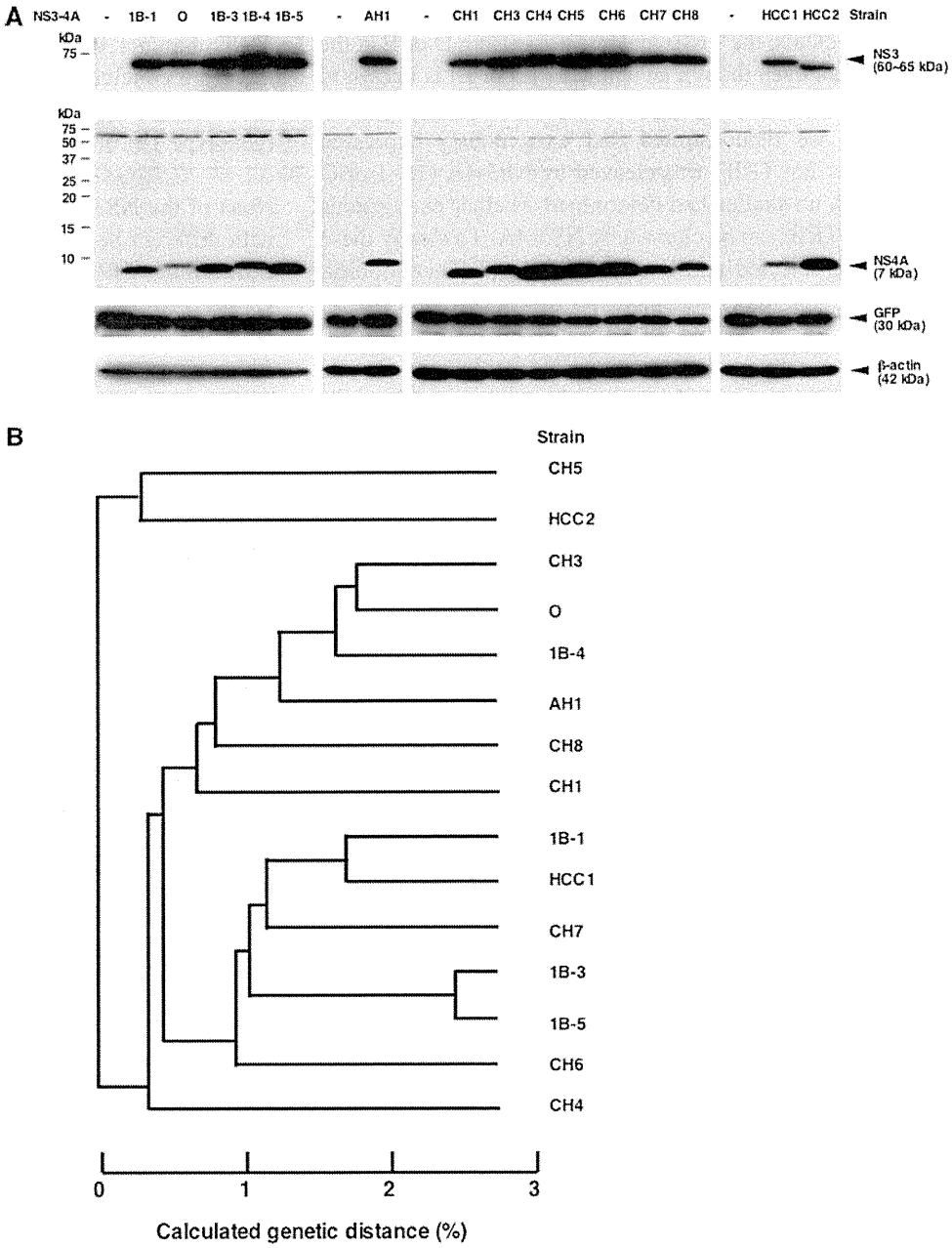


Fig. 3 Characterization of NS3-4As derived from patients with different hepatic disease diagnoses. **a** Expression of NS3 and NS4A in PH5CH8 cells. PH5CH8 cells were transfected with the expression vectors of NS3-4As derived from 15 different HCV strains and pEGFP-C1 (internal control reporter). Production of NS3 and NS4A in PH5CH8 cells was analyzed by immunoblot analysis using anti-NS3 and anti-NS4A antibody, respectively. PH5CH8 cells transfected with the pCX4bsr vector were used as a control (–). GFP was used to estimate the efficiency of transfection. β-actin was used as a control for the amount of protein loaded per lane. **b** Phylogenetic tree based on the amino acid sequences of NS3-4As used in this study

in the catalytic triad (H-57, D-81, and S-139), substrate recognition (L-135, F-154, A-157, and R-161), and metal coordination (C-97, C-99, C-145, and H-149) were well conserved among the NS3-4As (data not shown). In addition, the aa sequences (aa 626–631 in NS3, aa 1–5 in NS4A) surrounding the *cis*-cleavage site and aa 1–20 in NS4A, which is important for the stability of the NS3/4A complex, were also well conserved. The nucleotide sequences in the NS3-4 regions of these HCV strains showed differences of 6.62% (1B-4 strain)–10.47% (HCC2 strain) from those of the O strain. Similarly, the aa sequences in the NS3-4A regions of these HCV strains showed differences of 1.90% (CH3 strain)–5.11% (HCC2 strain) from those of the O strain. The phylogenetic tree based on the aa sequences of all NS3-4As examined is not indicative of any disease-stage-specific clusters (Fig. 3b).

Using these NS3-4A expression vectors, we examined the inhibitory effects of NS3-4As on dsRNA-induced IFN-β transcription in PH5CH8 cells. As described previously [6, 28], IFN-β transcription is induced via two pathways; one is mediated by the intracellular dsRNA (mainly the Cardif-mediated pathway), and the other is mediated by the extracellular dsRNA (TRIF-mediated pathway). Therefore, two different methods were used for the analysis, as described previously [6, 28]; one is to examine NS3-4A's inhibitory effects when the dsRNA

analog, poly(I-C), was introduced into cells using a liposome-mediated transfection procedure (the intracellular dsRNA, T-pIC), the other is to examine NS3-4A's inhibitory effects when poly(I-C) was added to the culture medium (the extracellular dsRNA, M-pIC). We observed that IFN-β gene promoter activity was strongly suppressed via the cleavage of Cardif by each NS3-4A when PH5CH8 cells were transfected with poly (I-C) (T-pIC) (Supplementary Fig. S1 in Electronic Supplementary Material). In contrast, IFN-β gene promoter activity was not significantly suppressed when poly (I-C) was added to the culture medium (M-pIC) (Fig. 4a). However, the promoter activity in cells expressing 1B-5-derived NS3-4A appeared to be slightly suppressed (Fig. 4a). Therefore, we next determined the levels of IFN-β mRNA by quantitative RT-PCR. The results revealed that IFN-β mRNA expression was not suppressed in cells expressing 1B-5-derived NS3-4A (Fig. 4b). We further showed that none of the NS3-4As examined cleaved the exogenously expressed TRIF (Fig. 4c). In addition, we showed that 1B-5, CH1, HCC1, or HCC2-derived NS3 interacted with endogenous TRIF, as was also observed with 1B-1-, O-, and JFH-1-derived NS3 (Figs. 2a, c, 4d). These results suggest that the suppressive effects of NS3-4As on dsRNA-induced IFN-β transcription and the interaction of NS3 with TRIF were not dependent on the HCV strain and genotype or associated with the stage or progression of hepatic disease.

Fig. 4 None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented M-pIC-induced IFN-β transcription via the TRIF-mediated pathway. **a** Effects of 15 NS3-4As on the activity of the IFN-β gene promoter. PH5CH8 cells transiently expressing NS3-4As from various HCV strains were subjected to M-pIC treatment. PH5CH8 cells transfected with pCX4bsr vector were used as a control (strain, –). The dual luciferase assay was performed as described in Materials and Methods. Data are expressed as the mean ± SD from three independent experiments, each of which was performed in triplicate. **b** Effect of NS3-4As on IFN-β mRNA induction by M-pIC treatment. PH5CH8 cells transiently expressing NS3-4As from several HCV strains containing 1B-5 were subjected to M-pIC treatment. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (strain, –). Quantitative RT-PCR for IFN-β mRNA was performed in triplicate. The IFN-β mRNA level was calculated relative to the level in control PH5CH8 cells, which was set at 100. **c** None of the NS3-4As cleaved exogenous TRIF. PH5CH8 cells were transfected with myc-TRIF and NS3-4A expression vectors. The production of myc-TRIF and NS3 was analyzed by immunoblot analysis using anti-myc and anti-NS3 antibody, respectively. PH5CH8 cells transfected with the pCX4bsr and pCX4pur vectors were used as a control (–). β-actin was used as a control for the amount of protein loaded per lane. **d** Endogenous TRIF interacts with NS3-4As from various HCV strains but not is cleaved by NS3-4As in PH5CH8 cells. The cell lysates were prepared and subjected to immunoprecipitation using anti-TRIF antibody, followed by immunoblot analysis using anti-TRIF or anti-NS3 antibody, as described in Fig. 2a. Cardif, NS3, and β-actin in the cell lysates were detected by anti-Cardif, anti-NS3, and anti-β-actin antibody, respectively. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (strain, –)

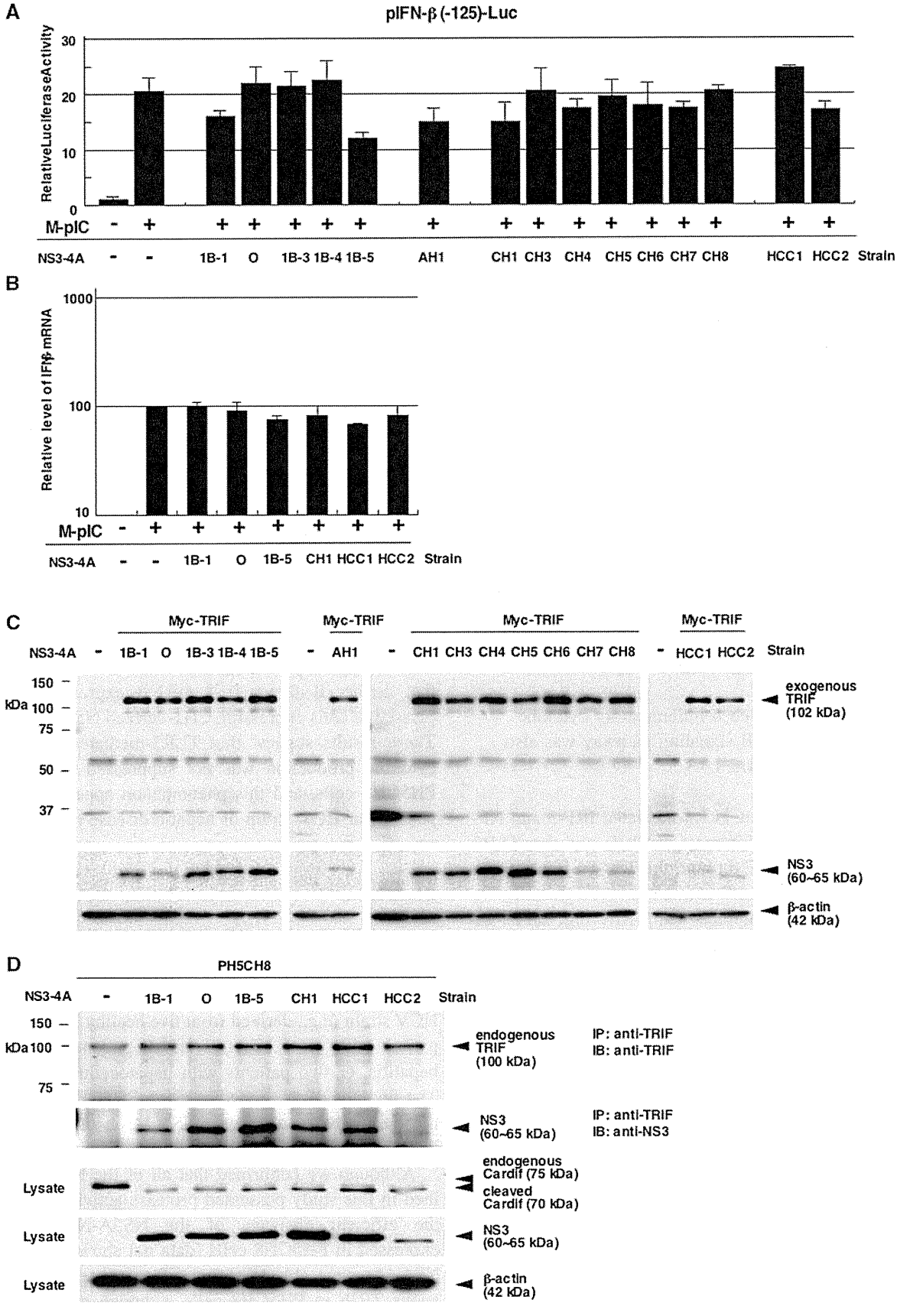
Extracellular dsRNA-induced inflammatory cytokine production via the NF-κB signaling pathway was also not suppressed by NS3-4A

It was already known that TLR3-mediated IRF-3 and NF-κB activation pathways bifurcate at TRIF, and that TLR3 recruits TRAF6 via TRIF through the TRAF6-binding site of TRIF, resulting in NF-κB activation [17, 34]. Since we demonstrated that NS3 interacts with TRIF (Fig. 2a, c), we expected that NS3-4A might interfere with the recruitment of TRAF6 by TRIF (Fig. 5a). To examine this possibility, we considered whether or not NS3-4A affects M-pIC-induced NF-κB activation in PH5CH8 cells. Initially, we demonstrated that NF-κB-inducing promoter activity was also enhanced with M-pIC treatment and that this enhancement was mediated by TLR3 and TRIF, as promoter activity was found to be substantially suppressed by TLR3 or TRIF siRNA (Fig. 5b). The results revealed that none of the NS3-4As examined significantly suppressed M-pIC-induced NF-κB activation (Fig. 6a). However, the enhancement of promoter activity in cells expressing CH1-derived NS3-4A was slightly lower than that in cells expressing other strain-derived NS3-4As (Fig. 6a). Therefore, we performed quantitative RT-PCR analysis to examine the levels of IL-6 and IL-8 mRNAs, both of which were induced by NF-κB activation. The results revealed that neither IL-6 nor IL-8 mRNA expression was suppressed in cells expressing CH1-derived NS3-4A (Fig. 6b). These results suggest that TLR3-mediated inflammatory cytokine production was not suppressed by NS3-4A in PH5CH8 cells, and this phenomenon appears to be independent of HCV strain or hepatic disease type.

Discussion

In the present study, we demonstrated that neither IFN-β transcription nor NF-κB activation by extracellular dsRNA was suppressed by NS3-4A, regardless of the source of the HCV strain (e.g., derived from five healthy HCV carriers, a patient with acute hepatitis, seven patients with chronic hepatitis, or two patients with hepatocellular carcinoma). The findings of these studies using PH5CH8 cells suggest that the inhibitory activity of NS3-4A on antiviral signaling pathways is limited to the Cardif-mediated pathway.

Although we confirmed that all of the NS3-4As examined in this study possessed protease activity that enabled the efficient cleavage of the NS5A-NS5B substrate expressed in PH5CH8 cells (data not shown), none of the NS3-4As were able to cleave either exogenous or endogenous TRIF in PH5CH8 cells, although all were able to cleave Cardif. These results suggest that both the non-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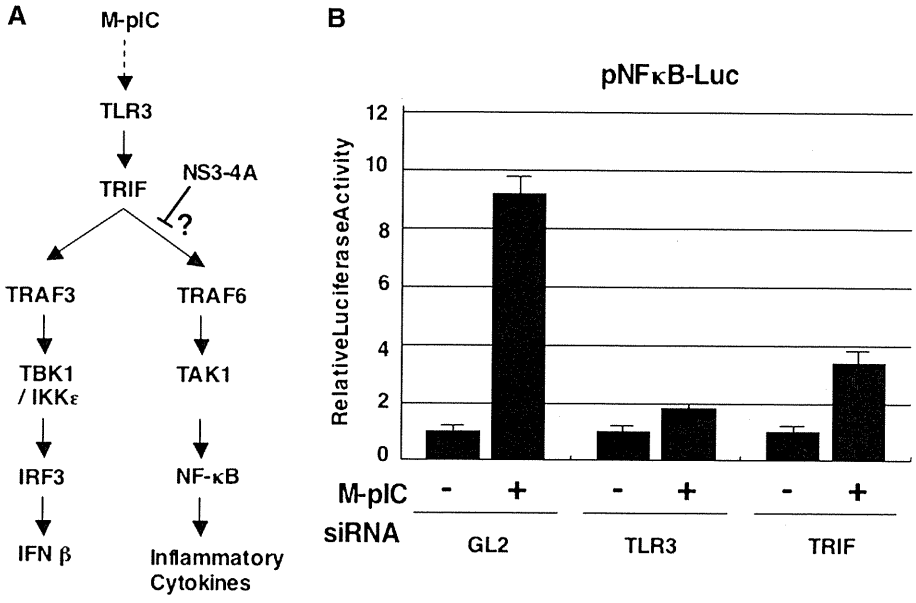

Fig. 5 TLR3-mediated activation of IRF-3 and NF-κB bifurcate at TRIF. **a** Model of TLR3-mediated signaling pathways. **b** Dual luciferase reporter assay of the NF-κB-inducing promoter using siRNA-transfected PH5CH8 cells treated with M-pIC

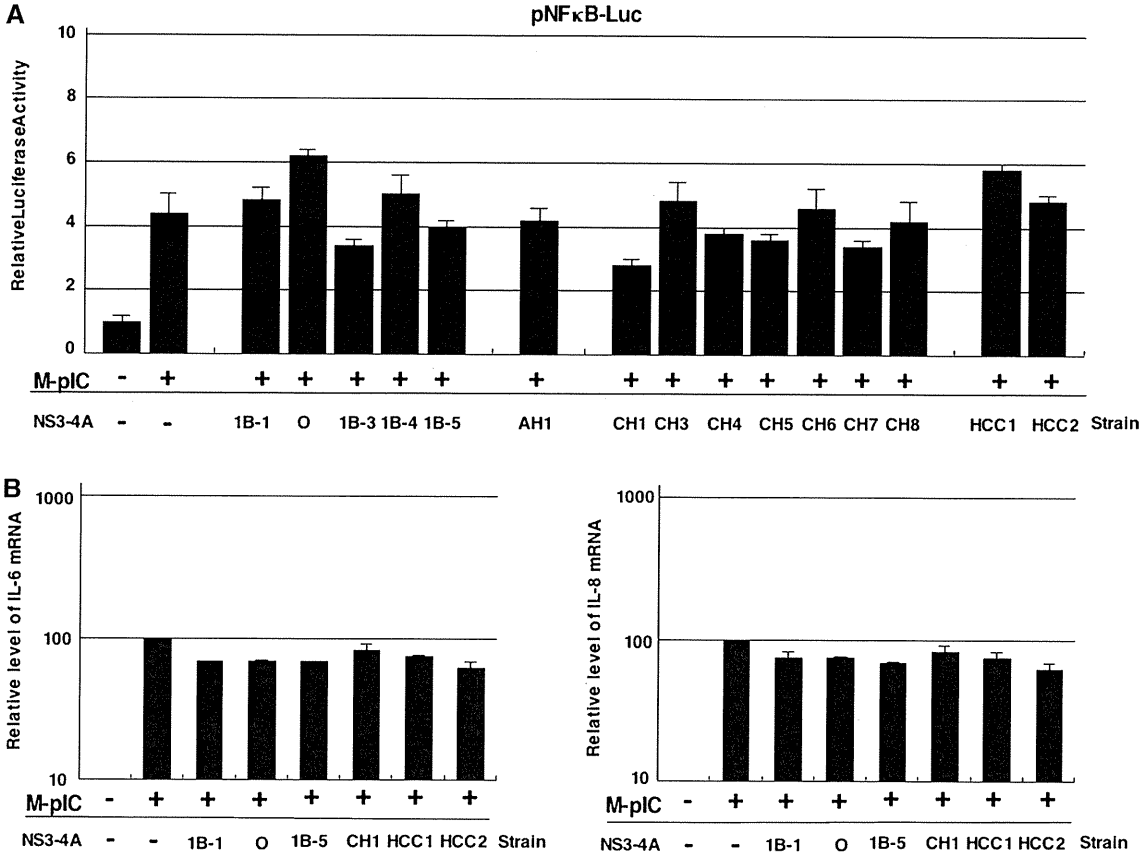

Fig. 6 None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented M-pIC-induced NF-κB activation. **a** Effect of 15 NS3-4As on the activity of NF-κB-inducing promoter. PH5CH8 cells transiently expressing NS3-4As from various HCV strains were subjected to M-pIC treatment. PH5CH8 cells transfected with pCX4bsr vector were used as a control (strain, –). Data are expressed as the mean ± SD from three independent experiments, each of which was performed in triplicate. **b** Effect of NS3-4As on IL-6 or IL-8 mRNA induction by M-pIC treatment. PH5CH8 cells transiently expressing NS3-4A from several strains containing CH1 were subjected to M-pIC treatment. PH5CH8 cells infected with pCX4bsr retrovirus were used as a control (strain, –). Quantitative RT-PCR for IL-6 or IL8 mRNA was performed as described in Fig. 4b

cleavage of TRIF and the cleavage of Cardif by NS3-4A remain unaffected by the genetic diversity observed in NS3-4As derived from 15 different HCV strains (genotype 1b) derived from patients with different stages of hepatic disease as well as different genotypes (1b and 2a). However, other group [10, 27] previously reported that NS3-4A (N strain of genotype 1b) was able to inhibit IFN-β production via the cleavage of TRIF. Although we also observed the interaction of NS3 and TRIF in PH5CH8 and O cells as well as JFH-1-infected RSc cells, the reasons for conflicting results regarding the cleavage of TRIF by NS3-4A are still unclear. To clarify why TRIF is not cleaved by NS3-4A, further analysis will be necessary.

On the other hand, there appear to be some conflicting effects of different HCV proteins on IFN production, as we previously found that the NS5B protein induced IFN-β production in PH5CH8 cells [9, 31] and that the combination of NS5B with the core protein synergistically enhanced IFN-β production [9]. In that study, we showed that enhanced IFN-β production depended on the RNA-dependent RNA polymerase activity of NS5B and on aas 12 and 13 of the core protein, and we observed that NS3-4A significantly inhibited IFN-β production through a combination of the core and NS5B proteins [9]. However, in that case as well, IFN-β production was not completely suppressed by NS3-4A. This may be because NS3-4A is unable to suppress dsRNA-induced and TRIF-mediated IFN-β production, although Cardif-mediated IFN-β production has been shown to be completely suppressed under the same experimental conditions. To clarify the mechanisms underlying the conflicting effects of HCV proteins on IFN-β production mechanisms, an HCV proliferation system using PH5CH8 cells is still needed. However, a HuH-7-cell-based HCV proliferation system [42] would be unsuitable for such purposes due to the functional loss of TLR3 and/or the RIG-I signaling pathway(s) [26, 35]. Studies employing a cell system for HCV proliferation possessing functional TLR3 and/or RIG-I signaling pathways could enhance our understanding of the mechanisms of persistent HCV infection.

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Oncostatin M synergistically inhibits HCV RNA replication in combination with interferon-α

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ABSTRACT

Oncostatin M (OSM), a member of the interleukin-6 family, possesses various functions, including hepatocyte differentiation and suppression of melanoma cell growth. Here, we report anti-hepatitis C virus (HCV) activity of OSM as a new function of this cytokine. OSM possessed marked anti-HCV activity (50% effective concentration: 0.71 ng/ml) in an HCV RNA replication cell culture system. The most striking finding is that OSM exhibited synergistic inhibitory activity on interferon (IFN)-α even at a low concentration with weak anti-HCV activity, such as 25 pg/ml. OSM is a candidate anti-HCV reagent and may improve the current IFN therapy for patients with chronic hepatitis C.

1. Introduction

Currently the combination therapy of pegylated-interferon-α (PEG-IFN-α) with ribavirin (RBV) is available for patients with chronic hepatitis C (CH C). However, the sustained virological response (SVR) rate is still approximately 55% [1]. There is thus an urgent need for novel partners for IFN.

Oncostatin M (OSM) belongs to the interleukin (IL)-6 family, which also includes IL-6, IL-11, IL-27, ciliary neurotrophic factor, cardiotrophin-like cytokine, cardiotrophin-1, neuropoietin and leukemia-inhibitory factor (LIF) [2,3]. OSM was first reported as a cytokine produced from U-937 lymphoma cells, when it was found to inhibit the growth of melanoma cells [4]. The IL-6 family members share glycoprotein 130 (gp130) for signal transduction, and the OSM receptor consists of gp130 and its unique OSMR [5]. Recently it was reported that the IL-31 receptor also contains OSMR and forms a heterodimer with IL31RA [6]. OSMR and gp130 are highly expressed in liver, and OSM plays a significant role in the differentiation and regeneration of liver [7,8]. Therefore, OSM was used as a reagent for the differentiation of hepatocytes in vitro.

Here, we have found that OSM's anti-hepatitis C virus (HCV) activity is a new function of this cytokine. OSM synergistically inhibited HCV RNA replication in combination with IFN-α even at a low concentration with weak anti-HCV activity (20% inhibition). OSM may improve the current PEG-IFN-α and RBV therapy for patients with CH C and provide a clue toward understanding the diverse sensitivity to IFN therapy.

2. Materials and methods

2.1. Compounds and antibodies

IFN-α was purchased from Sigma (St. Louis, MO). OSM and IL-31 were purchased from R&D Systems (Minneapolis, MN). IL-6 was purchased from Acris Antibodies (Herford, Germany). LIF was purchased from Chemicon International (Temecula, CA). Anti-HCV core antibody (CP11) was purchased from the Institute of Immunology (Tokyo, Japan), and anti-HCV non-structural 5A (NS5A) antibody was the generous gift of Dr. A. Takamizawa (Research Foundation for Microbial Diseases, Osaka University). Anti-β-actin antibody was purchased from Sigma. Anti-signal transducer and activator of transcription (STAT) 1 and anti-STAT3 antibodies were purchased from BD Bioscience (San Jose, CA). Anti-phospho-STAT1 (Y701) and anti-phospho-STAT3 (Y705) were purchased from Cell Signaling Technology (Danvers, MA).

Abbreviations: SVR, sustained virological response; CH C, chronic hepatitis C; EC_{50}, 50% effective concentration; EMCV, encephalomyocarditis virus; gp130, glycoprotein 130; HCV, hepatitis C virus; PEG-IFN, pegylated-interferon; IL, interleukin; IRES, internal ribosomal entry site; LIF, leukemia-inhibitory factor; NS, non-structural; OSM, oncostatin M; RBV, ribavirin; RL, *Renilla* luciferase; RT-PCR, reverse transcription-polymerase chain reaction; STAT, signal transducer and activator of transcription

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2.2. Cell culture

The OR6 cell line is cloned from ORN/C-5B/KE (strain O of genotype 1b) RNA replicating HuH-7 cells, as described previously [9]. OR6c cells are cured OR6 cells from which HCV RNA was eliminated by IFN-α treatment, as previously described [10]. HCV-O/RLGE (strain O) is the authentic HCV RNA containing adaptive mutations of Q1112R, P1115L, E1202G, and K1609E in the NS3 region and replicates efficiently in OR6c cells [11]. Li23 and PH5CH cells were cultured as previously described [12].

2.3. OR6 reporter assay

For the *Renilla* luciferase (RL) assay, 1.5×10^4 OR6 cells were plated onto 24-well plates in triplicate and pre-cultured for 24 h. The cells were treated with OSM and/or IFN-α for 72 h. After the treatment, the cells were harvested with *Renilla* lysis reagent (Promega, Madison, WI) and subjected to RL assay according to the manufacturer's protocol.

2.4. Cell growth assay

To examine OSM's activity in OR6 cell growth, 6.0×10^4 OR6 cells were plated onto 6-well plates in triplicate and were pre-cultured for 24 h. The cells were treated with OSM for 72 h, and then the number of viable cells was counted after trypan blue dye treatment, as previously described [13].

2.5. Reverse transcription and polymerase chain reaction (RT-PCR)

RT-PCR for gp130, OSMR, LIFR, IL6R, IL31RA and glyceraldehyde-3-phosphate dehydrogenase was performed by a method described previously [14]. Briefly, using cellular total RNAs (2 μg), cDNA was synthesized using M-MLV reverse transcriptase with oligo dT primer. One-tenth of the synthesized cDNA was subjected to PCR with the specific primer pairs (Supplementary materials).

2.6. Western blot analysis

For Western blot analysis to detect the expression of core and NS5A, 4×10^4 OR6c cells harboring HCV-O/RLGE RNA were plated onto 6-well plates and cultured for 24 h, and then were treated with IFN-α and/or OSM for 72 h. To detect the STATs and phosphorylated STATs, 5×10^5 OR6 cells were plated onto 6-well plates and cultured for 24 h, and then were treated with IFN-α and/or OSM. Preparation of the cell lysates, sodium dodecyl sulfate–polyacrylamide gel electrophoresis and immunoblotting were then performed as previously described [15].

3. Results

3.1. OSM inhibited HCV RNA replication in hepatoma cell line

We have tried to develop differentiated hepatocytes from mesenchymal stem cells using OSM as the differentiation reagent to establish the cell culture system for HCV RNA replication. We tested the reagents needed for differentiation, including OSM, to rule out negative activity for HCV RNA replication. In the course of this procedure, we happened to find that OSM possessed marked anti-HCV activity by using our developed full-length HCV RNA replication reporter system (OR6 assay system) [9]. This system enabled the prompt and precise evaluation of HCV RNA replication levels (Fig. 1A). OSM exhibited marked anti-HCV activity at a low concentration (50% effective concentration (EC_{50}): 0.71 ng/ml) (Fig. 1B) without cytotoxicity (Fig. 1C). OSM's anti-HCV activity was maintained at least until 96 h after a single administration of the reagent (Fig. 1D). These results indicate that OSM possesses anti-HCV activity at a concentration that

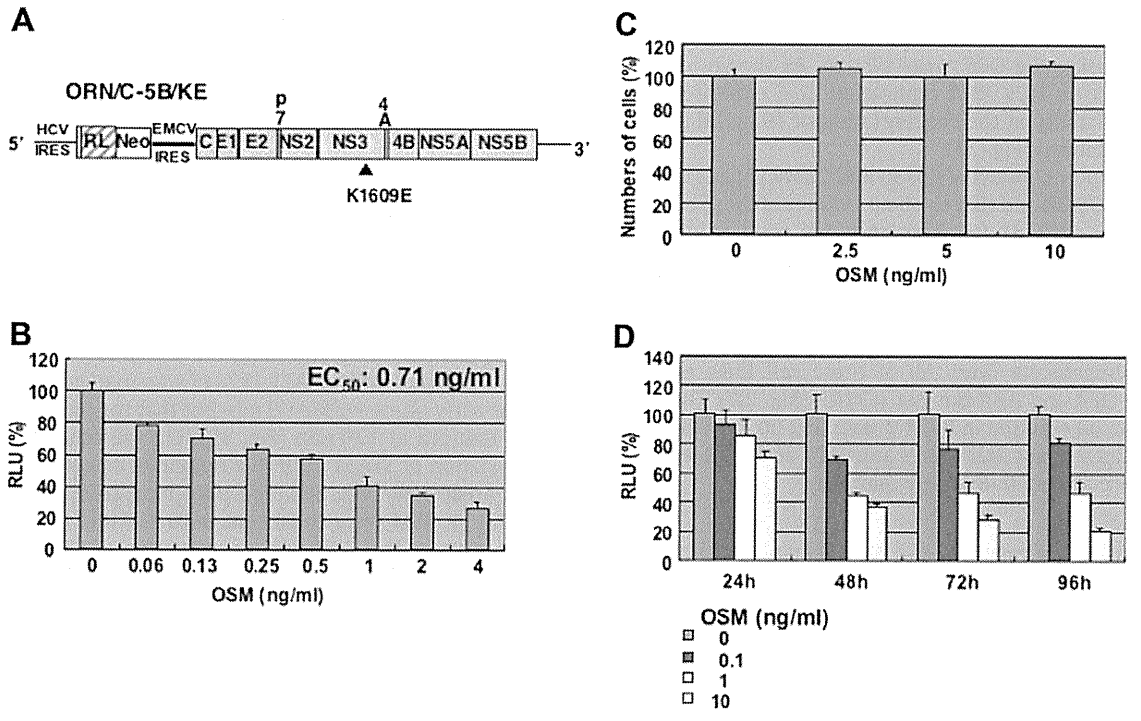

Fig. 1. Anti-HCV activity of OSM in HCV RNA replicating OR6 cells. (A) Schematic gene organization of the genome-length HCV RNA replicating in OR6 cells. The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) OR6 cells were treated with OSM for 72 h and subjected to RL assay. Relative luciferase unit (RLU) was calculated when the RL activity of the control was assigned as 100%. (C) OR6 cells were treated with OSM for 72 h and subjected to a cell viability assay with trypan blue staining. (D) OR6 cells were treated with OSM and harvested at 24, 48, 72, and 96 h and subjected to RL assay.

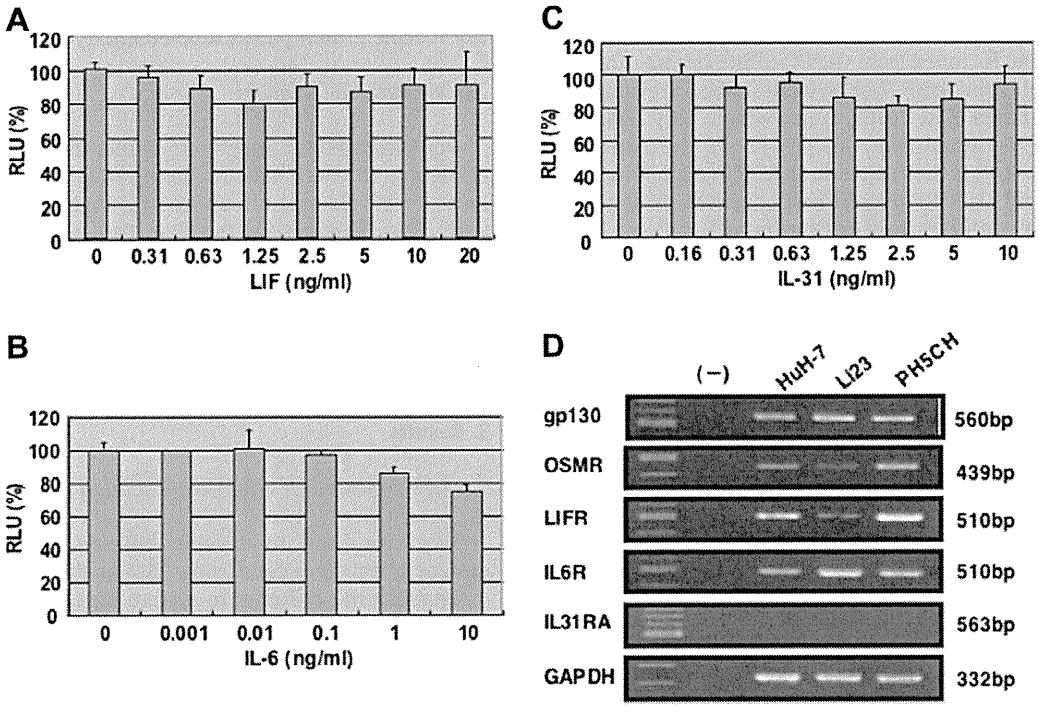

Fig. 2. The activities of LIF, IL-6 and IL-31 on HCV RNA replication. OR6 cells were treated with LIF (A), IL-6 (B) and IL-31 (C) for 72 h and subjected to RL assay. (D) RNAs from hepatocytes (HuH-7, Li23 and PH5CH) were subjected to RT-PCR with specific primer pairs to gp130, OSMR, LIFR, IL6R, IL31RA and GAPDH.

does not affect cell growth and is a new class of antiviral cytokine.

3.2. Anti-HCV activity of OSM is a unique feature in the IL-6 family

OSM belongs to the IL-6 family, whose members share the common gp130 molecule in each receptor [5]. Therefore, we next examined the activities of other representative IL-6 family members (LIF, IL-6) using the OR6 assay system. As shown in Fig. 2A, LIF had no effect on HCV RNA replication. IL-6 exhibited only a weak anti-HCV activity at the concentration of 10 ng/ml (approximately 20% inhibition) (Fig. 2B).

The OSM receptor consists of gp130 and OSMR [5]. IL31RA is another partner of OSMR and that the heterodimer of these molecules forms a receptor of IL-31 [6]. Therefore, we tried to determine whether or not IL-31 possesses anti-HCV activity in OR6 cells. The result revealed that IL-31 exhibited no anti-HCV activity. Next we examined the expression levels of the receptors in HuH-7, Li23 (a human hepatoma cell line) and PH5CH (an immortalized primary human hepatocyte line). All of these cell lines expressed gp130, OSMR, LIFR and IL6R but not IL31RA (Fig. 2D). The lack of 1L31RA expression resulted in IL-31 possessing no anti-HCV activity. These results suggest that OSM's anti-HCV activity seems to be a unique feature among IL-6 family members.

3.3. OSM synergistically enhanced anti-HCV activity of IFN-α

As HCV RNA contains three exogenous genes (RL, Neo and encephalomyocarditis virus (EMCV)-internal ribosomal entry site (IRES)) (Fig. 1A), we tried to determine whether OSM inhibits authentic HCV RNA replication in order to rule out the possibility that OSM's anti-HCV activity is not due to the inhibition of these exogenous genes. OSM inhibited core and NS5A expression in a dose-dependent manner (Fig. 3A, lanes 1–3). We next examined OSM's anti-HCV activity in combination with IFN-α using authentic HCV-O/RLGE RNA-replicating cells. OSM (1 and 10 ng/ml) drastically inhibited core and NS5A expression in combination with IFN-α (2.5, 5, and 10 IU/ml) (Fig. 3A, lanes 4–12).

OSM exhibited anti-HCV activity even at low concentrations, such as 62 pg/ml, and enhanced the anti-HCV activity of IFN-α (Fig. 3B). We also examined anti-HCV activity of CsA (0, 0.25, 0.5, and 1.0 μg/ml) alone or in combination with OSM (10 ng/ml) (Supplementary Fig. 1). OSM enhanced CsA's anti-HCV activity. Anti-HCV activity of OSM at 10 ng/ml was almost equal to that of CsA at 0.5 μg/ml. Then, we performed isobole plot analysis for EC_{50} of OSM and IFN-α. In Fig. 3C, dotted line means that the interaction of two reagents is evaluated as additive effect (or zero interaction). Points below this line correspond to synergistic interaction (or positive interaction) and points above this line indicate antagonism (or negative interaction) [16]. Therefore, isobole plot analysis of EC_{70} for OSM and IFN-α revealed that the combination of OSM and IFN-α exhibited striking synergistic inhibition of HCV RNA replication (Fig. 3C). Then we investigated whether or not OSM enhanced the IFN signaling pathway, since OSM activates STATs [17]. A kinetic study regarding the phosphorylation of STAT1 and STAT3 revealed that STAT1 (Y701) was markedly phosphorylated in the early phase within 60 min but that the phosphorylation level was reduced at 120 min (Fig. 3D). On the other hand, the phosphorylation of STAT1 by IFN-α remained consistent until 120 min after treatment (Fig. 3D). The phosphorylation kinetics of STAT3 (Y705) by OSM were consistent until 120 min (Fig. 3D). These results suggest that early-phase activation of STAT1 by OSM may trigger the synergistic activity in HCV RNA replication in combination with IFN-α.

3.4. OSM enhanced anti-HCV activity of IFN-α at even the low effective concentration by itself

As OSM exhibited marked synergistic anti-HCV activity with IFN-α, we tried to determine whether a low concentration of OSM could synergistically enhance the anti-HCV activity of IFN-

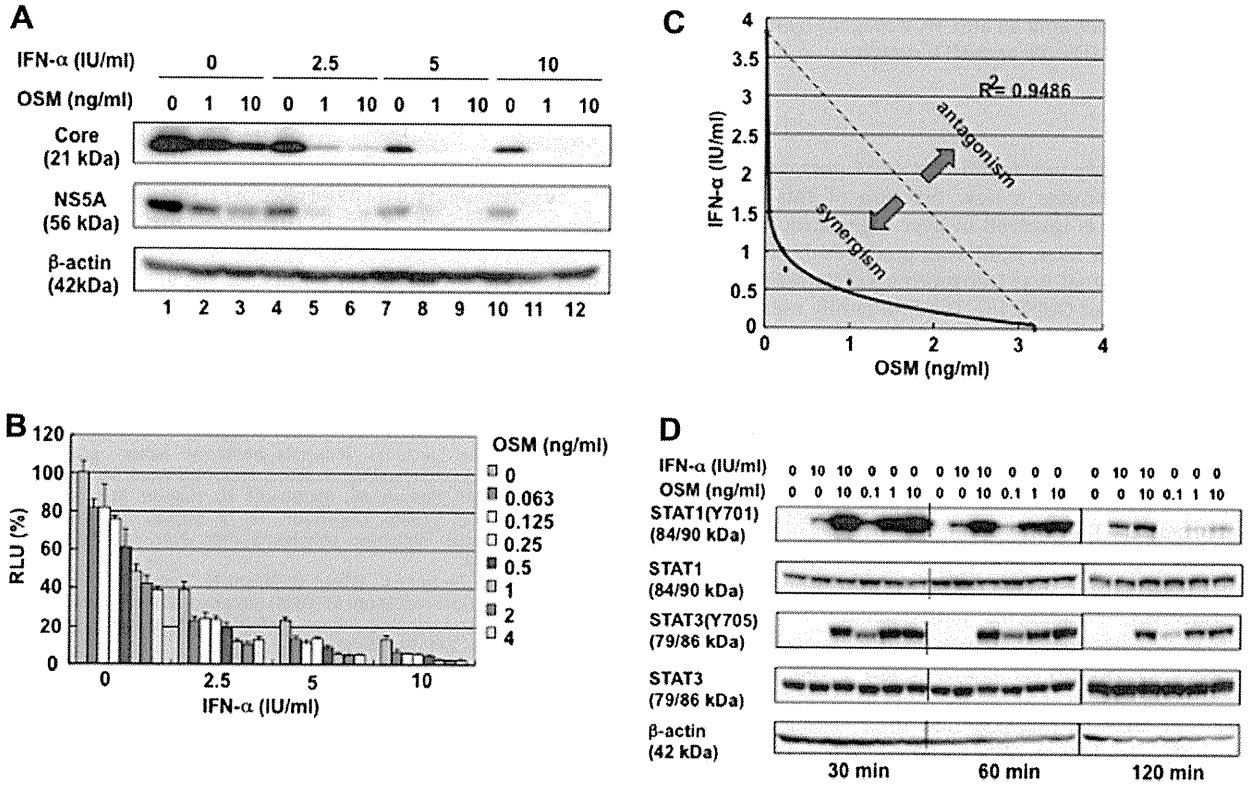

Fig. 3. Anti-HCV activity of OSM in combination with IFN-α. (A) HCV-O/RLGE-replicating OR6c cells were treated with OSM in combination with IFN-α for 96 h and subjected to Western blot analysis using anti-core, anti-NS5A and anti-β-actin antibodies. (B) OR6 cells were treated with OSM in combination with IFN-α for 72 h and subjected to RL assay. (C) Isobole plot analysis (EC_{70}) for OSM and IFN-α in OR6 cells after treatment for 72 h. (D) OR6 cells were treated with OSM and IFN-α for 30, 60 and 120 min and subjected to Western blot analysis using anti-STAT1, anti-phospho-STAT1 (Y701), anti-STAT3, anti-phospho-STAT3 (Y705) and anti-β-actin antibodies.

α. For this purpose, we treated OR6 cells with OSM at 25 pg/ml or 50 pg/ml in combination with IFN-α (0, 1, 2, 4, and 8 IU/ml). OSM alone at 25 pg/ml or 50 pg/ml exhibited only 20% inhibitory activity (Fig. 4A). However, OSM at these concentrations enhanced the anti-HCV activity of IFN-α up to 60% inhibition, when IFN-α at 8 IU/ml was treated with OSM at 25 pg/ml (Fig. 4B). These results

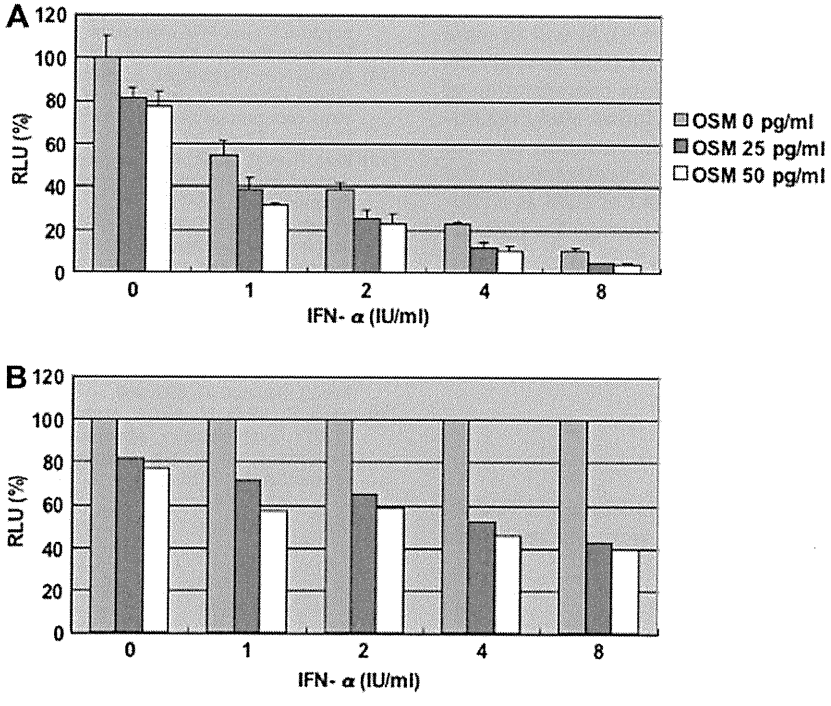

Fig. 4. OR6 cells were treated with OSM and IFN-α for 72 h and subjected to RL assay (A). Relative RL activity was adjusted when the RL activities of the cells treated with only IFN-α were assigned as 100% (B).

indicate that OSM is not only an anti-HCV reagent by itself but also a strong adjuvant for IFN-α's anti-HCV activity.

4. Discussion

In the present study, we found that OSM possesses anti-HCV activity, which constitutes a new function of this multi-functional cytokine. OSM is involved in liver regeneration and differentiation [7,8]. In the liver, OSM was produced by Kupffer cells [18], and the OSM signal was transmitted via its receptor, which consisted of gp130 and OMSR [5]. The IL-6 family members share gp130 in their receptors; it forms the heterodimer with a unique partner; for example IL6R in IL-6 and LIFR in LIF [2]. We tested the activity of LIF and IL-6 on HCV RNA replication. However, LIF did not exhibit anti-HCV activity, and IL-6 showed only weak anti-HCV activity compared to the OSM. These results suggest that OSM's anti-HCV activity is achieved via OSMR or the combination of gp130 and OSMR rather than via gp130. Recently, it was reported that IL31RA was another partner of OSMR in the IL-31 receptor [6]. If IL-31 could exhibit anti-HCV activity, OSMR seems to be significant in the signal transduction of anti-HCV activity. However, hepatocytes didn't seem to be a natural target of IL-31, because hepatocytes didn't express IL31RA. Further study is needed to clarify OSMR's role in anti-HCV activity.

Isobole plot analysis revealed that OSM exhibited a striking synergistic effect in the anti-HCV activity of IFN-α [19]. This synergistic activity of OSM may be caused by early strong activation of STAT1 by OSM. Furthermore, OSM enhanced the activity of 2′–5′ oligoadenylate synthetase promoter in combination with IFN-α (data not shown). These results suggest that STAT1 may be the key player in the synergy between OSM and IFN-α.

In this study, we found OSM's synergistic activity in the anti-HCV activity of IFN-α, when OSM was used at a low concentration (25 pg/ml) with only 20% inhibitory activity against HCV RNA replication. Surprisingly, OSM at 25 pg/ml enhanced the anti-HCV activity of IFN-α by up to 60%. RBV is the only adjuvant to the current PEG-IFN-α therapy for patients with CH C, and the combination therapy of PEG-IFN-α/RBV achieved only approximately 55% of the SVR rate. Therefore, OSM will become a strong partner to the current IFN therapy. As OSM strongly affected the anti-HCV activity of IFN-α, the serum concentration of OSM will affect the SVR in IFN therapy. The future study regarding the relationship between the serum concentration of OSM and SVR may provide a clue toward understanding the resistance to IFN therapy, and the development of OSM as a clinical reagent will serve as a breakthrough in therapy for patients with CH C.

In conclusion, we found OSM's anti-HCV activity a newly identified function of this multifunctional cytokine. The highlight of this study is that OSM exhibited a synergistic effect on the anti-HCV activity of IFN-α even at a low concentration with weak anti-HCV activity by itself.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.03.054.

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Short Communication

Gene expression profile of Li23, a new human hepatoma cell line that enables robust hepatitis C virus replication: Comparison with HuH-7 and other hepatic cell lines

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Aim: Human hepatoma cell line HuH-7-derived cells are currently the only cell culture system used for robust hepatitis C virus (HCV) replication. We recently found a new human hepatoma cell line, Li23, that enables robust HCV replication. Although both cell lines had similar liver-specific expression profiles, the overall profile of Li23 seemed to differ considerably from that of HuH-7. To understand this difference, the expression profile of Li23 cells was further characterized by a comparison with that of HuH-7 cells.

Methods: cDNA microarray analysis using Li23 and HuH-7 cells was performed. Li23-derived ORL8c cells and HuH-7-derived RSc cells, in which HCV could infect and efficiently replicate, were also used for the microarray analysis. For the comparative analysis by reverse transcription polymerase chain reaction (RT–PCR), human hepatoma cell lines (HuH-6, HepG2, HLE, HLF and PLC/PRF/5) and immortalized hepatocyte cell line (PH5CH8) were also used.

Results: Microarray analysis of Li23 versus HuH-7 cells selected 80 probes to represent highly expressed genes that have ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23). Among them, 17 known genes were picked up for further analysis. The expression levels of most of these genes in Li23 and HuH-7 cells were retained in ORL8c and RSc cells, respectively. Comparative analysis by RT–PCR using several other hepatic cell lines resulted in the classification of 17 genes into three types, and identified three genes showing Li23-specific expression profiles.

Conclusion: Li23 is a new hepatoma cell line whose expression profile is distinct from those of frequently used hepatic cell lines.

Key words: hepatitis C virus, hepatoma cell line, HuH-7, Li23, microarray

INTRODUCTION

HUH-7, A HUMAN hepatoma cell line,[1] is frequently used in the research of hepatitis C virus (HCV), since an HCV replicon system enabling HCV subgenomic RNA replication was developed using HuH-7 cells.[2] Even with the use of an efficient HCV production system developed in 2005,[3] HuH-7-derived cells are still used as the only cell line for persistent HCV production systems.

We previously developed HCV replicon systems[4,5] and an HCV production system[6] using HuH-7-derived cells. Furthermore, we recently found a new human hepatoma cell line, Li23, that enables robust HCV RNA replication and persistent HCV production.[7] In that study, using microarray analysis, we excluded the possibility that the obtained Li23-derived cells were derived from contamination of HuH-7-derived cells used for HCV replication.[7] In addition, we noticed that the gene expression profile of Li23 cells seemed considerably different from that of HuH-7 cells. Therefore, we assumed that the Li23 cell line possesses a unique expression profile among widely used human hepatoma cell lines. To evaluate this assumption, we further characterized the expression profile of Li23 cells by comparing it with those of other human hepatoma cell lines, including HuH-7,[1] HuH-6,[8] HepG2,[9] HLE,[10] HLF[10] and PLC/PRF/5.[11] Human immortalized hepatocyte cell line

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PH5CH8[12] was also used for the comparison. Here, we show that the Li23 cell line possesses a distinct expression profile among hepatic cell lines.

METHODS

Cell culture

HUH-7, HUH-6, HEPG2, HLE, HLF and PLC/PRF/5 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Li23 and PH5CH8 cells were maintained as described previously.[7] Cured cells (Li23-derived ORL8c and HuH-7-derived RSc), from which the HCV RNA had been eliminated by interferon (IFN) treatment, were also maintained as described previously.[7]

cDNA microarray analysis

Li23, ORL8c, HuH-7 and RSc cells (1×10^6 each) were plated onto 10-cm diameter dishes and cultured for 2 days. Total RNA from these cells were prepared using the RNeasy extraction kit (QIAGEN, Hilden, Germany). cDNA microarray analysis was performed according to the methods described previously.[7] Differentially expressed genes were selected by comparing the arrays from Li23 and HuH-7 cells. The selected genes were further compared with the array from ORL8c or RSc cells.

Reverse transcription polymerase chain reaction

Reverse transcription polymerase chain reaction (RT-PCR) was performed to detect cellular mRNA as described previously.[13] Briefly, total RNA (2 μg) was reverse-transcribed with M-MLV reverse transcriptase (Invitrogen, San Diego, CA, USA) using an oligo dT primer (Invitrogen) according to the manufacturer's protocol. One-tenth of the synthesized cDNA was used for PCR. The primers arranged for this study are listed in Table 1. In addition, we used primer sets for New York esophageal squamous cell carcinoma 1 (NY-ESO-1), β-defensin-1 (DEFB1), lectin, galactoside-binding, soluble 3 (LGALS3)/Galectin-3, melanoma-specific antigen family A6 (MAGEA6), UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4), transmembrane 4 superfamily member 3 (TM4SF3), insulin-like growth factor binding protein 2 (IGFBP2), arylacetamide deacetylase (AADAC), albumin and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), as described previously.[7]

RESULTS

Genes showing pronounced differences in gene expression between Li23- and HuH-7-derived cells

WE RECENTLY ESTABLISHED several Li23-derived cell lines showing robust HCV RNA replication.[7] In convenient microarray analysis using these cell lines, we noticed that the gene expression profile of Li23 cells differed considerably from that of HuH-7 cells, and that several genes, including cancer antigens such as NY-ESO-1 and MAGEA6, were highly expressed in Li23 cells but were not expressed in HuH-7 cells.[7] However, it

Table 1 Primers used for reverse transcription polymerase chain reaction analysis

Gene (accession no.)	Direction	Nucleotide sequence (5′–3′)	Products (bp)
Cancer antigen 45, A5	Forward	TGGAGATGACCTAGAATGCAG	218
(CT45A5); NM_001007551	Reverse	CTCGTCTCATACATCTTGCTG	
Four-and-a-half LIM domain 1	Forward	GGAATCACTTACCAGGATCAG	243
(FHL1; NM_001449)	Reverse	TTTGCAGTGGAAGCAGTAGTC	
Thymosin β4, X-linked	Forward	ACCAGACTTCGCTCGTACTC	179
(TMSB4X; NM_021109)	Reverse	TCGCCTGCTTGCTTCTCCTG	
Lectin, galactoside-binding, soluble 1	Forward	CAACACCATCGTGTGCAACAG	253
(LGALS1; NM_002305)	Reverse	CAGCTGCCATGTAGTTGATGG	
Interferon-induced transmembrane protein 2	Forward	CCTCTTCATGAACACCTGCTG	184
(IFITM2; NM_006435)	Reverse	CACTGGGATGATGATGAGCAG	
Apolipoproteins A1	Forward	ACTGTGTACGTGGATGTGCTC	273
(APOA1; X02162)	Reverse	CTTCTTCTGGAAGTCGTCCAG	
α-2-HS-glycoprotein	Forward	AACCGAACTGCGATGATCCAG	248
(AHSG; NM_001622)	Reverse	TTCGACAGCATGCTCCTTCAG	
Gap junction protein-α 1	Forward	CATCTTCATGCTGGTGGTGTC	253
(GJA1; NM_000165)	Reverse	GTTTCTGTCGCCAGTAACCAG	

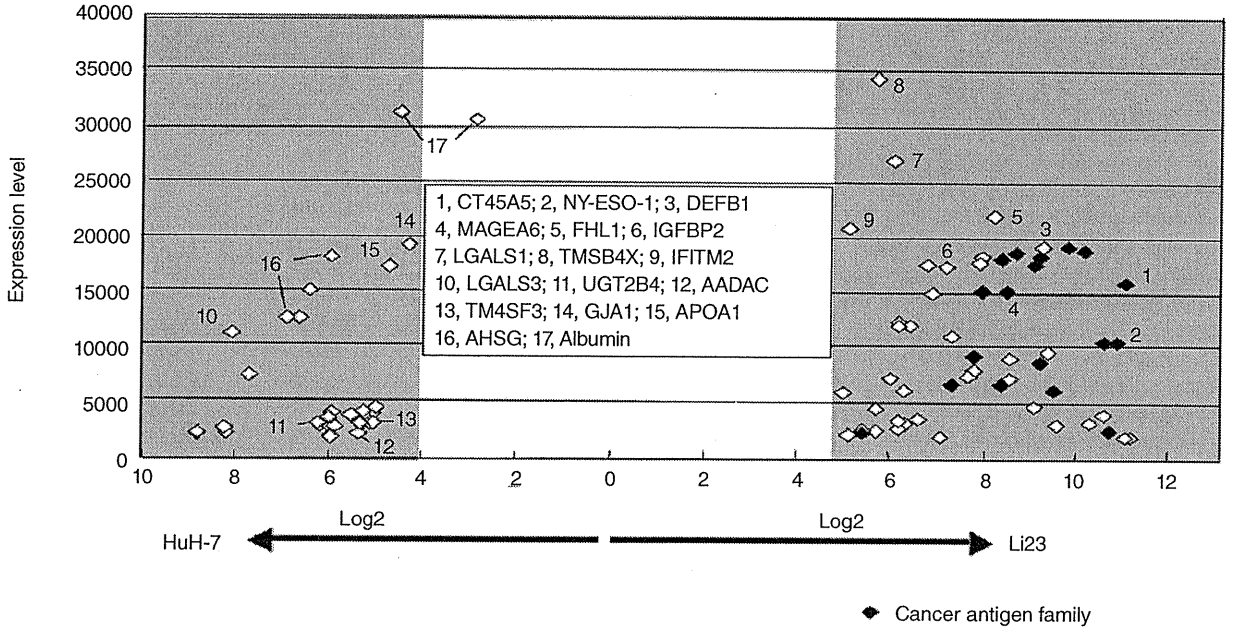

Figure 1 Genes showing pronounced differences in gene expression between Li23 and HuH-7 cells. The probes showing expression levels of more than 2000 and ratios of more than 30 (Li23/HuH-7) or 20 (HuH-7/Li23) are presented.

is unclear whether the expression profiles of these genes are characteristics of Li23 cells.

To clarify this point, comprehensive microarray analysis using Li23 and HuH-7 cells was performed. This revealed 4119 and 3570 probes whose expression levels were upregulated and downregulated at ratios of more than 2 and less than 0.5 in Li23 versus HuH-7 cells, respectively. From among these probes, we selected those showing ratios of more than 30 (Li23/HuH-7) and 20 (HuH-7/Li23), and further selected the probes showing expression levels of more than 2000 (actual value of measurement). By these selections, 80 probes were assigned (Fig. 1). The most distinguishing characteristic of the comparison is that the cancer antigen family (18 probes) was highly expressed in Li23 cells but was not highly expressed in HuH-7 cells (Fig. 1). From these probes, 14 known genes showing expression levels above 10 000 (#1–10 and #14–17 in Fig. 1) and three additional known genes (#11–13 in Fig. 1) were chosen as representative genes for further analysis.

Regarding the total of 17 genes, the expression levels in Li23 versus ORL8c or HuH-7 versus RSc were compared. The expression levels of most of the 17 genes were maintained between Li23 and ORL8c cells or between HuH-7 and RSc cells (Table 2). These results indicate that ORL8c and RSc cells retained the characteristics of parent Li23 and HuH-7 cells, respectively. However, it was notable that the expression levels of apolipoprotein A1 (APOA1), α-2-HS-glycoprotein (AHSG), and albumin were significantly higher in ORL8c cells than in Li23 cells, suggesting that ORL8c is selected as a specific clone from Li23 cell populations.

Expression profiles of representative genes whose expression levels showed drastic differences between Li23 and HuH-7 cells among human hepatic cell lines

Regarding the 17 genes selected above, we performed comparative analyses by RT–PCR using Li23, HuH-7, HuH-6, HepG2, HLE, HLF, PLC/PRF/5 and PH5CH8 cells in order to clarify whether or not these genes exhibit Li23-specific expression profiles. The results of the RT–PCR performed after optimization of PCR conditions in each gene resulted in the classification of the 17 genes into three types (A, B and C in Fig. 2). NY-ESO-1 and DEFB1 (high expression in Li23 only), and LGALS3/Galectin-3 (no expression in Li23 only) belonged to type A, which showed a Li23-specific feature. Type B showed that the expression levels in Li23, HLE, HLF, PLC/PRF/5 and/or PH5CH8 cells were greatly higher or lower than those in HuH-7, HuH-6 and HepG2 cells. Type B consisted of cancer antigen 45, A5

Table 2 Representative genes showing pronounced differences in gene expression between Li23 and HuH-7 cells

Gene	Accession no.	Li23	Li23-derived ORL8c	HuH-7	HuH-7-derived RSc
Cancer antigen 45, A5 (CT45A5)	NM_001007551	15 857†	10 508	8	23
Cancer testis antigen 1A (NY-ESO-1/CTAG1A)	U87459	9 005	5 503	5	8
β-Defensin-1 (DEFB1)	U73945	18 311	8 326	31	7
Melanoma-specific antigen family A6 (MAGEA6)	U10691	15 168	17 050	42	35
Four-and-a-half LIM domain 1 (FHL1)	NM_001449	21 851	13 428	77	79
Insulin-like growth factor binding protein 2 (IGFBP2)	NM_000597	17 429	8 931	117	13
Lectin, galactoside-binding, soluble 1 (LGALS1)	NM_002305	26 694	27 098	379	11
Thymosin β4, X-linked (TMSB4X)	NM_021109	34 273	26 199	648	307
IFN-induced transmembrane protein 2 (IFITM2)	NM_006435	20 762	9 645	595	637
Lectin, galactoside-binding, soluble 3 (LGALS3/Galectin 3)	BC001120	41	70	10 973	6 020
UDP glycosyltransferase 2 family polypeptide B4 (UGT2B4)	NM_021139	40	57	2 863	7 546
Arylacetamide deacetylase (AADAC)	NM_001086	57	73	2 282	4 746
Transmembrane 4 superfamily member 3 (TM4SF3)	NM_004616	95	51	3 220	1 265
Gap junction protein-α 43 KDa (GJA1)	NM_000165	951	2	19 090	19 485
Apolipoprotein A1 (APOA1)	X02162	673	7 230	16 920	15 202
α-2-HS-glycoprotein (AHSG)	NM_001622	308	6 373	18 436	26 000
Albumin	AF116645	4 304	30 111	30 234	33 140
	D16931	1 387	23 615	30 668	39 144

†Signal intensity in human genome U133 Plus 2.0 array.

(CT45A5), MAGEA6, four-and-a-half LIM domains 1 (FHL1), Thymosin B4, X-linked (TMSB4X), lectin, galactoside-binding, soluble 1 (LGALS1) and IFN-induced transmembrane protein 2 (IFITM2) – all of which were highly expressed in Li23 cells – and APOA1, AHSG and UGT2B4, which were highly expressed in HuH-7 cells. The remaining five genes were assigned to type C and showed more complex expression profiles (Fig. 2). For instance, Gap junction protein-α 43 kDa (GJA1) expression was observed in HuH-7, HLE, HLF, PLC/PRF/5 and PH5CH8 cell lines, but not in Li23, HuH-6 or HepG2 cell lines. In addition, IGFBP2 expression was observed in Li23, HuH-6 and PH5CH8 cell lines, but not in the other cell lines. Together, these results indicate that the Li23 cell line possesses a distinct expression profile among frequently used hepatic cell lines.

DISCUSSION

IN THIS STUDY, we assigned 17 known genes that showed drastic differences between Li23 and HuH-7 cells, and classified the expression profiles of these genes into at least three types among frequently used hepatic cell lines. Three genes (NY-ESO-1, DEFB1 and LGALS3/Galectin-3) were identified as the representative showing Li23-specific expression.

NY-ESO-1 is a well-characterized cancer-testis antigen (CTAG) that appears to be the most immunogenic CTAG known to date.[14] NY-ESO-1 is expressed in malignant tumors such as melanoma, lung carcinoma and bladder cancer, which are called "CTAG-rich" tumor types, but are expressed solely in the testis among normal adult tissues.[15] Because a spontaneous immune response to NY-ESO-1 is frequently observed in patients with malignant tumors including hepatocellular carcinoma,[16] cancer vaccine trials based on NY-ESO-1 are currently underway.[15] However, the biological role of NY-ESO-1 in both tumors and testis remains poorly understood. Accordingly, the Li23 cell line may be useful for the study of the biological role of NY-ESO-1.

Human defensins, which are small cationic peptides produced by neutrophils and epithelial cells, form two genetically distinct subfamilies, α-defensin and β-defensin. DEFB1, identified in this study, is one of six members belonging to β-defensins and appears to be involved in the antimicrobial defense of the epithelia of surfaces.[16,17] Although α-defensins consisting of six members are known to be expressed in a variety of tumors, DEFB1 is downregulated in some tumor types in which it could behave as a tumor suppressor protein.[18] Our study revealed that except DEFB1 in Li23 cells, no α- or β-defensin members were expressed in the

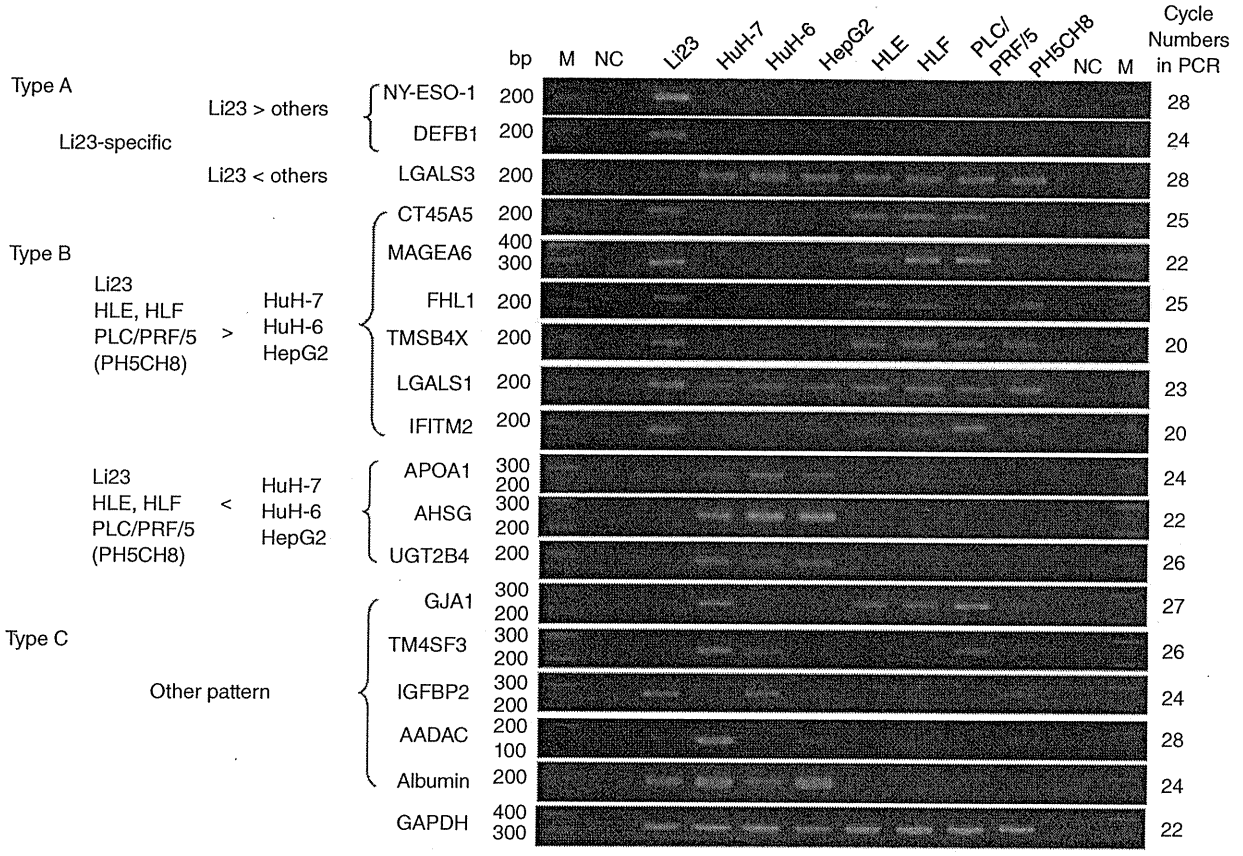

Figure 2 Expression profiles of representative genes, whose expression levels showed drastic differences between Li23 and HuH-7 cells, among human hepatic cell lines. Reverse transcription polymerase chain reaction (RT–PCR) analysis was performed as described in Methods. PCR products were detected by staining with ethidium bromide after separation by electrophoresis on 3% agarose gels.

hepatic cell lines tested in this study (data not shown). Because the molecular mechanism underlying DEFB1 expression or its role in oncogenesis remains to be clarified, Li23 cells may be useful for a study like that.

LGALS3/Galectin-3 is the most studied member of the galectin family, which is characterized by specific binding of β-galactosides through the carbohydrate-recognition domain.[19] LGALS3/Galectin-3 is ubiquitously expressed in numerous cell and tissue types; it is located in both nuclei and cytoplasm, and is secreted through a non-classical pathway. To date, LGALS3/Galectin-3 was found to be involved in many regulations including development, immune reaction, tumorigenesis, and tumor growth and metastasis.[19,20] Indeed, the overexpression of LGALS3/Galectin-3 in cirrhotic and hepatocellular carcinoma has also been reported.[21] In such situations, the absence of LGALS3/Galectin-3 expression in the Li23 cell line is a unique feature among hepatic cell lines, which show high expression levels. Accordingly, the Li23 cell line might be useful as a LGALS3/Galectin-3-null cell line for various studies including those on tumor growth and metastasis.

Although we identified Li23-specific genes showing distinct expression levels among hepatic cell lines examined, microarray analysis revealed that the expression profiles of Li23 and HuH-7 cells, both of which possess an environment for robust HCV replication, differed considerably. Accordingly, such differences may affect the properties or multiplications of HCV, such as susceptibility to anti-HCV reagents, the mutation rate of the HCV genome and the efficiency of HCV replication. Further comparative analysis using Li23 and HuH-7 cells will help to resolve these uncertain subjects.

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The ESCRT System Is Required for Hepatitis C Virus Production

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Abstract

Background: Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for hepatitis C virus (HCV) production. However, the mechanisms of HCV assembly, budding, and release remain poorly understood. Retroviruses and some other enveloped viruses require an endosomal sorting complex required for transport (ESCRT) components and their associated proteins for their budding process.

Methodology/Principal Findings: To determine whether or not the ESCRT system is needed for HCV production, we examined the infectivity of HCV or the Core levels in culture supernatants as well as HCV RNA levels in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, expressing short hairpin RNA or siRNA targeted to tumor susceptibility gene 101 (TSG101), apoptosis-linked gene 2 interacting protein X (Alix), Vps4B, charged multivesicular body protein 4b (CHMP4b), or Brox, all of which are components of the ESCRT system. We found that the infectivity of HCV in the supernatants was significantly suppressed in these knockdown cells. Consequently, the release of the HCV Core into the culture supernatants was significantly suppressed in these knockdown cells after HCV-JFH1 infection, while the intracellular infectivity and the RNA replication of HCV-JFH1 were not significantly affected. Furthermore, the HCV Core mostly colocalized with CHMP4b, a component of ESCRT-III. In this context, HCV Core could bind to CHMP4b. Nevertheless, we failed to find the conserved viral late domain motif, which is required for interaction with the ESCRT component, in the HCV-JFH1 Core, suggesting that HCV Core has a novel motif required for HCV production.

Conclusions/Significance: These results suggest that the ESCRT system is required for infectious HCV production.

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Introduction

Hepatitis C virus (HCV) is a causative agent of chronic hepatitis, which progresses to liver cirrhosis and hepatocellular carcinoma. HCV is an enveloped virus with a positive single stranded 9.6 kb RNA genome, which encodes a large polyprotein precursor of approximately 3,000 amino acid residues. This polyprotein is cleaved by a combination of the host and viral proteases into at least 10 proteins in the following order: Core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1]. HCV Core, a highly basic RNA-binding protein, forms a viral capsid and is targeted to lipid droplets [2–6]. The Core is essential for infectious virion production [7]. NS5A, a membrane-associated RNA-binding phosphoprotein, is also involved in the assembly and maturation of infectious HCV particles [8,9]. Intriguingly, NS5A is a key regulator of virion production through the phosphorylation by casein kinase II [9]. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [4]. Indeed, NS5A is known to colocalize with the Core on lipid droplets [5], and the interaction between NS5A and the Core is critical for the production of infectious HCV particles [3]. However, the host factor involved in HCV assembly, budding, and release remains poorly understood.

Budding is an essential step in the life cycle of enveloped viruses. Endosomal sorting complex required for transport (ESCRT) components and associated factors, such as tumor susceptibility gene 101 (TSG101, a component of ESCRT-I), charged multivesicular body protein 4b (CHMP4b, a component of ESCRT-III), and apoptosis-linked gene 2 interacting protein X (ALIX, a TSG101- and CHMP4b-binding protein), have been found to be involved in membrane remodeling events that accompany endosomal protein sorting, cytokinesis, and the budding of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1) [10–12]. The ESCRT complexes I, II, and III are sequentially, or perhaps concentrically recruited to the endosomal membrane to sequester

cargo proteins and drive vesicularization into the endosome. Finally, ESCRT-III recruits Vps4 (two isoforms, Vps4A and Vps4B), a member of the AAA-family of ATPase that disassembles and thereby terminates and recycles the ESCRT machinery.

Since HCV is also an enveloped RNA virus, we hypothesized that the ESCRT system might be required for HCV production. To test this hypothesis, we examined the release of HCV Core into culture supernatants from cells rendered defective for ESCRT components by RNA interference. The results provide evidence that the ESCRT system is required for HCV production.

Materials and Methods

Cell Culture

293FT cells (Invitrogen, Carlsbad, CA) were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (FBS). The HuH-7-derived cell line, RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [13] could infect and effectively replicate [14–16] and OR6c and OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [17,18].

Plasmid Construction

To construct pcDNA3-FLAG-Alix, a DNA fragment encoding Alix was amplified from total RNAs derived from RSc cells by RT-PCR using the follwing pairs of primers: Forward 5′-CGGG-ATCCAAGATGGCGACATTCATCTCGGT-3′ and reverse 5′-CCGGCGGCCGCTTACTGCTGTGGATAGTAAG-3′. The obtained DNA fragment was subcloned into *Bam*HI-*Not*I of pcDNA3-FLAG vector [19], and the nucleotide sequences were determined by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The plasmid of pJRN/3-5B was based on pJFH1 [13] and was constructed as previously described [20].

RNA synthesis, RNA transfection, and Selection of G418-resistant cells

Plasmid pJRN/3-5B were linearized by *Xba*I and used for the RNA synthesis with the T7 MEGAScript kit (Ambion, Austin, TX). *In vitro* transcribed RNA was transfected into OR6c cells by electroporation [17,18]. The transfected cells were selected in culture medium containing G418 (0.3 mg/ml) for 3 weeks. We referred to them as OR6c/JRN 3-5B cells.

Immunofluorescence and Confocal Microscopic Analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS) and permeabilized in 0.1% Nonidet P-40 (NP-40) in PBS at room temperature as previously described [21]. Cells were incubated with anti-HCV Core antibody (CP-9 and CP-11 mixture; Institute of Immunology, Tokyo, Japan), anti-Myc-Tag antibody (PL14; Medical & Biological Laboratories, MBL, Nagoya, Japan), anti-Alix antibody (Covalab, Villeurbanne, France), and/or anti-FLAG polyclonal antibody (Sigma, St. Louis, MO) at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37°C for 30 min. Cells were then stained with fluorescein isothiocyanate (FITC)-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA) or anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch) at a 1:300 dilution in PBS containing BSA at 37°C for 30 min. Lipid droplets and nuclei were stained with BODIPY 493/503 (Molecular Probes, Invitrogen) and DAPI (4′,6′-diamidino-2-phenylindole), respectively. Following extensive washing in PBS, cells were mounted on slides using a mounting media of 90% glycerin/10% PBS with 0.01% *p*-phenylenediamine added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (LSM510; Zeiss, Jena, Germany).

RNA Interference

The following siRNAs were used: human TSG101 (siGENOME SMARTpool M-003549-01-0005 and 5′-CCUCCAGU-CUUCUCUCGUCUU-3′ sense, 5′-GACGAGAGAAGACUG-GAGGUU-3′ antisense), human Alix/PDCD6IP (siGENOME SMARTpool M-004233-02-0005), human Vps4B (siGENOME SMARTpool M-013119-02-0005), human CHMP4b (siGENOME SMARTpool M-018075-00-0005), and siGENOME Non-Targeting siRNA Pool#1 (D-001206-13-05) (Dharmacon, Thermo Fisher Scientific, Waltham, MA) as a control. siRNAs (50 nM final concentration) were transiently transfected into either RSc cells [14–16] or OR6 cells [17,18] using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin (sh) RNA-encoding sequences against TSG101, Alix, Vps4B, or CHMP4b in lentiviral vector: TSG101i, 5′-GATCCCC GGAGGAAATGGATCGTGCCTT-CAAGAGAGGCACGATCCATTTCCTCCTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAAGGAGGAAATGGATCGTG-CCTCTCTTGAAGGCACGATCCATTTCCTCCGGG-3′ (antisense); Alixi, 5′-GATCCCC GGAGGTGTTCCCTGTCTTG-TTCAAGAGACAAGACAGGGAACACCTCCTTTTTGGAA-A-3′ (sense), 5′-AGCTTTTCCAAAAAGGAGGTGTTCCCTG-TCTTGTCTCTTGAACAAGACAGGGAACACCTCCGGG-3′ (antisense); Vps4Bi, 5′-GATCCCC GGAGAATCTGATGATC-CTGTTCAAGAGACAGGATCATCAGATTCTCCTTTTTG-GAAA-3′ (sense), 5′-AGCTTTTCCAAAAAGGAGAATCT-GATGATCCTGTCTCTTGAACAGGATCATCAGATTCTC-CGGG-3′ (antisense); CHMP4bi, 5′-GATCCCC GAGGAG-GACGACGACATGATTCAAGAGATCATGTCGTCGTCC-TCCTCTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAA-GAGGAGGACGACGACATGATCTCTTGAATCATGTCG-TCGTCCTCCTCGGG-3′ (antisense); Broxi, 5′-GATCCCCG-GATGACAGTACTAAACCCTTCAAGAGAGGGTTTAGTA-CTGTCATCCTTTTTGGAAA-3′ (sense), 5′-AGCTTTTC-CAAAAAGGATGACAGTACTAAACCCTCTCTTGAAGGG-TTTAGTACTGTCATCCGGG-3′ (antisense). The oligonucleotides above were annealed and subcloned into the *Bgl*II-*Hin*dIII site, downstream from an RNA polymerase III promoter of pSUPER [22], to generate pSUPER-TSG101i, pSUPER-Alixi, pSUPER-Vps4Bi, and pSUPER-CHMP4bi, respectively. To construct pLV-TSG101i, pLV-Alixi, pLV-Vps4Bi, and pLV-CHMP4bi, the *Bam*HI-*Sal*I fragments of the corresponding pSUPER plasmids were subcloned into the *Bam*HI-*Sal*I site of pRDI292 [23], an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistant marker allowing for the selection of transduced cells, respectively.

Lentiviral Vector Production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [24–26]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [24–26] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pRDI292 into 293FT cells with FuGene6 (Roche Diagnostics, Basel, Switzerland).

HCV Infection Experiments

The supernatants was collected from cell culture-generated HCV-JFH1 [13]-infected RSc cells [14–16] at 5 days post-

infection and stored at −80°C after filtering through a 0.45 μm filter (Kurabo, Osaka, Japan) until use. For infection experiments with HCV-JFH1 virus, RSc cells (1×10^5 cells/well) were plated onto 6-well plates and cultured for 24 hours (hrs). Then, we infected the cells with 50 μl (equivalent to a multiplicity of infection [MOI] of 0.1) of inoculum. The culture supernatants were collected and the levels of HCV Core were determined by enzyme-linked immunosorbent assay (ELISA) (Mitsubishi Kagaku Bio-Clinical Laboratories, Tokyo, Japan). Total RNA was isolated from the infected cellular lysates using RNeasy mini kit (Qiagen, Hilden, Germany) for quantitative RT-PCR analysis of intracellular HCV RNA. The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 hrs post-infection. The HCV infected cells were detected using anti-HCV Core antibody (CP-9 and CP-11). Intracellular HCV infectivity was determined by a focus-forming assay at 48 hrs post-inoculation of lysates by repeated freeze and thaw cycles (three times).

Quantitative RT-PCR Analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [17,18]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: TSG101, 5′-ATGGCGGTGTCGGAGAGCCA-3′ (forward), 5′-AACAGGTTTGAGATCTTTGT-3′ (reverse); Alix, 5′-ATGGCGACATTCATCTCGGT-3′ (forward), 5′-TACTGGGCCTGCTCTTCCCC-3′ (reverse); Vps4B, 5′-ATGTCATCCACTTCGCCCAA-3′ (forward), 5′-ATACTGCACAGCATGCTGAT-3′ (reverse); CHMP4b, 5′-ATGTCGGTGTTCGGGAAGCT-3′ (forward), 5′-ATCTCTTCCGTGTCCCGCAG-3′ (reverse); Brox, 5′-ATGACCCATTGGTTTCATAG-3′ (forward), 5′-CCTGGATGACCTCAAGTCAT-3′ (reverse); β-actin, 5′-TGACGGGGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAGCCGCGCTCGGT-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGTCTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse).

MTT Assay

Cells (5×10^3 cells/well) were plated onto 96-well plates and cultured for 24, 48 or 72 hrs, then, subjected to the colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromid (MTT) assay according to the manufacturer's instructions (Cell proliferation kit I, Roche). The absorbance was read using a microplate reader (Multiskan FC, Thermo Fisher Scientific) at 550 nm with a reference wavelength of 690 nm.

Renilla Luciferase (RL) Assay

OR6 cells (1.5×10^4 cells/well) [17,18] were plated onto 24-well plates and cultured for 24 hrs. The cells were transfected with siRNAs (50 nM) using Oligofectamine and incubated for 72 hrs, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

Western Blot Analysis

Cells (2×10^5 cells/well) were plated onto 6-well plates and cultured for 24 or 48 hrs. Cells were lysed in buffer containing 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% NP-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol (DTT) and 1 mM phenylmethylsulfonyl fluoride (PMSF). Supernatants from these lysates were subjected to SDS-polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-TSG101 antibody (BD Transduction Laboratories, San Jose, CA), anti-Alix antibody, anti-Vps4B antibody (Abnova, Taipei, Taiwan) (A302-078A; Bethyl Laboratories, Montgomery, TX), anti-CHMP4B antibody (sc-82557; Santa Cruz Biotechnology, Santa Cruz, CA), anti-HCV Core antibody, anti-β-actin antibody (Sigma), anti-Myc-Tag antibody, anti-FLAG antibody (M2; Sigma), anti-Chk2 antibody (DCS-273; MBL), anti-heat shock protein (HSP) 70 antibody (BD), Living Colors A.v. monoclonal antibody (JL-8; Clontech, Mountain View, CA), anti-HCV NS5A monoclonal antibody (no. 8926; a generous gift from A Takamizawa, The Research Foundation for Microbial Diseases of Osaka University, Japan), or anti-HCV NS5A polyclonal antibody (a generous gift from K Shimotohno, Chiba Institute of Technology, Chiba, Japan).

Immunoprecipitation Analysis

Cells were lysed in buffer containing 10 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% NP-40, 1 mM PMSF, and protease inhibitor cocktail containing 104 μM 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride, 80 nM aprotinin, 2.1 μM leupeptin, 3.6 μM bestatin, 1.5 μM pepstatin A, and 1.4 μM E-64 (Sigma). Lysates were pre-cleaned with 30 μl of protein-G-Sepharose (GE Healthcare Bio-Sciences). Pre-cleaned supernatants were incubated with 5 μl of Living Colors A.v. monoclonal antibody or anti-FLAG antibody at 4°C for 1 hr. Following absorption of the precipitates on 30 μl of protein-G-Sepharose resin for 1 hr, the resin was washed four times with 700 μl lysis buffer. Proteins were eluted by boiling the resin for 5 min in 1× Laemmli sample buffer. The proteins were then subjected to SDS-PAGE, followed by immunoblotting analysis using either anti-FLAG antibody, Living Colors A.v. monoclonal antibody or anti-HCV Core antibody.

Statistical Analysis

Statistical comparison of the infectivity of HCV in the culture supernatants between the knockdown cells and the control cells was performed using the Student's *t*-test. *P* values of less than 0.05 were considered statistically significant. All error bars indicate standard deviation.

Results

The ESCRT system is required for HCV production

To investigate the potential role(s) of the ESCRT system in the HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown the ESCRT components, including TSG101, Alix, Vps4B, or CHMP4b in HuH-7-derived RSc cured cells that cell-culture-generated HCVcc (HCV-JFH1, genotype 2a) [13] could infect and effectively replicate [14–16]. We used puromycin-resistant pooled cells 10 days after the lentiviral transduction in all experiments. Western blot and real-time LightCycler RT-PCR analyses for TSG101, Alix, Vps4B, or CHMP4b demonstrated a very effective knockdown of each ESCRT component in RSc cells transduced with lentiviral vectors expressing the corresponding shRNAs (Fig. 1A–E). Importantly, we noticed that the depletion of ESCRT components did not affect the levels of several cellular proteins, including HSP70, Chk2, and b-actin (Fig. 1A). To test the cell toxicity of each shRNA, we examined colorimetric MTT assay. In this context, we demonstrated that the shRNAs did not affect the cell viabilities (Fig. 1F). We next examined the levels of HCV Core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in the TSG101, Alix, Vps4B, or CHMP4b stable knockdown RSc cells 97 h after HCV-JFH1 infection at an MOI of 0.1. The results showed that the release of HCV Core into the culture supernatants