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Production and characterization of HCV particles from serum-free culture

Daisuke Akazawa[a,b], Kenichi Morikawa[b,1], Noriaki Omi[a,b], Hitoshi Takahashi[a,b,2], Noriko Nakamura[a], Hidenori Mochizuki[a], Tomoko Date[b], Koji Ishii[b], Tetsuro Suzuki[b,3], Takaji Wakita[b,*]

[a] Pharmaceutical Research Laboratories, Toray Industries, Inc., Kanagawa, Japan
[b] Department of Virology II, National Institute of Infectious Diseases, 1-23-1 Toyama, Shinjuku-ku, Tokyo, Japan

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ABSTRACT

Hepatitis C virus (HCV) is a major cause of liver cancer, and it is therefore important to develop a prophylactic strategy for HCV infection. In recent years, a system for cell culture of the infectious HCV particle has been established, and the inactivated particle has potential as an antigen for vaccine development. In this study, we aimed to establish highly efficient HCV particle purification procedures using the following serum-free culture of HCV particles. First, naïve human hepatoma Huh7 cells were grown in serum-free medium that was supplemented with human-derived insulin, transferrin and sodium selenite. Then, *in vitro* transcribed JFH-1 or J6/JFH-1 chimeric HCV-RNA was transfected into the serum-free conditioned Huh7 cells. Infectious HCV was secreted into the culture supernatant with the same efficiency as that from cells cultured in FBS-containing medium. The HCV-core protein and RNA continued to be detected in the culture supernatant when the infected cells were subcultured in serum-free medium. Sucrose gradient centrifugation analyses indicated that the profiles of HCV-core, HCV-RNA and the infectivity of HCV particles were almost identical between HCV from FBS-supplemented and serum-free cultures. We further determined that anti-CD81, anti-SR-BI and anti-E2 antibodies inhibited infection by serum-free cultured HCV to a greater extent than infection by HCV from FBS-supplemented cultures. These HCV particles also differed in the level of associated apoplipoproteins: the ApoE level was lower in serum-free cultured HCV. ApoB and ApoE antibody-depletion assays suggested that infection of serum-free cultured HCV was independent of ApoB and ApoE proteins. These data suggest that lipids conjugated with HCV affect infection and neutralization.

1. Introduction

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. HCV is a human pathogen that is a major cause of chronic hepatitis, liver cirrhosis and hepatic carcinoma. HCV therapy mainly involves treatment with pegylated-interferon and rivabirin; however, these agents are not very effective for patients with high titer HCV-RNA and genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive care treatments for HCV. It was discovered that a genotype 2a strain, JFH-1, efficiently replicated in Huh7 cells [1]. Moreover, an *in vitro* culture system that generates infectious HCV has also been successfully developed using the JFH-1 genome [2–4]. Recently, it has been shown to be possible to produce various chimeric HCVs by replacement of the JFH-1 structural protein region with the same region from other strains [5]. These chimeric HCV particles are expected to lead to a HCV vaccine as well as to new pharmaceuticals.

Huh7 is a human hepatoma cell line that was established in 1982 [6]. This cell line can be cultured in serum-free medium supplemented with selenium. Serum-free culture has advantages for the simple purification and preparation of animal-origin-free virus particles. In this study, we successfully produced HCV particles in serum-free culture and compared the properties of these particles to those from FBS-supplemented cultures. Interestingly, serum-free cultured HCV was susceptible to CD81-, SR-BI- and HCV-E2-neutralizing antibodies. It was recently suggested that HCV particles associate with lipids to form viro-lipo particles [7–9], and it has also been shown that HCV particles can associate with

Abbreviations: HCV, hepatitis C virus; ITS, insulin-transferrin-selenium; MOI, multiplicity of infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; VLDL, very-low-density lipoprotein.

* Corresponding author. Tel.: +81 3 5285 1111; fax: +81 3 5285 1161.
E-mail address: wakita@nih.go.jp (T. Wakita).
[1] Present address: Institute of Microbiology, University of Lausanne, and Division of Gastroenterology and Hepatology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland.
[2] Present address: Center for Influenza Virus Research, National Institute of Infectious Diseases, Tokyo, Japan.
[3] Present address: Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan.

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lipids to form exosomes [10,11]. We examined apolipoprotein association of serum-free cultured HCV. We found that this virus had a lower ApoE level than HCV from serum-supplemented cultures and that infection by this virus was apolipoprotein-independent.

2. Materials and methods

2.1. Cell culture

Huh7, Huh7.5.1 ([4], a generous gift from Dr. Francis V. Chisari), Huh7-25 and Huh7-25-CD81 [12] cell lines were cultured in 5% CO_2 at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (DMEM-10). Our previous FACS analysis indicated that Huh7-25 cells do not express CD81, and that Huh7-25-CD81 constitutively express CD81, on the cell surface [12]. For serum-free culture, the cells were conditioned and cultured in DMEM/F-12 supplemented with Insulin-Transferrin-Selenium-X (ITS) (Invitrogen, Carlsbad, CA).

2.2. Establishment of serum-free cultured cells

Sub-confluent Huh7 cells that were cultured in DMEM-10 were passaged in 10-cm dishes (Nunc, Rochester, NY) in DMEM containing 5% FBS. The cells were then sequentially passaged in DMEM containing 2, 1 and 0.5% FBS and were ultimately passaged in serum-free medium. The cells were detached for passage in serum-free culture using TrypLE Sellect (Invitrogen).

2.3. Cell growth assay

Cell growth was assayed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using the CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. In brief, 1×10^4 cells were seed into a 96-well culture plate (IWAKI, Tokyo, Japan) in 100 μL of media, and 20 μL of the assay solution was added into each well at the appropriate time. After incubation for 1 h at 37 °C, the absorbance of the solution at 490 nm was measured.

2.4. Plasmids

pJFH1 and pJ6/JFH1 were generated as previously reported [2,13].

2.5. RNA synthesis

RNA synthesis was performed as described previously [14]. Briefly, the pJFH1 and pJ6/JFH1 plasmids were digested with *Xba* I and were treated with Mung Bean nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment was then purified and was used as a template for RNA synthesis. HCV-RNA was synthesized *in vitro* using a MEGAscript™ T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

2.6. RNA transfection

RNA transfection was performed as described previously [15]. Briefly, trypsinized cells were washed with Opti-MEM I™ reduced-serum medium (Invitrogen) and were resuspended at a density of 7.5×10^6 cells/mL in Cytomix buffer [1]. RNA (10 μg) that was synthesized from pJFH1 or pJ6/JFH1 was mixed with 400 μL of the cell suspension and was transferred into an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950 μF with the Gene Pulser II™ apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to a 6-well plate, in which each well contained 3 mL of culture medium.

2.7. Infectivity titration

Huh7.5.1 cells were employed to determine the infectivity titer using end point dilution and immunofluorescence as described below. Briefly, each sample was serially diluted 5-fold in DMEM-10 and a 100-μL aliquot was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-Core antibody 2H9 and Alexa 488-conjugated secondary anti-mouse IgG antibody. The infectious foci were counted. The titer was then calculated and is indicated as focus forming units per mL (FFU/mL).

2.8. HCV inhibition assay

To analyze the inhibitory effects of anti-CD81 and anti-SR-BI against virus infection, naïve Huh7.5.1 cells (2×10^4) were seeded into a 48-well plate and were incubated for 1 h at 37 °C with JS-81 or rat anti-SR-BI serum ([16], a generous gift from Dr. H. Barth) that was serially diluted with DMEM. Mouse IgG1 (Sigma, St. Louis, MO) and rat pre-immune serum were used as controls for JS-81 and anti-SR-BI, respectively. Antibodies were removed and the cells were washed once with PBS. The cells were then inoculated with viruses (MOI 0.1) from FBS-supplemented or serum-free culture for 3 h, and were then washed with PBS and cultured with DMEM-10 for 72 h. The cells were washed three times with PBS and 100 μL of Passive Lysis Buffer (Promega) was added into each well. Cell lysates were collected and HCV-core concentrations were measured as described below.

To analyze the inhibitory effects of anti-E2 against HCV particles, viruses that were purified from FBS-supplemented or serum-free culture (2×10^3 FFU) were mixed with mouse anti-E2 (AP33, a kind gift from Genentech, Inc.) antibody, and were then incubated for 30 min at room temperature. Naïve Huh7.5.1 cells (1×10^4) were seeded into a poly-D-lysine coated 96-well plate, and cells were inoculated with the virus-antibody mixtures, which were serially diluted with DMEM-10, and, after 3 h, the mixtures were removed and the cells were washed once with PBS. DMEM-10 was added into each well, and the cells were cultured for 72 h. The cells were fixed with methanol for 15 min at −20 °C, and the infected cells were stained with rabbit anti-NS5A antibody using immunofluorescence as described above [17]. Percentage infection was calculated from the infectious titer of each diluted virus.

2.9. Sucrose density gradient analysis and HCV purification

Supernatants (4 mL) of J6/JFH-1 HCV cells were layered on top of a preformed continuous 10–60% sucrose gradient in 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA (TNE buffer). HCV-core levels, HCV-RNA titer and infectious titers of the media are shown in the supplementary table. The gradients were centrifuged using an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (500 μL each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100-μL drop from fractions of a gradient run.

Partially purified HCV was prepared by collecting the peaks of HCV-core and HCV-RNA and was used for the infection assay and for characterization.

2.10. Quantification of HCV-core protein and RNA

To estimate the levels of HCV-core proteins, the concentration of HCV-core proteins was measured. Aliquots of samples were

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assayed using the HCV Core ELISA kit (Ortho Clinical Diagnostics, Tokyo, Japan). Viral RNA was isolated from harvested culture media or from sucrose density gradient fractions using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). Copy numbers of HCV-RNA were determined by the real-time detection reverse transcription-polymerase chain reaction (RTD-PCR) using an ABI Prism 7500 fast sequence detector system (Applied Biosystems, Tokyo, Japan) [18].

2.11. Immunoprecipitation of HCV particles

Protein G-Sepharose (GE Healthcare, Little Chalfont, UK) was mixed with DMEM-10 for 1 h at 4 °C, and was spun down by centrifugation for 1 min at 5000 rpm (TOMY, Tokyo, Japan). HCV particles (1×10^7 copies HCV-RNA) were mixed with the resin and were incubated overnight at 4 °C with rotation. The sample was centrifuged for 1 min at 5000 rpm, and the supernatant was then collected. A 7.5 μL aliquot of anti-human ApoB (AB742, Millipore, Billerica, MA) or anti-human ApoE polyclonal antibody (AB947, Millipore) was added into the pre-cleared virus fluid (100 μL), and the mixture was incubated overnight at 4 °C. Mouse IgG (5 μg, Sigma) was used as a control. The mixture was mixed with the resin and incubated for 1 h at 4 °C, with rotation. The supernatants were collected following centrifugation and the pellets were then washed twice with PBS and suspended in DMEM-10. Viral RNA was eluted from both the supernatants and the suspended pellets using the QIAamp Viral RNA mini kit (Qiagen). The HCV-RNA titer present in each total RNA from the supernatant and the pellet was evaluated, and the infectivity of the supernatant was measured by inoculation of naïve Huh7.5.1 cells.

3. Results

3.1. Establishment of serum-free cultured Huh7 cells

Huh7 cells are routinely maintained in our laboratory by culture in 10% FCS-supplemented medium. To examine HCV particles produced from infected cells cultured under serum-free conditions, we first established a serum-free culture system which allowed the proliferation of Huh7 cells. It was previously reported that Huh7 cells could be cultured in serum-free media that contains selenium [6]. We therefore examined the growth of Huh7 cells following gradual reduction of the level of FBS and ultimately culture in completely serum-free, selenium-supplemented (ITS-containing) media. The cells could be passaged and cultured over a long period in this medium, although the observed growth, as assayed using an MTS assay, was slightly lower than that of FBS-supplemented cultures for all the cell lines used in this study (Fig. 1 and Supplementary Fig. S1). Based on this result, we used ITS-supplemented media for the evaluation of serum-free cultured HCV.

3.2. Production of HCV particles from serum-free cultures

We next tested the efficiency of HCV particle production in serum-free culture. *In vitro* transcribed HCV-RNAs were transfected into the CD81-negative Huh7-25, and the CD81 positive Huh7-25-CD81 cell lines. The re-infection rate is known to be negligible when Huh7-25 is used [19]. When synthetic RNAs of JFH-1 or J6/JFH-1 strains were transfected, the HCV-core protein and HCV-RNA were detected in the culture media, and each medium was infectious for naïve Huh7 cells (Fig. 2, Supplementary Table). The specific infectivity of each medium (the values of the infectivity titer divided by the values of the HCV-core protein or of HCV-RNA) of J6/JFH-1 HCV was higher than that of JFH-1 (Fig. 2C, Supplementary Table). These results showed that infectious HCV was secreted into the culture medium of both cell lines. The slightly higher HCV production of Huh7-25-CD81 cells may be due to re-infection of secreted virus particles. To determine if HCV-infected cells could be cultured for several passages in serum-free medium, serum-free cultured Huh7 cells were inoculated with infectious J6/JFH-1 chimeric HCV at multiplicity of infection (MOI) of 0.2 and were then cultured for a long period, following which the HCV-core protein and HCV-RNA in the culture medium was analyzed. The HCV-core protein and HCV-RNA were continuously detected in serum-free media, and their level was almost equal to that of infected FBS-supplemented Huh7 culture (Fig. 3).

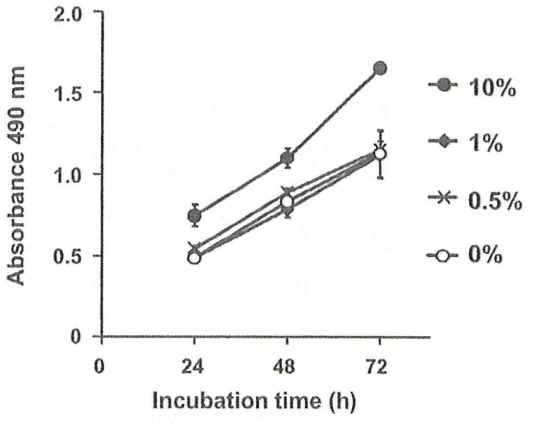

Fig. 1. Cell proliferation assay of serum-free cultured Huh7 cells. Huh7 cells that were seeded into a 96-well plate (1×10^4/well) were sequentially grown in DMEM/F-12 media containing 10%, 1%, 0.5% and 0% fetal bovine serum. At indicated times, an MTS cell proliferation assay was performed using a commercial kit.

3.3. Characterization of serum-free cultured HCV by sucrose density gradient analysis

We next compared the characteristics of HCV viruses produced under serum-free and serum-supplemented conditions by density gradient analysis. Each infectious supernatant was layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Eighteen fractions were obtained and HCV-core and RNA titers of each fraction were determined. The detected virus titers in each density fraction were different mainly due to differences in the amount of input virus, as shown in the supplementary table. As previously reported, infectivity of all viruses was observed in fractions of lower density (approximately 1.10 g/mL sucrose) than those in which the peaks of HCV-core and HCV-RNA were detected (Fig. 4), although the specific infectivity of serum-free cultured HCV was slightly lower than that of FBS-supplemented HCV. These results suggested that the infectious HCV produced in the media by serum-free cultures had similar characteristics to those of HCV produced by serum-supplemented cultures. In addition, the virus particles produced from CD81-positive and -negative cells exhibited similar density profiles (Compare Fig. 4A, B vs. C, D).

3.4. Antibodies differentially inhibit HCV from serum-free and serum supplemented cultures

We next examined antibody inhibition of cell infection by HCV derived from serum-free or serum-supplemented cultures. CD81 and SR-BI are candidate cellular receptors for HCV infection. We first determined the inhibitory effect of anti-CD81 and anti-SR-BI antibodies on infection of serum-free cultured HCV. Interestingly, HCV infection by HCV derived from serum-free and serum-supplemented cultures was differently inhibited by these

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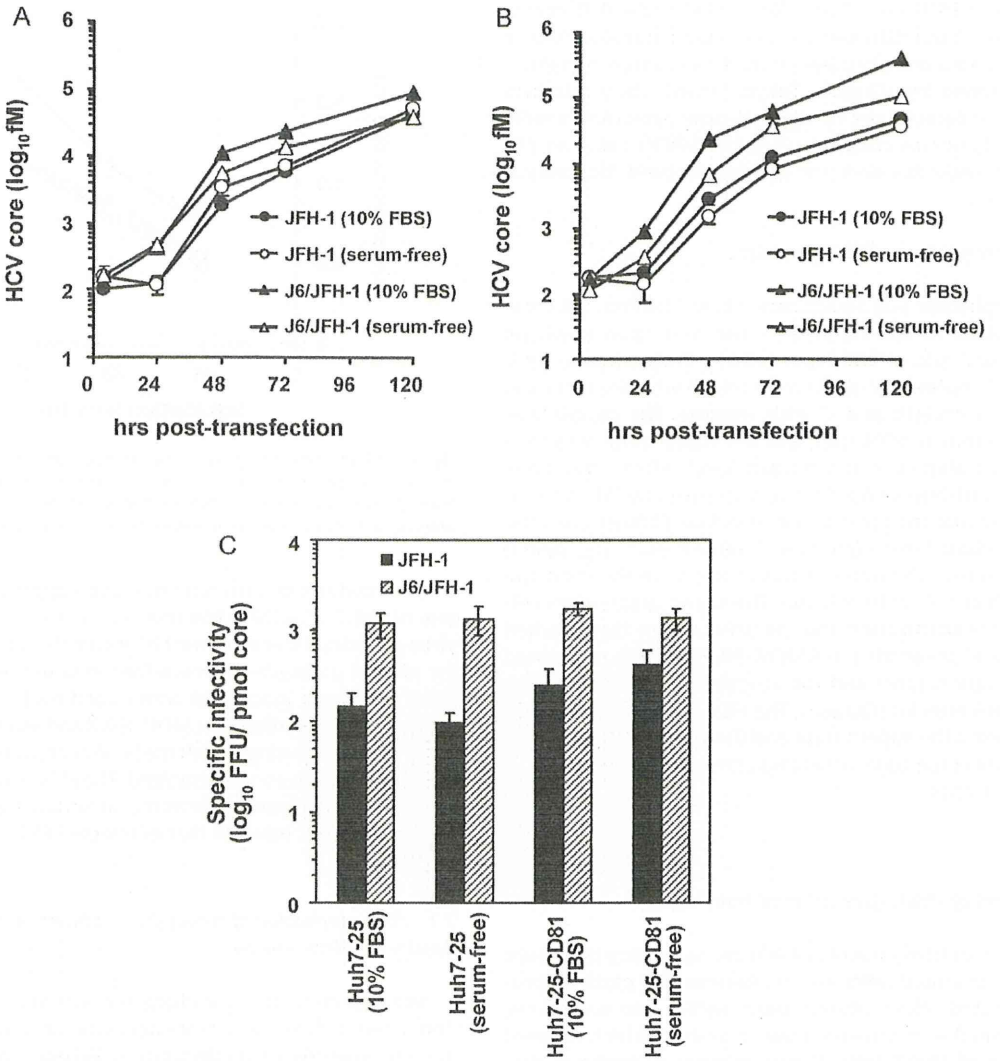

Fig. 2. HCV production from serum-free cultured Huh7 cells transfected with *in vitro* transcribed JFH-1 and J6/JFH-1 RNA. *In vitro* transcribed JFH-1 and J6/JFH-1 RNA was transfected into Huh7-25 (A) and Huh7-25-CD81 (B) cells that were grown under the indicated serum conditions. The culture supernatant was collected 4, 24, 48, 72 and 120 h post-transfection, and the HCV-core protein levels were analyzed using ELISA. All data were measured in triplicate, and are shown as means ± SD. Infectivity of each supernatant that was collected 120 h post-transfection was analyzed by infectivity titration, and specific infectivity was calculated by dividing the mean value of the infectivity titer by that of the HCV-core protein (C). All data were measured in triplicate, and are shown as means ± SD. Profiles of HCV-core, HCV-RNA and infectivity are indicated in the Supplementary Table.

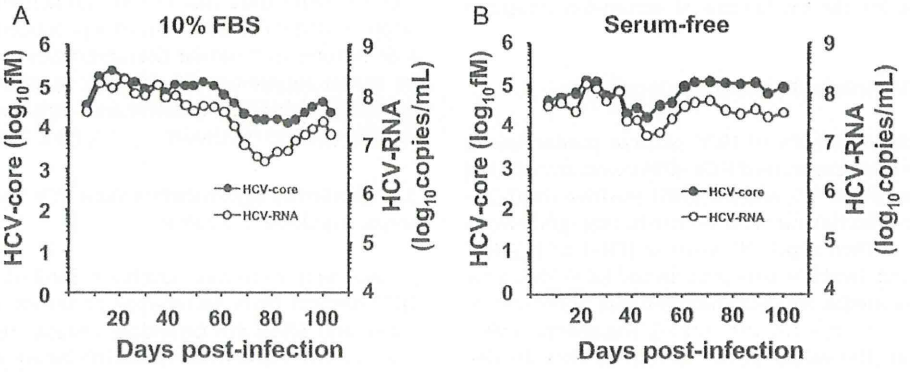

Fig. 3. HCV production from serum-free cultured Huh7 cells infected with J6/JFH-1 HCV. Huh7 cells that were grown in 10% FBS-supplemented (A) or serum-free (B) DMEM/F-12 were inoculated with the J6/JFH-1 virus (MOI, 0.2), and media of sub-cultures were collected. The HCV-core (closed circles) and RNA (open circles) were analyzed using ELISA and RTD-PCR, respectively.

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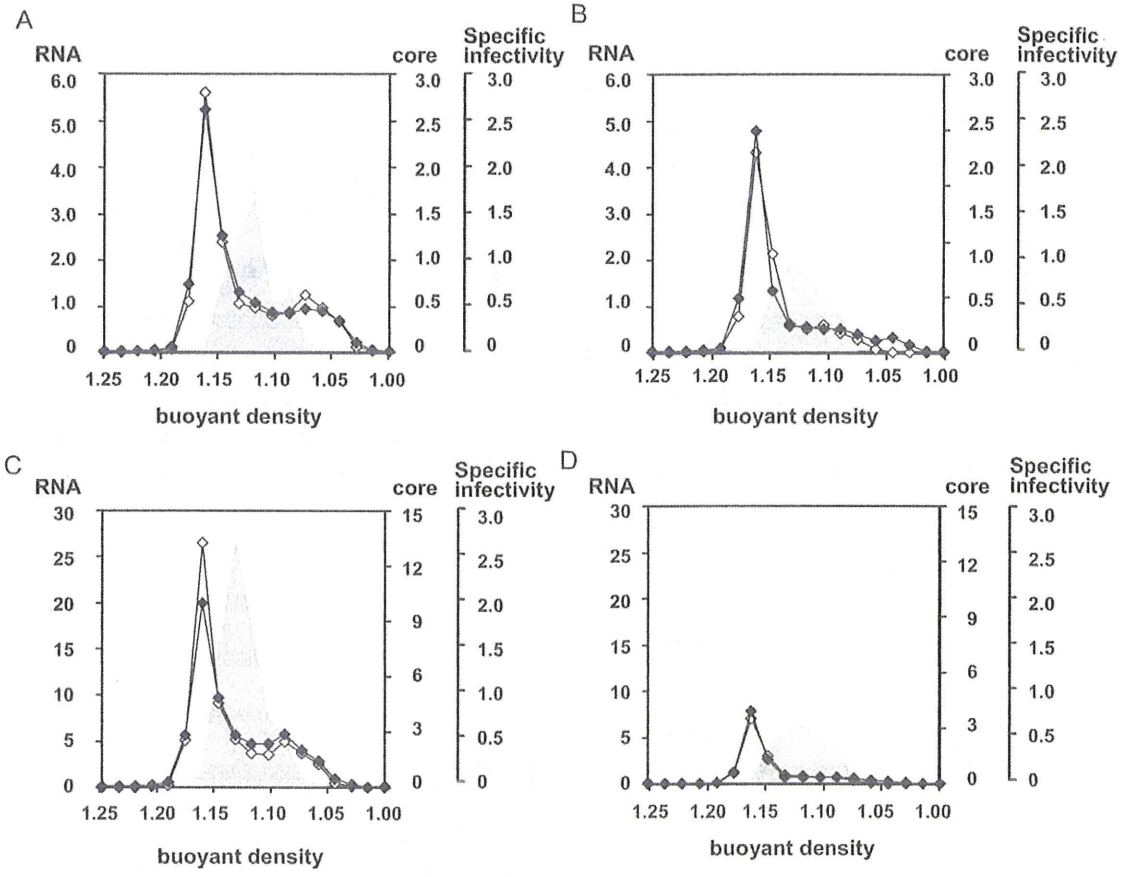

Fig. 4. Density gradient analysis of the supernatants derived from infected serum-free cultured Huh7 cells. *In vitro* transcribed J6/JFH-1 RNA was transfected into Huh7-25 (A and B) and Huh7-25-CD81 (C and D) cells that were cultured under 10% FBS-supplemented (A and C) or serum-free (B and D) conditions. Culture supernatants (4 mL) were collected 5 days post-transfection, and were then layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged using an SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (500 μL each) were collected from the bottom of the tube. The buoyant density (g/mL, *x*-axis), the levels of HCV-core ($\times 10^2$ pM, closed diamonds) and HCV-RNA ($\times 10^8$ copies/mL, open diamonds), and the specific infectivity for naïve Huh7.5.1 cells (FFU/pmol core, shown in gray) of each fraction were analyzed as described in Section 2.

antibodies (Fig. 5A and B). Next, to confirm that the anti-E2 antibody, which has been shown to bind HCV particles, inhibits HCV infection, HCV was pre-incubated with the anti-E2 antibody AP33 and inoculated into Huh7 cells. As shown in previous reports [20,21], AP33 inhibited HCV infection. However, its inhibitory effect was different for serum-free and serum-supplemented cultured HCV. Thus, infection of serum-free cultured HCV displayed the highest susceptibility to this antibody (Fig. 5C).

It has also been recently reported that VLDL associates with HCV and affects infectious particle formation and infection [7–9,22,23]. We therefore determined whether apolipoproteins associate with serum-free cultured HCV by immunoprecipitation of apolipoproteins from the culture media with anti-human apolipoprotein antibodies, followed by analysis of the viral titer in the pellet and the supernatant. HCV particles from both serum-free and serum-supplemented cultures were associated with both ApoB and ApoE (Fig. 6A). The percent of HCV from FBS-supplemented and serum-free cultures respectively that was associated with ApoB was $13.22 \pm 0.09\%$ and $16.84 \pm 0.08\%$ ($p < 0.05$, *t*-test) and the percent associated with ApoE was $20.77 \pm 0.33\%$ and $10.04 \pm 0.04\%$ ($p < 0.005$, *t*-test). Thus, serum-free HCV particles had a larger amount of associated ApoB, and a smaller amount of ApoE, than HCV from serum-supplemented cultures. We next determined whether depletion of ApoE affects viral infectivity by measurement of the infectivity titers of the virus in the supernatant following ApoE precipitation. This experiment showed that the infectivity of HCV from FBS-supplemented cultures, but not of HCV from serum-free cultures, was down-regulated by depletion of ApoB and ApoE (Fig. 6C). These results indicated that apolipoprotein associates differently with viral particles derived from FBS-supplemented and serum-free cultures, and, further, that the infectivity of HCV derived from serum-free culture is only weakly affected by the associated apolipoprotein. These data therefore suggest that, unlike HCV from serum supplemented culture, and in contrast to previous reports regarding HCV infection, infection of HCV derived from serum-free culture may be apolipoprotein-independent. However, further studies are required to confirm this possibility.

4. Discussion

In this study, we established a serum free cell culture system for the production of HCV particles, and compared the characteristics of these particles to those of HCV particles derived from serum-supplemented cultures. The particles derived from serum-free culture were infectious, suggesting that these particles would provide an appropriate antigen for the development of antibodies and vaccines. The serum-free cultured HCV could infect naïve Huh7 cells. Furthermore, sucrose density gradient analysis indicated that the profiles of HCV-core protein and HCV-RNA of serum-free cultured HCV were almost the same as those of HCV from FBS-supplemented cultures. Under serum-free conditions, HCV components (core protein and RNA) tended to be

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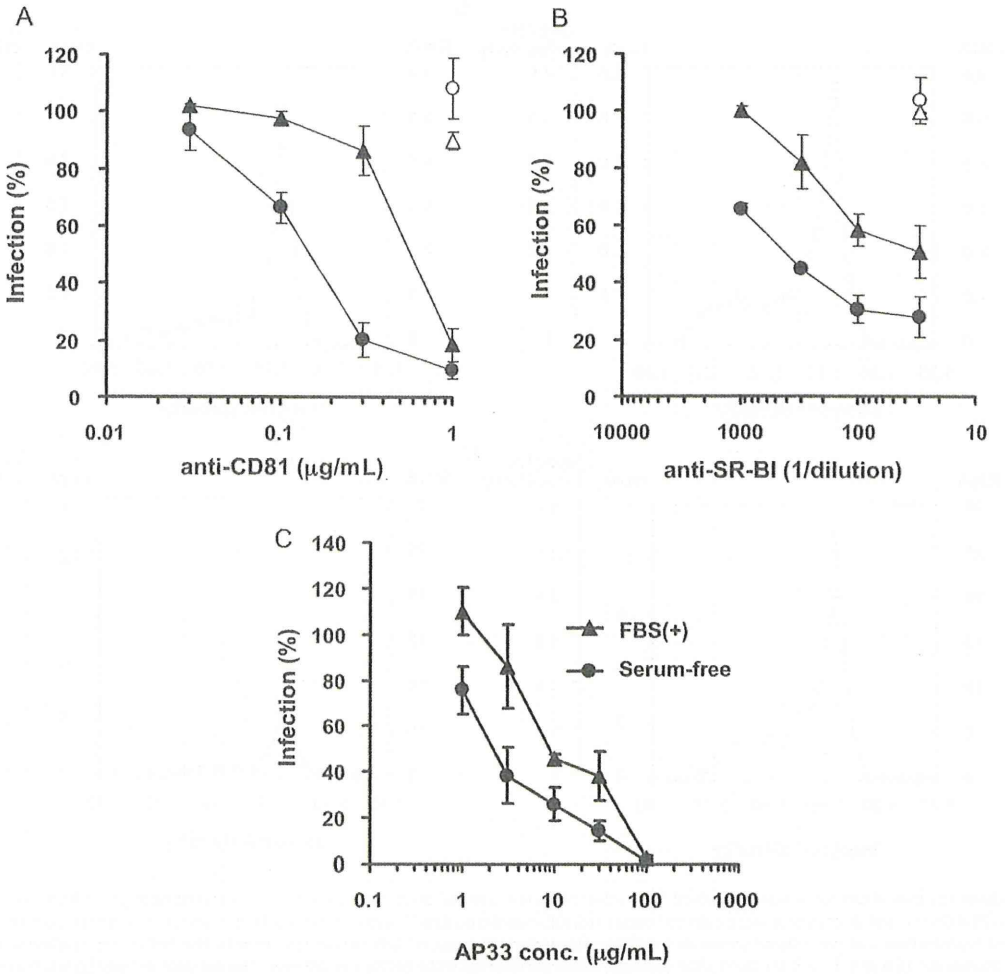

Fig. 5. Inhibition of serum-free cultured HCV infection by anti-CD81, anti-SR-BI and anti-E2 antibodies. Huh7.5.1 cells were pre-incubated with serially diluted anti-CD81 (JS-81, panel A) or anti-SR-BI (panel B) antibody for 1 h, and infectious J6/JFH-1 virus (MOI 0.1) obtained from FBS-supplemented (triangles) or serum-free (circles) culture was inoculated into each well for 72 h. Cells were lysed and HCV-core protein was measured. As controls, 1 μg/mL of mouse IgG (open triangles) and 30-fold diluted rat serum (open circles) were used. For incubation of virus particles with antibody, infectious J6/JFH-1 virus obtained from FBS-supplemented (closed triangle) or serum-free (closed circle) cultures was mixed with the indicated concentration of anti-E2 (AP33, panel C) antibody, and was then inoculated into naïve Huh7.5.1 cells. Infectivity was calculated by titration.

lost from fractions of lower density. Since exosome-like multivesicular particles have been reported to be present in lower density fractions [10], production of multivesicular particles may be different between serum-free and serum-supplemented cultures. When culture supernatants were used, the specific infectivity of serum-free cultured HCV was almost identical to that of FBS-supplemented HCV. However, infectivity of fractions separated on sucrose density gradients was lower for serum-free cultured HCV than for FBS-supplemented HCV cultures. These data may indicate that host factors that contribute to HCV infection differed under different culture conditions. Following infection and subsequent long-term culture under either serum-free or serum-supplemented conditions, HCV-core and RNA levels were identical for the first 40 days, but after this time period the ratio of HCV-core protein:RNA increased in both cultures. This result suggested that long-term culture may induce HCV-core protein which did not have HCV-RNA. As shown in Fig. 3, virus production under serum-free conditions seems to be more stable over 100 days than production under 10% FBS conditions. Fluctuation of viral titer during continuous cultivation of HCV-infected cells has been previously reported [24]. This fluctuation is dependent on the appearance of non-permissive cells with low or no CD81 expression. It is thus likely that these non-permissive cells appear more rapidly under 10% FBS culture conditions than under serum-free conditions. This result may be due to the higher cellular growth rate of these cells in serum-supplemented culture as shown in Fig. 1 and Supplementary Fig. S1.

Our study highlighted some differences between HCV that was produced under serum-free and serum-supplemented conditions. Thus, interestingly, serum-free cultured HCV showed a higher susceptibility to antibody inhibition of viral infection than HCV from serum-supplemented cultures. The multiplicity of infection (MOI) of partially purified HCV was calculated and an MOI of 0.02 was used for HCV infection of naïve Huh7 cells. Although infection by HCV produced under both culture conditions was inhibited by anti-CD81, anti-SR-BI and anti-E2 antibodies in a dose-dependent manner, the degree of antibody inhibition at each dose was stronger for serum-free cultured HCV than for HCV from serum-supplemented cultures (Fig. 5). The anti-CD81 and anti-SR-BI antibody targets the host cells, since CD81 and SR-BI are candidate cellular receptors for HCV. In contrast, the anti-E2 antibody AP33 is a neutralizing antibody for HCV [20,21], and targets virus particles. The antibody inhibition result obtained therefore suggested that HCV infection was affected by contaminating pro-

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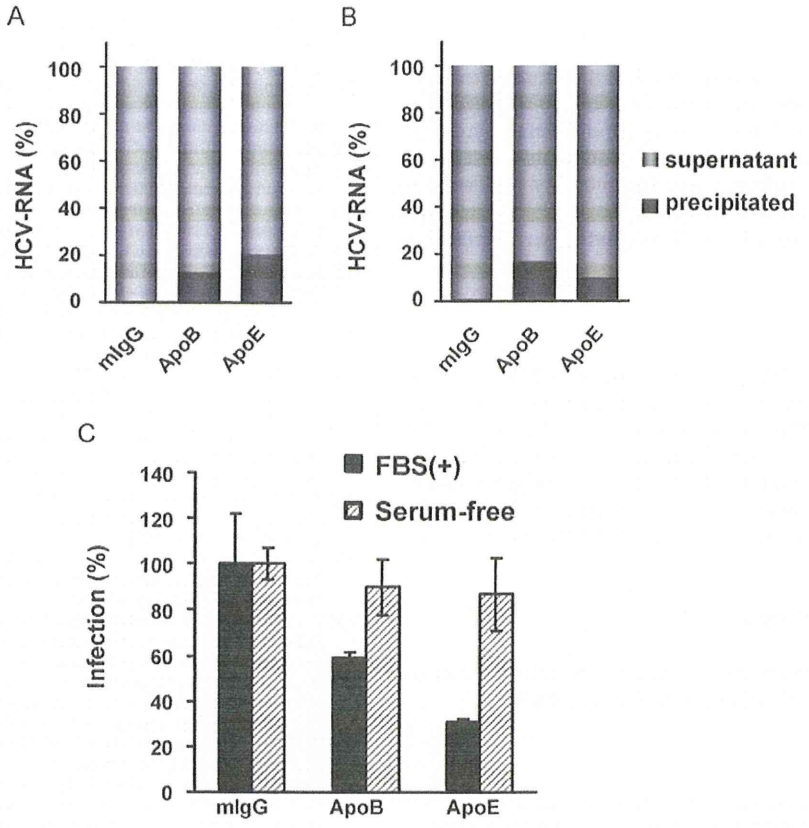

Fig. 6. Immunoprecipitation of cell-cultured HCV with anti-ApoB and ApoE antibodies. 1×10^7 HCV-RNA copies of the J6/JFH-1 virus were incubated with mouse IgG, anti-ApoB or anti-ApoE antibodies, and were then precipitated using protein G-Sepharose. Virus RNA was extracted from the supernatant and the precipitated resin, and HCV-RNA was analyzed using RTD-PCR. All experiments were performed in triplicate, and data are shown as percentages of the mean (A: serum-supplemented, B: serum-free cultured, HCV). Infectivity of the antibody-depleted virus (supernatant) was determined by inoculation into naïve Huh7.5.1 cells. All experiments were performed in triplicate, and data are shown as means ± SD. All data are indicated as percentage of infection, in which the infectivity (FFU/mL) of the supernatant from the mouse IgG (mIgG) precipitation was designated as 100% (C).

teins or by characteristics of HCV that arose due to the different culture conditions used for HCV production. Since CD81-negative Huh7 cells were also able to produce infectious HCV, and since anti-CD81 and anti-E2 antibodies similarly inhibited infection by HCV produced in serum-free culture (data not shown), it is highly unlikely that viral incorporation of CD81 that is produced by CD81-expressing virus-producing cells modulates anti-CD81-inhibition of viral infection. An alternative possibility was that the different antibody inhibition results may have been due to differences in the level, or type of apolipoprotein associated with HCV. HCV associates with lipoproteins in human sera [25], and it has been reported that the envelope proteins of HCV interact with lipoproteins [26]. Moreover, in a recent study, VLDL was reported to associate with HCV particles and affect viral infection and virus secretion [22,23]. Mature VLDL is composed of the apolipoproteins B, C-II and E, as well as lipid, and ApoB and ApoE have been reported to be important for the infectivity of HCV particles [22,23,27–29]. We found that the content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different (Fig. 6A). Thus, serum-free HCV particles had a greater amount of associated ApoB, and a lesser amount of ApoE, than HCV from serum-supplemented cultures. Since ApoE is usually only found in mature VLDL, the serum-free cultured HCV may associate with immature VLDL. A second difference related to associated apolipoprotein of HCV grown under different serum conditions is that the infectivity of HCV from serum-supplied culture, but not that of serum-free cultured HCV, was down-regulated by antibody depletion of ApoB and ApoE. This result suggested that the infection of HCV from serum-supplemented culture, but not infection of serum-free cultured HCV, depended on apolipoproteins. It is possible that production of HCV in serum-free culture results in the formation of HCV particles that differ in lipid and lipoprotein composition from particles produced under serum-supplemented conditions. Consequently, the affinity of serum-free cultured HCV for its cellular receptor might have been altered, and its susceptibility to antibody inhibition of infectivity may have been increased. However, further analysis of the lipid content of HCV grown under different serum conditions is necessary to confirm this point.

Production of HCV using a serum-free culture system has advantage for vaccine development because there is low protein contamination, it facilitates simple viral purification procedures, and it does not involve the use of animals. Furthermore, lipoproteins associated with virus particles may shield conserved epitopes. Purified virus particles produced under serum-free conditions may have these shielded epitopes exposed and thus potentially become stronger immunogens for the induction of neutralizing antibodies. In this study, serum-free cultured HCV was infectious *in vitro*, and the viral particles had a form similar to that of native enveloped viruses. However, we have not yet confirmed the antigenic or immunogenetic properties of serum-free cultured HCV, which would be important for its potential use as a vaccine. Nevertheless, we have recently designed a system for the purification of HCV particles (Morikawa, unpublished data) which will allow testing of these parameters. If these purified particles have high antigenicity, then a HCV vaccine that is derived from HCV produced in cell culture may be available in the future.

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5. Conclusions

Infectious HCV could be efficiently produced in serum-free culture. However, the serum-free cultured HCV was highly susceptible to anti-CD81, anti-SR-BI and anti-E2 inhibition of infectivity. The content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different and the infectivity of serum-free cultured HCV appeared to be independent of apolipoproteins. Therefore, lipids conjugated with HCV may affect virus infection and neutralization.

Acknowledgements

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.069.

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In Vivo Adaptation of Hepatitis C Virus in Chimpanzees for Efficient Virus Production and Evasion of Apoptosis

Mohsan Saeed,[1,2] Masaaki Shiina,[3] Tomoko Date,[1] Daisuke Akazawa,[1] Noriyuki Watanabe,[1] Asako Murayama,[1] Tetsuro Suzuki,[1] Haruo Watanabe,[2,4] Nobuhiko Hiraga,[5] Michio Imamura,[5] Kazuaki Chayama,[5] Youkyung Choi,[6] Krzysztof Krawczynski,[6] T. Jake Liang,[7] Takaji Wakita,[1] and Takanobu Kato[1]

Hepatitis C virus (HCV) employs various strategies to establish persistent infection that can cause chronic liver disease. Our previous study showed that both the original patient serum from which the HCV JFH-1 strain was isolated and the cell culture–generated JFH-1 virus (JFH-1cc) established infection in chimpanzees, and that infected JFH-1 strains accumulated mutations after passage through chimpanzees. The aim of this study was to compare the *in vitro* characteristics of JFH-1 strains emerged in each chimpanzee at early and late stages of infection, as it could provide an insight into the phenomenon of viral persistence. We generated full-genome JFH-1 constructs with the mutations detected in patient serum-infected (JFH-1/S1 and S2) and JFH-1cc–infected (JFH-1/C) chimpanzees, and assessed their effect on replication, infectious virus production, and regulation of apoptosis in cell culture. The extracellular HCV core antigen secreted from JFH-1/S1-, S2-, and C-transfected HuH-7 cells was 2.5, 8.9, and 2.1 times higher than that from JFH-1 wild-type (JFH-1/wt) transfected cells, respectively. Single cycle virus production assay with a CD81-negative cell line revealed that the strain JFH-1/S2, isolated from the patient serum-infected chimpanzee at a later time point of infection, showed lower replication and higher capacity to assemble infectious virus particles. This strain also showed productive infection in human hepatocyte–transplanted mice. Furthermore, the cells harboring this strain displayed lower susceptibility to the apoptosis induced by tumor necrosis factor α or Fas ligand compared with the cells replicating JFH-1/wt. *Conclusion:* The ability of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis may be important for prolonged infection *in vivo*. Such control of viral functions by specific mutations may be a key strategy for establishing persistent infection. (HEPATOLOGY 2011;00:000–000)

Currently, approximately 200 million people are infected with hepatitis C virus (HCV) and are at continuous risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and hepatocellular carcinoma.[1,2] Although acute HCV infection elicits innate and adaptive immune responses, the virus successfully evades clearance in approximately 75% of infected individuals.[3,4] The mechanisms by

Abbreviations: Ag, antigen; CTL, cytotoxic T lymphocytes; FasL, Fas ligand; HCV, hepatitis C virus; JFH-1cc, cell culture–generated JFH-1 virus; JFH-1/wt, JFH-1 wild-type; MFI, mean fluorescence intensity; NK, natural killer; NS, nonstructural; PARP, poly(adenosine diphosphate ribose) polymerase; TNF-α, tumor necrosis factor α; TUNEL, terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling.

From the [1]Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan; the [2]Department of Infection and Pathology, Graduate School of Medicine, The University of Tokyo, Tokyo, Japan; the [3]Division of Gastroenterology, Tohoku University Graduate School of Medicine, Sendai, Japan; the [4]National Institute of Infectious Diseases, Tokyo, Japan; the [5]Department of Medicine and Molecular Science, Division of Frontier Medical Science, Programs for Biomedical Research, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan; the [6]Division of Viral Hepatitis, Center for Disease Control and Prevention, Atlanta, GA; and the [7]Liver Diseases Branch, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, MD.

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Tetsuro Suzuki is currently affiliated with the Department of Infectious Diseases, Hamamatsu University School of Medicine, Hamamatsu, Japan.

Address reprint requests to: Takanobu Kato, M.D., Ph.D., Department of Virology II, National Institute of Infectious Diseases, Tokyo, 162-8640, Japan. E-mail: takato@nih.go.jp; fax: (81)-3-5285-1161.

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which HCV leads to persistent infection at a high frequency are not yet fully understood. Lack of appropriate animal models, except chimpanzees, has rendered such studies difficult. Human hepatocyte-transplanted mice,[5,6] a useful small animal model to study HCV infection, are unsuitable to study the mechanisms of virus persistence because of a lack of B and T cell–mediated immunity.

HCV is a noncytopathic positive-stranded RNA virus of the *Flaviviridae* family. It primarily infects hepatocytes of humans and chimpanzees, where, thanks to error-prone RNA-dependent RNA polymerase, the infected virus accumulates a high number of mutations rapidly, thus providing opportunity for selection of viruses that have the ability to escape the immune system and establish persistent infection. Deciphering the strategies employed by HCV to establish persistence can be helpful in the development of new strategies to eradicate the virus and to stop disease progression. Until recently, the lack of an HCV strain having the ability to establish infection *in vivo* and *in vitro* was a substantial hindrance in studying the molecular mechanisms of virus persistence. This problem was solved by the identification of an HCV strain, JFH-1, that was isolated from a fulminant hepatitis patient and found to be capable of replicating and assembling infectious virus particles in chimpanzees as well as in cell culture.[7-10] This clone can be used to study the molecular mechanisms by which HCV evades the host immune system and causes chronic infection.

In a previous report, we inoculated patient serum from which the JFH-1 strain was originally isolated and cell culture–generated JFH-1 virus (JFH-1cc) into two different chimpanzees.[11] HCV established infection in both animals within 3 days of inoculation. In the JFH-1cc–infected chimpanzee, genome sequence of predominant infecting virus at week 2 was identical to JFH-1 wild-type (JFH-1/wt [in this study, this abbreviation was used instead of JFH-1 to distinguish it from other variant strains]), and the infecting virus has four synonymous and seven nonsynonymous mutations at week 7. In the JFH-1 patient serum–infected chimpanzee, 19 synonymous and six nonsynonymous mutations were observed in predominantly circulating virus at week 2, and this number increased to 35 synonymous and 17 nonsynonymous mutations at the later stage of infection course (week 23).[11] From these observations, we presumed that the isolates evolved in each chimpanzee at later stages of infection might have some advantage over the viruses isolated at earlier time points for survival in infected animals. Thus, in this study, we generated JFH-1 variants containing the mutations observed in these animals and assessed their effect on replication and infectious virus production in cell culture. Furthermore, we examined the effects of infection of these strains to tumor necrosis factor α (TNF-α)– or Fas ligand (FasL)–mediated apoptosis.

Materials and Methods

The complete Materials and Methods are provided in the Supporting Information.

Results

Effects of Mutations Identified in Chimpanzees. To investigate the effect of mutations on virus phenotype, we generated constructs containing the mutations observed in JFH-1 patient serum–infected chimpanzee and JFH-1cc–infected chimpanzee at various time points. The JFH-1 variants JFH-1/S1 and JFH-1/S2 contain the mutations observed in the patient serum–infected chimpanzee at week 2 and week 23, respectively, and JFH-1/C contains the mutations observed in the JFH-1cc–infected chimpanzee at week 7 (Supporting Table 1). The replication and virus production capacity of these variants in HuH-7 cells was compared with that of JFH-1/wt. After electroporation of *in vitro*–synthesized full-genome RNA of JFH-1/wt and variant strains, extracellular and intracellular HCV RNA and core antigen (Ag) were measured (Fig. 1). At day 5 posttransfection, all constructs displayed similar intracellular HCV RNA levels. However, extracellular HCV RNA level of JFH-1/C was 1.6 times higher than that of JFH-1/wt. Likewise, extracellular HCV RNA level of JFH-1/S2 was 3.4 times higher than that of JFH-1/S1 (Fig. 1A). Intracellular HCV core Ag levels of JFH-1/S2 and C were 240.9 ± 58.2 and 189.8 ± 42.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/S1 (526.1 ± 58.2 fmol/mg protein) and JFH-1/wt (511.7 ± 32.9 fmol/mg protein) at day 1, but reached comparable levels at day 5 posttransfection. On the other hand, extracellular HCV core Ag level of JFH-1/C was 2.2 times higher than that of JFH-1/wt, and that of JFH-1/S2 was 3.6 times higher than that of JFH-1/S1 at day 5 posttransfection (Fig. 1B). Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

Single Cycle Virus Production Assay. For detailed analysis of the effects of these mutations on different stages of the virus lifecycle, we used a Huh7-25 cell

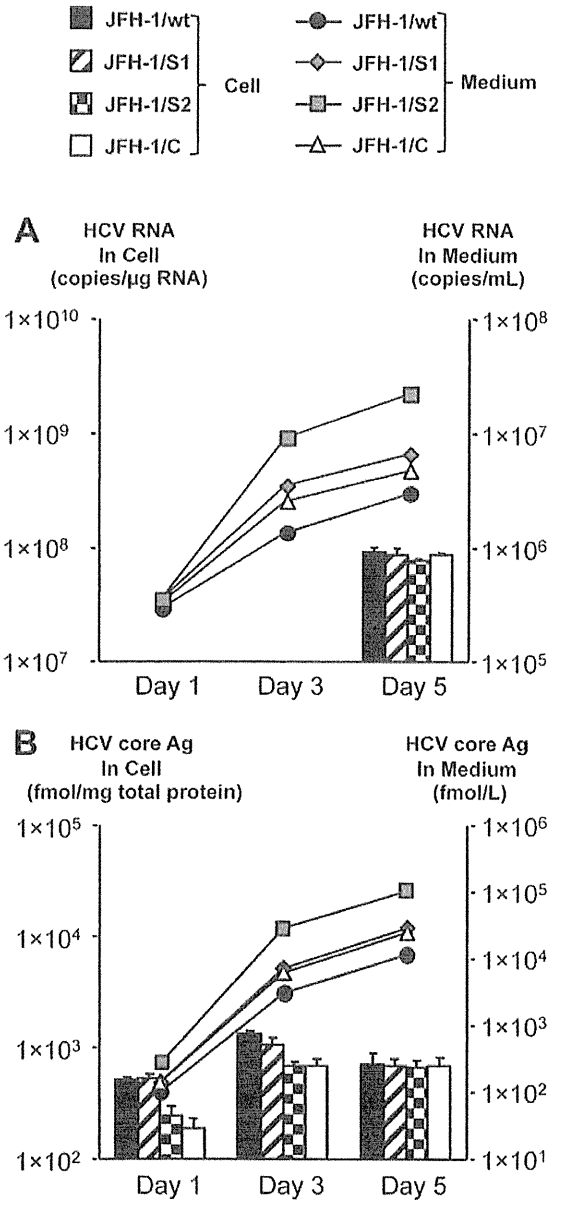

Fig. 1. Effects of *in vivo* adaptive mutations on virus production in HuH-7 cells. One million cells were transfected with 10 μg *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean ± SD.

line that lacks the surface expression of CD81, one of the cellular receptors for HCV entry. Three days after transfection with full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, HCV RNA levels and infectivity titer were measured, and the specific infectivity was calculated (Table 1). Intracellular HCV RNA levels of JFH-1/C and JFH-1/S2 were lower than those of JFH-1/wt and S1, suggesting lower replication efficiency of these strains. However, the intracellular infectivity titers of JFH-1/C and JFH-1/S2 were 2.03 and 11.0 times higher than those of JFH-1/wt and JFH-1/S1, respectively ($P < 0.005$). Intracellular-specific infectivities (infectivity titer/HCV RNA copy number) of JFH-1/C and JFH-1/S2 showed more pronounced difference from those of JFH-1/wt and JFH-1/S1 (3.92 times and 12.9 times higher, respectively; $P < 0.005$). The infectious virus secretion rate (extracellular infectivity titer/intracellular infectivity titer) was not significantly different between JFH-1/wt and variant strains. These data indicate that mutations identified in chimpanzees at the later time point of infection led to reduced viral replication and increased assembly of infectious virus particles without any effect on viral release in cell culture.

Subgenomic Replicon Assay. To further confirm the replication efficiencies of strains observed in chimpanzees, we generated subgenomic replicons of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C carrying the firefly luciferase reporter gene (SGR-JFH-1/Luc/wt, SGR-JFH-1/Luc/S1, SGR-JFH-1/Luc/S2, and SGR-JFH-1/Luc/C). *In vitro*–transcribed RNAs of these constructs were transfected into HuH-7 cells, and luciferase activity was measured to assess their replication capacity. The luciferase activities of SGR-JFH-1/Luc/C and SGR-JFH-1/Luc/S2 replicons were 7.30 and 7.33 times lower than those of SGR-JFH-1/Luc/wt and SGR-JFH-1/Luc/S1, respectively, at day 1 ($P < 0.00005$), suggesting attenuated replication capacities of variant replicons isolated from each animal at later time points of infection (Supporting Fig. 1A). The luciferase activity 4 hours after transfection was comparable, indicating similar levels of transfection efficiency (data not shown). Based on these data, we found that the mutations that emerged in nonstructural (NS)3-NS5B of JFH-1/S2 and JFH-1/C reduced the replication efficiency in cell culture.

Genomic Regions Responsible for Lower Replication and Higher Assembly of JFH-1/S2. To further clarify the genomic region responsible for lower replication efficiency and higher assembly rate of JFH-1/S2, we generated the chimeric constructs JFH-1/S2-wt and JFH-1/wt-S2 as described in the Supporting Materials and Methods. *In vitro*–transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 were introduced into HuH-7 cells by electroporation and intracellular and extracellular HCV RNA and core Ag were measured. At day 5 posttransfection, all constructs displayed comparable intracellular HCV RNA levels (Fig. 2). However, extracellular HCV RNA levels of JFH-1/S2 and JFH-1/S2-wt were significantly

Table 1. Infectious Virus Production and Release of JFH-1/wt and Variants in Huh7-25 Cells

Strain	Intracellular			Extracellular	Secretion Ratio (Extracellular/ Intracellular)
	HCV RNA (copies/μg RNA)	Infectivity Titer (ffu/well)	Specific Infectivity (ffu/copies)	Infectivity Titer (ffu/well)	
JFH-1/wt	$7.75 \times 10^8 \pm 1.04 \times 10^8$	$4.21 \times 10^2 \pm 4.32 \times 10^1$	$2.09 \times 10^{-7} \pm 7.06 \times 10^{-8}$	$1.94 \times 10^3 \pm 3.76 \times 10^1$	4.6 ± 1.3
JFH-1/S1	$7.04 \times 10^8 \pm 8.49 \times 10^7$	$4.72 \times 10^2 \pm 5.63 \times 10^1$	$2.91 \times 10^{-7} \pm 6.00 \times 10^{-8}$	$3.02 \times 10^3 \pm 2.77 \times 10^2$	5.4 ± 2.0
JFH-1/S2	$4.16 \times 10^{8}** \pm 7.47 \times 10^6$	$5.19 \times 10^{3}** \pm 8.24 \times 10^1$	$3.76 \times 10^{-6}** \pm 7.01 \times 10^{-7}$	$3.23 \times 10^{4}** \pm 3.52 \times 10^3$	6.2 ± 3.0
JFH-1/C	$3.15 \times 10^{8}* \pm 5.02 \times 10^7$	$8.59 \times 10^{2}* \pm 4.81 \times 10^1$	$8.19 \times 10^{-7}* \pm 5.68 \times 10^{-8}$	$3.68 \times 10^3 \pm 3.02 \times 10^3$	4.3 ± 1.4
JFH-1/S2-wt	$7.07 \times 10^8 \pm 8.43 \times 10^7$	$4.40 \times 10^{3}* \pm 9.5 \times 10^1$	$2.73 \times 10^{-6}* \pm 2.35 \times 10^{-7}$	$3.0 \times 10^{4}* \pm 1.1 \times 10^3$	6.7 ± 0.7
JFH-1/wt-S2	$4.21 \times 10^{8}* \pm 1.97 \times 10^7$	$2.7 \times 10^2 \pm 2.9 \times 10^1$	$2.02 \times 10^{-7} \pm 4.0 \times 10^{-8}$	$1.7 \times 10^3 \pm 1.3 \times 10^2$	4.5 ± 0.4

Abbreviation: ffu, focus-forming units.
*$P < 0.005$ versus JFH-1/wt.
**$P < 0.005$ versus JFH-1/S1.

higher ($P < 0.0005$) than that of JFH-1/wt. On the other hand, extracellular RNA level of JFH-1/wt-S2 chimeric construct was lower than that of JFH-1/S2 and JFH-1/S2-wt and similar to that of JFH-1/wt. Likewise, extracellular core Ag levels of JFH-1/S2 and JFH-1/S2-wt were also significantly higher than that of JFH-1/wt. Intracellular HCV core Ag levels of JFH-1/S2 and JFH-1/wt-S2 on day 1 posttransfection were 240.9 ± 58.2 and 134.3 ± 17.1 fmol/mg protein, respectively, and were significantly lower ($P < 0.005$) than that of JFH-1/wt (526.1 ± 58.2 fmol/mg protein), whereas intracellular HCV core Ag level of JFH-1/S2-wt was comparable to that of JFH-1/wt. Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

To further elucidate, we transfected Huh7-25 cells with *in vitro*–transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2 and measured HCV RNA, core Ag, and infectivity titer in the cells and culture medium. Intracellular HCV RNA levels of JFH-1/S2 and JFH-1/wt-S2 were similar and lower than those of JFH-1/wt and JFH-1/S2-wt, suggesting mutations in NS3-NS5B were responsible for lower replication efficiency of JFH-1/S2 (Table 1). Intracellular infectivity titer of JFH-1/S2 and JFH-1/S2-wt was 12.3 and 10.4 times higher, respectively, than that of JFH-1/wt ($P < 0.005$) on day 3 posttransfection. The intracellular specific infectivities of JFH-1/S2 and JFH-1/S2-wt were significantly higher than that of JFH-1/wt (18 times and 13.1 times higher, respectively; $P < 0.005$). On the other hand, intracellular specific infectivity of JFH-1/wt-S2 was comparable to that of JFH-1/wt. The infectious virus secretion rate was not significantly different among all the constructs (Table 1). These data indicate that mutations emerged in the core-NS2 region of JFH-1/S2 are responsible for the enhanced assembly of infectious virus particles compared with JFH-1/wt.

Mapping Study for JFH-1/S2 Strain. Because our experiments with JFH-1/S2 subgenomic replicon and JFH-1/wt-S2 chimeric construct showed that mutations emerged in the NS3-NS5B region are responsible for reduced replication efficiency of JFH-1/S2, we performed mapping studies by generating various JFH-1 subgenomic replicons, each containing the mutations observed in individual nonstructural protein. Although mutations in NS4B and NS5A were associated with attenuated replication capacity of JFH-1, the most significant decrease in replication was observed with NS5B mutations (Supporting Fig. 1B).

For detailed analysis of mutations responsible for higher assembly, *in vitro*–transcribed RNAs of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, JFH-1/N397S, JFH-1/L752V, JFH-1/S2-NS2 (containing mutations G838R, A878V, and V881A), JFH-1/G838R, and JFH-1/A878V were transfected into Huh7-25 cells, and intracellular-specific infectivities were compared (Supporting Table 2). As reported previously, JFH1/G838R showed higher intracellular specific infectivity than that of JFH-1/wt, but could not reach the level of JFH-1/S2 or JFH-1/S2-wt. Among the mutants, intracellular specific infectivities of JFH1/L752V, JFH1/NS2, and JFH1/G838R were 4.02, 5.42, and 3.07 times higher than that of JFH-1/wt, but those of JFH1/N397S and JFH1/A878V were similar to that of JFH-1/wt. Thus, the combination of mutations in P7 and NS2 was found to contribute to the higher assembly of the JFH-1/S2 strain.

Human Hepatocyte–Transplanted Mouse Assay. To assess the *in vivo* infectivity of these strains, we inoculated culture medium containing 10^7 copies (HCV RNA titer measured by RTD-PCR) of JFH-1/wt, JFH-1/S1, JFH-1/S2, and C viruses into human

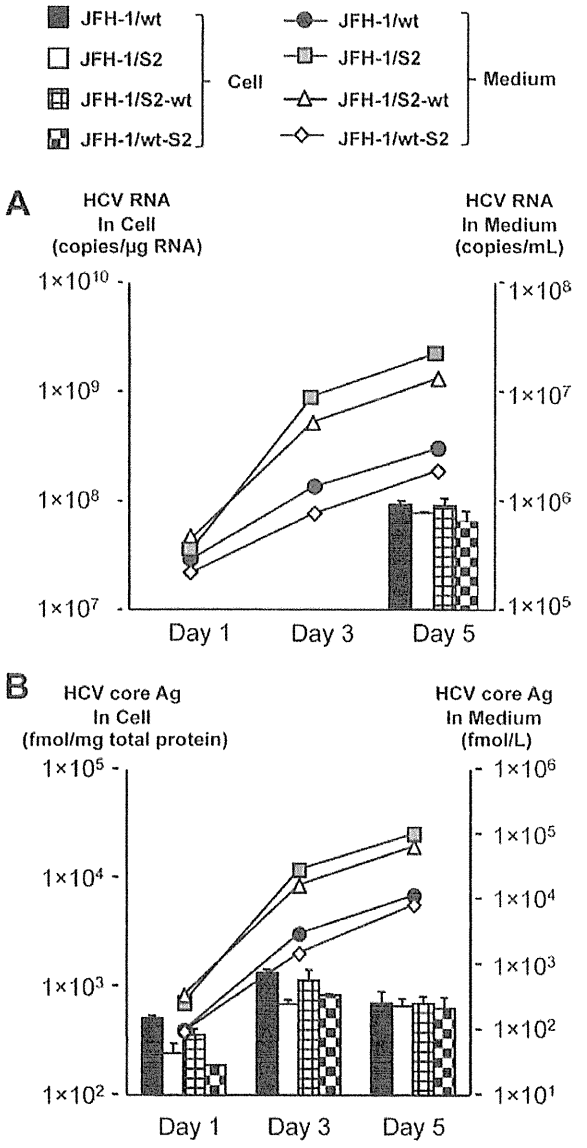

Fig. 2. Virus production of JFH-1/S2 chimeric constructs in HuH-7 cells. One million cells were transfected with 10 μg *in vitro*-transcribed RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. (A) HCV RNA and (B) core Ag levels in cell lysates and medium were measured at the indicated time points. Assays were performed in triplicate, and data are presented as the mean ± SD.

hepatocyte-transplanted mice. Two mice were used for each virus. Two weeks after intravascular inoculation, all mice but one became HCV RNA–positive (Fig. 3). Two mice died 3 weeks after inoculation; one was inoculated with JFH-1/wt and had developed infection, and the other was inoculated with JFH-1/C and died without developing infection. HCV RNA levels in infected mice fluctuated, ranging from 10^6 to 10^9 copies/mL. We could not observe much difference of infected HCV RNA titer among these inoculated mice. Sequence analyses of the complete open reading frames revealed that infecting JFH-1/wt virus and variant strains had no nonsynonymous mutations at the time of development of infection. From these data, we concluded that not only JFH-1/wt virus but also JFH-1/S1, JFH-1/S2, and JFH-1/C viruses were able to establish productive infection in human hepatocyte-transplanted mice.

Apoptosis Induction Assay. To investigate the survival strategy against the host defense system, we examined the susceptibility of JFH-1/wt and variant strains to TNF-α–mediated apoptosis induction. After transfection with *in vitro*–transcribed RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C, Huh-7.5.1 cells were exposed to TNF-α plus actinomycin D. Without exposure, apoptosis was observed in a limited number of HCV-positive cells (Supporting Fig. 2A). Forty-eight hours later, cells were harvested, fixed, and

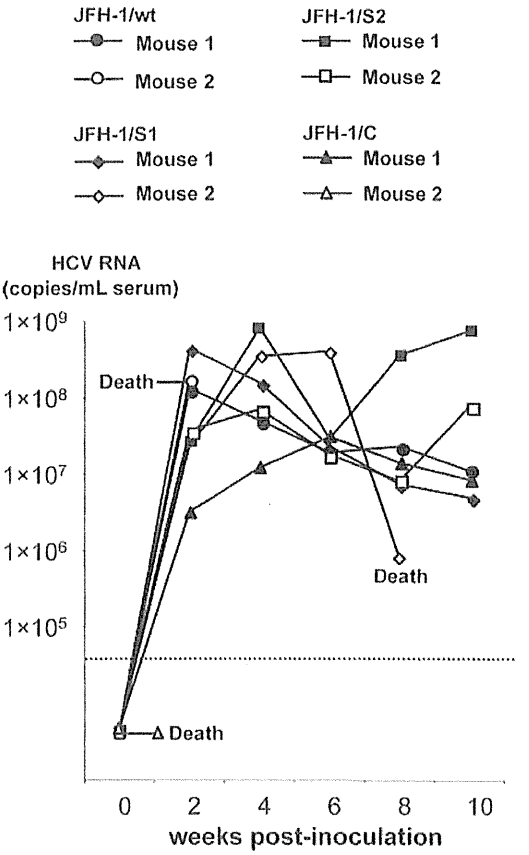

Fig. 3. *In vivo* infection study of JFH-1/wt and its variants in human hepatocyte-transplanted mice. Cell culture medium containing 1 × 10^7 HCV RNA copies of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C were inoculated into human hepatocyte-transplanted mice, and HCV RNA levels in mice serum were monitored.

subjected to terminal deoxynucleotidyl transferase–mediated deoxyuridine triphosphate nick-end labeling (TUNEL) assay and anti-HCV NS5A staining. The effects of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C transfection on apoptosis induction were determined by calculating the ratio of apoptosis between HCV-positive and HCV-negative populations and expressed as an apoptosis induction index. After treatment of JFH-1/wt–transfected cells with TNF-α, apoptosis was observed in 36.8% of the HCV-positive population and in 19.3% of the HCV-negative population, and the apoptosis induction index was 1.85 ± 0.06 (Fig. 4). The apoptosis induction indexes of JFH-1/S1–transfected and JFH-1/C– transfected cells were 1.23 ± 0.06 and 1.16 ± 0.10, respectively, suggesting lower susceptibility to apoptosis induction compared with JFH-1/wt. On the other hand, the apoptosis induction index of JFH-1/S2 was 0.74 ± 0.17, which was substantially lower than that of JFH-1/wt, demonstrating the more reduced apoptosis in the cells harboring this strain. Similar results were obtained by treatment with FasL plus actinomycin D (Supporting Fig. 2B). To confirm the lower susceptibility of JFH-1/S2–transfected cells, apoptosis was also detected by staining with anticleaved poly(adenosine diphosphate ribose) polymerase (PARP) antibody. The apoptosis induction indexes of JFH-1/wt and JFH-1/S2–transfected cells were 2.28 ± 0.24 and 1.15 ± 0.14, respectively, and were consistent with TUNEL assay (Fig. 5). Although the HCV NS5A-positive rate in JFH-1/S2–transfected cells was higher than that in JFH-1/wt, the mean fluorescence intensity of the NS5A-positive population in JFH-1/S2–transfected cells was significantly lower (185.0 ± 8.7) than that in JFH-1/wt–transfected cells (395.0 ± 98.0), corresponding to the observed phenotype of the JFH-1/S2 strain in the single cycle virus production assay (i.e., lower replication efficiency and rapid spread to surrounding cells).

To clarify the genomic region responsible for lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, we examined the effect of TNF-α on the cells carrying subgenomic reporter replicons. The apoptosis induction index of SGR-JFH1/Luc/S2–transfected cells was lower than that of SGR-JFH1/Luc/wt–transfected cells (Supporting Fig. 2C); however, the difference was not as pronounced as with full-genome constructs, indicating that mutations in the NS3-NS5B region contribute to lower susceptibility of JFH-1/S2 to cytokine-induced apoptosis, but they are not sufficient to explain the difference between JFH-1/wt and JFH-1/S2. We confirmed these results by use of the chimeric

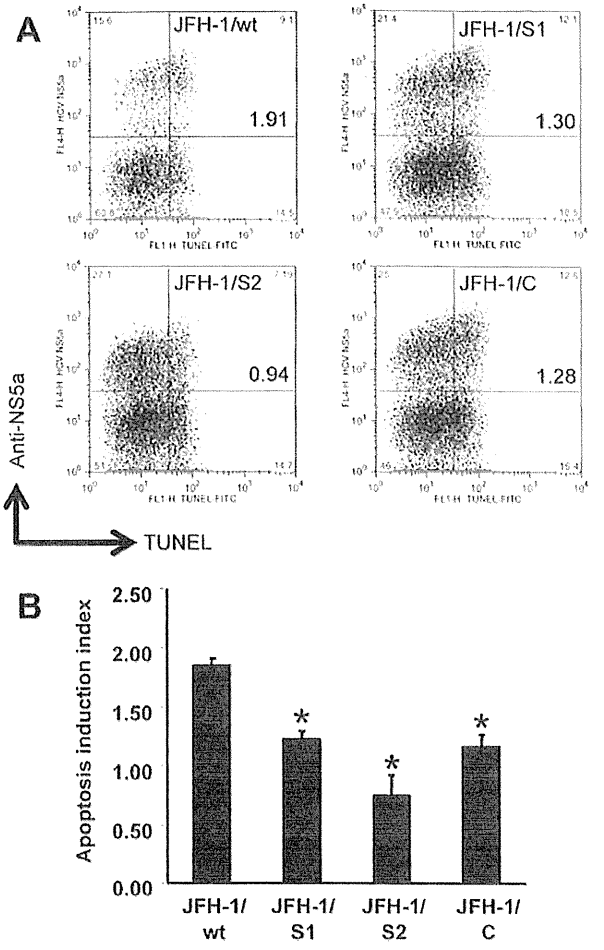

Fig. 4. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt and its variants. (A) Three million cells were transfected with 3 μg *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S1, JFH-1/S2, and JFH-1/C. Forty-eight hours later, apoptosis was induced by exposing cells to 20 ng/mL TNF-α plus 50 ng/mL actinomycin D. Cells were harvested after 48 hours of treatment and subjected to TUNEL and anti-HCV NS5A staining. Dot plots show HCV replication and apoptosis at the single cell level. Quadrant gates were determined using unstained and a terminal deoxynucleotidyltrasferase-untreated control in each culture condition. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt–, JFH-1/S1–, JFH-1/S2–, and JFH-1/C– transfected cells. The mean ± SD of three independent experiments is shown. *$P < 0.005$ versus JFH-1/wt.

constructs JFH-1/S2-wt and JFH-1/wt-S2. The apoptosis induction indexes of JFH-1/S2-wt–transfected and JFH-1/wt-S2–transfected cells were 1.42 ± 0.13 and 1.71 ± 0.08, respectively (Fig. 5). These data indicate that both structural and nonstructural regions of JFH-1/S2 were associated with lower susceptibility to cytokine-induced apoptosis, although mutations in core-NS2 seemed to have higher contribution toward this phenotype. Together, these results indicate that the JFH-1/S2 strain, which was selected after passage in

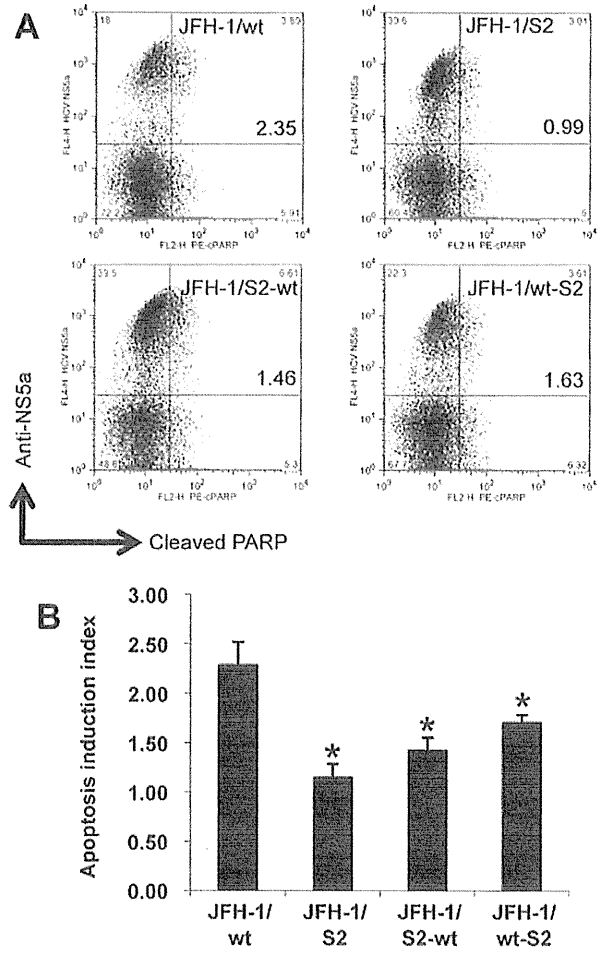

Fig. 5. Apoptosis induction in Huh-7.5.1 cells transfected with JFH-1/wt, JFH-1/S2, and their chimeric constructs. (A) Three million cells were transfected with 3 μg *in vitro*-transcribed full-genome RNA of JFH-1/wt, JFH-1/S2, JFH-1/S2-wt, and JFH-1/wt-S2. Apoptosis was induced by exposing cells to 20 ng/mL TNF-α plus 50 ng/mL actinomycin D and detected by anticleaved PARP staining. The clone names and apoptosis induction indexes are indicated in the upper right box. (B) Apoptosis induction indexes of JFH-1/wt–, JFH-1/S2–, JFH-1/S2-wt–, and JFH-1/wt-S2–transfected cells. The mean ± SD of three independent experiments is shown. *$P < 0.05$ versus JFH-1/wt.

the patient serum–infected chimpanzee, acquired less susceptibility to the cytokine-induced apoptosis.

Discussion

HCV develops chronic infection in the vast majority of infected patients[1]; however, the mechanisms of its persistence are still under investigation. Many viruses have evolved different strategies to cope with host immune systems, thus causing the development of persistent infection. For example, some viruses interfere with the major histocompatibility complex class I presentation of viral antigens, whereas others modulate lymphocyte and macrophage functions, including cytokine production.[12-16] In our previous study, we detected an increasing number of mutations in the HCV genome isolated from JFH-1 patient serum–infected chimpanzees. Thus, we reasoned that these detected mutations might have imparted some advantage to this virus for long-time survival. To examine this hypothesis, we compared the phenotypes of JFH-1 variant strains emerged at early and late stages of infection in JFH-1 patient serum–infected and JFH-1cc–infected chimpanzees and found that the JFH-1/S2 strain isolated from the patient serum–infected chimpanzee at a later time point of infection replicated slowly, produced more infectious viruses, and displayed reduced susceptibility to cytokine-induced apoptosis.

The JFH-1 variant strain JFH-1/C, which contains seven nonsynonymous mutations identified in the JFH-1cc–infected chimpanzee at week 7, showed comparatively slower replication kinetics and slightly enhanced infectious virus production in cell culture. The intracellular specific infectivity of this strain in Huh7-25 cells was 3.9 times higher than that of JFH-1/wt (Table 1). These characteristics might have imparted some advantage to this strain for establishing productive infection in the chimpanzee. The other JFH-1 variant strains, JFH-1/S1 and JFH-1/S2, contain 6 and 17 nonsynonymous mutations identified in the JFH-1 patient serum–infected chimpanzee at weeks 2 and 23 postinfection, respectively. Replication kinetics and infectious virus production of the JFH-1/S1 strain were comparable to that of JFH-1/wt in cultured cells (Fig. 1, Table 1). In contrast, the JFH-1/S2 strain showed lower replication efficiency. Although the intracellular HCV RNA level of this strain in Huh7-25 cells was lower than that of JFH-1/wt and JFH-1/S1, and almost the same as that of JFH-1/C (Table 1), intracellular specific infectivity was 18.0 and 12.9 times higher than that of JFH-1/wt and JFH-1/S1, respectively, suggesting a significant increase in the assembly of infectious virus particles ($P < 0.005$, Table 1). The enhanced capacity of this strain to assemble infectious virus particles resulted in a higher extracellular infectivity titer that contributed to the rapid spread of virus to surrounding cells. Flow cytometry analyses of cells transfected with JFH-1/wt and variant strains revealed that the percentage of the HCV NS5A-positive population in JFH-1/S2–transfected cells was higher, but the mean fluorescence intensity of the anti-NS5A signal was lower than that in JFH-1/wt–transfected cells, thus confirming higher spread and lower replication of this strain. Taken together, both JFH-1/C and JFH-1/S2 exhibited a tendency toward

decreased replication and increased infectious virus production. However, the extent of enhanced virus production was substantially lower in JFH-1/C than in JFH-1/S2, which might have led to the earlier elimination of infection in the JFH-1cc–infected chimpanzee. In other words, the potency of infectious virus production and spread seems to correspond to the duration of infection in infected animals.

The association between a lower replication efficiency and persistent infection is still unclear. It has been reported that an escape mutant with an amino acid substitution at the cytotoxic T lymphocyte (CTL) epitope in the NS3 region exhibits lower NS3/4 protease activity and replication capacity *in vitro*.[17,18] The JFH-1/S2 strain contains the T1077A mutation in the NS3 region (Supporting Table 1), and this mutation is located close to mutations reported to be associated with immune evasion and lower replication.[17] Thus, the lower replication efficiency of the JFH-1/S2 strain may be a result of an immune escape mutation at the expense of viral fitness. Meanwhile, we cannot deny the advantage of lower replication in establishing persistent infection. Lower replication may contribute to the avoidance of major histocompatibility class I–mediated antigen presentation and to escape from the host immune system. Either way, by acquiring the ability to produce more viral particles, the JFH-1/S2 strain could rapidly spread to surrounding cells, irrespective of its lower replication efficiency. Importantly, these emerged mutations did not attenuate *in vivo* infectivity, unlike cell culture adaptive mutations reported to cause attenuated infection *in vivo*.[19] Upon inoculation into human hepatocyte–transplanted mice, JFH-1/S1, JFH-1/S2, and JFH-1/C strains could establish infection without any mutations, produced levels of viremia similar to JFH-1/wt, and persisted for a similar observed period of infection (Fig. 2). This observation is different from that in chimpanzees, where JFH-1/wt and JFH-1/C strains were eliminated earlier than JFH-1/S2. In contrast to chimpanzees, human hepatocyte–transplanted mice lack a CTL and natural killer (NK) cell–mediated immune system, which could be responsible for this difference.[6] Taken together, our results suggest that along with efficient infectious virus production, the JFH-1/S2 strain might have acquired an advantage that helps it evade the CTL and NK cell–mediated immune system.

Apoptosis of virus-infected cells by the immune system is crucial as a general mechanism of clearing infections.[20,21] The J6/JFH-1 chimeric virus has been reported to exhibit proapoptotic characteristics in cell culture.[22] However, because HCV needs to escape the host immune system in order to establish chronic infection, immune cell–mediated apoptosis may be inhibited in infected hepatocytes. In the liver, HCV-infected hepatocytes are eliminated by targeted apoptosis induced by NK cells, macrophages, and CTLs with ligand-mediated and receptor-mediated signals such as TNF-α, FasL, and TNF-related apoptosis–inducing ligand.[23-26] Thus, we used TNF-α to mimic natural immunomediated apoptosis and found that the JFH-1/S2–replicating cells have lower susceptibility to the apoptosis induced by these cytokines. In JFH-1/S2–transfected cells, TNF-α–induced apoptosis detected by TUNEL assay was substantially lower than that of JFH-1/wt–transfected cells (Fig. 4). We confirmed it by staining with anticleaved PARP. In complete agreement with the results produced by way of TUNEL assay, the number of anticleaved PARP stained cells among JFH-1/S2–infected cells was significantly lower than that among JFH-1/wt–infected cells (Fig. 5). In our previous study, we reported that HCV-specific immune responses with T cell proliferation and interferon-γ production were maintained until the disappearance of viremia in the patient serum–infected chimpanzee.[11] This finding indicates that continuous selection pressure in the infected chimpanzee might have contributed to the emergence of a clone with an ability to escape the cytokine-induced apoptosis. We are not sure whether this phenotype of JFH-1/S2 is due to its lower replication efficiency and thus lower production of HCV proteins. The accumulation of viral proteins might predispose cells to the apoptosis induced by TNF-α. To answer this question, it will be necessary to investigate the genomic regions of JFH-1/S2 and cellular host factors responsible for the ability of this strain to escape the apoptosis.

By way of mapping analysis for JFH-1/S2, we could determine responsible regions; NS5B was for lower replication efficiency (Supporting Fig. 1B), and P7 and NS2 were for enhanced viral particle assembly (Supporting Table 2). For the evasion of apoptosis, we could not specify the responsible region, because both chimeric constructs, JFH-1/S2-wt and JFH-1/wt-S2, showed less susceptibility to cytokine-induced apoptosis to a certain extent. These data indicate that both structural and nonstructural regions might have contributed to the acquisition of this phenotype. Previously, a potent antiapoptotic effect of the HCV NS5A protein was described.[27] NS5A interacts with Bin1, which is a nucleocytoplasmic c-Myc–interacting protein with tumor suppressor and apoptotic properties, thus inhibiting Bin1-

associated apoptosis. Because JFH-1/S2 contains several mutations in the NS5A region (Supporting Table 1), one or more mutations in this protein may be associated with antiapoptotic effects.

In conclusion, we demonstrated that the JHF-1/S2 strain acquired phenotypes of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis. These phenotypes were associated with mutations that emerged 23 weeks after infection in a chimpanzee, and might have contributed to long-term infection *in vivo*. Such control of viral functions by specific mutations may be a key viral strategy to establish persistent infection.

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Malnutrition Impairs Interferon Signaling Through mTOR and FoxO Pathways in Patients With Chronic Hepatitis C

MASAO HONDA,*,‡ KENJI TAKEHANA,§ AKITO SAKAI,* YUSUKE TAGATA,§ TAKAYOSHI SHIRASAKI,‡ SHINOBU NISHITANI,§ TAKAHIKO MURAMATSU,‖ TATSUYA YAMASHITA,* YASUNARI NAKAMOTO,* EISHIRO MIZUKOSHI,* YOSHIO SAKAI,* TARO YAMASHITA,* MIKIKO NAKAMURA,* TETSURO SHIMAKAMI,¶ MINKYUNG YI,# STANLEY M. LEMON,¶ TETSUO SUZUKI,** TAKAJI WAKITA,** SHUICHI KANEKO,* and the Hokuriku Liver Study Group

*Department of Gastroenterology, ‡Department of Advanced Medical Technology, Kanazawa University Graduate School of Medicine, Kanazawa, Japan; §Exploratory Research Laboratories, Research Center, Ajinomoto Pharmaceuticals, Co, Ltd, Kanagawa, Japan; ‖Frontier Research Labs, Institute for Innovation, Ajinomoto, Co, Inc, Kanagawa, Japan; ¶Division of Infectious Diseases, School of Medicine, The University of North Carolina at Chapel Hill, Chapel Hill, North Carolina; #Center for Hepatitis Research, Institute for Human Infections and Immunity, and Department of Microbiology and Immunology, University of Texas Medical Branch, Galveston, Texas; and **Department of Virology II, National Institute of Infectious Diseases, Tokyo, Japan

BACKGROUND & AIMS: Patients with advanced chronic hepatitis C (CH-C) often are malnourished, but the effects of malnutrition on interferon (IFN) signaling and response to treatment have not been determined. We assessed the importance of the nutritional state of the liver on IFN signaling and treatment response. **METHODS:** We studied data from 168 patients with CH-C who were treated with the combination of pegylated-IFN and ribavirin. Plasma concentrations of amino acids were measured by mass spectrometry. Liver gene expression profiles were obtained from 91 patients. Huh-7 cells were used to evaluate the IFN signaling pathway, mammalian target of rapamycin complex 1 (mTORC1), and forkhead box O (FoxO). Antiviral signaling induced by branched-chain amino acids (BCAAs) was determined using the in vitro hepatitis C virus replication system. **RESULTS:** Multivariate logistic regression analysis showed that Fischer's ratio was associated significantly with nonresponders, independent of interleukin-28B polymorphisms or the histologic stage of the liver. Fischer's ratio was correlated inversely with the expression of BCAA transaminase 1, and was affected by hepatic mTORC1 signaling. IFN stimulation was impaired substantially in Huh-7 cells grown in medium that was low in amino acid concentration, through repressed mTORC1 signaling, and increased Socs3 expression, which was regulated by Foxo3a. BCAA could restore impaired IFN signaling and inhibit hepatitis C virus replication under conditions of malnutrition. **CONCLUSIONS: Malnutrition impaired IFN signaling by inhibiting mTORC1 and activating Socs3 signaling through Foxo3a. Increasing BCAAs to up-regulate IFN signaling might be used as a new therapeutic approach for patients with advanced CH-C.**

Keywords: HCV; Liver Disease; Therapy; Diet.

Interferon (IFN) and ribavirin (RBV) combination therapy is a popular modality for treating patients with chronic hepatitis C (CH-C), but approximately 50% of patients usually relapse, particularly those with hepatitis C virus (HCV) genotype 1b and a high viral load.[1]

Recent landmark studies of genome-wide associations identified genomic loci associated with treatment responses to pegylated (Peg)-IFN and RBV combination therapy,[2,3] and a polymorphism in the interleukin (IL)-28B gene was found to predict hepatitis C treatment–induced viral clearance. Moreover, we previously showed that expression of hepatic IFN-stimulated genes (ISGs) was associated with the IL-28B polymorphism and might contribute to the treatment response.[4] In addition to the IL-28B polymorphism, host factors such as fibrosis stage and metabolic status of the liver might be associated with the treatment outcome[4,5]; however, the significance of these factors in conjunction with the IL-28B polymorphism has not been evaluated fully.

In CH-C livers, prolonged liver cell damage, fibrosis development, and microcirculation failure can lead to a state of malnutrition in hepatocytes, resulting in the impairment of multiple metabolic pathways. In patients with advanced stage CH-C, hypoalbuminemia and decreased plasma values for the Fischer's ratio of branched-amino acids (BCAA; leucine, isoleucine, and valine) to aromatic amino acids (tyrosine and phenylalanine) commonly are observed. BCAA are the essential amino acids necessary for ammonium metabolism in muscle when the liver is unable to perform this function. Recent reports have shown that BCAA activates albumin synthesis in rat

Abbreviations used in this paper: BCAA, branched-chain amino acid; BCAT1, branched chain amino-acid transaminase 1; CH-C, chronic hepatitis C; ChIP, chromatin immunoprecipitation; DMEM, Dulbecco's modified Eagle medium; FBE, Foxo binding element; FBEmut, Foxo binding element mutant; FoxO, forkhead box, subgroup O; GLuc, Gaussia luciferase; IFN, interferon; IL, interleukin; ISG, interferon-stimulated genes; mTOR, mammalian target of rapamycin; mTORC1, mammalian target of rapamycin complex 1; NR, no response; PCR, polymerase chain reaction; Peg, pegylated; p-mTOR, phosphorylated form of mammalian target of rapamycin; pS6K, phosphorylated form of p70 S6 protein kinase; pSTAT1, phosphorylated form of signal transducer and activator of transcription 1; Raptor, regulatory associated protein of mTOR; RBV, ribavirin; S6K, p70 S6 protein kinase; siRNA, small interfering RNA; SVR, sustained viral response; TR, transient response.

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primary hepatocytes[6] and cirrhotic rat liver[7] through mammalian target of rapamycin (mTOR) signaling, a central regulator of protein synthesis, by sensing nutrient conditions.[8] Thus, peripheral amino acid composition is closely related to signaling pathways in the liver.

In addition to metabolic aspects, recent reports have elucidated new functional roles for mTOR in the IFN signaling pathway. Targeted disruptions of tuberous sclerosis 2 and eukaryotic translation initiation factor 4E binding protein 1, which both inhibit mTOR complex 1 (mTORC1) signaling, substantially enhanced IFN-alfa–dependent antiviral responses.[9,10] Therefore, mTORC1 signaling might be involved in the antiviral response as well as in metabolic processes. However, these issues have not yet been addressed in terms of IFN treatment for CH-C. In the present study, therefore, we evaluated the clinical relevance of the nutritional state of the liver, as estimated by the plasma Fischer's ratio, on Peg-IFN and RBV combination therapy. We also evaluated antiviral signaling induced by BCAA using an in vitro HCV replication system.

Materials and Methods

Patients

A total of 168 patients with CH-C at the Graduate School of Medicine at Kanazawa University Hospital (Kanazawa, Japan) and its related hospitals in Japan (Table 1, Supplementary Table 1) were evaluated in the present study. The clinical characteristics of these patients have been described previously.[4] All patients were administered Peg–IFN-alfa 2b (Schering-Plough K.K., Tokyo, Japan) and RBV combination therapy for 48 weeks. The definition of the treatment response was as follows: sustained viral response (SVR), clearance of HCV viremia 24 weeks after the cessation of therapy; transient response (TR), no detectable HCV viremia at the cessation of therapy but relapse during the follow-up period; and no response (NR). Genetic variation of the IL-28B polymorphism at rs8099917 was evaluated in all patients using TaqMan Pre-Designed SNP Genotyping Assays (Applied Biosystems, Carlsbad, CA) as described previously.[4] Gene expression profiling in the liver was performed in 91 patients using the Affymetrix Human 133 Plus 2.0 microarray chip (Affymetrix, Santa Clara, CA) as described previously (Supplementary Table 1).[4]

Plasma Amino Acid Analysis

Amino acid concentrations in plasma samples were measured by high-performance liquid chromatography–electrospray ionization–mass spectrometry, followed by derivatization.[11] Detailed experimental procedures are described in the Supplementary Materials and Methods section.

Culture Medium

Huh-7 and Huh-7.5 cells (kindly provided by Professor C. M. Rice, Rockefeller University, New York, NY) were maintained in Dulbecco's modified Eagle medium (DMEM; Gibco BRL, Gaithersburg, MD) containing 10% fetal bovine serum and 1% penicillin/streptomycin (normal medium). Amino acid-free medium (ZERO medium) was prepared by mixing 5.81 g nutrition-free DMEM (Nacalai Tesque, Kyoto, Japan), 1.85 g $NaHCO_3$, 1 g glucose, and 0.5 mL 1M (mol/L) sodium pyruvate in 500 mL Milli-Q water, then sterilizing with a 0.22-μm filter (Millipore, Billerica, MA). Low amino acid media ($\times$1/5, $\times$1/10, $\times$1/30, and $\times$1/100 DMEM) were prepared by diluting $\times$1 DMEM with ZERO medium. Powdered BCAA (leucine-isoleucine-valine, 2:1:1.2) (Ajinomoto Pharma, Tokyo, Japan) was freshly dissolved with distilled water at 100 mmol/L, then applied to cultured medium at 2 mmol/L, 4 mmol/L, or 8 mmol/L.

Western Blotting and Immunofluorescence Staining

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium and maintained for up to 24 hours. Western blotting was performed as previously described.[12] Cells were washed in phosphate-buffered saline (PBS) and lysed in RIPA buffer containing complete Protease Inhibitor Cocktail and PhosSTOP (Roche Applied Science, Indianapolis, IN). The membranes were blocked in Blocking One-P (Nacalai Tesque). The antibodies used for Western blotting are summarized in the Supplementary Materials and Methods section.

For immunofluorescence staining, cells were fixed with 4% paraformaldehyde in PBS, then permeabilized with 0.1% Triton-X 100 in PBS. The primary anti–forkhead box O (Foxo)3a antibody (Abcam, Cambridge, MA) was used at a final concentration of 2 μg/mL in PBS containing 2% fetal bovine serum at 4°C for 16 hours. Incubation with the Alexa Fluor 488–conjugated secondary antibody (Invitrogen, Carlsbad, CA) at a 500-fold dilution in PBS containing 3% fetal bovine serum antibody was performed for 4 hours, and cells were stained with Hoechst 33258 to visualize nuclear DNA (Vector Laboratories, Burlingame, CA).

Quantitative Real-Time Detection Polymerase Chain Reaction

A total of 1.5×10^5 Huh-7 cells were seeded in normal medium 24 hours before performing the experiments. The medium was changed to low-amino-acid medium, to which IFN-alfa and/or BCAA was added, and maintained for 24 hours. Rapamycin treatment (100 nmol/L) was performed for 30 minutes in normal medium before a medium change. RNA was isolated using TriPure isolation reagent (Roche Applied Science), and complementary DNA (cDNA) was synthesized using the High Capacity cDNA reverse transcription kit (Applied Biosystems, Carlsbad, CA). Real-time detection polymerase chain reaction (PCR) was performed using the 7500 Real-Time PCR System (Applied Biosystems) and Power SYBR Green PCR Master Mix (Applied Biosystems) containing specific primers according to the manufacturer's

Table 1. Comparison of Clinical Factors Between Patients With and Without NR

Clinical category	SVR+TR	NR	Univariate *P* value	Multivariate odds (95% CI)	Multivariate *P* value
Patients, n	125	43		—	
Age and sex					
Age, y	57 (30–72)	56 (30–73)	.927	—	
Sex, male vs female	68 vs 57	24 vs 19	.872	—	
Liver histology					
F stage (F1–2 vs F3–4)	95 vs 30	20 vs 23	.001	6.35 (2.02–23.7)	.001
A grade (A0–1 vs A2–3)	68 vs 57	19 vs 24	:248	—	
Host gene factors					
IL-28B (TT vs TG/GG)[a]	109 vs 12	12 vs 31	<.001	19.7 (5.74–82.7)	<.001
ISGs (Mx, IFI44, IFIT1), (<3.5 vs ≥3.5)	103 vs 22	12 vs 31	<.001	5.26 (1.65–17.6)	.005
Metabolic factors					
BMI, kg/m^2	23.2 (16.3–34.7)	23.4 (19.5–40.6)	.439	—	
TG, mg/dL	98 (30–323)	116 (45–276)	.058	—	
T-Chol, mg/dL	167 (90–237)	160 (81–214)	.680	—	
LDL-Chol, mg/dL	82 (36–134)	73 (29–123)	.019	—	
HDL-Chol, mg/dL	42 (20–71)	47 (18–82)	.098	—	
FBS, mg/dL	94 (60–291)	96 (67–196)	.139	—	
Insulin, μU/mL	6.6 (0.7–23.7)	6.8 (2–23.7)	.039	—	
HOMA-IR	1.2 (0.3–11.7)	1.2 (0.4–7.2)	.697	—	
Fischer ratio	2.3 (1.5–3.3)	2.1 (1.5–2.8)	.005	8.91 (1.62–55.6)	.011
Other laboratory parameters					
AST level, IU/L	46 (18–258)	64 (21–283)	.017	—	
ALT level, IU/L	60 (16–376)	82 (18–345)	.052	—	
γ-GTP level, IU/L	36 (4–367)	75 (26–392)	<.001	—	
WBC, /mm^3	4800 (2100–11100)	4800 (2500–8200)	.551	—	
Hb level, g/dL	14 (9.3–16.6)	14.4 (11.2–17.2)	.099	—	
PLT, ×104/mm^3	15.7 (7–39.4)	15.2 (7.6–27.8)	.378	—	
Viral factors					
ISDR mutations ≤1 vs ≥2	80 vs 44	34 vs 9	.070	4.12 (1.25–15.9)	.019
HCV-RNA, KIU/mL	2300 (126–5000)	1930 (140–5000)	.725	—	
Treatment factors					
Total dose administered					
Peg-IFN, μg	3840 (960–7200)	3840 (1920–2880)	.916	—	
RBV, g	202 (134–336)	202 (36–336)	.531	—	
Achieved administration rate					
Peg-IFN, %					
≥80%	84	28	.975	—	
<80%	42	14			
RBV (%)					
≥80%	76	24	.745	—	
<80%	50	18			
Achievement of EVR	101/125 (81%)	0/43 (0%)	<.001	—	

BMI, body mass index; CI, confidence interval; FBS, fasting blood sugar; γ-GTP, gamma-glutamyl transpeptidase; Hb, hemoglobin; HDL-chol, high density lipoprotein cholesterol; LDL-chol, low density lipoprotein cholesterol; PLT, platelets; T-chol, total cholesterol; TG, triglycerides; WBC, leukocytes.

[a]IL-28B SNP at rs8099917.

instructions. The primer sequence for real-time detection PCR is given in the Supplementary Materials and Methods section. HCV RNA was detected as described previously[12] and expression was standardized to that of glyceraldehyde-3-phosphate dehydrogenase.

Reporter Assay

Construction of the interferon stimulated response element (ISRE)-luc reporter plasmid and Socs3-luc or Socs3 (FoxO binding element mutant [FBEmut])-luc reporter plasmids is described in the Supplementary Materials and Methods section.

Huh-7 cells were transfected with the ISRE-luc reporter plasmid 24 hours before IFN-alfa treatment. Cells were treated with IFN-alfa (0 or 100 U/mL) and BCAA (2 mmol/L) in low-amino-acid media. After 24 hours, luciferase activities were measured using the Dual Luciferase assay system (Promega, Madison, WI). For Socs3 promoter activities, Huh-7 cells were transfected with Socs3-luc or Socs3 (FBEmut)-luc reporter plasmids together with the Foxo3a expression plasmid, and luciferase activities were measured after 24 hours. Values were normalized to the luciferase activity of the co-transfected pGL4.75 Renilla luciferase-expressing plasmid (Promega).

Knockdown Experiments

Huh-7 cells were transfected with Ctrl (Stealth RNAi Negative Control Low GC Duplex #2; Invitrogen) or