absorbance at 360 nm is directly proportional to the amount of Pi generated in the reaction.

Transient Transfection. Transient transfection was performed using Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol.

Luciferase Assays. Luciferase activity was measured with a Lumat LM9501 luminometer (Promega) using a Bright-Glo Luciferase Assay System (Promega).

Western Blot Analysis. Western blotting was performed as described.[10] Briefly, 10 μg of total cell lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), and blotted onto a poly-