The resulting material was fractionated as an EtOAc- and water-soluble fraction. The water-soluble fraction was dried up and solubilized in MeOH. The EtOAc- and the MeOH-soluble fractions were used for screening. All samples were dried and then solubilized in dimethyl sulfoxide (DMSO) before testing.

Cell Lines and Virus

The following Huh-7-derived cell lines used in this study were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum and 0.5 mg/ml G418. The Lunet/Con1 LUN Sb #26 cell line, which harbors the subgenomic replicon RNA of the Con1 strain (genotype 1b), was kindly provided by Ralf Bartenschlager [26]. Huh7/ORN3-5B #24 cell line, which harbors the subgenomic replicon RNA of the O strain (genotype 1b) was reported previously [54] and used for screening in this study (Table 1). HCV replicon cell line derived from genotype 2a strain JFH1 was described previously [55]. The surviving cells were infected with the JFH-1 virus at a multiplicity of infection (moi) of 0.05. The viral RNA derived from the plasmid pJFH1 was transcribed and introduced into Huh7OK1 cells according to the method of Wakita et al. [56]. The infectivity of the JFH1 strain was determined by a focus-forming assay [56].

Quantitative Reverse-transcription PCR (qRT-PCR) and Estimation of Core Protein

The estimation of viral RNA genome was carried out by the method described previously [57] with slight modification. Total RNAs were prepared from cells and culture supernatants by using an RNeasy mini kit (QIAGEN, Tokyo, Japan) and QIAamp Viral RNA mini kit (QIAGEN), respectively. First-strand cDNA was synthesized by using a high capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA, USA) with random primers. Each cDNA was estimated by using Platinum SYBR Green qPCR SuperMix UDG (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Fluorescent signals of SYBR Green were analyzed by using an ABI PRISM 7000 (Applied Biosystems). The HCV internal ribosomal entry site (IRES) region was amplified using the primer pair 5′-GAGTGTCGTGCAGCCTCCA -3′ and 5′- CACTCGCAAG-CACCCTATCA -3′. Expression of HCV core protein was determined by an enzyme-linked immunosorbent assay (ELISA) as described previously [57].

Determination of Luciferase Activity and Cytotoxicity in HCV Replicon Cells

HCV replicon cells were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment. C-29EA was added to the culture medium at various concentrations. The treated cells were harvested 72 h post-treatment and lysed in cell culture lysis reagent (Promega, Madison, WI, USA) or *Renilla* luciferase assay lysis buffer (Promega). Luciferase activity in the harvested cells was estimated with a luciferase assay system (Promega) or a *Renilla* luciferase assay system (Promega). The resulting luminescence was detected by the Luminescencer-JNR AB-2100 (ATTO, Tokyo, Japan) and corresponded to the expression level of the HCV replicon. Cell viability was measured by a dimethylthiazol carboxymethoxy-phenylsulfophenyl tetrazolium (MTS) assay using a CellTiter 96 aqueous one-solution cell proliferation assay kit (Promega).

Effects on Activities of Internal Ribosome Entry Site (IRES)

Huh7 cells were transfected with pEF.Rluc.HCV.IRES.Feo or pEF.Rluc.EMCV.IRES.Feo and then were established in medium containing 0.25 mg/ml G418, as described previously [58]. These cell lines were seeded at 2×10^4 cells per well in a 48-well plate 24 h before treatment, treated with 15 μg/ml extract C-29EA, and then harvested at 72 h post-treatment. The firefly luciferase activities were measured with a luciferase assay system (Promega). The total protein concentration was measured using the BCA Protein Assay Reagent Kit (Thermo Scientific, Rockford, IL, USA) to normalize luciferase activity.

Western Blotting and Reverse-transcription Polymerase Chain Reaction (RT-PCR)

Western blotting was carried out by a method described previously [30]. The antibodies to NS3 (clone 8G-2, mouse monoclonal, Abcam, Cambridge, UK), NS5A (clone 256-A, mouse monoclonal, ViroGen, Watertown, MA, USA), and beta-actin were purchased from Cell Signaling Technology (rabbit polyclonal, Danvers, MA, USA) and were used as the primary antibodies in this study. RT-PCR was carried out by a method described previously [30,58].

Assays for RNA Helicase, ATPase, and RNA-binding Activities

A continuous fluorescence assay based on photoinduced electron transfer (PET) was described previously [29] and was slightly modified with regard to the reaction mixture [30]. The NS3 RNA unwinding assay was carried out by the method of Gallinari et al. [59] with slight modifications [30]. NS3 ATPase activity was determined by the method of Gallinari et al. [59] with slight modifications [30]. RNA binding to NS3 helicase was analyzed by a gel mobility shift assay [30,31]. The gene encoding NS3 helicase was amplified from the viral genome of genotype 1b and was introduced into a plasmid for the expression of a recombinant protein [38,60]. The radioactive band was visualized with the Image Reader FLA-9000 and quantified by Multi Gauge V 3.11 software.

NS3 Protease Assay

The fluorescence NS3 serine protease assay based on fluorescence resonance energy transfer (FRET) was carried out by the modified method using the SensoLyte™ 520 HCV protease assay kit (AnaSpec, Fremont, CA, USA). In brief, NS3 protein with a two-fold excess of NS4A cofactor peptide (Pep4AK) was prepared in 1× assay buffer provided with the kit. HCV NS3/4A protease was mixed with increasing concentrations of C-29EA and incubated at 37°C for 15 min. The reaction was started by adding the 5-FAM/QXL 520 substrate to the reaction mixture containing 180 nM HCV NS3/4A protease and various concentrations (0–400 μg/ml) of C-29EA. The resulting mixture (20 μl) was incubated at 37°C for 120 min using a LightCycler 1.5 (Roche Diagnostics, Basel, Switzerland). The fluorescence intensity was recorded every minute for 120 min. The NS3 serine protease activity was calculated as the initial reaction velocity and presented as a percentage of relative activity to that of the control examined with DMSO solvent but not C-29EA, in the same way as described in the fluorescence helicase assay [29].

Analysis of Drug-drug Interaction

The effects of drug combinations were evaluated using the isobologram method [33]. Various doses of C-29EA and interferon-alpha on 90% inhibition of HCV replication were combined to generate an isoeffect curve (isobole) to determine drug–drug interaction. Concave, linear, and convex curves exhibit synergy, additivity, and antagonism, respectively.

Statistical Analysis

The results are expressed as the mean ± standard deviation. The significance of differences in the means was determined by Student's *t*-test.

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Author Contributions

Conceived and designed the experiments: MN MT YS ST NA NN AY JT KM. Performed the experiments: YF KAS AF YM OF HT AY. Analyzed the data: MI NK NS SM NE. Wrote the paper: YF AY JT KM. Collected marine organisms: JT. Identified the sponge: NJdV.

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Original Article

Eradication of Hepatitis C Virus Subgenomic Replicon by Interferon Results in Aberrant Retinol-Related Protein Expression

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Hepatitis C virus (HCV) infection induces several changes in hepatocytes, such as oxidative stress, steatosis, and hepatocarcinogenesis. Although considerable progress has been made during recent years, the mechanisms underlying these functions remain unclear. We employed proteomic techniques in HCV replicon-harboring cells to determine the effects of HCV replication on host-cell protein expression. We examined two-dimensional electrophoresis (2-DE) and mass spectrometry to compare and identify differentially expressed proteins between HCV subgenomic replicon-harboring cells and their "cured" cells. One of the identified proteins was confirmed using enzyme-linked immunosorbent assay (ELISA) and Western blot analysis. Full-length HCV genome RNA replicating and cured cells were also assessed using ELISA. Replicon-harboring cells showed higher expression of retinal dehydrogenase 1 (RALDH-1), which converts retinol to retinoic acid, and the cured cells showed higher expression of retinol-binding protein (RBP), which transports retinol from the liver to target tissues. The alteration in RBP expression was also confirmed by ELISA and Western blot analysis. We conclude that protein expression profiling demonstrated that HCV replicon eradication affected retinol-related protein expression.

Key words: hepatitis C virus, retinol-binding protein

Hepatitis C virus (HCV) is the most common hepatitis virus in Japan. Approximately 85% of cases progress to chronic infection, resulting in liver cirrhosis and hepatocellular carcinoma. HCV is a member of the *Flaviviridae* family and possesses a single-stranded, sense RNA genome of about 9.6kb that encodes a single polyprotein. This precursor protein is cleaved co- and post-transcriptionally into at least 10 proteins: core, envelope (E) 1, E2, p7, nonstructural protein (NS) 2, NS3, NS4A, NS4B, NS5A, and NS5B [1, 2]. Although many studies have examined the functions of single proteins, the function of the polyprotein has not been sufficiently studied, owing to the lack of reproducible HCV proliferation in cell culture systems.

Recent advances in cell culture have enabled the reproducible implementation of the HCV replication system. An HCV replicon system that contained HCV nonstructural proteins and showed autologous replication of the HCV proteins was first established in

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1999 [3]. Recently, a full-length HCV genome containing replication system was introduced, followed by an *in vitro* viral replication system that can produce HCV viral particles, has also been established [4-8].

Recent advances in genomic and proteomic technologies have provided powerful tools for studying the global characteristics of host cell protein responses to HCV *in vitro*. Refined multidimensional liquid chromatographic (LC) separation, coupled with mass spectrometry (MS) for proteome analysis, has enabled global analysis using less protein and with increased sensitivity, throughput, and dynamic range than with previous proteomic techniques. Although the efficient replication of an HCV subgenomic replicon is thought to affect the gene expression profiles of host cells [9, 10], few proteomic analyses of this system have been reported [11].

In the present study, a proteomic approach was utilized to compare global protein expression profiles in HCV subgenomic replicon-harboring cells with "cured" cells, from which the replicons had been eliminated by prolonged treatment with interferon (IFN) alpha.

Materials and Methods

Cell cultures. The sO cells, harboring HCV subgenomic replicons derived from genotype 1b strains, were produced from HuH-7 cells [12]. sAH1 cells harboring the HCV subgenomic replicons were used to establish a cloned cell line, using an HCV replicon RNA library constructed with the HCV AH1 strain [7]. AH1 cells, harboring HCV full genome RNA were established from a full-length HCV genome RNA by the transfection of HCV RNA into sAH1c cells. sAH1c cells were "cured sAH1 cells" which were created by eliminating HCV RNA from transfected subgenomic replicon-harboring sAH1 cells by prolonged IFN alpha treatment. Cured cells were used because these enhanced the colony formation of the subgenomic replicon more than did parental HuH-7 cells [8].

The sO and sAH1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and G418 (300 μg/ml; Geneticine, Invitrogen, Carlsbad, CA, USA). HCV RNA-replicating cells are G418-resistant due to the production of neomycin phosphotransferase (Neo^R) from the efficient replication of HCV RNA. The presence of G418 is toxic when HCV RNA is excluded from the cells or its levels are decreased. Therefore, cured cells obtained from sO and sAH1 cells were maintained in the absence of G418.

IFN treatment (establishment of cured cells). The sO and sAH1 cells are sensitive to IFN [7, 12]. To prepare cured cells, sO and sAH1 cells (1×10^6) were plated onto 10-cm plates and cultured for 1 day immediately prior to IFN treatment. Human IFN alpha (Sigma, St. Louis, MO, USA) was added to the cells at a final concentration of 3,000 IU/ml. The cells were incubated in the absence of G418 for 3 weeks with the addition of IFN alpha (3,000 IU/ml) at 4-day intervals. The cured cells obtained from sO and sAH1 cells were named sOc and sAH1c, respectively. Negativity for HCV-RNA was confirmed by RT-PCR and defined as "cured" cells.

Protein extraction and two-dimensional electrophoresis (2-DE). Cells were washed with phosphate-buffered saline (PBS), and harvested by mechanical scraping during the exponential growth phase. Cells were centrifuged, and the cell pellets were dissolved in lysis buffer consisting of 5 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), 2% sulfobetaine (SB) 3-10, 1% dithiothreitol (DTT) and a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). After three freeze-thaw cycles, the pellets were sonicated for 30 sec and ultracentrifuged at 75,000 g for 30 min at 10℃ using an Optima™ TLF Ultracentrifuge (Beckman Coulter, Brea, CA, USA). The supernatant was transferred to a new tube and treated with a ReadyPrep 2D Cleanup Kit (Bio-Rad, Hercules, CA, USA) to remove ions, DNA, and RNA. The protein concentration was estimated using an RC-DC Protein Assay (Bio-Rad), according to a standard two-washed protocol. Pharmalyte 3-10 for isoelectric focusing (IEF) was formulated to increase the resolution at the basic end of a flatbed isoelectric focusing gel.

The first dimension IEF was performed using a 17-cm immobilized pH gradient (IPG) DryStrip (Bio-Rad), nonlinear pH 3-10. After rehydration for 15 h in 300 μL buffer consisting of 5 M urea, 2 M thiourea, 2% CHAPS, 3% SB3-10, 1% DTT, and 0.2% Bio-Lyte® 3/10 ampholyte (Bio-Rad), the protein

samples, 60 μg each, were loaded onto the strips. Focusing was accomplished with the following conditions: 250 V for 40 min, 10,000 V for 4 h, a third step with a total 70,000 V-h, and finally maintenance at 500 V as needed.

The focused strips were then equilibrated in buffer I (6 M urea, 2% sodium dodecyl sulfate (SDS), 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2% DTT) for 30 min and then in buffer II (6 M urea, 2% SDS, 0.375 M Tris-HCl pH 8.8, 20% glycerol, 2.5% iodoacetamide) for 15 min with gentle shaking. The second-dimension separation was carried out on 12% SDS-polyacrylamide gels using PROTEAN II Cell (Bio-Rad) at 20℃ using 40 mA/gel constant amps for 4 h. After 2-DE, the gels were stained with SYPRO Ruby (Invitrogen) according to the manufacturer's protocol.

Image analysis of 2-DE gels. Images of SYPRO Ruby-stained gels were obtained using an FLA-3000 (Fujifilm, Tokyo, Japan) image analyzer. Background subtraction, spot detection, and volume normalization were performed with PDQuest Advanced Version 8.0 software (Bio-Rad). The gels were then destained and restained with a silver staining kit (Dodeca Silver Stain kit, Bio-Rad). The spot intensity of each sample was determined and analyzed with PDQuest Advanced Version 8.0 software (Bio-Rad). The intensities of the matched spots were compared, and differences > 1.5-fold were confirmed by visual inspection. Three independent experiments were performed.

Protein identification using mass spectrometry. Overexpressed protein spots were selected based on Sypro Ruby staining intensity. Spots of interest were manually excised after silver staining. Gel spots were washed and digested with sequencing-grade trypsin and the resulting peptides were extracted using standard protocols. Peptide sequencing was accomplished by using a nanoflow HPLC, with electronic flow control (1100 Series nanoflow LC system, Agilent Technologies, Palo Alto, CA, USA), interfaced to an ion trap mass spectrometer (LC-MSD Trap SL, Agilent Technologies). A reverse-phase column (75 mm × 150 mm, C18 Zorbax StableBond) was used as the analytical column. The MS data were searched against a subset of human proteins in the Spectrum Mill for MassHunter Workstation software, protein sequence database. Positive protein identification was based on a total MS/MS search score of > 21.

Analysis of retinol-binding protein (RBP) levels using ELISA. Conditioned medium was collected from sO and sOc cells. Cell extracts were prepared by treatment with cell lysis buffer (MBL, Nagoya, Japan) and centrifuged at 15,000 rpm for 5 min. Protein concentrations were determined by the colorimetric BioRad protein assay. The protein concentrations in the sO cells and sOc samples were equalized. RBP concentrations in culture supernatants were measured by an RBP ELISA kit (Assaypro, St. Charles, MO, USA) according to the manufacturer's instructions.

Analysis of RBP levels using Western blot analysis. Cultured cells were washed twice with ice-cold PBS and lysed with lysis buffer (0.1 M Tris-HCl, 4% SDS, 10% glycerol, 0.004% bromophenol blue, 10% 2-mercaptoethanol). The lysates were collected and boiled for 5 min. Samples were electrophoresed on a 15% SDS-polyacrylamide gel, and transferred to a PVDF transfer membrane (Millipore, Bedford, MA, USA). Membranes were blocked in 5% BSA in 20 mM Tris-HCl (pH 7.6), 137 mM NaCl, and 0.1% Tween 20 (TBS-T) for 1 h at 37℃, and then probed at 4℃ overnight with antibodies in TBS-T containing 1% BSA. The primary antibodies were rabbit anti-beta actin (Sigma) and goat anti-RBP (Abcam, Cambridge, MA, USA). After washing, the membranes were incubated with a peroxidase-conjugated secondary antibody (anti-rabbit: Amersham Biosciences, Piscataway, NJ, USA) (anti-goat: R&D Systems, Minneapolis, MN, USA) at room temperature for 1 h, and visualized with an enhanced chemiluminescence detection system (Amersham Biosciences).

Analysis of RBP and retinal dehydrogenase 1 (RALDH-1) mRNA expression by reverse transcription polymerase chain reaction (RT-PCR). Expression levels of RBP and RALDH-1 genes were analyzed using RT-PCR. Total RNA was extracted from cell lines by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). Two micrograms of total RNA was reverse-transcribed into cDNA using ReverTra Ace (Toyobo, Osaka, Japan) at 42℃ for 20 min followed by 99℃ for 5 min using oligo (dT) primer according to the manufacturer's instructions. The resulting cDNA was subjected to PCR using the following primers (for-

ward PCR and reverse PCR primers, respectively):
RBP (F: 5'-TTCCGAGTCAAAGAGAACTTCG
R: 5'-TCATAGTCCGTGTCGATGATCC)
RALDH-1 (F: 5'-TACTCACCGATTTGAAGATT
R: 5'-TTGTCAACATCCTCCTTATC)
GAPDH (F: 5'-CACCCACTCCTCCACCTTTG
R: 5'-GTCCACCACCCTGTTGCTGT)

PCR reactions were performed using the KOD Dash DNA polymerase (Toyobo). PCR temperature conditions for RBP amplification were as follows: 30 cycles at 94℃ for 30 sec, 60℃ for 2 sec, and 74℃ for 30 sec. PCR temperature conditions for RALDH-1 amplification were as follows: 35 cycles at 94℃ for 30 sec, 51℃ for 2 sec, and 72℃ for 30 sec. To define the best amplification conditions for these genes, we tried 25, 30, 35, and 40 cycles of PCR. We found that 30 cycles for RBP and 35 cycles for RALDH-1 were the best conditions. For each primer set, the sense and antisense primer pairs were located on different exons to avoid amplification of contaminating genomic DNA. The housekeeping gene GAPDH (with the following amplifications: 30 cycles of 94℃ for 30 sec, 61℃ for 2 sec, and 72℃ for 30 sec) was used as an internal control to confirm the success of the RT-PCR. PCR products were analyzed on 2% agarose gels stained with ethidium bromide.

Statistical analysis. Results were expressed as means ± standard deviation (SD). All data were compared using Student's t test (Stat View, Cary, NC, USA). Data were considered statistically significant at $p < 0.05$.

Results

Proteomic profiling analysis of sO and sOc cells. Fig. 1 shows representative images of proteomic profiling of sO and sOc cells. Five spots were identified as reduced in sOc (spots: 1 to 5) and 3 spots were identified as enhanced in sOc (spots: 6 to 8) (Fig. 1).

Protein identification of detected spots. The results of LC/MS analysis are shown in Table 1. Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1), hemoglobin beta subunit, T-complex

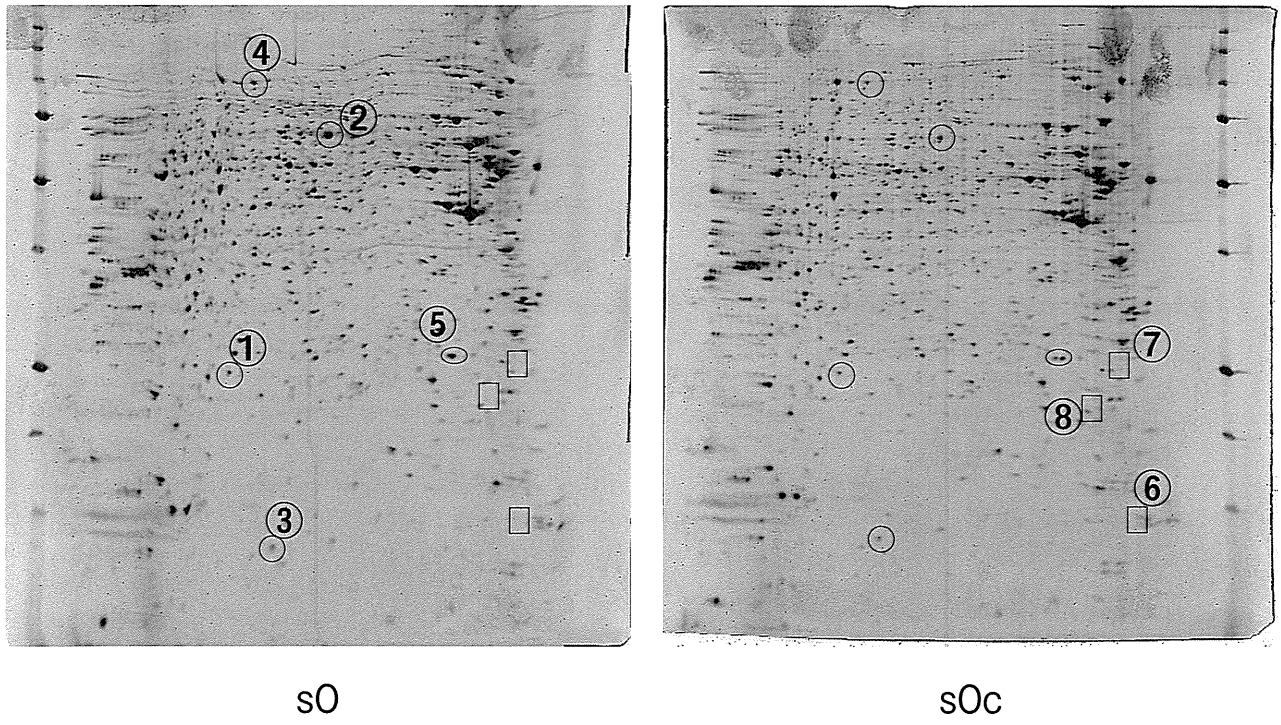

Fig. 1 2-DE analyses of proteins extracted from sO and sOc cells. Total protein extracts from the sO and sOc cells were separated on nonlinear IPG strips (pH 3-10) in the first dimension followed by 12% SDS-PAGE in the second dimension, and then were visualized by SYPRO Ruby staining. The boxed areas were identical between sO and sOc.

protein 1 subunit gamma (TCP1-gamma), RALDH-1, and Elongation factor 2 were down-regulated in sOc cells, *i.e.*, interferon-induced or HCV elimination-induced. UCH-L1 was divided into 2 spots that might be modified. Conversely, myosin light polypeptide 6, Rho GDP-dissociation inhibitor 1 (Rho-GDI alpha), and the plasma retinol-binding protein (RBP) precursor were upregulated in sOc cells.

Table 1

A) Proteins upregulated in sO	
1	Ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCH-L1)
2	Hemoglobin subunit beta
3	T-complex protein 1 subunit gamma (TCP1-gamma)
4	Retinal dehydrogenase 1 (RALDH-1)
5	Elongation factor 2
B) Proteins upregulated in sOc	
6	Myosin Light Polypeptide 6
7	Rho GDP-dissociation inhibitor 1
8	Plasma retinol binding protein

Of these proteins, retinol metabolism-related proteins were included in both downregulated (RALDH-1) and upregulated (RBP) by HCV eradication. Thus, we further examined the expression of these proteins.

RBP and RALDH-1 mRNA expression. We examined the expression levels of RBP and RALDH-1 in sO and sOc cells by RT-PCR in order to explore retinol's role in HCV replicon-harboring cells. No significant differences in RBP and RALDH-1 mRNA expression were found between sO and sOc cell lines (Fig. 2A).

RBP expression. Western blot analysis and ELISA were employed for RBP analysis. The Western blot results, shown in Fig. 2B, indicate that RBP expression was higher in sOc cells than in sO cells. The ELISA results, shown in Fig. 2C, indicated that the RBP level in sOc cells was significantly (1.6 times) higher than that in sO cells ($p<$

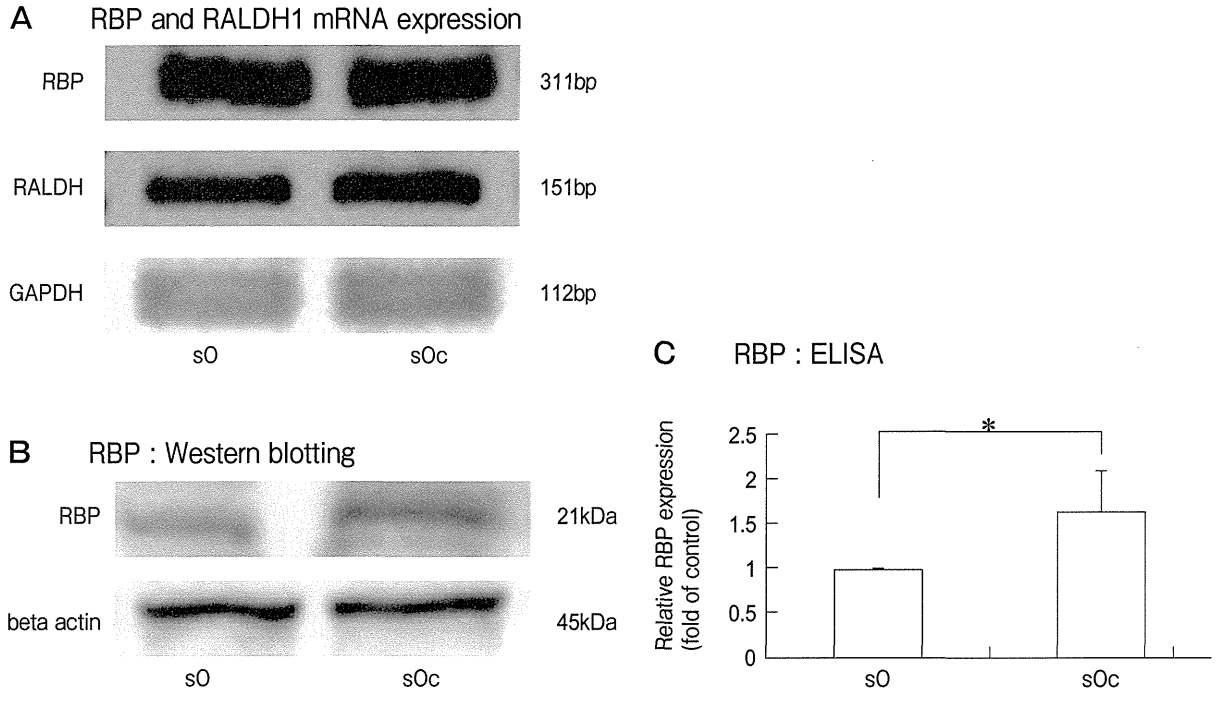

Fig. 2 (A) RT-PCR analysis of RBP and RALDH-1 in sO and sOc cells. The expression of GAPDH was used as an internal control. (B) Western blot analysis of RBP in sO and sOc cells. Actin was used as a loading control. (C) Analysis of RBP levels by ELISA in sO and sOc cells. The results are expressed as fold increases compared to sO cells. The data represent mean ± SD of triplicate measurements. $^*p < 0.01$

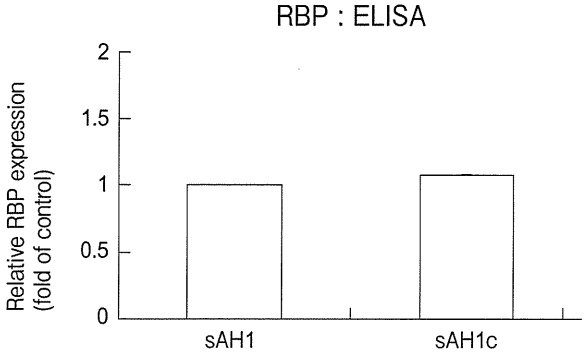

Fig. 3 RBP levels as determined by ELISA in sAH1 and sAH1c cells. The results are expressed as fold increases compared to sAH1 cells. The data represent mean ± SD of triplicate measurements. $^{*}p = 0.53$

0.01).

Additionally, we employed ELISA to compare RBP levels in sAH1 and sAH1c cells. RBP expression levels did not differ between sAH1 and sAH1c cells (Fig. 3).

Discussion

We have shown that, compared with cured cells, HCV subgenomic replicon-harboring cells bear more Hemoglobin subunit beta, TCP1-gamma, RALDH-1, Elongation factor 2, and UCH-L1. Furthermore, when the replicon-harboring cells were treated with interferon, thereby eliminating HCV proteins, myosin light polypeptide 6, Rho GDP-dissociation inhibitor 1 (Rho-GDI alpha), and plasma RBP precursor were upregulated.

Since these proteins have many functions and do not belong to a single functional category, we concentrated our next step on retinol-related proteins RBP and RALDH-1, which HCV elimination both upregulated and downregulated.

Other molecules were used as follows, and we did not subject them to further analysis. UCH-L1 is a member of a group of deubiquitinating enzymes and is one of the most abundant proteins in the brain [13]. It has been linked to Parkinson's disease, neuronal degeneration, and neuropathic pain. Additional evidence implicates it in other organ cancers. UCH-L1 is expressed in certain lung cancer cell lines and in breast cancer cells, especially in high grade tumors [14, 15]. Hemoglobin (Hb) is a heterotetramer, consisting of 2α-chain and 2β-chain subunits that form 2 semirigid $\alpha\beta$ dimers ($\alpha_1\beta_1$ and $\alpha_2\beta_2$). Hemoglobin beta is a hemoglobin subunit. TCP1-gamma is a member of the group II chaperonin family. The substrates for TCP1-gamma are cytoskeletal proteins such as tubulins, actins, and cyclin E. It is also thought to be involved in cell growth, since TCP1-gamma is strongly upregulated during the G1/S phase transition of the cell cycle through the early S phase [16]. Disruption of the TCP1-gamma function using siRNA results in the inhibition of cell proliferation, decreased cell viability, cell-cycle arrest, and cellular apoptosis [17]. Elongation factor 2 is known to regulate protein synthesis. Elongation factor 2 catalyzes the ribosomal translocation reaction, resulting in movement of ribosomes along mRNA during protein translation, thereby increasing protein synthesis. Elongation factor 2 also has anti-apoptotic effects against TNF-alpha or HIV-1 viral protein R-induced apoptosis [18].

The non-structural HCV proteins in our replicon-harboring cells may increase retinoic acid as a result of RALDH-1 upregulation.

In our proteomic profiling analysis of sO and sOc cells, 2 proteins involved in retinol (vitamin A) metabolism were markedly altered. The liver is the major site of vitamin A-loaded RBP production for the purpose of vitamin A delivery to peripheral organs. Eradication of the HCV replicon resulted in RALDH-1 downregulation and RBP upregulation.

Dietary retinol is absorbed in the intestine, processed into retinyl esters, and transferred into the circulation. The circulating retinyl esters are taken to the liver and converted to retinol. A portion of the hepatic retinol is bound to a cellular binding protein (CRBP) and stored. The remainder of the hepatic retinol is bound to RBP and transported to target cells. Retinol is oxidized to retinal by a subfamily of alcohol dehydrogenase (ADH) enzymes. Retinal is oxidized to retinoic acid (RA) by 3 retinaldehyde dehydrogenases (RALDHs): RALDH-1, RALDH-2, and RALDH-3. These enzymes are expressed in different patterns and play specific roles in various tissues [19]. RA has many functions and can modulate a variety of important processes, such as cell growth and differentiation, the induction of apoptosis, and the prevention of angiogenesis. All-trans- and 9-cis-

retinoic acid regulate transcription and target gene expression through binding to the retinoic acid receptor (RAR) and the retinoid X receptor (RXR), within the nucleus.

Retinol is stored in hepatic stellate cells as retinyl ester and secreted into the blood bound to RBP, which is synthesized mainly in the liver [20]. Therefore, the concentration of plasma vitamin A is strongly related to the synthesis of RBP in the liver. Moreover, RBP synthesis depends on vitamin A levels in the hepatocyte, and the levels of vitamin A and RBP are correlated. RBP is eliminated by the kidney with a half-life of 12h in the circulation.

The parental HuH-7 cells may not be appropriate for use as control cells in proteomic analysis, because the HCV subgenomic replicon cells used are derived from a single cloned cell. Therefore, it is very important to avoid clone-based differences for the proteomic analysis. From this point of view, we compared HCV replicon-harboring cells and their cured cells. In HCV replicon-harboring cells, RALDH-1 was increased and might induce retinoic acid production. When the HCV is eliminated, the activation of retinol metabolism is reduced and may result in an increase in retinol that could induce RBP precursor upregulation. Although RALDH-1 was increased, proteins downstream from retinol metabolism, such as retinoic acid or retinoic acid receptors, were not upregulated in our experiment. This might be explained by either 1) the effects being minimal, or 2) the downstream pathway being compromised. In infection with another hepatotropic virus, hepatitis B, HBx protein is known to promote oncogenesis. The HBx gene has been reported to induce promoter hypermethylation in the retinoic acid receptor $\beta 2$ (RAR-$\beta 2$) gene, resulting in decreased susceptibility to retinoic acid-induced cell growth inhibition [21]. Both the hepatitis B and C viruses have pathologic effects on the liver, resulting in chronic hepatitis and hepatocarcinogenesis. Retinol metabolism dysfunction could be involved in this common pathology.

In our experiment, RBP expression in full-length HCV genome-containing sAH1 cells was not different from that in cured cells, whereas the subgenomic replicon-harboring sO cells containing non-structural proteins exhibited decreased RBP levels. The HCV core protein has been reported to affect retinol metabolism. A comprehensive analysis of gene expression in the liver of core gene-expressing transgenic mice revealed the downregulation of RBP [22]. The HCV core protein is reported to bind Sp110b, a transcriptional suppressor of retinoic acid receptor (RAR), and to suppress the function of Sp110b, which results in the activation of retinoic acid-related functions, such as apoptosis [23]. The core protein has been found to interact with RXRα in its DNA-binding domain [24]. These results led us to understand that the core protein and the non-structural proteins both decrease RBP. However, their co-expression might interfere with these effects. Chronic infection with hepatitis C virus results in hepatocarcinogenesis 30 to 40 years post-infection. This may illustrate that this virus's carcinogenic effects are not strong enough to directly induce early cell proliferation. Our present results indicate that the carcinogenic effect of individual HCV proteins may be controlled by mutual interference.

Proteomic analysis of hepatitis B virus (HBV) replicon-harboring cells, compared with parent cells, revealed that retinol metabolism-related proteins were differentially expressed [25]. In the HBV replicon-harboring cells, ALDH, RBP and CRBP1 were upregulated. Although the expression pattern was different, HBV, another chronic hepatitis and hepatocarcinogenic virus, induced alterations in retinol metabolism-related proteins. DNA microarray analysis experiments comparing the same HCV subgenomic replicon cells (sO) with cured cells indicated that there was no effect on retinol metabolism-related genes [26]. This illustrates that the HCV replicon's effects on retinol metabolism-related proteins are post-transcriptional.

In conclusion, we have employed proteomic techniques to elucidate the mechanisms underlying the replication and pathogenesis of HCV in HCV replicon-harboring cells. By comparing the protein expression profiles of HCV subgenomic replicon-harboring cell lines with cured cells, we observed several alterations in proteins that are correlated with cell proliferation and apoptosis control, including retinol metabolism. Such an analysis of the protein expression profile in HCV replicon-harboring cells has extended our understanding of the mechanisms underlying HCV pathogenesis.

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PML tumor suppressor protein is required for HCV production

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ABSTRACT

PML tumor suppressor protein, which forms discrete nuclear structures termed PML-nuclear bodies, has been associated with several cellular functions, including cell proliferation, apoptosis and antiviral defense. Recently, it was reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML. However, role(s) of PML in HCV life cycle is unknown. To test whether or not PML affects HCV life cycle, we examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in HuH-7-derived RSc cells, in which HCV-JFH1 can infect and efficiently replicate, stably expressing short hairpin RNA targeted to PML. In this context, the level of secreted HCV core and the infectivity in the supernatants from PML knockdown cells was remarkably reduced, whereas the level of HCV RNA in the PML knockdown cells was not significantly affected in spite of very effective knockdown of PML. In fact, we showed that PML is unrelated to HCV RNA replication using the subgenomic HCV-JFH1 replicon RNA, JRN/3-5B. Furthermore, the infectivity of HCV-like particle in the culture supernatants was significantly reduced in PML knockdown JRN/3-5B cells expressing core to NS2 coding region of HCV-JFH1 genome using the *trans*-packaging system. Finally, we also demonstrated that INI1 and DDX5, the PML-related proteins, are involved in HCV production. Taken together, these findings suggest that PML is required for HCV production.

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1. Introduction

Hepatitis C virus (HCV) is the 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 3000 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, non-structural 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B [1,2]. HCV core protein forms a viral capsid and is essential for infectious virion production. The core protein is targeted to lipid droplets. Recently, lipid droplets have been found to be involved in an important cytoplasmic organelle for HCV production [3].

In addition, HCV core has been reported to facilitate cellular transformation as well as development of hepatocellular carcinoma in HCV core-transgenic mice [4]. Interactions of core with tumor suppressor proteins such as p53 and DDX3 may lead to enhanced cellular proliferation [4]. Indeed, HCV core interacts with promyelocytic leukemia (PML) protein and inhibits the PML tumor suppressor pathway through interfering with the PML-mediated apoptosis-inducing function [5]. PML forms discrete nuclear structures termed PML-nuclear bodies (PML-NBs) and associates with several cellular functions, including cell proliferation, apoptosis and antiviral defense [6,7]. In acute promyelocytic leukemia (APL) patient, the PML gene is fused with the retinoic acid receptor-α (RARα) gene, thus resulting in expression of an oncogenic PML-RARα fusion protein [6,7]. Conversely, treatment of APL patient with arsenic trioxide leads to reformation of PML-NBs and results in disease remission [6,7], indicating that PML is a target of arsenic trioxide. Interestingly, we have recently demonstrated that arsenic trioxide strongly inhibited HCV infection and HCV RNA replication without cell toxicity [8]. However, the role of PML in HCV life cycle yet remains unclear. To investigate the possible involvement of PML in HCV life cycle, we examined the accumulation of HCV RNA as well as the release of HCV core into culture

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supernatants from cells rendered defective for PML by RNA interference. The results provide evidence that PML is required for HCV production.

2. Materials and methods

2.1. Cell culture

293FT cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (FBS). The three HuH-7-derived cell lines: RSc cured cells that cell culture-generated HCV-JFH1 (JFH1 strain of genotype 2a) [9] could infect and effectively replicate [10–13], OR6c cells is cured cells of OR6 cells harboring the genome-length HCV-O RNA with luciferase as a reporter [14] or OR6c JRN/3-5B cells harboring the subgenome HCV-JFH1 RNA with luciferase as a reporter were cultured in DMEM with 10% FBS as described previously [13].

2.2. RNA interference

Oligonucleotides with the following sense and antisense sequences were used for the cloning of short hairpin RNA (shRNA)-encoding sequences targeted to DDX5 in a lentiviral vector: 5′-GATCCCCCTCTAATGTGGAGTGCGACTTCAAGAGAGTCGCACTCCACA TTAGAGTTTTTGGAAA-3′ (sense), 5′-AGCTTTTCCAAAAACTCTAATGT GGAGTGCGACTCTCTTGAAGTCGCACTCCACATTAGAGGGG-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 [15], to generate pSUPER-DDX5i. To construct pLV-DDX5i, the *Bam*HI-*Sal*I fragments of the pSUPER-DDX5i were subcloned into the *Bam*HI-*Sal*I site of pRDI292, an HIV-1-derived self-inactivating lentiviral vector containing a puromycin resistance marker allowing for the selection of transduced cells [16]. We previously described pLV-PMLi [8] and pLV-INI1i [17], respectively.

2.3. Lentiviral vector production

The vesicular stomatitis virus (VSV)-G-pseudotyped HIV-1-based vector system has been described previously [18,19]. The lentiviral vector particles were produced by transient transfection of the second-generation packaging construct pCMV-ΔR8.91 [18,19] and the VSV-G-envelope-expressing plasmid pMDG2 as well as pLV-PMLi into 293FT cells with FuGene6 (Roche Diagnostics, Mannheim, Germany).

2.4. HCV infection experiments

The supernatants was collected from cell culture-generated HCV-JFH1-infected RSc cells 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 or J6/JFH1 [20], RSc cells (5×10^4 cells/well) were plated onto 6-well plates and cultured for 24 h (hrs). We then infected the cells at a multiplicity of infection (MOI) of 0.05. The culture supernatants were collected at the indicated time post-infection and the levels of the core protein were determined by enzyme-linked immunosorbent assay (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-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection.

2.5. Quantitative RT-PCR analysis

The quantitative RT-PCR analysis for HCV RNA was performed by real-time LightCycler PCR (Roche) as described previously [14]. We used the following forward and reverse primer sets for the real-time LightCycler PCR: PML, 5′-GAGGAGTTCCAGTTTCT GCG-3′ (forward), 5′-GCGCCTGGCAGATGGGGCAC-3′ (reverse); DDX5, 5′-ATGTCGGGTTATTCGAGTGA-3′ (forward), 5′-TTTCTCC CCAGGGTTTCCAA-3′ (reverse); INI1, 5′-ATGATGATGATGGCGCTG AG-3′ (forward), 5′-TCGGAACATACGGAGGTAGT-3′ (reverse); β-actin, 5′-TGACGGGGTCACCCACACTG-3′ (forward), 5′-AAGCTGTAG CCGCGCTCGGT-3′ (reverse); and HCV-JFH1, 5′-AGAGCCATAGTGGT CTGCGG-3′ (forward), 5′-CTTTCGCAACCCAACGCTAC-3′ (reverse).

2.6. Western blot analysis

Cells were lysed in buffer containing 50 mM Tris–HCl (pH 8.0), 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 0.1% sodium dodecyl sulfate (SDS), 1 mM dithiothreitol and 1 mM phenylmethylsulfonyl fluoride. Supernatants from these lysates were subjected to SDS–polyacrylamide gel electrophoresis, followed by immunoblot analysis using anti-HCV core (CP-9 and CP-11; Institute of Immunology, Tokyo, Japan) or anti-β-actin antibody (Sigma).

2.7. WST-1 assay

RSc or OR6c JRN/3-5B cells (1×10^3 cells/well) were plated onto 96-well plates and cultured. The cells were subjected to the WST-1 cell proliferation assay (Takara Bio, Otsu, Japan) according to the manufacturer's protocol. The absorbance was read using a microplate reader at 440 nm with a reference wavelength of 690 nm.

2.8. Renilla luciferase (RL) assay

OR6c JRN/3-5B cells (1.5×10^4 cells/well) were plated onto 24-well plates and cultured for 72 h, then, subjected to the RL assay according to the manufacturer's instructions (Promega, Madison, WI, USA). A lumat LB9507 luminometer (Berthold, Bad Wildbad, Germany) was used to detect RL activity.

2.9. RNA synthesis and transfection

Plasmid pJRN/3-5B was linearized by digestion with *Xba*I and was used for RNA synthesis with T7 MEGAscript (Ambion) as previously described [13]. *In vitro* transcribed RNA was transfected into OR6c cells by electroporation as described previously [14].

2.10. Immunofluorescence and confocal microscopic analysis

Cells were fixed in 3.6% formaldehyde in phosphate-buffered saline (PBS), permeabilized in 0.1% Nonidet P-40 in PBS at room temperature, and incubated with anti-PML antibody (PM001, MBL) and anti-HCV core at a 1:300 dilution in PBS containing 3% bovine serum albumin (BSA) at 37 °C for 30 min. They were then stained with anti-Cy3-conjugated anti-mouse antibody (Jackson ImmunoResearch, West Grove, PA) or Alexa Fluor 647-conjugated anti-rabbit antibody (Molecular Probes, Invitrogen) 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, the cells were mounted on slides using a mounting media of SlowFade Gold antifade reagent (Invitrogen) added to reduce fading. Samples were viewed under a confocal laser-scanning microscope (FV1000; Olympus, Tokyo, Japan).

3. Results

3.1. PML is involved in the propagation of HCV

To investigate the potential role(s) of PML in HCV life cycle, we first used lentiviral vector-mediated RNA interference to stably knockdown PML in HuH-7-derived RSc cured cells that HCV-JFH1 [9] could infect and effectively replicate [10–13]. Real-time RT-PCR analysis for PML demonstrated a very effective knockdown of PML in RSc cells transduced with lentiviral vector expressing shRNA targeted to PML (Fig. 1A). To test the cell toxicity of shRNA, we examined WST-1 assay. In spite of very effective knockdown of PML, we demonstrated that the shRNA targeted to PML did not affect the cell viabilities (Fig. 1B). We next examined the level of secreted HCV core and the infectivity of HCV in the culture supernatants as well as the level of HCV RNA in PML knockdown RSc cells 24, 48, or 72 h after HCV-JFH1 infection at an MOI of 0.05. The results showed that the level of HCV RNA in PML knockdown cells was not affected until 72 h post-infection (Fig. 1C), while the release of HCV core protein into the culture supernatants was significantly suppressed in PML knockdown cells at 48 or 72 h post-infection (Fig. 1D). Consistent with this finding, the infectivity of HCV in the culture supernatants was also significantly suppressed in the PML knockdown cells at 48 or 72 h post-infection (Fig. 1E). We also obtained similar results using siRNA specific for human PML (siGENOME SMRT pool M-006547-01-0005, Dharmacon, Thermo Fisher Scientific, Waltham, MA) (data not shown). These results suggested that PML is associated with propagation of HCV.

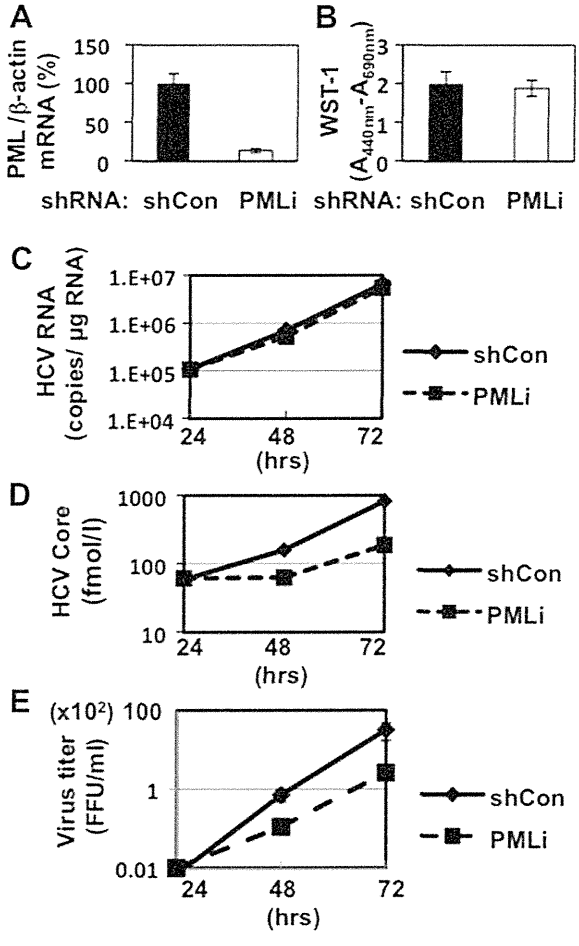

Fig. 1. PML is required for infectious HCV production. (A) Inhibition of PML mRNA expression by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for PML was performed as well as for β-actin mRNA. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (shCon) which was assigned as 100%. (B) WST-1 assay of the PML knockdown (PMLi) or the control (shCon) RSc cells. (C) The levels of intracellular genome-length HCV-JFH1 RNA in the PML knockdown or the control cells at 24, 48 or 72 h post-infection at an MOI of 0.05 were monitored by real-time LightCycler RT-PCR. (D) The levels of HCV core in the culture supernatants from the PML knockdown or the control RSc cells 24, 48 or 72 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. All experiments were done in triplicate.

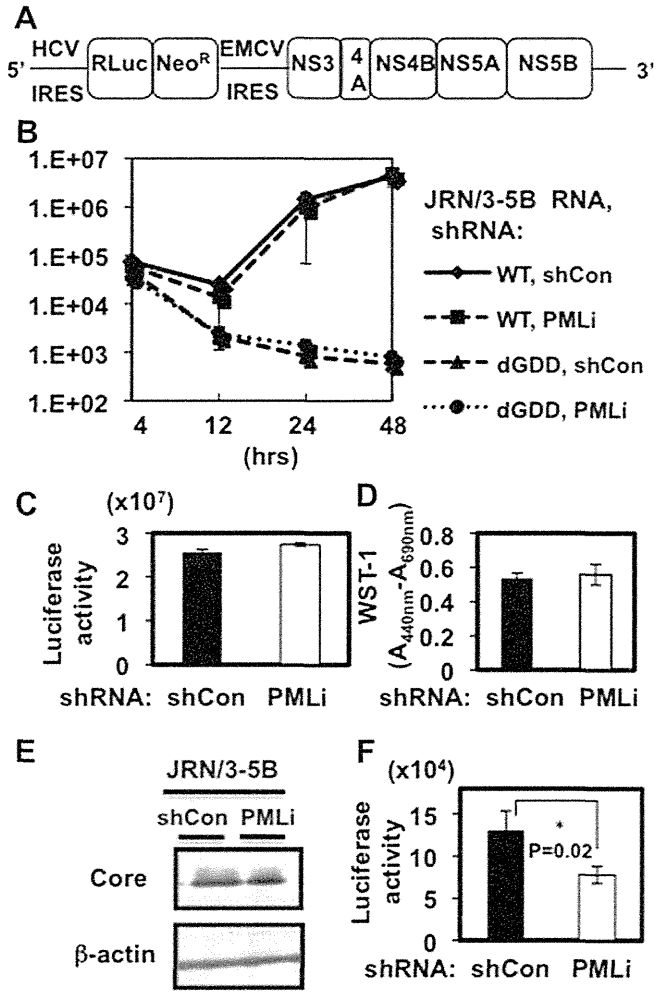

Fig. 2. PML is unrelated to the HCV RNA replication. Schematic gene organization of subgenomic JFH1 (JRN/3-5B) RNA encoding *Renilla* luciferase (RL) gene. *Renilla* luciferase gene (RLuc) is depicted as a box and is expressed as a fusion protein with Neo. (B) The transient replication of subgenomic HCV-JFH1 replicon in the PML knockdown (PMLi) or the control OR6c cells (shCon) after electroporation of *in vitro* transcribed JRN/3-5B RNA (10 μg) was monitored by RL assay at the indicated time. The results of *Renilla* luciferase activity are shown. dGDD indicates the deletion of the GDD motif in the NS5B polymerase, and the subgenomic HCV replicon with the deletion of GDD was used as a negative control. (C) The level of HCV RNA replication in PML knockdown (PMLi) or the control (shCon) OR6c JRN/3-5B cells was monitored by RL assay. The results shown are means from three independent experiments. (D) WST-1 assay of the PML knockdown or the control JRN/3-5B cells. (E) The level of HCV core protein in OR6c JRN/3-5B cells by expression of HCV core to NS2 coding region of HCV-JFH1 using mouse retroviral vector. pCX4bsr-JFH1-myc-C-NS2 and pMDG2 were cotransfected into Plat-E cells, mouse retroviral packaging cells. Mouse retroviral vector was obtained from their culture supernatants and transduced into OR6c JRN/3-5B PML knockdown or the control cells. The results of Western blot analysis of cellular lysates with anti-HCV core or an anti β-actin antibody are shown. (F) The level of HCV RNA replication in RSc cells 72 h after inoculation of HCV-like particles produced using *trans*-packaging system was monitored by RL assay. Asterisk indicates significant difference compared to the control. $^*P = 0.02$.

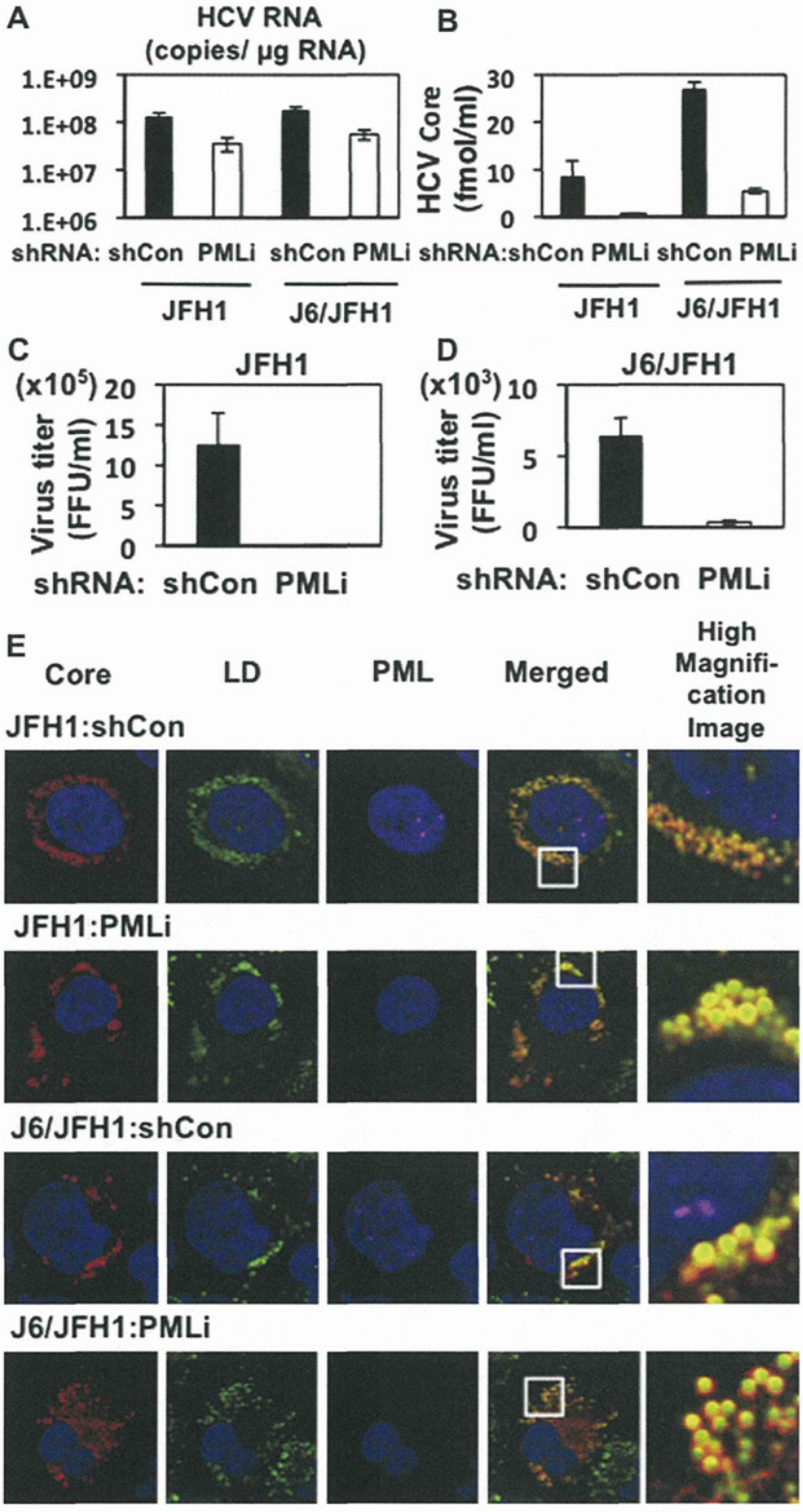

Fig. 3. PML is dispensable for the localization of HCV core to lipid droplet. (A) The levels of intracellular HCV RNA in PML knockdown or the control RSc cells 96 h after inoculation of HCV-JFH1 or HCV-J6/JFH1 were monitored by real-time LightCycler RT-PCR. Results from three independent experiments are shown (A–C). (B) The levels of HCV core in the culture supernatants from the PML knockdown RSc cells at 96 h post-infection were determined by ELISA. (C, D) The infectivity of HCV in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. (E) HCV core localizes to lipid droplet (LD) in the PML knockdown (PMLi) or the control (shCon) cells after infection with either HCV-JFH1 or HCV-J6/JFH1. Cells were fixed 72 h post-infection and were then examined by confocal laser scanning microscopy.

3.2. PML is unrelated to HCV RNA replication

To examine whether or not PML is involved in HCV RNA replication, we used the subgenomic replicon RNA of HCV-JFH1, JRN/3-5B, encoding *Renilla* luciferase gene for monitoring the HCV RNA replication (Fig. 2A). *In vitro* transcribed JRN/3-5B RNA was transfected into the PML knockdown OR6c cells by electroporation and we examined the luciferase activity. Consequently, the

luciferase activity in the PML knockdown cells was similar to that of the control cells (Fig. 2B), indicating that shRNA targeted to PML could not affect the transient HCV RNA replication. As well, the level of HCV RNA in PML knockdown HuH-7-derived OR6c JRN/3-5B cells harboring the subgenomic replicon RNA of HCV-JFH1 and the cell growth was not affected (Fig. 2C and D), suggesting that PML is unrelated to the HCV RNA replication. To further confirm whether or not PML is involved in HCV production, we used *trans*-packaging system [21,22], that HCV subgenomic replicon was efficiently encapsidated into infectious virus-like particles by expression of HCV core to NS2 coding region. In fact, infectious HCV-like particles were produced and released into the culture medium from PML knockdown JRN/3-5B cells stably expressing core to NS2 coding region of HCV-JFH1 genome by mouse retroviral vector (Fig. 2E). We could monitor the HCV RNA replication by *Renilla* luciferase assay in target naïve RSc cells after the inoculation of infectious HCV-like particles. Consequently, the release of infectious HCV-like particles into the culture supernatants was significantly suppressed in PML knockdown cells at 72 h post-infection (Fig. 2F). Thus, we conclude that PML is associated with HCV production.

3.3. PML is required for the late step in the HCV-JFH1 life cycle

To avoid the possibility of specific finding when we only used HCV-JFH1, we examined another strain of HCV-J6JFH1 [20]. For this, we analyzed the level of HCV core and the infectivity in the culture supernatant as well as the level of HCV RNA in the PML knockdown RSc cells 96 h after inoculation of HCV-J6/JFH1. In this context, the level of HCV RNA in PML knockdown cells was only somewhat decreased (Fig. 3A), while the level of core and the infectivity in the culture supernatants was remarkably reduced (Fig. 3B–D), indicating that PML is required for infectious HCV-J6/JFH1 production as well as HCV-JFH1.

Since lipid droplets have been shown to be involved in an important cytoplasmic organelle for HCV production [3], we performed immunofluorescence and confocal microscopic analyses to determine whether or not HCV core misses localization into lipid droplets in the PML knockdown cells. We found that the core protein was targeted into lipid droplets even in PML knockdown RSc cells as well as in the control RSc cells after infection with either HCV-JFH1 or HCV-J6/JFH1 (Fig. 3E). This suggests that PML plays a role in the late step after the core is targeted into lipid droplet in the HCV life cycle. Importantly, HCV did not disrupt the formation of PML-NBs in response to HCV infection (Fig. 3E) unlike HIV-1 and other DNA viruses [6,7,23].

3.4. INI1 and DDX5, PML-related proteins, are involved in HCV production

Finally, we established the INI1 or DDX5, PML-related protein [23,24], knockdown RSc or OR6c JRN/3-5B cells by lentiviral vector expressing shRNA target to INI1 [17] or DDX5 to examine potential role of INI1 and DDX5 in HCV life cycle. Consequently, we found that the release of HCV core or the infectivity of HCV into the culture supernatants was significantly suppressed in the INI1 or DDX5 knockdown RSc cells 96 h after HCV-JFH1 infection, while the RNA replication in the knockdown cells was only somewhat decreased in spite of the very effective knockdown of INI1 or DDX5 mRNA without growth inhibition (Fig. 4A–F), suggesting that INI1 and DDX5 are involved in HCV life cycle. To confirm whether or not these proteins are involved in HCV RNA replication, we examined the luciferase assay in the INI1 or DDX5 knockdown OR6c JRN/3-5B cells. In this context, the shRNA target to INI1 or DDX5 did not affect the luciferase activity and the cell growth in these knockdown cells (Fig. 4G and H), suggesting that both INI1 and DDX5 are required for HCV production like PML.

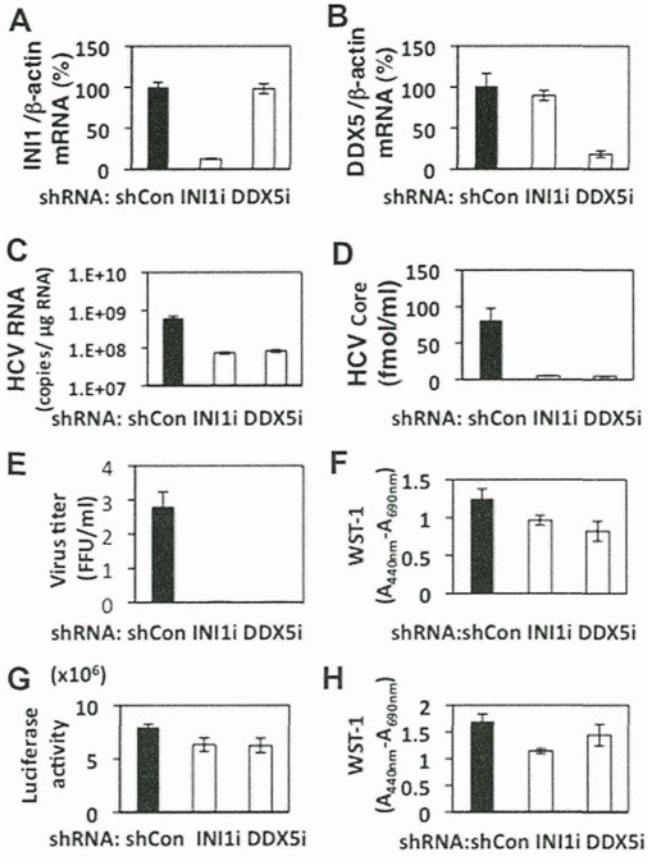

Fig. 4. INI1 and DDX5, PML-related proteins, are required for HCV production. (A, B) Inhibition of INI1 and DDX5 mRNA expressions by the shRNA-producing lentiviral vector. Real-time LightCycler RT-PCR for INI1 and DDX5 was performed as well as for β-actin mRNA in triplicate. Each mRNA level was calculated relative to the level in RSc cells transduced with a control lentiviral vector (Con) which was assigned as 100%. (C) The levels of intracellular genome-length HCV-JFH1 RNA in each knockdown cells at 96 h post-infection at an MOI of 0.05 were monitored by real-time LightCycler RT-PCR. (D) The levels of HCV core in the culture supernatants from the INI1 (INI1i) or DDX5 knockdown (DDX5i) RSc cells 96 h after inoculation of HCV-JFH1 were determined by ELISA. (E) The infectivity of HCV-JFH1 in the culture supernatants was determined by a focus-forming assay at 48 h post-infection. Virus titer is shown as ($x10^7$) FFU/ml. (F) WST-1 assay of each knockdown RSc cells at 96 h post-infection. (G) The HCV RNA replication level in INI1 and DDX5 knockdown OR6c JRN/3-5B cells was monitored by RL assay. (H) WST-1 assay of each knockdown OR6c JRN/3-5B cells. All results shown are means from three independent experiments.

4. Discussion

So far, the PML tumor suppressor protein, which forms PML-NBs, has been implicated in host antiviral defenses [6,7]. In fact, PML is induced by interferon after viral infection and suppresses some viral replication [6,7]. In contrast, PML-NBs are often disrupted or sequestered in the cytoplasm by infection with several DNA or RNA viruses to protect from the antiviral function of PML [6,7,23]. In case of HCV, Herzer et al. recently reported that the HCV core protein colocalizes with PML in PML-NBs and abrogates the PML function through interaction with PML isoform IV by over-expression studies [5]. However, we did not observe such colocalization of HCV core with PML and HCV did not affect the formation of PML-NBs in response to HCV-JFH1 infection (Fig. 3E). Interestingly, Watashi et al., previously demonstrated the HCV core modulates the retinoid signaling pathway through sequestration of

Sp110b, PML-related potent transcriptional corepressor of retinoic acid receptor, in the cytoplasm from nucleus [25].

In contrast, we have demonstrated that PML is required for infectious HCV production (Fig. 1). However, the molecular mechanism(s) how PML regulates HCV production yet remains unclear. At least, PML seems to be unrelated to the HCV RNA replication (Fig. 2). In this regard, several host factors including apolipoprotein E, components of ESCRT system, and PA28γ have been implicated in infectious HCV production [13,26,27]. Indeed, PA28γ, a proteasome activator, interacts with HCV core and affects nuclear retention and stability of the core protein. Importantly, PA28γ participates in the propagation of infectious HCV by regulation of degradation of the core protein [27]. Intriguingly, Zannini reported that PA28γ interacts with PML and Chk2 and affects PML-NBs number [28]. Accordingly, we demonstrated that ATM and Chk2, which phosphorylates PML and regulates the PML function, are involved in HCV life cycle [11]. In addition, other PML-related proteins such as INI1 and DDX5 seem to be involved in HCV production (Fig. 4). Indeed, INI1, also known as hSNF5, is incorporated into HIV-1 virion and is required for efficient HIV-1 production [29]. On the other hand, cytoplasmic PML may be involved in HCV production, since endoplasmic reticulum (ER) and lipid droplets are important cytoplasmic organelle for the HCV life cycle. In this regard, Giorgi et al. recently reported that cytoplasmic PML specifically enriches at ER [30], suggesting that cytoplasmic PML may be associated with HCV production. Altogether, the PML pathway seems to be involved in infectious HCV production.

Acknowledgments

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Research Paper

Peginterferon Alfa-2a plus Ribavirin in Japanese Patients Infected with Hepatitis C Virus Genotype 2 Who Failed Previous Interferon Therapy

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Abstract

Some patients infected with hepatitis C virus (HCV) genotype 2 could be cured with treatment shorter than 24 weeks using peginterferon plus ribavirin, but there are still treatment-refractory patients. Direct-acting antivirals (DAAs) are not currently available for HCV genotype 2 patients, different from genotype 1 patients, in clinical practice. We investigated 29 HCV genotype 2-infected Japanese patients who had been previously treated and failed to clear HCV. We retreated them with peginterferon alfa-2a plus ribavirin and measured HCV RNA level to assess the efficacy and safety of this treatment in patients who had failed previous therapy. We found that retreatment of HCV genotype 2-infected Japanese patients with peginterferon alfa-2a plus ribavirin for 24-48 weeks led to 60 to 66.6% sustained virological response (SVR) in patients previously treated with (peg-)interferon monotherapy and to 69.9% SVR in relapsers previously treated with peginterferon plus ribavirin. Attention should be paid to certain patients with unique features. Selection of patients according to their previous treatment could lead to optimal therapy in HCV genotype 2 treatment-experienced patients.

Key words: Retreatment, HCV G2, Japanese

INTRODUCTION

Hepatitis C virus (HCV) infection causes acute and chronic hepatitis, cirrhosis and hepatocellular carcinoma (HCC) [1]. HCV is also a major causative agent of HCC in Japan [2]. HCV is a positive-sense single stranded RNA virus with ~9.6 kb length, belonging to the genus Hepacivirus, a member of *the family Flaviviridae*. It is known that there exist at least 6 main genotypes of HCV [3]. These approximately equidistant genetic groups each contain a variable number of more closely related, genetically and epidemiologically distinct "subtypes". Genotypes differ from each other by 31 to 33% at the nucleotide level,

compared with 20 to 25% between subtypes [3]. In Japan, HCV genotype 1b, 2a and 2b, respectively, are observed in ~70, 20 and 10% of HCV-infected patients [4].

Treatment with peginterferon plus ribavirin for 24 weeks leads to 70-80% sustained virological response (SVR) in treatment-naïve patients infected with HCV genotype 2 [5-7]. Combination of peginterferon with ribavirin for 24 weeks is the current standard of care (SOC) for treatment-naïve patients infected with HCV genotype 2 or 3. Some selected HCV genotype 2-infected patients achieved SVR with treatment periods shorter than 24 weeks [5-9]. However, in treatment-naïve patients infected with HCV genotype 1, treatment with peginterferon plus ribavirin for 48 weeks leads to only ~50% SVR [7]. Thus, HCV genotype is one of the important factors influencing the outcome of interferon treatments [7,10].

Retreatment of chronic hepatitis C patients failing to achieve SVR with combination peginterferon plus ribavirin could only obtain 10 to 15% SVR in non-responders and 40 to 50% SVR in relapsers [11]. In North America and European countries, retreatment for HCV genotype 2 or 3 patients failing to achieve SVR with combination peginterferon plus ribavirin could lead to 37 to 46% SVR in non-responders and 52 to 63% in relapsers [12, 13], even though retreatment with SOC was performed for 48 weeks. In our previous study [14], we observed that retreatment for HCV genotype 2 Japanese patients who failed to achieve SVR with combination peginterferon alfa-2b plus ribavirin for 16, 24 or 48 weeks resulted in 71.4% SVR, but the proportions of non-responders and relapsers were unclear and HCV RNA was measured with COBAS AMPLICOR HCV Monitor Test v. 2.0 (range: 0.5 - 850 kIU/mL) (Roche Diagnostics, Tokyo, Japan).

In the present study, we investigated 29 HCV genotype 2-infected Japanese patients who had been previously treated and failed to clear HCV. We retreated them with peginterferon alfa-2a plus ribavirin and measured HCV RNA with the more sensitive COBAS TaqMan HCV test (Roche) to assess the efficacy and safety of peginterferon alfa-2a with ribavirin in patients who had failed previous therapy in clinical practice. We focused on 3 females retreated with peginterferon alfa plus ribavirin and resulting in non-SVR, in whose sera a single very low-titer fluctuation of HCV RNA from negative to positive was detected after HCV RNA had been undetected. This would indicate that treatment with SOC should be stopped in HCV genotype 2 female patients with these features.

MATERIALS AND METHODS

Patients

Patients were recruited from Chiba University Hospital and 29 hospitals in Chiba, Ibaraki, and Saitama Prefectures between March 2008 and September 2011. Patients were eligible if they met the following inclusion criteria: (i) infected with HCV genotype 2 alone, (ii) age ≥ 20 years, (iii) diagnosed as chronic hepatitis C, (iv) negative for HBs antigen, (V) negative for human immunodeficiency virus, (vi) no autoimmune liver diseases, (vii) no severe renal disease, (viii) no severe heart disease, (ix) no mental disorders, (x) no current intravenous drug abuse, and (xi) no pregnancy. Thirty-four of the patients had previously been included in an investigation of the incidence of HCC during and immediately after peginterferon alfa-2a and ribavirin treatment in patients with chronic hepatitis C in Japan [2].

Study design

We recruited previously treated patients infected with HCV genotype 2. In Japan, combination therapy for treatment-naïve patients infected with HCV genotype 2 was not supported by the Japanese health insurance system at that time [15]. Concerning previously treated patients, they had to have failed previous treatment with either conventional interferon monotherapy, peginterferon monotherapy, conventional interferon/ribavirin combination therapy, or peginterferon/ribavirin combination therapy, different from the previous study by Sherman et al. [12]. Twenty-nine consecutive patients were enrolled in this study. Informed consent was obtained from all patients prior to enrolment. The Ethics Committee of Chiba University School of Medicine approved the study protocol. In this study, 180 μg of peginterferon alfa-2a per week plus 600-800 mg ribavirin per day were usually given in the treatment of patients for as long as 24, 48, or 72 weeks, according to the patient's will, as combination therapy for retreated patients infected with HCV genotype 2 was supported for only 24 weeks by the Japanese health insurance system at that time [15]. Clinical and laboratory assessments were performed at least every 4 weeks during treatment and a 12-week follow-up period. Adverse events were noted by oral inquiry (patient interview), physical examinations and laboratory tests.

Determination of HCV RNA titers and HCV genotype

Serum HCV RNA titer was measured using COBAS TaqMan HCV test (Roche), with levels ranging from 1.2 to 7.8 log IU/mL [16]. HCV genotype was

determined by the antibody serotyping method of Tukiyama-Kohara et al. [17,18]. According to this assay, HCV serotypes 1 and 2 correspond to HCV genotypes 1a/1b and 2a/2b [3]. HCV RNA titer and HCV genotype were determined before treatment, and HCV RNA was measured every 4 weeks before, during, and for at least 24 weeks after the end of treatment.

Serum liver function tests and hematology tests

Serum aminotransferase concentrations, other liver function tests and hematology tests were performed according to standard methods every 1 to 3 months before, during, and for at least 24 weeks after the end of treatment.

Assessment of efficacy

SVR was defined as undetectable serum HCV RNA at 24 weeks after the end of treatment. Relapse was defined as undetectable HCV RNA at the end of therapy, followed by the reappearance of HCV RNA [11]. Non-response was defined as detectable HCV RNA at the end of therapy. Patients with undetectable HCV RNA within the initial 4 weeks of treatment were considered to have had rapid virological response (RVR). Patients who had undetectable HCV RNA within the initial 12 weeks of treatment were considered to have had complete early virological response (cEVR) (described as EVR in this article) [16].

Statistical analysis

Data were expressed as mean ± standard deviation (SD). Differences were evaluated by Student's *t*-test, chi-square test, or Fisher's exact test. $P < 0.05$ was considered statistically significant.

RESULTS

Patient characteristics

The characteristics of the 29 patients in the present study are shown in Table 1. They had a history of peginterferon/conventional interferon with or without ribavirin, and 4 were unknown regarding previous treatment response (Table 1). In these 29 patients, 3 received conventional interferon monotherapy, 10 peginterferon alfa-2a monotherapy, 12 peginterferon alpha-2b plus ribavirin, 3 peginterferon alfa-2a plus ribavirin, and 1 had details unknown. HCV RNA levels (≥5log IU/mL, <5log IU/mL, and unknown) were 24, 4 and 1, respectively. Concerning virological response, 18 (62.0%) had SVR, 9 (31.0%) relapsed, and 2 (6.8%) discontinued treatment due to side effects.

Table 1. Baseline and demographic characteristics of patients in the present study

Number of patients	29
Age (years)	60.1 ± 8.6
Gender (male/female)	15/14
Body mass index (kg/m^2)	26.2 ± 3.6
HCV RNA (log IU/mL)	5.5 ± 2.0
ALT (IU/L)	57.8 ± 50.7
γ-GTP (IU/L)	46.0 ± 40.7
AFP (ng/mL)	5.7 ± 3.4
Leukocyte count (/mm^3)	4940 ± 1670
Hemoglobin (g/dL)	14.0 ± 1.6
Platelet count (x10^4/mm^3)	16.2 ± 5.2
Treatment response	
Duration of treatment (~24/48/72 wks)	9/18/2
RVR rates, %	34.4 (10/29)
HCV RNA negativity at 8 wks	81.4 (22/27)
EVR rates, %	88.8 (24/27)
SVR rates, %	62.0 (18/29)

Data are expressed as mean ± SD. ALT, alanine aminotransferase; γ-GTP, gamma-glutamyl transferase; AFP, alpha-fetoprotein; RVR, rapid virological response; EVR, early virological response; SVR, sustained virological response.

Comparison of SVR patients with non-SVR patients among previously treated patients

Next, we compared 18 SVR patients with 11 non-SVR patients among the previously treated patients (Table 2). The platelet count of SVR patients tended to be higher than that of non-SVR patients ($P = 0.061$). We did not see any differences in the baselines of other factors and treatment responses (Table 2). In the 18 SVR patients previously treated, 3 received conventional interferon monotherapy, 5 peginterferon alfa-2a monotherapy, 7 peginterferon alpha-2b plus ribavirin, 2 peginterferon alfa-2a plus ribavirin and 1 with details unknown. In the 11 non-SVR patients previously treated, 5 received peginterferon alfa-2a monotherapy, 5 peginterferon alpha-2b plus ribavirin, and 1 peginterferon alfa-2a plus ribavirin. Concerning previous treatment response of the 29 previously treated patients, 18 were relapsers, 7 non-responders, and 4 had details unknown. In the 18 SVR patients, 12 were relapsers, 4 non-responders, and 2 had details unknown. In 11 non-SVR patients, 6 were relapsers, 3 non-responders, and 2 had details unknown.