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ORIGINAL ARTICLE

Double-stranded RNA-induced interferon-beta and inflammatory cytokine production modulated by hepatitis C virus serine proteases derived from patients with hepatic diseases

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Abstract We previously demonstrated that hepatitis C virus (HCV) serine protease NS3-4A was unable to cleave TRIF (adaptor protein of Toll-like receptor 3), resulting in a lack of suppression of the TRIF-mediated pathway, whereas NS3-4A cleaved Cardif (adaptor protein of retinoic acid-inducible gene I or melanoma differentiation-associated gene-5), resulting in an interruption of the Cardif-mediated pathway in non-neoplastic human hepatocyte PH5CH8 cells. To elucidate these observations, we examined the cleavage potential of NS3-4A for TRIF in PH5CH8 cells, genome-length HCV RNA-replicating O cells, and HCV-infected cells, and we demonstrated that NS3-4A lacked the ability to cleave endogenous TRIF, regardless of HCV strains derived from patients with different stages of hepatic disease. Furthermore, we demonstrated that inflammatory cytokine production by NF-κB activation via the TRIF-mediated pathway also remained unsuppressed by NS3-4A. These results suggest that the inhibitory effects of NS3-4A on antiviral signaling pathways are limited to the Cardif-mediated pathway in human hepatocytes.

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Introduction

Hepatitis C virus (HCV) infection causes a number of liver diseases such as acute hepatitis, chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma [5, 24, 32, 33]. The progression of liver disease from chronic hepatitis to hepatocellular carcinoma by persistent HCV infection is a serious health problem [37]. In order to elucidate the relationship between the mechanism of persistent HCV infection and liver disease progression, it will be necessary to examine the virus life cycle and develop more effective anti-HCV reagents based on these observations. HCV is an enveloped positive single-stranded RNA (9.6 kb) virus belonging to the family *Flaviviridae* [20, 36]. The HCV genome encodes a large polyprotein precursor of approximately 3,000 amino acid (aa) residues, which is cleaved co- and post-translationally into at least ten proteins in the following order: core, envelope 1 (E1), E2, p7, non-structural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B. These cleavages are mediated by the host and a virally encoded serine protease located in the amino-terminal domain of NS3. The serine protease activity of NS3 requires NS4A, a protein that consists of 54 aa residues, to form a stable complex with NS3 [11, 12, 19].

During infection by RNA viruses such as HCV, double-stranded RNA (dsRNA) is produced by viral RNA replication in virus-infected cells, and dsRNA is in turn recognized by Toll-like receptor (TLR) 3, which is expressed on the cell surface or in endosome vesicles [3, 13]. Additionally, dsRNA is recognized by retinoic-acid-inducible gene I (RIG-I) and/or melanoma differentiation-associated gene 5 (MDA5), which are both localized in the cytoplasm [18, 40, 41]. The stimulation of TLR3 by extracellular dsRNA leads to the activation of two signaling pathways that bifurcate at TRIF [17, 34], i.e., interferon

(IFN)-β production is induced via activation of the TRIF/TRAF3/TBK1/IRF-3 pathway, and inflammatory cytokines such as IL-6 or IL-8 are produced via activation of the TRIF/TRAF6/TAK1/NF-κB pathway (see Fig. 5a). On the other hand, the stimulation of RIG-I or MDA5 by intracellular dsRNA may induce both IFN-β and inflammatory cytokine production by similar signaling pathways that bifurcate at Cardif (i.e., the Cardif/TRAF3/TBK1/IRF-3 pathway and the Cardif/TRAF6/TAK1/NF-κB pathway) [39]. IFN-β and the inflammatory cytokines are upregulated to induce an antiviral state in virus-infected cells, and then these production levels return to a steady state in virus-eliminated cells.

Several groups, including ours, have previously reported that the HCV serine protease NS3-4A inhibited intracellular dsRNA-induced IFN-β production via the cleavage of Cardif [6, 29, 30]. The findings of these reports have indicated that Cardif is a key molecule for establishing persistent HCV infection. On the other hand, we also previously demonstrated that NS3-4A (1B-1 and O strains of genotype 1b) was not able to inhibit extracellular dsRNA-induced IFN-β production due to a lack of ability to cleave TRIF [6]; however, in another previous report, it was demonstrated that NS3-4A (N strain of genotype 1b) was able to inhibit IFN-β production via the cleavage of TRIF [27]. These latter results, taken together, suggest that among HCV strains, NS3-4A possesses a range of ability to cleave TRIF. In the present study, NS3-4As derived from patients with different stages of liver disease were used to compare the potential of NS3-4As to inhibit IFN-β production and NF-κB activation via intracellular or extracellular dsRNA.

Materials and methods

Cell culture

Non-neoplastic human hepatocyte PH5CH8 cells susceptible to HCV infection and supportive of HCV replication were cultured as reported previously [16]. Genome-length HCV RNA-replicating O cells [14], their cured Oc cells [14] and other HuH-7-derived RSc cells [25] were cultured in Dulbecco's modified Eagle's medium (Invitrogen, Carsbad, CA, USA) supplemented with 10% fetal bovine serum.

Construction of expression vectors

Retroviral vectors pCX4bsr and pCX4pur [1], which contain the resistance gene for blasticidin and puromycin, respectively, were used to construct various expression vectors. pCX4pur/myc-TRIF(P367D), (P367E), (P367D/S368C), or (P367E/S368D) mutants were constructed using PCR mutagenesis with primers containing base alterations. pCX4pur/myc-TRIF [6] was used as the template for PCR mutagenesis. The NS3-4A expression vectors used in this study were constructed using oligonucleotides (Supplementary Table S1 in Electronic Supplementary Material) as described below. RNA was extracted using an ISOGEN extraction kit (Nippon Gene, Toyama, Japan) and serum from 13 HCV-infected patients: three healthy carriers (1B-3, 1B-4, and 1B-5 strains [15]), a patient with acute hepatitis (AH1 strain [21]), and seven patients with chronic hepatitis (CH1, CH3, CH4, CH5, CH6, CH7, and CH8 strains). In addition, serum was obtained from two patients with hepatocellular carcinoma (HCC1 and HCC2 strains [2]). Informed consent was obtained from each patient before the study. The DNA fragments, including the NS3-4A region, were amplified by RT-nested PCR using KOD-plus DNA polymerase (Toyobo, Osaka, Japan) and oligonucleotides for cDNA synthesis, first-round PCR, and second-round PCR (Supplementary Table S1). The obtained DNA fragments were subcloned into the *Xba*I site of pBR322MC [22], and the nucleotide sequences of the NS3-4A regions were determined. The oligonucleotides for the construction of the NS3-4A expression vector were designed from the nucleotide sequences of the NS3-4A regions (Supplementary Table S1). The DNA fragments encoding NS3-4A were amplified by PCR using KOD-plus DNA polymerase and the specifically designed oligonucleotides, and the amplified fragments were cloned into the *Eco*RI and *Not*I sites of pCX4bsr. The nucleotide sequences of the constructed expression vectors were confirmed by Big Dye termination cycle sequencing using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA).

Molecular evolutionary analysis

Molecular evolutionary trees were constructed from the aa sequences of the NS3-4A regions using the UPGMA method and the program GENETYX-MAC (Software Development, Tokyo, Japan).

JFH-1 infection experiments

The infection of RSc cells with JFH-1 was performed as described previously [25]. Briefly, 1.0×10^5 RSc cells were seeded onto 6-well plates 24 h before infection. Then, an inoculum of JFH-1 was added to the cells at a multiplicity of infection of 0.1. After 96 h of JFH-1 infection, cell lysates were prepared as described below.

Immunoprecipitation and Western blot analysis

The preparation of cell lysates from PH5CH8 cells stably expressing NS3-4A and two mutants (S1165A and W1528A) [6] was performed as described previously [31].

Cell lysates were subjected to immunoprecipitation using anti-TRIF antibody (Exalpha Biologicals, Maynard, MA, USA) or anti-Cardif antibody (Bethyl Laboratories, Montgomery, TX, USA). Bound proteins were collected from cell lysates using Protein G Sepharose (Amersham) and were subjected to immunoblot analysis. Anti-NS3 antibody (polyclonal R212; a generous gift from Dr. M. Kohara, Tokyo Metropolitan Institute of Medical Science), and anti-NS4A antibody (C14II3-3; also a generous gift from Dr. Kohara) were used to detect NS3 and NS4A proteins. Anti-myc antibody (PL14; Medical and Biological Laboratories, Nagoya, Japan), anti-EGFP antibody (JL-8; Clontech), and anti-β-actin antibody were used in this study as primary antibodies. Immunocomplexes were detected using a Renaissance enhanced chemiluminescence assay (Perkin-Elmer Life Sciences, Boston, MA, USA).

Luciferase reporter assay

For the dual luciferase assay, we used a firefly luciferase reporter vector, pIFN-β (−125)-Luc [4], containing the IFN-β gene promoter region (−125 to +19) and pNF-κB-Luc (Stratagene). The reporter assay was carried out as described previously [8]. Briefly, a total of 0.3×10^5 cells were seeded onto 24-well plates 24 h before transfection. Then, PH5CH8 cells were transfected with 0.1 μg pIFN-β (−125)-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.2 ng pRL-CMV (Promega, Madison, WI, USA), used as an internal control reporter, for the measurement of IFN-β promoter activity. For the measurement of NF-κB promoter activity, PH5CH8 cells were transfected with 0.01 μg pNF-κB-Luc, 0.2 μg NS3-4A expression pCX4bsr vectors (NS3-4A series), and 0.02 ng pRL-CMV. The cells were cultured for 48 h, and then a dual luciferase assay was performed according to the manufacturer's protocol (Promega). In some cases, the cells were cultured for 42 h, and then poly(I-C) (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA) was added to the medium for 6 h at 50 μg/ml (M-pIC) before the reporter assay. Three independent triplicate transfection experiments were conducted in order to verify the reproducibility of the results. Relative luciferase activity was normalized to the activity of *Renilla* luciferase (internal control). A manual Lumat LB 9501/16 luminometer (EG&G Berthold, Bad Wildbad, Germany) was used to detect luciferase activity.

RNA interference and real-time LightCycler PCR

siRNA duplexes targeting the coding regions of human TLR3 [31], TRIF (Dharmacon; catalog no. M-012833-00), and luciferase GL2 (Dharmacon), used as a control, were chemically synthesized. Using PH5CH8 cells with drastically decreased TLR3 or TRIF mRNA levels [6], NF-κB promoter activity was measured as described above, and dsRNA-induced inflammatory cytokine production levels were examined by using a primer set for IL-6 or IL-8 [38]. Total cellular RNA extraction and real-time LightCycler PCR were performed as described previously [6, 7].

Results

NS3-4A lacks the ability to cleave endogenous TRIF

We recently reported that NS3-4A serine protease (1B-1 strain of genotype 1b) was unable to cleave TRIF expressed in human PH5CH8 hepatocyte cells [6]. To account for this lack of cleavage ability, we examined the ability of NS3-4A to cleave TRIF mutants converted to a sequence similar to the consensus sequence required for cleavage ability by NS3-4A (Fig. 1a). The results obtained with

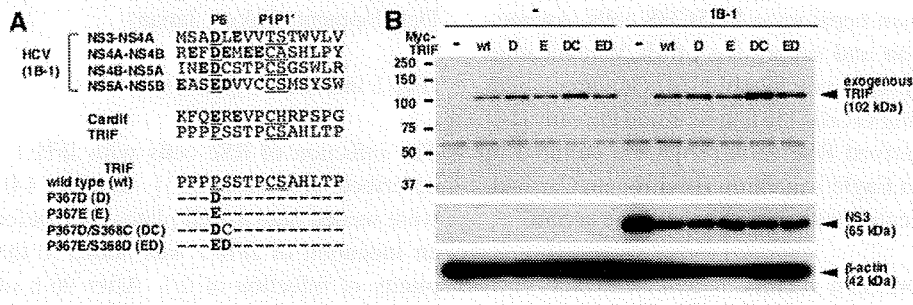


Fig. 1 NS3-4A does not cleave exogenous TRIF or its mutants at the P6 position. **a** The alignment of amino acid sequences surrounding the site cleaved *in trans* or *in cis* by NS3-4A. The consensus sequences required for cleavage by NS3-4A are underlined (P6, P1, and P1′ positions). The amino acids with a negative charge are indicated in boldface type. **b** TRIF mutants with a negative charge at the P6 position also remain uncleaved by NS3-4A. Wild-type TRIF and TRIF mutants (P367D, P367E, P367D/S368C, and P367E/S368D) are indicated as wt, D, E, DC, and ED, respectively. PH5CH8 cells stably expressing NS3-4A (1B-1) were transfected with the pCX4pur vector (as a control, –) or myc-TRIF expression vectors (wild-type strain or mutants). Production of myc-TRIF and NS3 in the cells was analyzed by immunoblot analysis using anti-myc and anti-NS3 antibody, respectively. PH5CH8 cells infected with retrovirus pCX4bsr were used as a control (–). β-actin was used as a control for the amount of protein loaded per lane

PH5CH8 cells revealed that NS3-4A (1B-1 strain) was still unable to cleave the TRIF mutants possessing D or E at the P6 position, even though an acidic aa (D or E) is known to be important for cleavage by NS3-4A [23] (Fig. 1b). Although we demonstrated that exogenously expressed Cardif, but not TRIF, was cleaved by NS3-4A (1B-1 or O strain) [6], no studies had determined whether endogenous Cardif or TRIF can be cleaved by NS3-4A. To clarify these issues, we selected anti-Cardif and anti-TRIF antibodies, and we immunoprecipitated lysates from PH5CH8 cells [in which NS3-4A (1B-1 or O strain) was overexpressed] and lysates from genome-length HCV RNA-replicating O cells [14]. We then performed immunoblot analyses using anti-Cardif, anti-TRIF, or NS3 antibodies. The results revealed that endogenous TRIF was also not cleaved by the NS3-4A expressed in PH5CH8 and O cells, whereas endogenous Cardif (75 kDa) was efficiently cleaved to the expected size (70 kDa) in PH5CH8 and O cells (Fig. 2a). On the other hand, we observed that NS3 interacted with TRIF, but not with Cardif, in both PH5CH8 and O cells (Fig. 2a), as had also been observed previously by another group [10]. In addition, we demonstrated that endogenous Cardif was cleaved by the NS3-4A/W1528A mutant, which lacks RNA helicase activity, but not by the NS3-4A/S1165A mutant, which lacks the serine protease activity (Fig. 2b). Furthermore, we examined whether or not endogenous Cardif and TRIF are cleaved in JFH-1-infected RSc cells. The results revealed that endogenous TRIF was also not cleaved in JFH-1-infected RSc cells, whereas endogenous Cardif was efficiently cleaved in these cells (Fig. 2c). In addition, we also observed that NS3 interacted weakly with TRIF, but not with Cardif, in these cells (Fig. 2c). We therefore concluded that endogenous TRIF is not cleaved by NS3-4A derived from at least the 1B-1 (genotype 1b), O (genotype 1b) or JFH-1 (genotype 2a) strain.

None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented extracellular dsRNA-induced IFN-β transcription via the TRIF-mediated pathway

Although we demonstrated that NS3-4As derived from healthy carriers (1B-1 and O) was unable to suppress IFN-β production induced by the TRIF-mediated pathway [6], there is still no evidence that NS3-4As derived from patients with various hepatic disease diagnoses carry out such suppression. To obtain more evidence, we first amplified NS3-4A-encoding regions by RT-PCR using sera derived from five HCV-positive healthy carriers (including strains 1B-1 and O), one patient with acute hepatitis, seven patients with chronic hepatitis, and two patients with hepatocellular carcinoma; using these samples, we next constructed 15 types of NS3-4A expression vector. Although we observed that all of the NS3-4As expressed in PH5CH8 cells were processed into NS3 and NS4A by an intramolecular reaction, there were some size differences (60–65 kDa) of NS3 and NS4A (Fig. 3a). These size differences may be related to the aa sequence variation, as described below. Sequence analysis of these NS3-4A-encoding regions revealed that the aa sequences involved

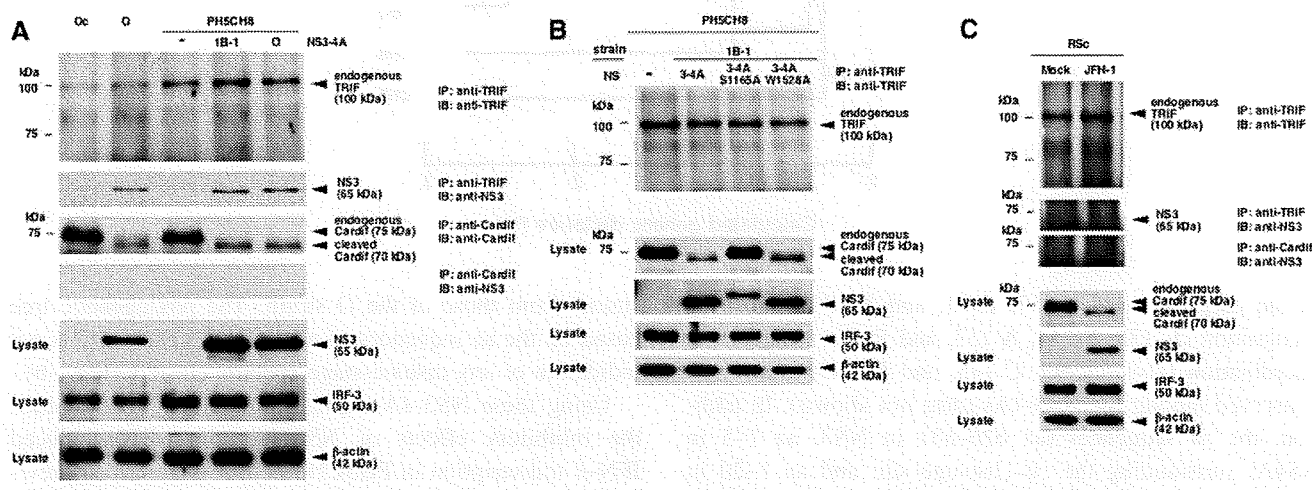


Fig. 2 NS3-4A lack the ability to cleave endogenous TRIF, but not Cardif. **a** Endogenous Cardif, but not TRIF is cleaved by NS3-4As from 1B-1 and O strains. Cell lysates were prepared and subjected to immunoprecipitation using anti-TRIF or anti-Cardif antibody. Bound proteins were collected from cell lysates using Protein G Sepharose and were subjected to immunoblot analysis using anti-TRIF, anti-Cardif, or anti-NS3 antibody. NS3, IRF3, and β-actin in the cell lysates were detected by anti-NS3, anti-IRF3, and anti-β-actin antibody, respectively. **b** Endogenous Cardif is cleaved by the serine protease activity of NS3-4A. The cell lysates were prepared and subjected to immunoprecipitation and followed by immunoblot analysis as described in **a**. **c** Endogenous Cardif, but not TRIF, is cleaved in JFH-1-infected RSc cells. The cell lysates were prepared and subjected to immunoprecipitation followed by immunoblot analysis as described in **a**

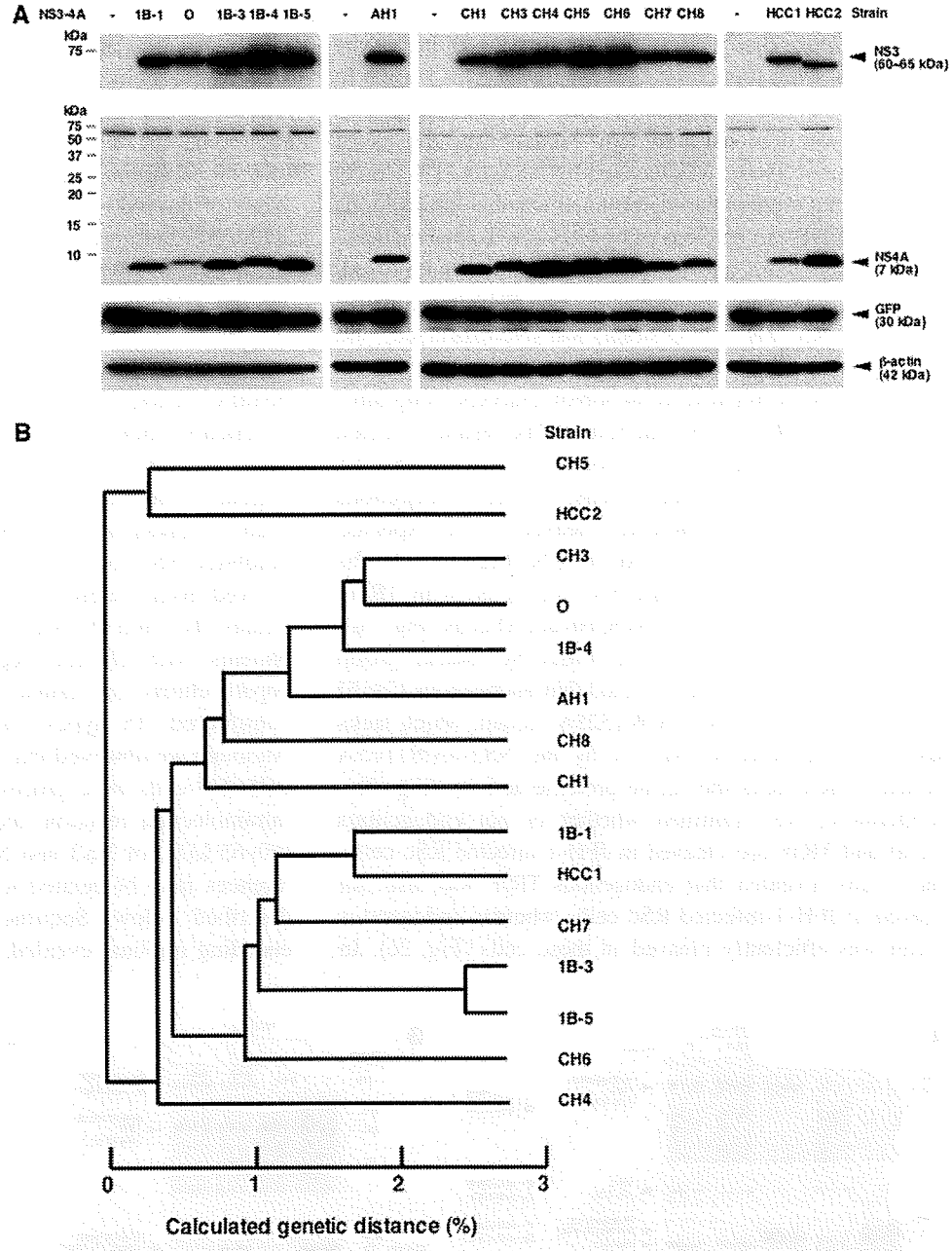


Fig. 3 Characterization of NS3-4As derived from patients with different hepatic disease diagnoses. **a** Expression of NS3 and NS4A in PH5CH8 cells. PH5CH8 cells were transfected with the expression vectors of NS3-4As derived from 15 different HCV strains and pEGFP-C1 (internal control reporter). Production 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.

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

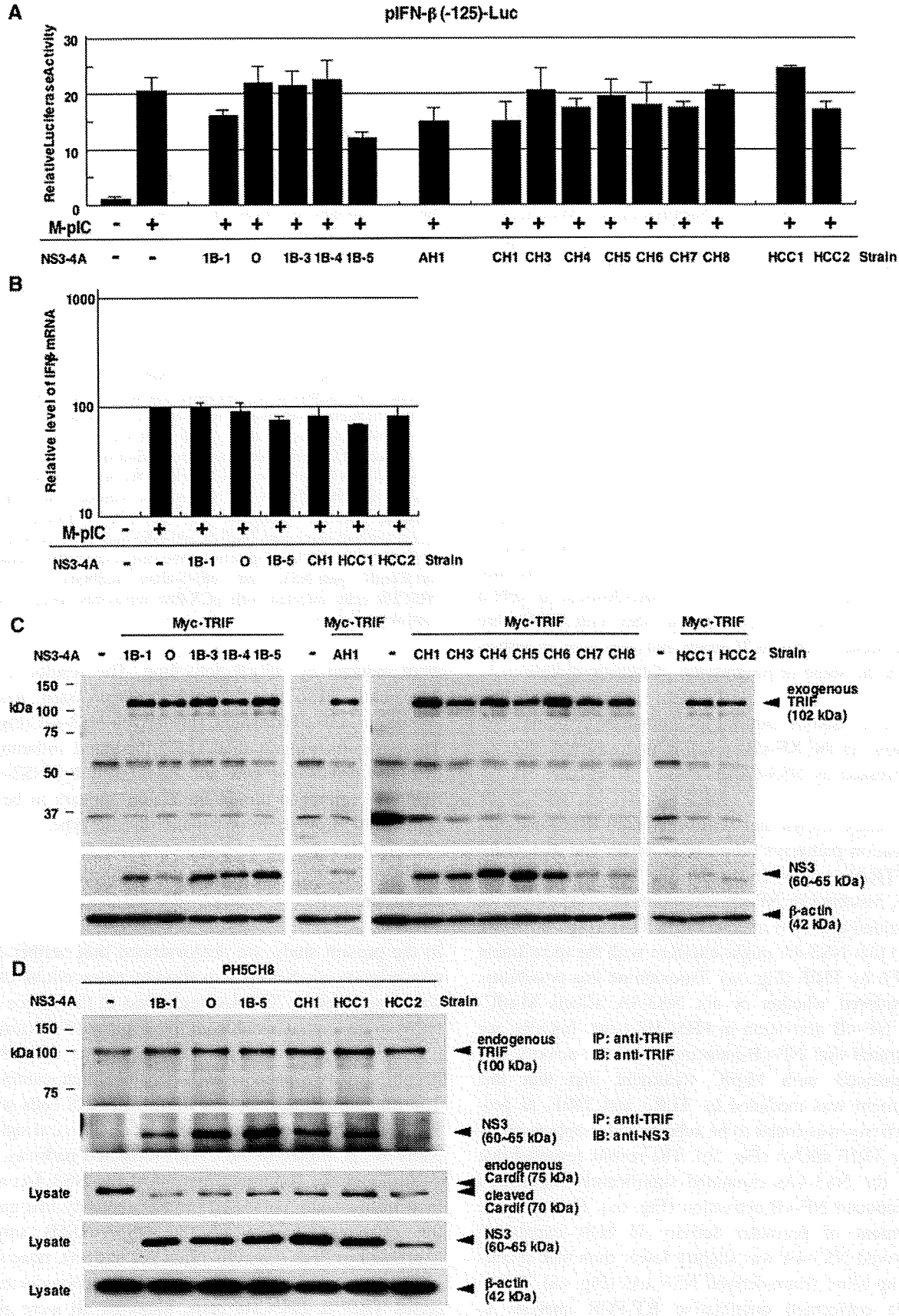
It was already known that TLR3-mediated IRF-3 and NF-κB activation pathways bifurcate at TRIF, and that TLR3 recruits TRAF6 via TRIF through the TRAF6-binding site of TRIF, resulting in NF-κB activation [17, 34]. Since we demonstrated that NS3 interacts with TRIF (Fig. 2a, c), we expected that NS3-4A 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.

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, –)

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-


A
pIFN-β(-125)-Luc
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M-pIC - + + + + + + + + + + + + + + + +
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B
Relative level of IFNβ mRNA
1000
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10
M-pIC - + + + + + + +
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Myc-TRIF
Myc-TRIF
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Myc-TRIF
NS3-4A - 1B-1 O 1B-3 1B-4 1B-5 - AH1 - CH1 CH3 CH4 CH5 CH6 CH7 CH8 - HCC1 HCC2 Strain
kDa 150 100 75 50 37
exogenous TRIF (102 kDa)
NS3 (60~65 kDa)
β-actin (42 kDa)
D
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NS3-4A - 1B-1 O 1B-5 CH1 HCC1 HCC2 Strain
kDa 150 100 75
endogenous TRIF (100 kDa)
IP: anti-TRIF
IB: anti-TRIF
NS3 (60~65 kDa)
IP: anti-TRIF
IB: anti-NS3
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Lysate
NS3 (60~65 kDa)
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β-actin (42 kDa)

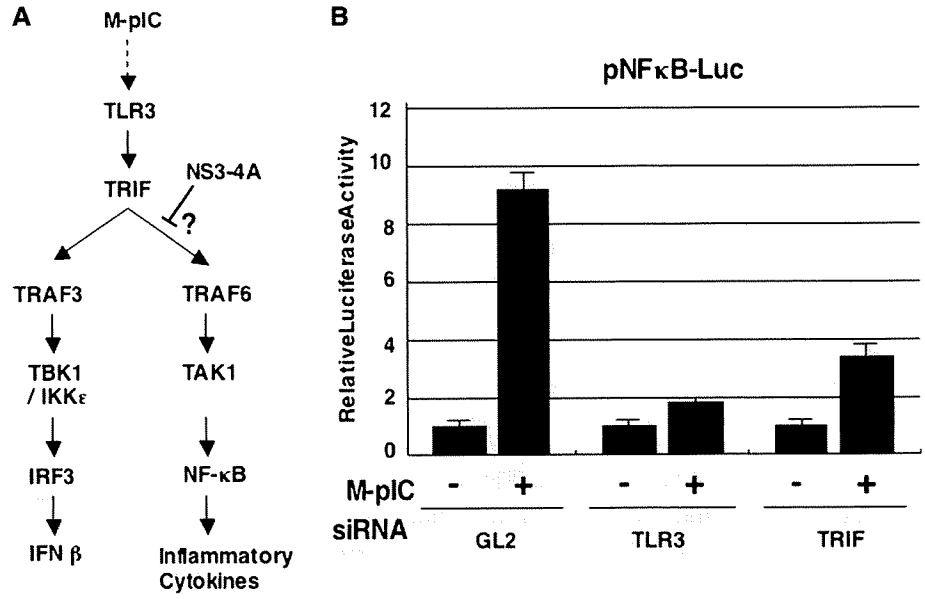

Fig. 5 TLR3-mediated activation of IRF-3 and NF-κB bifurcate at TRIF. **a** Model of TLR3-mediated signaling pathways. **b** Dual luciferase reporter assay of the NF-κB-inducing promoter using siRNA-transfected PH5CH8 cells treated with M-pIC

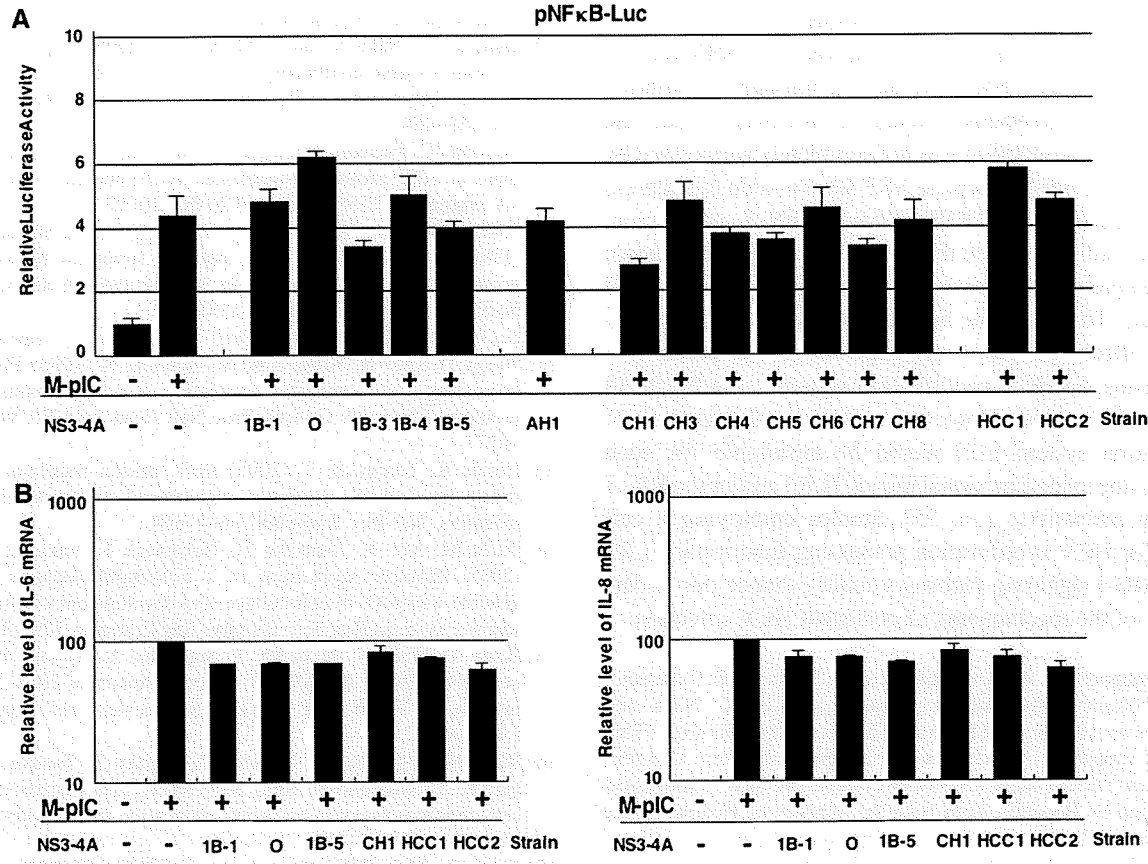

Fig. 6 None of the NS3-4As derived from patients with different hepatic disease diagnoses prevented M-pIC-induced NF-κB activation. **a** Effect of 15 NS3-4As on the activity of NF-κB-inducing promoter. PH5CH8 cells transiently expressing NS3-4As from various HCV strains were subjected to M-pIC treatment. 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

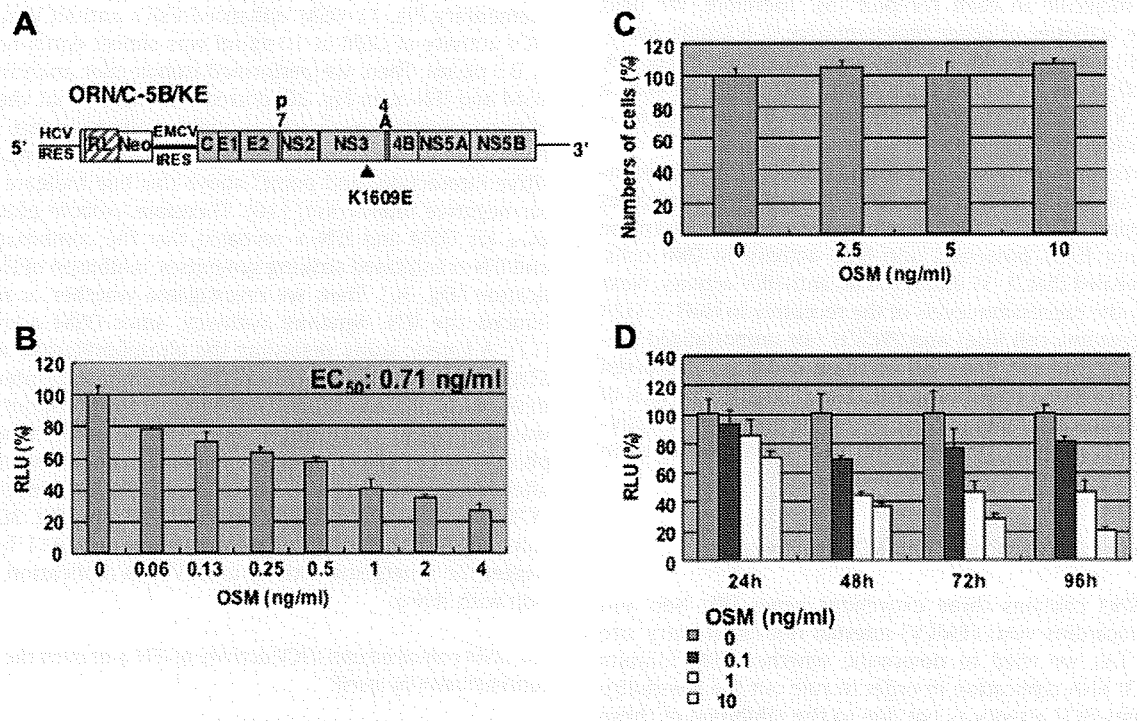

Fig. 1. Anti-HCV activity of OSM in HCV RNA replicating OR6 cells. (A) Schematic gene organization of the genome-length HCV RNA replicating in OR6 cells. The position of an adaptive mutation, K1609E, is indicated by a black triangle. (B) OR6 cells were treated with OSM for 72 h and subjected to RL assay. 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.

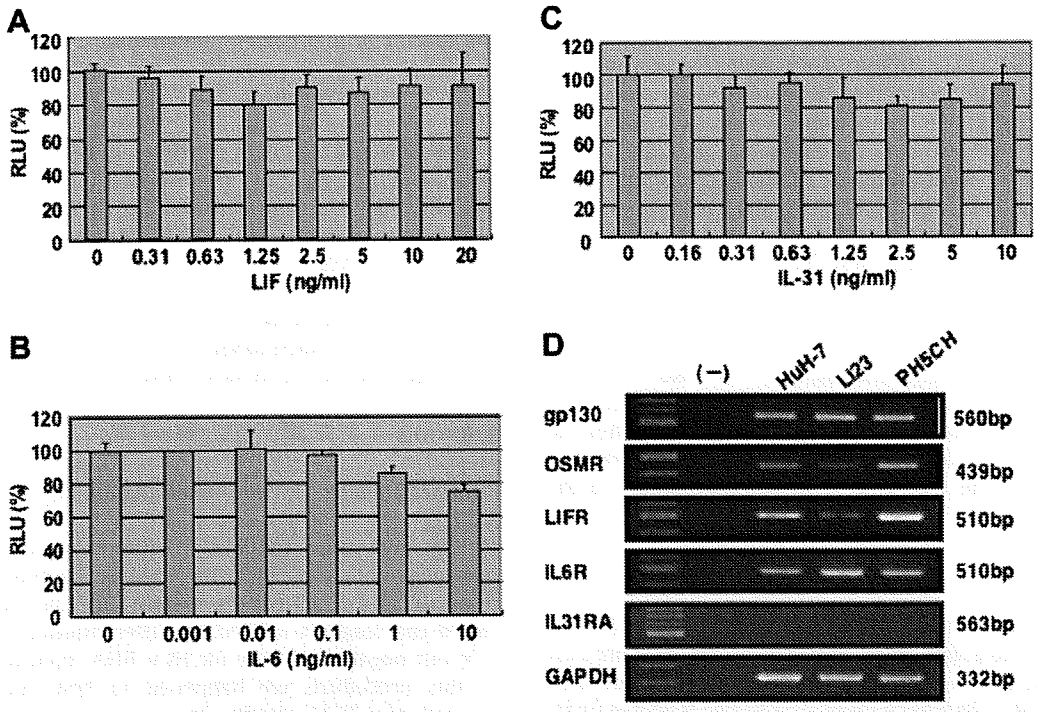

Fig. 2. The activities of LIF, IL-6 and IL-31 on HCV RNA replication. OR6 cells were treated with LIF (A), IL-6 (B) and IL-31 (C) for 72 h and subjected to RL assay. (D) RNAs from hepatocytes (HuH-7, Li23 and PH5CH) were subjected to RT-PCR with specific primer pairs to gp130, OSMR, LIFR, IL6R, IL31RA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

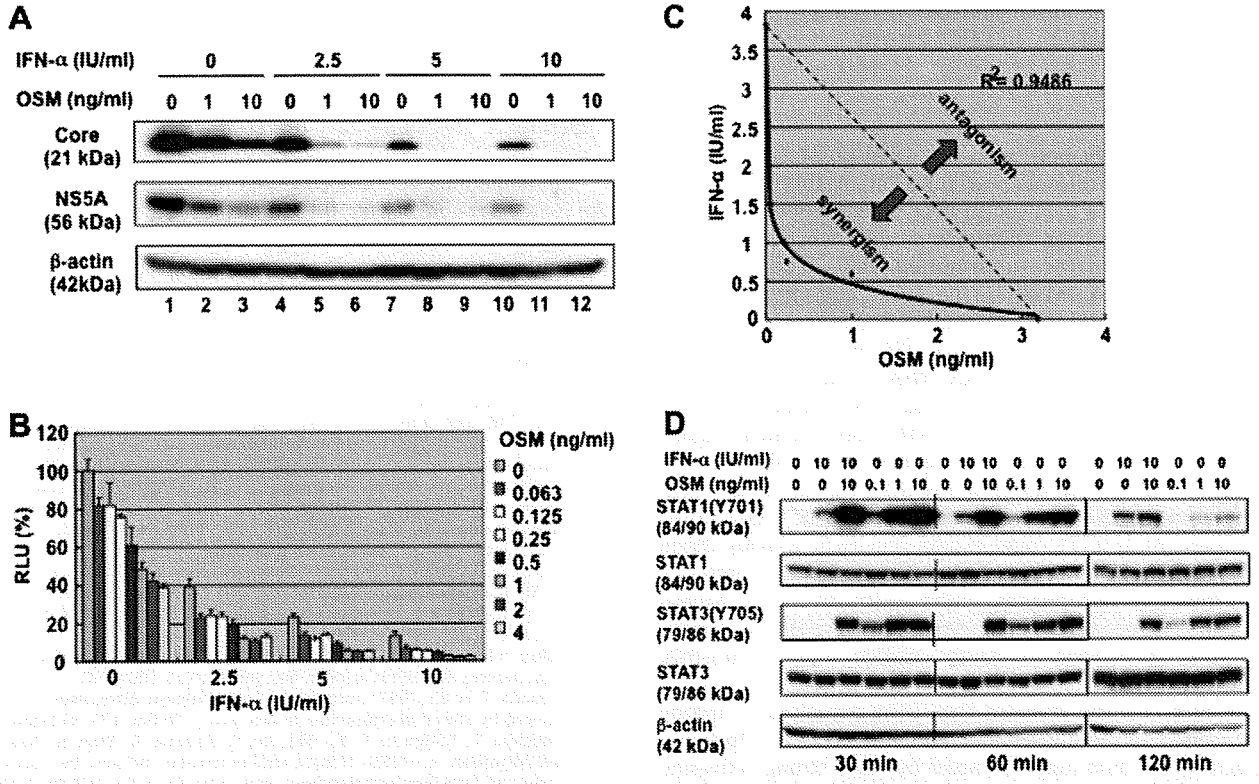

Fig. 3. Anti-HCV activity of OSM in combination with IFN-α. (A) HCV-O/RLGE-replicating OR6c cells were treated with OSM in combination with IFN-α for 96 h and subjected to Western blot analysis using anti-core, anti-NS5A and anti-β-actin antibodies. (B) OR6 cells were treated with OSM in combination 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

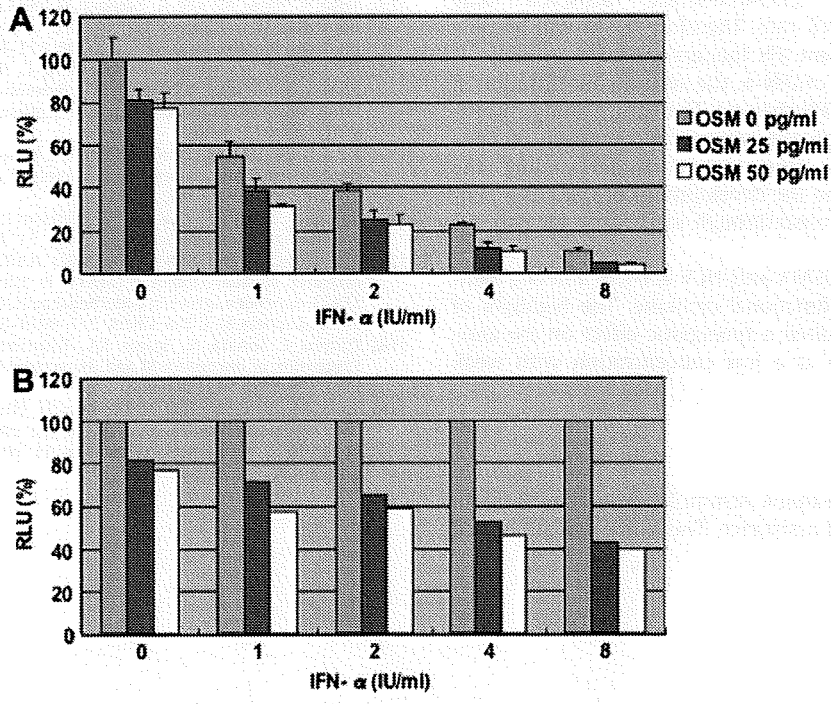

Fig. 4. OR6 cells were treated with OSM and IFN-α for 72 h and subjected to RL assay (A). Relative RL activity was adjusted when the RL activities of the cells treated with only IFN-α were assigned as 100% (B).

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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Review Article

Animal models for hepatitis C and related liver disease

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Persistent infection with hepatitis C virus (HCV) is a major risk toward development of hepatocellular carcinoma (HCC). The elucidation of pathogenesis of HCV-associated liver disease is hampered by the absence of appropriate animal models: there has been no animal model for HCV infection/pathogenesis except for the chimpanzee. In contrast, a number of transgenic mouse lines carrying the cDNA of the HCV genome have been established and evaluated in the study of HCV pathogenesis. The studies using transgenic mouse models, in which the HCV proteins such as the core protein are expressed, indicate the direct pathogenicity of HCV, including oncogenic activities. HCV transgenic mouse models also show a close relationship between HCV and some hepatic and extrahepatic manifestations such as hepatic steatosis, insulin resistance or Sjögren's syndrome. A crucial role of hepatic steatosis and insulin resistance in the pathogenesis of liver disease in HCV infection has been demonstrated, implying hepatitis C to be a metabolic disease. Besides the data connecting liver fibrosis progression and the disturbance in lipid and glucose metabolisms in hepatitis C patients, a series of evidence was found showing the association between these two conditions and HCV infection, chiefly using transgenic mouse carrying the HCV genome. Furthermore, the persistent activation of peroxisome proliferator-activated receptor (PPAR)-α has recently been found, yielding dramatic changes in the lipid metabolism and oxidative stress overproduction in cooperation with the mitochondrial dysfunction. These results would provide a clue for further understanding of the role of lipid metabolism in pathogenesis of hepatitis C including liver injury and hepatocarcinogenesis.

Key words: core protein, hepatitis C, hepatocellular carcinoma, insulin resistance, steatosis, transgenic mouse.

INTRODUCTION

HEPATITIS C VIRUS (HCV) infection frequently evolves into a persistent state, leading to the development of chronic hepatitis, cirrhosis and, eventually, hepatocellular carcinoma (HCC). For understanding of the mechanism of entry into hepatocytes, replication and the pathogenesis of HCV, an *in vitro* replication system or animal models for HCV infection/pathogenesis have been eagerly awaited. An *in vitro* HCV replication system was not established until the development of a subgenomic, non-structural region HCV replicon system or an infectious genotype 2a HCV clone, JFH-1.[1] There has been no animal model for HCV infection/pathogenesis except for the chimpanzee.[2] Recently, however, several small animal models for HCV infection have been evaluated, including *Tupaia*[3] and genetically engineered mice that are chimeric for human hepatocytes.[4] On the other hand, a number of transgenic mouse lines carrying the cDNA of HCV genome have been established and evaluated in the study of HCV pathogenesis, as described hereafter. These mice, including those that are transgenic for the core gene of HCV, show the features resembling those of chronic hepatitis C patients, such as hepatic steatosis, insulin resistance and HCC. These animal models provide us a molecular understanding of the pathogenesis of HCV infection and a perspective for the future development of treatment and prophylaxis of liver disease occurring in HCV infection.

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THE CHIMPANZEE MODEL

AS EARLY AS the discovery of the cDNA clone of HCV, or even before that, the chimpanzee has been known to be susceptible to HCV (or the non-A, non-B

hepatitis agent), and has long been used as a sole animal model for HCV infection.[2] However, due to ethical reasons and vast costs, the use of this animal for HCV research is limited: the data on this animal model were obtained from the studies chiefly conducted in the USA. The serum samples from hepatitis C patients were inoculated to chimpanzees, and the natural course was evaluated in biochemical, virological or histological methods. These studies demonstrated that the course of HCV infection in this animal is similar to that in human beings, warranting the chimpanzee to be a good animal model for HCV infection, albeit HCC being a rare occurrence in chimpanzees.

In 1997, potential infectious HCV clones, which were produced by several study groups, were evaluated for *in vivo* infectivity using chimpanzees. The chimpanzees were also used for the evaluation of a role of cellular immunity in acute HCV infection: intrahepatic $CD4^+$ or $CD8^+$ T-cell response was found to play a crucial role in the eradication of HCV from the liver. Recently, this animal is also used for the evaluation of candidates for HCV vaccines and the assessment of *in vivo* infectivity of JFH-1 HCV viral clone, which shows a robust replication in human HCC-derived HuH-7 cells.[1] Immunization with virus-like particles of chimpanzees induced an HCV-specific immune response of $CD4^+$ or $CD8^+$ T cells, thereby suppressing the development of high viral loads in chimpanzees that were challenged with HCV.[5] Also, inoculation of the non-structural proteins of HCV using recombinant adenovirus vector induced HCV-specific immune T-cell response, leading to a significant suppression of replication of genotype 1a HCV that was challenged after immunization.[6]

In general, the liver lesions observed in HCV-inoculated chimpanzees are milder than those in human chronic hepatitis C patients, for example, cirrhosis or HCC rarely develops, but the morphological changes and inflammatory responses are similar to those in humans.[2] Therefore, the studies using chimpanzees are indispensable now and in the future for the analyses of viral replication, pathogenesis of liver disease and the evaluation of candidates for HCV vaccines.

THE SMALL PRIMATES MODEL

TUPAIA (*TUPAIA BELANGERI chinensis*), a small primate resembling the squirrel, has been reported to be susceptible to hepatitis B virus (HBV) infection in 1996,[3] and was used for the study of HCV infection.[7] However, only a quarter of inoculated individuals contracted HCV infection, and developed only a transient or intermittent viremia with low viral loads. Another study group reported on the usefulness of how a primary culture of hepatocytes from the liver of Tupaia can be infected with serum- or plasma-derived HCV from infected humans, as measured by de novo synthesis of HCV RNA, analysis of viral quasispecies evolution, and detection of viral proteins.[8,9]

While the development of liver disease (a cirrhosis-like lesion) in HCV-infected Tupaia was presented at scientific meetings, a scientific paper describing it has not appeared yet. In conclusion, the value of Tupaia in HCV research is limited, but it may be utilized for the analysis of viral entry or replication when HCV particles other than JFH-1 are used for the study.

HCV

THE DEVELOPMENT OF transgenic mouse technology was a great step forward in biotechnology in that this technology provides opportunities to examine *in vivo* an exceptionally wide variety of biological questions that were previously examined only *in vitro*. The selective addition of defined genes to the genome of a living animal is useful for investigating the consequences of expression of dominant genes, and thus a number of exogenous genes including oncogenes and humoral factor genes have been introduced into mouse eggs. Viral genes have also been transferred to define the complex cascades of events that can be triggered *in vivo* in response to the expression of a viral protein.

Hepatitis C virus is an enveloped RNA virus of the *Flavivirus* family, in which a positive-sense, single-stranded RNA genome of approximately 9600 nucleotides (nt) is contained within the nucleocapsid.[10] The genome consists of a large translational open reading frame (ORF) encoding a polyprotein of approximately 3010 amino acids (aa) (Fig. 1). The ORF is flanked by highly conserved untranslated regions (UTR) at both the 5′ and 3′ termini. The complete 5′ UTR consists of 341 nt and acts as an internal ribosomal entry site. This feature leads to the translation of the RNA genome using a cap-independent mechanism, rather than ribosome scanning from the 5′ end of a capped molecule.

The polyprotein is processed by both the cellular and viral proteases to generate the viral gene products, which are subdivided into the structural and non-structural proteins. The structural proteins, which are encoded by the NH_2-terminal quarter of the genome, include the

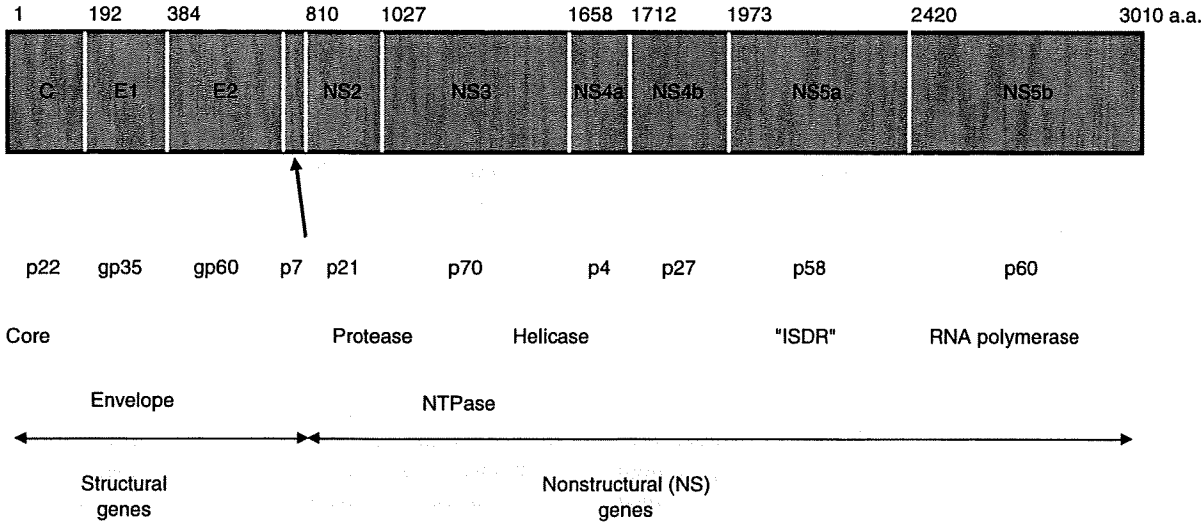

Figure 1 The structure of hepatitis C virus (HCV) genome. The HCV genome RNA encodes a polyprotein of 3010 amino acids (a.a.), which is processed to structural and non-structural proteins by the cellular or viral proteases. ISDR, interferon sensitivity-determining region.

core protein and the envelope proteins, E1 and E2. The E2 has an alternative form, E2-p7, though it is not clear whether or not the p7 composes the viral particle. The NS2, NS3, NS4A, NS4B, NS5A and NS5B are the non-structural proteins that are coded in the remaining portion of the polyprotein. These include serine protease (NS3/4A), NTPase/helicase (NS3) and RNA-dependent RNA polymerase (NS5B).

The core protein of HCV occupies residues 1–191 of the precursor polyprotein and is cleaved between the core and E1 protein by host signal peptidase. The C-terminal membrane anchor of the core protein is further processed by host signal peptide peptidase.[11] The mature core protein is estimated to consist of 177–179 amino acids and shares high homology among HCV genotypes. The HCV core protein possesses the hydrophilic N-terminal region "domain 1" (residues 1–117) followed by a hydrophobic region called "domain 2", which is located from residue 118–170. The domain 1 is rich in basic residues, and is implicated in RNA-binding and homo-oligomerization. The amphipathic helices I and II spanning from residue 119–136 and residue 148–164, respectively, in domain 2 are involved in the association of HCV core protein with lipid.[12] In addition, the region spanning from residue 112–152 is associated with membranes of the endoplasmic reticulum and mitochondria.[13] The core protein is also localized into the nucleus[14,15] and binds to the nuclear proteasome activator PA28γ/REGγ, resulting in PA28γ-dependent degradation of the core protein.[16]

A recent report suggests that ubiquitination and adenosine triphosphate (ATP) are not required for PA28γ-dependent proteasome activity.[17] HCV core protein is also known to be ubiquitinated by E3 ligase E6AP and degraded in the ubiquitin/ATP-dependent pathway.[14] Thus, the HCV core protein is degraded in at least two different ways. To further assess the pathological significance of the interaction of core protein with PA28γ, Core-Tg/PA28γ-knockout mice have been generated and analyzed as described below (section 9).[15]

POSSIBLE ROLE OF HCV IN HEPATOCARCINOGENESIS

THE MECHANISM UNDERLYING hepatocarcinogenesis in HCV infection is not fully understood yet, despite the fact that nearly 80% of patients with HCC in Japan are persistently infected with HCV.[18–20] HCV infection is also common in patients with HCC in other countries albeit to a lesser extent. These lines of evidence prompted us to seek to determine the role of HCV in hepatocarcinogenesis. Inflammation induced by HCV should be considered, of course, in a study on the hepatocarcinogenesis in hepatitis viral infection: necrosis of hepatocytes due to chronic inflammation followed by regeneration enhances genetic aberrations in host cells,

the accumulation of which culminates in HCC. This theory presupposes an indirect involvement of hepatitis viruses in HCC through hepatic inflammation. However, this context leaves us with a serious question: can inflammation alone result in the development of HCC in such a high incidence (90% in 15 years) or the multicentric nature of HCV infection?

The other role of HCV would have to be weighed against a rare occurrence of HCC in patients with autoimmune hepatitis in which severe inflammation in the liver persists indefinitely, even after the development of cirrhosis. These backgrounds and reasonings lead to a possible activity of viral proteins for inducing neoplasia. This possibility has been evaluated by introducing genes of HCV into hepatocytes in culture with little success. One of the difficulties in using cultured cells is the carcinogenic capacity of HCV, if any, which would be weak and would take a long time to manifest itself. Actually, it takes 30–40 years for HCC to develop in individuals infected with HCV. On the basis of these viewpoints, we started to investigate carcinogenesis in chronic hepatitis C, *in vivo*, by transgenic mouse technology.

TRANSGENIC MOUSE LINES CARRYING THE HCV GENOME

AS DESCRIBED ABOVE, the HCV proteins have been characterized chiefly using *in vitro* translation or cultured cells. Little is known, however, about the role of HCV or its proteins in the pathogenesis of hepatitis and subsequent liver diseases, cirrhosis and HCC. One of the major issues regarding the pathogenesis of HCV-associated liver lesions is whether the HCV proteins have direct effects on pathological phenotypes. Although several strategies have been used to characterize the hepatitis C viral proteins, the relationship between the protein expression and disease phenotype has not been clarified. For this purpose, several lines of mice have been established which were transgenic for the HCV cDNA (Table 1). They include the ones carrying the entire coding region of the HCV genome,[33] the core region only,[21,29] the envelope region only,[30,31] the core and envelope regions[33,34] and the core to NS2 regions.[41] Although detection of mRNA from the NS regions of the HCV cDNA has been reported,[33,37] the detection of HCV NS proteins in the transgenic mouse liver have not been successful. The reason for this failure in detecting NS proteins is unclear, but the expression of the NS enzymes may be harmful to mouse development and may allow the establishment of only low-expression mice.

In terms of expression system, two different ways have been applied; transient and constitutive expression systems. One transgenic mouse line has been reported which expresses the HCV genes using a transient expression system. Wakita *et al.* utilized the Cre/*lox*P system, by which a gene under silent can be switched on by the introduction of Cre recombinase. They established a transgenic mouse line that had the core, envelopes and NS2 genes of HCV in a silent state. After the injection of the recombinant adenovirus that had *Cre* recombinase in the mice, the HCV genes expressed transiently.[41] These mice developed acute hepatitis, which was blocked by the administration of anti-CD4 and CD8 antibodies. This mouse system would provide a good animal model for acute hepatitis C and be useful for the study of immunological aspects of hepatitis. The possibility, however, that the greatly overexpressed HCV proteins had caused the death of hepatocytes and provoked the immune response thereafter still remains.

We have engineered transgenic mouse lines carrying the HCV genome by introducing the genes from the cDNA of the HCV genome of genotype 1b.[21,22] Established are three different kinds of transgenic mouse lines, which carry the core gene, envelope genes or non-structural genes, respectively, under the same transcriptional regulatory element. Among these mouse lines, only the transgenic mice carrying the core gene developed HCC in two independent lineages (Fig. 2).[22] The envelope gene transgenic mice do not develop HCC, despite high expression levels of both E1 and E2 proteins,[31,32] and the transgenic mice carrying the entire non-structural genes have developed no HCC.

The core gene transgenic mice express the core protein of an expected size, and the level of the core protein in the liver is similar to that in chronic hepatitis C patients. Early in life, these mice develop hepatic steatosis, which is one of the histological characteristics of chronic hepatitis C, along with lymphoid follicle formation and bile duct damage.[43] Thus, the core gene transgenic mouse model well reproduces the feature of chronic hepatitis C. Of note, any pictures of significant inflammation are not observed in the liver of this animal model. Late in life, these transgenic mice develop HCC. Notably, the development of steatosis and HCC has been reproduced by other HCV transgenic mouse lines, which harbor the entire HCV genome or structural genes including the core gene.[29,33,34] These outcomes indicate that the core protein per se of HCV has an oncogenic potential when expressed *in vivo*.

Table 1 Transgenic mouse lines constitutively expressing hepatitis C virus proteins

HCV gene	Genotype	Promoter	Protein expression	Phenotypes	References
Core	1b	HBV	Similar to patients	Steatosis, HCC, insulin resistance, oxidative stress	Moriya 1997[21] & 1998[22] Tsutsumi 2002[23] & 2003[24] Moriishi 2003[16] & 2007[25] Shintani 2004[26] Miyamoto 2007[15] Tanaka 2008[27,28]
Core	1b	EF-1a	Similar to patients	Steatosis, adenoma, HCC, oxidative stress	Machida 2006[29]
Core, E2 truncated	1b	MUP	(–)	None	Pasquinelli 1998[30]
E1-E2	1b	HBV	Abundant	None in the liver	Koike 1995,[31] Koike 1997[32]
Core-E1-E2	1b	Albumin	Similar to patients	Steatosis, HCC, oxidative stress	Lerat 2003[33]
Core-E1-E2	1a	CMV	Similar to patients	Steatosis, HCC	Naas 2005[34]
Core or structural proteins	1b	Alb	Low	Larger tumor development with DEN treatment	Kamegaya 2005[35]
Structural proteins	1b	MUP	Low	None	Kawamura 1998[36]
Structural proteins	1b	MHC	Low in the liver	Hepatitis	Honda 1999[37]
Entire polyprotein	1b	Albumin	Only mRNA detectable	Steatosis, HCC	Lerat 2003[33]
Entire polyprotein	1a	A1-antitrypsin		Steatosis, intrahepatic T cell recruitment	Alonzi 2004[38]
NS3/4A	1a	MUP		None (modulation of immunity)	Frelin 2006[39]
NS5A	1a	apoE		None (resistance to TNF)	Majumder 2002[40]

HBV, hepatitis B virus; EF, elongation factor; MUP, major urinary protein; Alb, albumin; CMV, cytomegalovirus; MHC, major histocompatibility complex; AT, anti-trypsin; apo E, apolipoprotein E.