

Production and characterization of HCV particles from serum-free culture

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ARTICLE INFO

Article history:

Received 26 October 2010
Received in revised form 8 April 2011
Accepted 19 April 2011
Available online 7 May 2011

Keywords:

Hepatitis C virus
Cell culture
Serum-free
Apolipoprotein

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 apolipoproteins: 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.

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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 geno-

type 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.

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doi:10.1016/j.vaccine.2011.04.069

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% CO₂ 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 Select (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[®] AQueous 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 μ L of media, and 20 μ L 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 μ g) that was synthesized from pJFH1 or pJ6/JFH1 was mixed with 400 μ L 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 μ F 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- μ L 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 μ L 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 μ L each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100- μ L 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

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 μ L 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 μ L), and the mixture was incubated overnight at 4 °C. Mouse IgG (5 μ g, 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

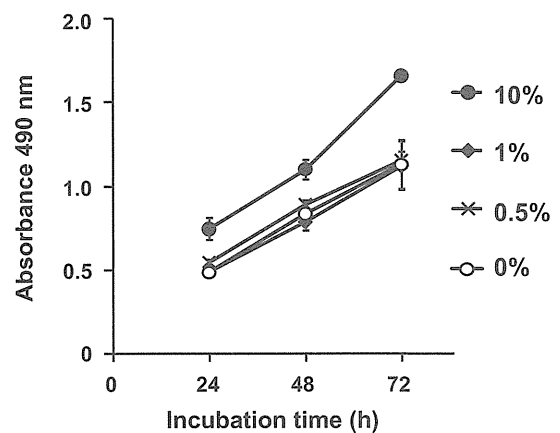


Fig. 1. Cell proliferation assay of serum-free cultured Huh7 cells. Huh7 cells that were seeded into a 96-well plate (1×10^4 /well) were sequentially grown in DMEM/F-12 media containing 10%, 1%, 0.5% and 0% fetal bovine serum. At indicated times, an MTS cell proliferation assay was performed using a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

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).

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

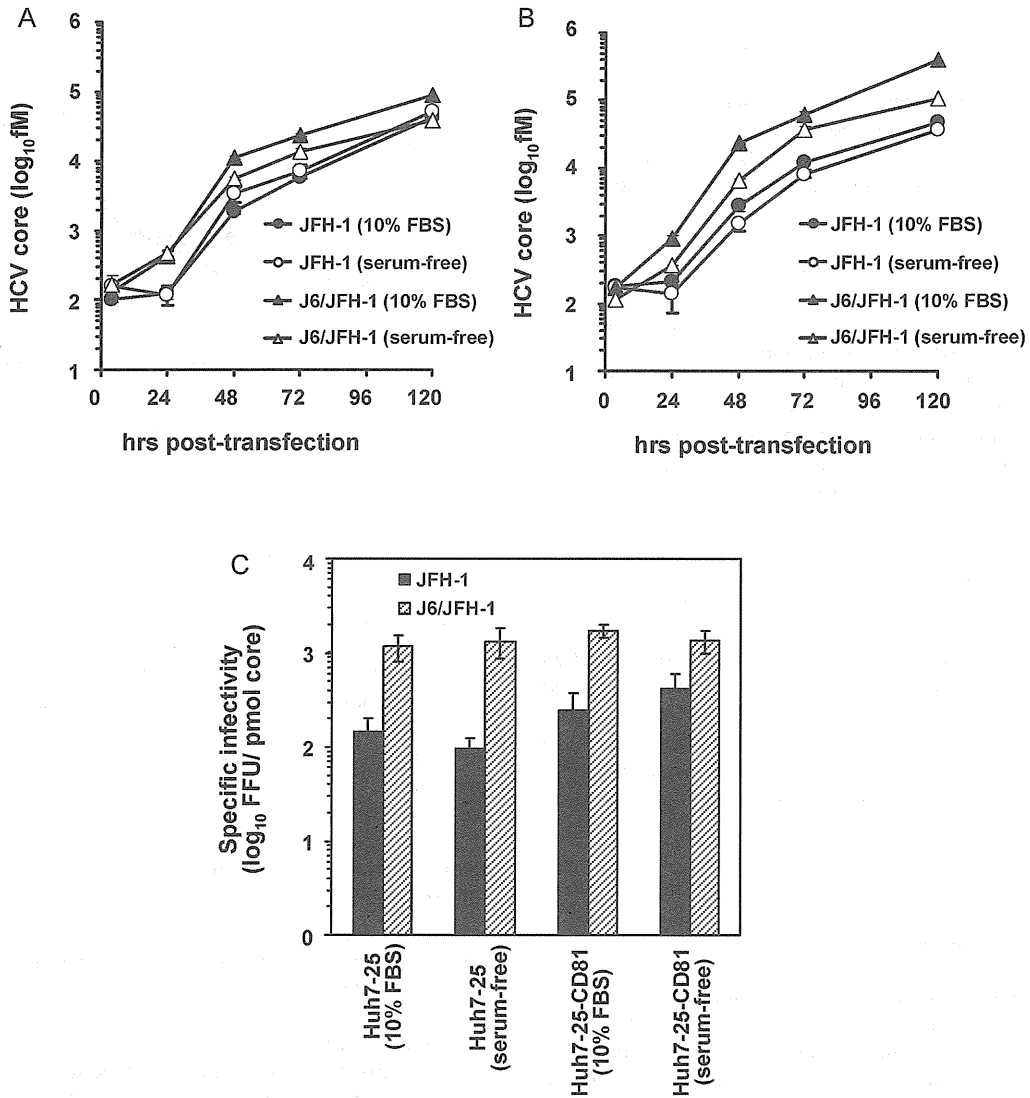


Fig. 2. HCV production from serum-free cultured Huh7 cells transfected with *in vitro* transcribed JFH-1 and J6/JFH-1 RNA. *In vitro* transcribed JFH-1 and J6/JFH-1 RNA was transfected into Huh7-25 (A) and Huh7-25-CD81 (B) cells that were grown under the indicated serum conditions. The culture supernatant 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 \pm 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 \pm SD. Profiles of HCV-core, HCV-RNA and infectivity are indicated in the Supplementary Table.

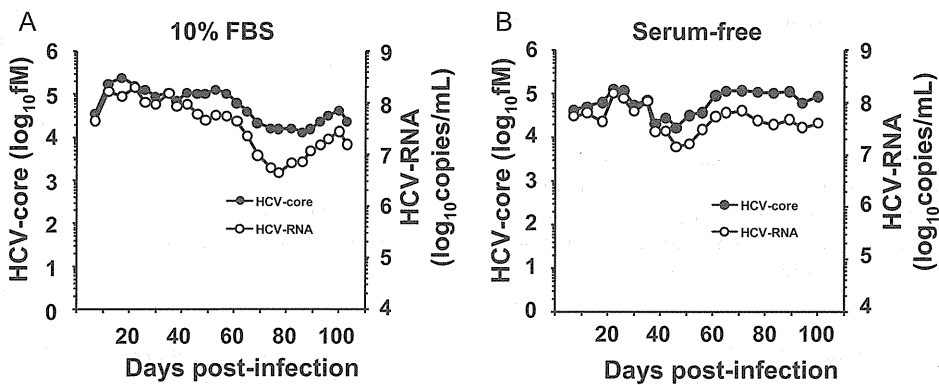


Fig. 3. HCV production from serum-free cultured Huh7 cells infected with J6/JFH-1 HCV. Huh7 cells that were grown in 10% FBS-supplemented (A) or serum-free (B) DMEM/F-12 were inoculated with the J6/JFH-1 virus (MOI, 0.2), and media of sub-cultures were collected. The HCV-core (closed circles) and RNA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

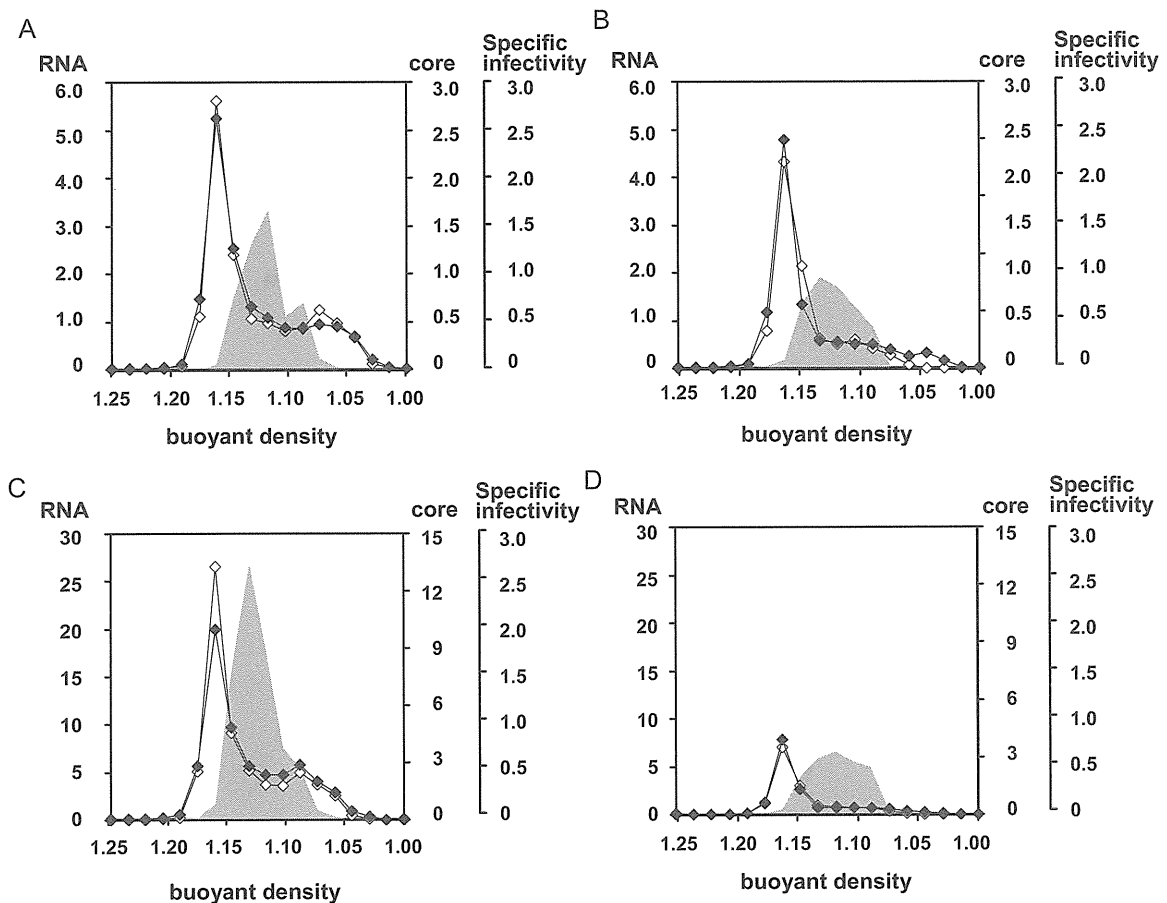


Fig. 4. Density gradient analysis of the supernatants derived from infected serum-free cultured Huh7 cells. *In vitro* transcribed J6/JFH-1 RNA was transfected into Huh7-25 (A and B) and Huh7-25-CD81 (C and D) cells that were cultured under 10% FBS-supplemented (A and C) or serum-free (B and D) conditions. 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 μ L 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

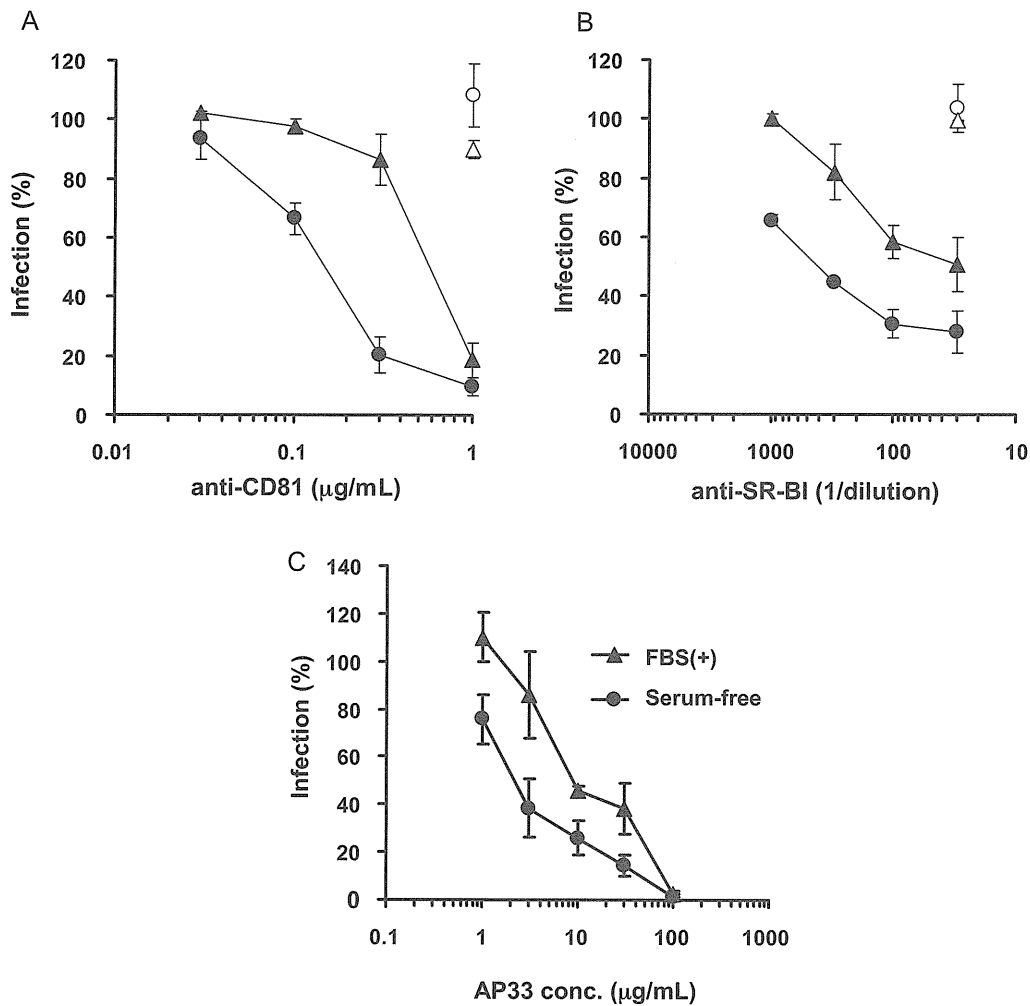


Fig. 5. Inhibition of serum-free cultured HCV infection by anti-CD81, anti-SR-BI and anti-E2 antibodies. Huh7.5.1 cells were pre-incubated with serially diluted anti-CD81 (JS-81, panel A) or anti-SR-BI (panel B) antibody for 1 h, and infectious J6/JFH-1 virus (MOI 0.1) obtained from FBS-supplemented (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-

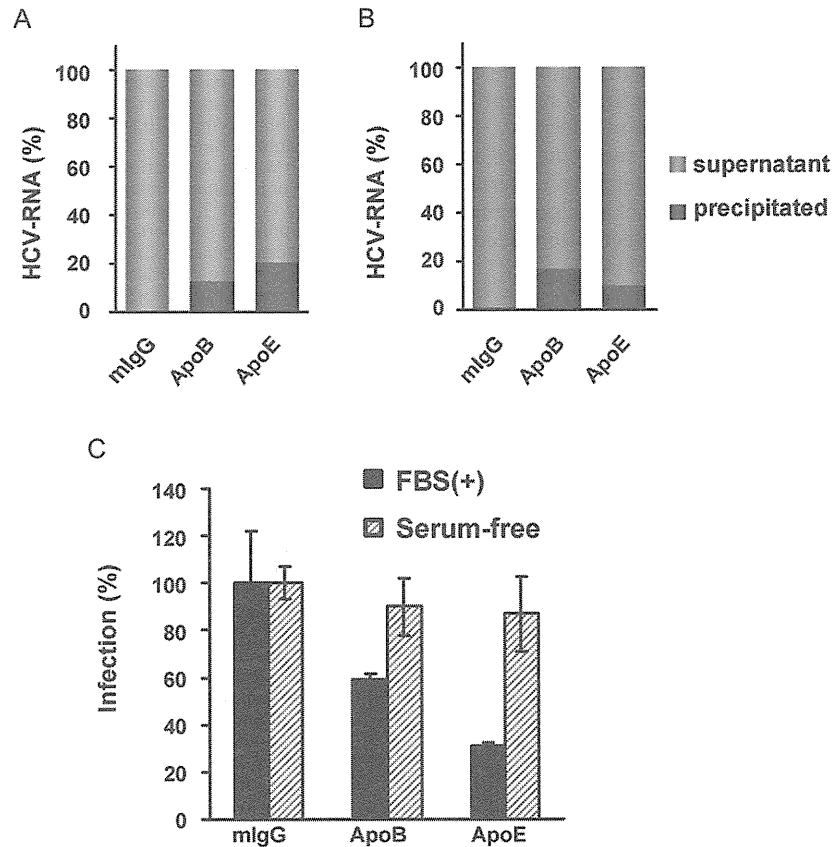


Fig. 6. Immunoprecipitation of cell-cultured HCV with anti-ApoB and ApoE antibodies. 1×10^7 HCV-RNA copies of the J6/JFH-1 virus were incubated with mouse IgG, anti-ApoB or anti-ApoE antibodies, and were then precipitated using protein G-Sepharose. Virus RNA was extracted from the supernatant and the 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 \pm SD. All data are indicated as percentage of infection, in which the infectivity (FFU/mL) of the supernatant from the mouse IgG (mlgG) 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 infec-

tion 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.

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

This work was partially supported by a grant-in-aid for Scientific Research from the Japan Society for the Promotion of Science and from the Ministry of Health, Labor, and Welfare of Japan by the Research on Health Sciences Focusing on Drug Innovation from the Japan Health Sciences Foundation. Huh7.5.1 was a kind gift from Dr. Francis V. Chisari. Anti-SR-BI antibody and rat serum were kind gifts from Dr. H. Barth. Antibody AP33 was a kind gift from Genentech, Inc.

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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Development of Mouse Hepatocyte Lines Permissive for Hepatitis C Virus (HCV)

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Abstract

The lack of a suitable small animal model for the analysis of hepatitis C virus (HCV) infection has hampered elucidation of the HCV life cycle and the development of both protective and therapeutic strategies against HCV infection. Human and mouse harbor a comparable system for antiviral type I interferon (IFN) induction and amplification, which regulates viral infection and replication. Using hepatocytes from knockout (ko) mice, we determined the critical step of the IFN-inducing/amplification pathways regulating HCV replication in mouse. The results infer that interferon-beta promoter stimulator (IPS-1) or interferon A receptor (IFNAR) were a crucial barrier to HCV replication in mouse hepatocytes. Although both IFNARko and IPS-1ko hepatocytes showed a reduced induction of type I interferons in response to viral infection, only IPS-1^{-/-} cells circumvented cell death from HCV cytopathic effect and significantly improved J6JFH1 replication, suggesting IPS-1 to be a key player regulating HCV replication in mouse hepatocytes. We then established mouse hepatocyte lines lacking IPS-1 or IFNAR through immortalization with SV40T antigen. Expression of human (h)CD81 on these hepatocyte lines rendered both lines HCVcc-permissive. We also found that the chimeric J6JFH1 construct, having the structure region from J6 isolate enhanced HCV replication in mouse hepatocytes rather than the full length original JFH1 construct, a new finding that suggests the possible role of the HCV structural region in HCV replication. This is the first report on the entry and replication of HCV infectious particles in mouse hepatocytes. These mouse hepatocyte lines will facilitate establishing a mouse HCV infection model with multifarious applications.

Citation: Aly HH, Oshiumi H, Shime H, Matsumoto M, Wakita T, et al. (2011) Development of Mouse Hepatocyte Lines Permissive for Hepatitis C Virus (HCV). PLoS ONE 6(6): e21284. doi:10.1371/journal.pone.0021284

Editor: Jacques Zimmer, Centre de Recherche Public de la Santé (CRP-Santé), Luxembourg

Received: May 13, 2011; **Accepted:** May 24, 2011; **Published:** June 22, 2011

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Funding: This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, and Culture (Specified Project for Advanced Research), the Ministry of Health, Labor, and Welfare of Japan, and the Hokkaido University Leader Development System in the Basic Interdisciplinary Research Areas (L station). Supports from Mitsubishi Foundation, Mochida Foundation, NorthTec Foundation Waxman Foundation and Yakult Foundation are gratefully acknowledged. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Chronic hepatitis C virus (HCV) infection is a major cause of mortality and morbidity throughout the world infecting around 3.1% of the world's population [1]. The development of much needed specific antiviral therapies and an effective vaccine has been hampered by the lack of a suitable small animal model. The determinants restricting HCV tropism to human and chimpanzee hosts are unknown. Replication of HCV strain JFH1 has been demonstrated in mouse cells only upon antibody selection [2], highlighting the very limited replication efficiency. Human CD81 and occludin have been implicated as important entry receptors for retrovirus particles bearing HCV glycoproteins, HCV pseudoparticles (HCVpp), into NIH3T3 murine cells [3]. However, HCV infection, spontaneous replication and particle production by mouse cells have not yet been reported.

In mammalian cells, the host detects and responds to infection by RNA-viruses, including HCV, by primarily recognizing viral RNA through several distinct pathogen recognition receptors (PRRs), including the cell surface and endosomal RNA sensors Toll-like receptors 3 and 7 (TLR3 and TLR7), and the cytoplasmic RNA sensors retinoic acid-inducible gene I (RIG-I

and melanoma differentiation associated gene 5 (MDA5) [4]. The detection of virus infection by these receptors leads to the induction of interferons (IFNs) and their downstream IFN-inducible anti-viral genes through distinct signaling pathways [5]. Type I IFN is an important regulator of viral infections in the innate immune system [6]. Another type of IFN, IFN-lambda, affects the prognosis of HCV infection, and its response to antiviral therapy [7,8].

Mutations impairing the function of the RIG-I gene and the induction of IFN were essential in establishing HCV infectivity in human HuH7.5 cells [9]. Similarly, the HCV-NS3/4a protease is known to cleave IPS-1 adaptor molecule, inducing further downstream blocking of the IFN-inducing signaling pathway [10]. These data clearly demonstrate that the host RIG-I pathway is crucial for suppressing HCV proliferation in human hepatocytes. Using a similar strategy, we investigated whether suppressing the antiviral host innate immune system conferred any advantage on HCV proliferation in mouse hepatocytes. We examined the possibility of HCV replication in mice lacking the expression of key factors that modulate the type I IFN-inducing pathways. Only gene silencing of the IFN receptor (IFNAR) or IPS-1 was sufficient to establish spontaneous HCV replication in

mouse hepatocytes. To establish a cell line permissive for HCV replication, which is required for further *in vitro* studies of the HCV life cycle in mouse hepatocytes, we immortalized IFNAR- and IPS-1-knockout (ko) mice hepatocytes with SV40 T antigen. Upon expression of the human (h)CD81 gene, these newly established cell lines were able to support HCV infection for the first time in mouse hepatocytes. Viral factors required for HCV replication in mouse hepatocytes were also analyzed.

Results

IPS-1-mediated IFN signaling is important for HCV replication in mouse hepatocytes

As a first step in establishing HCV infection in mice, we tested the susceptibility of mouse hepatocytes to persistent expression of HCV proteins after RNA transfection. *In vitro* transcribed chimeric J6JFH1 RNA, in which the HCV structural and non-structural regions were from J6 and JFH1 isolates respectively, was transfected into hepatocytes from wild-type mice. We used a highly sensitive polyclonal antibody derived from HCV-patient serum for the detection of HCV proteins. No HCV proteins were detected five days after transfection (Fig. 1 A), suggesting that wild-type mouse hepatocytes were unable to maintain HCV replication. We then tried to find and block the pathway used by mouse hepatocytes for the detection of viral-RNA and the induction of IFN response. Mouse hepatocytes did not show the expression of either TLR3 or TLR7 as detected by RT-PCR, unlike IPS-1 and RIG-I which was fairly detected (Fig. S1), suggesting that the cytoplasmic RIG-I/IPS-1 pathway is the main pathway utilized by mouse hepatocytes for the detection of RNA viruses. We then checked the susceptibility of hepatocytes from TICAM-1ko, IPS-1ko and IFNARko mice to the prolonged expression of HCV proteins (Fig. 1B–D). Only IPS-1- and IFNARko mouse hepatocytes showed expression of J6JFH1 proteins five days after transfection (Fig. 1), indicating the importance of impaired IPS-1 and/or IFNAR receptors for HCV persistence. Similarly, the detection of the J6JFH1-RNA in transfected hepatocyte lines from various knockout mice showed higher levels in IPS-1 or IFNAR knockout cells compared to TICAM-1knockout cells in which a rapid decline of J6JFH1-RNA levels was noticed similar to the non-replicating control JFH1GND construct (Fig. S2). These data

clearly suggest that the RIG-I/IPS-1 but not TLR3/TICAM-1 is the main pathway utilized for the detection of HCV-RNA and the induction of anti-viral immune response in mouse hepatocytes. Its suppression significantly improves HCV replication in mouse hepatocytes.

Establishment and characterization of immortalized mouse hepatocyte cell lines lacking expression of the IFNAR or IPS-1 gene

We further established mouse hepatocyte lines with disrupted IFNAR or IPS-1 genes through immortalization with SV40T antigen, and used these cell lines to study factors required for the HCV life cycle. Hepatocytes were transduced with SV40T-expressing lentivirus vectors. Six weeks after transduction, hepatocytes transduced with SV40T showed continuous proliferation and clonally proliferating hepatocyte lines were selected. SV40T-immortalized IFNARko and IPS-1ko clones were designated IRK (Fig. 2 A) and IPK (Fig. 2 B), respectively. 20 IRK and 19 IPK clones were picked up, of which IRK clones 2 and 4 (IRK2 and IRK4) and IPK clones 10 and 17 (IPK10 and IPK17) were most closely related to primary mouse hepatocytes in term of differentiation (Fig. 2 C) and were used in the following experiments. Expression of SV40T was confirmed by RT-PCR analysis (data not shown). IRK2, IRK4, IPK10 and IPK17, but not the non-hepatocytic NIH3T3 cells, displayed albumin and hepatocyte nuclear factor 4 (HNF4) expression similar to that observed in liver tissue, but did not express the bile duct marker, cytokeratin. IRK and IPK cells did not show expression of IFNAR and IPS-1 respectively (Fig. 2 C).

Replication of the HCV genome in IRK and IPK cells

To assess the permissiveness of the established cell lines to HCV replication, we transduced IRK4 and IPK17 cells with J6JFH1 RNA and monitored the HCV protein and RNA levels by IF (Fig. 3 A) and real time RT-PCR (Fig. 3 B). The number of cells expressing HCV proteins, as detected by IF, increased over time, indicating the continuous proliferation of J6JFH1 in these cells. However, the ratio between infected and non-infected cells did not significantly change over time for 7 days after transfection. Similarly, the amount of total J6JFH1 RNA in 1 µg of total cellular RNA was reasonably constant. By contrast, the level of

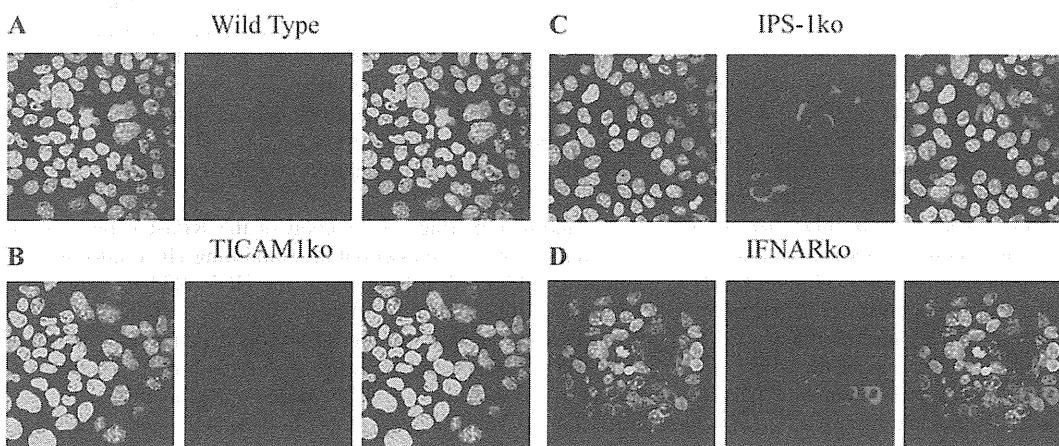


Figure 1. IF detection of of J6JFH1 proteins' expression 5 days after transfection of J6JFH1-RNA through electroporation into wild type (A), TICAM1ko (B), IPS-1ko (C), and IFNARko (D), freshly isolated primary hepatocytes. A highly sensitive polyclonal antibody extracted from HCV-patient serum (AbS3) was used for the detection. Staining of the uninfected hepatocytes from different Ko mice was also performed and they showed negative for HCV proteins (data not shown). doi:10.1371/journal.pone.0021284.g001

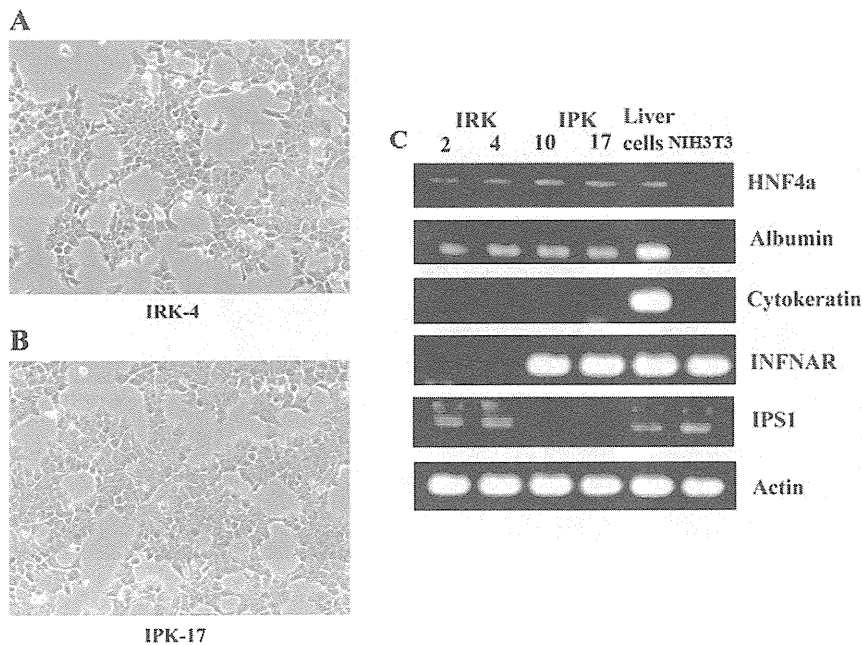


Figure 2. Morphological characteristics of IRK-4 (A) and IPK-17 (B) cells. (C) RT analysis for the expression of albumin, HNF4, cytokeratin, interferon A receptor, and IPS-1 in 2 IFNAR-KO cell lines (IRK2 and 4), 2 IPS-1-KO cell lines (IPK-10 and 17), total liver, and NIH3T3 cells. doi:10.1371/journal.pone.0021284.g002

JFH1GND RNA carrying a mutation in NS5B hampering HCV replication, rapidly declined, indicating the requirement of continuous HCV replication for the maintenance of HCV positivity in the transfected mouse hepatocytes. Similar data were obtained from IRK2 and IPK10 cells (data not shown).

IPS-1-dependent/Interferon-independent pathway is responsible for HCV's cytopathic effect

In comparison to IPS-1ko hepatocytes, J6JFH1-RNA in IFNARko were lower and decreased further after its transfection, while higher stable levels of J6JFH1-RNA were maintained in IPS-1ko cells (Fig. 3 B and Fig. S2). Similarly, larger numbers of HCV-positive cells were detected in IPS-1ko hepatocytes compared with their IFNARko counterparts (Fig. 3 A), suggesting that the IPS-1 disruption benefits HCV replication in a distinct manner from IFNAR disruption. To measure the interferon induction after RNA virus infection in those cells, we used a highly infectious RNA-Virus (VSV) and measured the induction of interferon after its infection. All the interferons measured showed similar suppression of induction in IFNARko and IPS-1ko hepatocytes (Fig. 4). Surprisingly, cellular cytopathic effect that was monitored after transfection of J6JFH1-RNA was markedly reduced in IPS-1ko but not in IFNARko hepatocytes after transfection (Fig. 5A). This suppression was accompanied by an increase of J6JFH1-RNA levels in IPS-1ko cells, suggesting that minimal cellular damage induced by HCV replication in IPS-1-/- cells led to the improvement of HCV proliferation in mouse hepatocytes (Fig. 5B). Reduction of HCV-induced cellular cytotoxicity (Fig. 5C), and improvement of HCV replication (Fig. 5D) in wild type, and IFNAR-KO cells were found when we cultured the cells with a pan-caspase inhibitor, zVAD-fmk, 2 days before and after HCV-RNA transfection. We reasoned that the IPS-1 pathway rather than the IFNAR pathway capacitates hepatocytes to induce HCV-derived apoptotic cell death and its disruption resulted in the circumvention of cell death.

Human CD81 is required for HCV infection of mouse hepatocytes

Similar to the primary mouse hepatocytes, immortalized mouse hepatocytes showed the expression of all the mouse counterparts of human HCV entry receptors (Fig. S3). Human CD81 and hOccludin, but not other human HCV receptors such as SR-B1 or claudin1, have previously been reported to be essential for HCVpp entry into NIH3T3 mouse cells [3]. We then expressed hCD81 and/or hOccludin in IRK2 and IRK4 cells using lentivirus vectors. Using a MOI of 10, 95% transfection efficiency was achieved (Fig. S4) with lentivirus vector. We next tested the effect of these proteins on HCV particle (HCVcc) infection. Human CD81 alone was found to be required for J6JFH1 infection into all IRK and IPK cells tested (Fig. S5 and Fig. 6 A, and B). For the first time in mouse hepatocytes, HCV proteins were detected in nearly 1% of the cells used for infection. These data demonstrated the importance of hCD81 in establishing HCVcc infection in mouse hepatocytes.

Viral factors affecting HCV replication in mouse hepatocytes

After successfully establishing J6JFH1 infection in mouse hepatocytes, we attempted to infect these cells with other strains of HCV. Human CD81-expressing IPK17 cells were infected with full-length JFH1FL, however, no infection was detected (data not shown). This might be due to a problem in infection and/or replication. We further examined the replication efficiency of JFH1FL, the subgenomic JFH1 replicon and the J6JFH1 chimera in two different mouse hepatocyte lines and the HuH7.5.1 cell line. The persistent expression of HCV proteins was detected seven days after RNA transfection. Although HCV proteins were detected in HuH7.5.1 cells in all cases (Fig. 7 C), only J6JFH1 proteins were detected in the mouse hepatocyte lines, suggesting for the first time the importance of the J6 structural region for the replication of HCV in mouse hepatocytes (Fig. 7 A, and B).

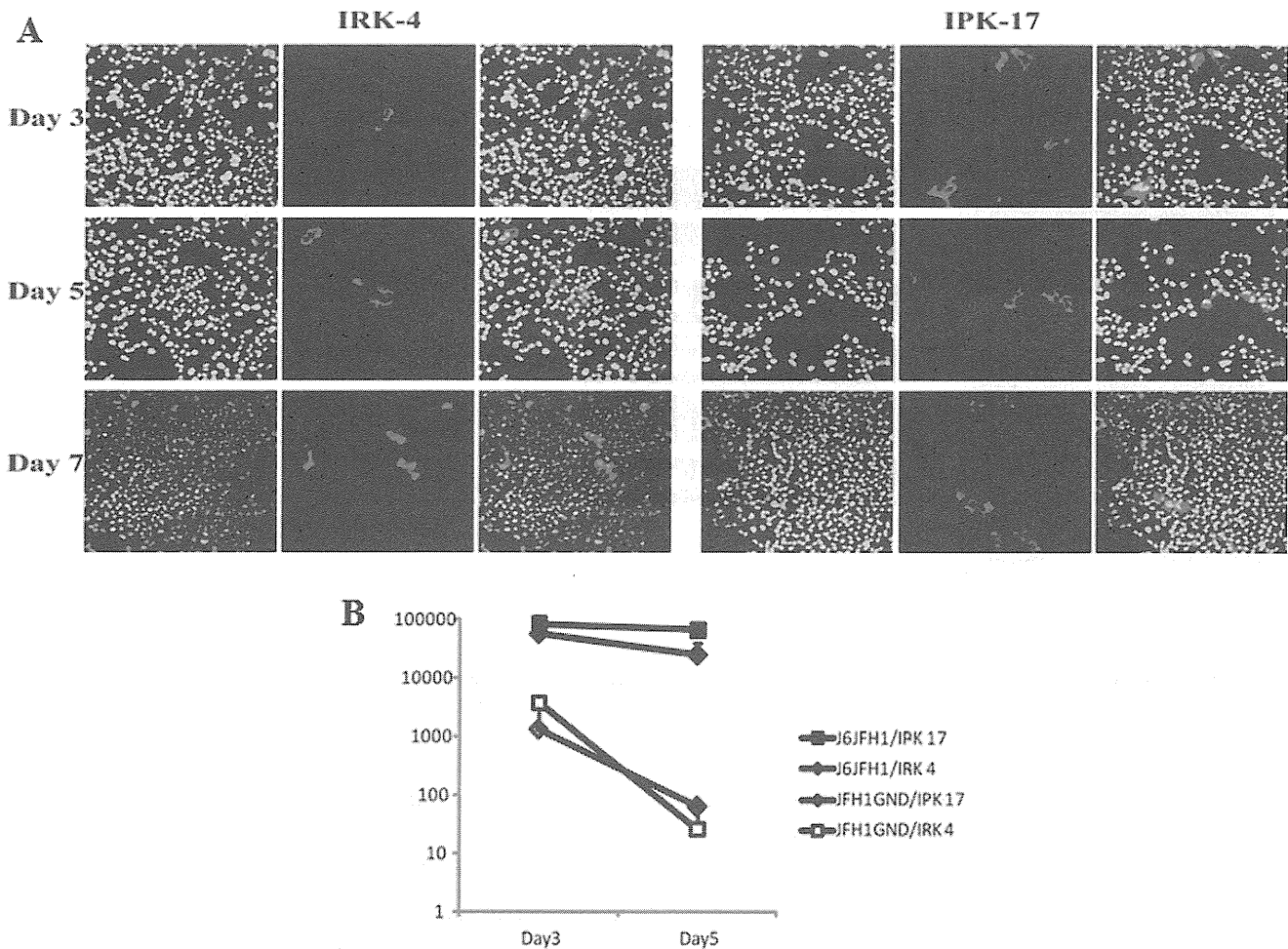


Figure 3. Proliferation of HCV in IRK4 and IPK17 cells over time as detected by immunofluorescence staining of NS5a protein using the CL1 rabbit polyclonal antibody (A) and by quantitative real-time RT-PCR analysis of HCV-RNA levels (B). JFH1GND was used as a negative control to exclude non replicating HCV-RNA. The data plotted represent the average \pm STD of 3 different experiments. doi:10.1371/journal.pone.0021284.g003

Discussion

Gene silencing of either IPS-1 or IFNAR significantly improves HCV replication and persistence in mouse hepatocytes compared with wild-type or TICAM-1ko mice. This result demonstrated the importance of the IPS-1 pathway rather than the TICAM-1 pathway in the induction of type I IFN by HCV infection, and revealed that the IFNAR amplification pathway confers resistance to HCV in mouse hepatocytes independently of TICAM-1. In accordance with our data, HCV-NS3/4A protease is known to cleave the IPS-1 and/or RIG-I-complement molecules including DDX3 and Riplet in humans to overcome the host innate immune response, showing the importance of RIG-I/IPS-1 pathway suppression in the establishment of HCV infection [10,11,12].

To further study factors affecting the HCV life cycle in mouse hepatocytes, we established IPK and IRK immortalized mouse hepatocyte lines by transduction with SV40T antigen. The established hepatocytes cell lines showed expression of HNF4, a major hepatocyte transcription factor, required for hepatocyte differentiation and liver-specific gene expression [13]. The maintenance of hepatocellular functions was demonstrated by continuous expression of hepatocyte specific differentiation marker, albumin, and the lack of expression of the bile duct marker, cytokeratin. The close resemblance of these cell lines to

primary mouse hepatocytes is crucial to ensure the physiological relevance of factors identified in these cell lines that affect the HCV life cycle.

It is worth noting that HCV replication in IPS-1ko was higher than that in IFNARko hepatocytes. Since IPS-1 is present upstream of IFNAR in the IFN-amplification pathway, this higher J6JFH1 replication efficiency in IPS-1ko hepatocytes suggested the presence of an additive factor affecting HCV replication other than the induction of IFNAR-mediated type I IFN. This enhanced replication efficiency was also not accompanied by the induction of other interferon types, but was correlated with the reduction of HCV-induced apoptosis in mouse hepatocytes. This data clearly demonstrates that IPS-1 is playing an important role in the regulation of HCV infection in mouse hepatocytes through two different pathways, the IFN-induction pathways and another new IFN-independent pathway, leading to apoptotic cell death and elimination of HCV-harboring hepatocytes. The cytopathic effect of HCV infection in human cells is still contradictory. Although, some reports showed the induction of apoptosis and cell death by HCV infection in human hepatocytes [14,15,16], others showed suppression of apoptosis by HCV proteins [17,18]. This difference may be due to the different cell lines used in the different studies. Almost all the studies reporting HCV-induced apoptosis used

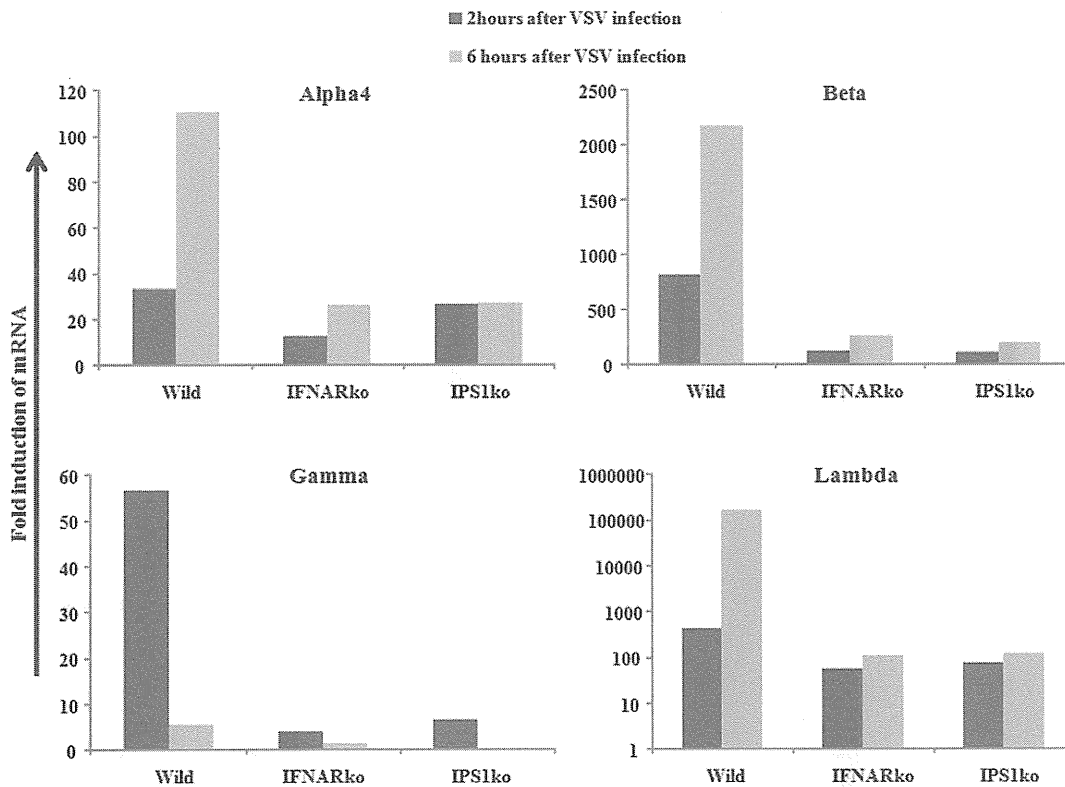


Figure 4. Wild type, IFNARko, and IPS-1ko mice hepatocytes were infected with mock or VSV virus, 2 and 6 hours later, total RNA was extracted from the cells, and interferon alpha, beta, gamma and lambda mRNA induction levels were measured by real-time RT-PCR. Similar results were obtained from 2 different experiments, each was performed in duplicates. The data plotted represent the mean duplicate readings in one of them. doi:10.1371/journal.pone.0021284.g004

hepatocellular carcinoma cell lines. Since it has been established that the inability to undergo apoptosis is essential for the development of cancer [19,20,21], our use of immortalized, non-cancerous hepatocytes may make it possible to reproduce the physiological response of the cells to HCV infection more closely. The IPS-1 regulation of cell death following the introduction of HCV-RNA may also regulate the effector cell function. It is likely that hepatocyte debris generated secondary to intrinsic production of viral dsRNA in HCV-infected hepatocytes affect the antiviral effector response of the immune system through maturation of dendritic cells [22]. Hence, the effector cell activation may be enhanced by the induction of cell death through the IPS-1 pathway in hepatocytes which may facilitate producing dsRNA-containing debris.

In comparison to the JFH1GND construct with deficient replication that showed a rapid reduction in its RNA levels over time after transfection into mouse hepatocytes, J6JFH1 RNA was detected at four-log higher levels and was maintained at a relatively stable levels in IPS-1ko hepatocytes. Although the number of mouse cells expressing HCV proteins was found to increase over time, as detected by IF, the ratio between HCV-negative and -positive cells did not show any significant change for 7 days after transfection and increased after 10 days (data not shown). This indicates a negative selection of HCV-bearing cells over time which may be due to slower cellular replication, or loss of HCV replication. Another possibility may be that HCV infection is affected by the presence of an inhibitory factor possibly triggered by HCV replication or the lack of a human host factor required for HCV replication. Due to the initial replication of

HCV in the transfected IPK and IRK mouse hepatocytes for the first 7 days and the establishment of infection, we favor the presence of a possible inhibitory factor that may be triggered by HCV replication. Another factor that also limits HCV spread in mouse hepatocytes is the failure of HCV to produce infectious particles in these cells (data not shown).

Using this newly established immortalized mouse hepatocyte line, we found that although J6JFH1, JFH1FL and the subgenomic JFH1 replicon all share a similar non-structural region derived from isolate JFH1 that is required for HCV replication, and although all of these constructs can replicate efficiently in HuH7.5.1 cells, strikingly, only J6JFH1 carrying the J6 structural region replicated in mouse hepatocytes. This indicates the importance of the J6 structural region and/or the chimeric construct between J6 and JFH1 for HCV replication in mouse hepatocytes. Structural regions are known to be important for HCV entry and/or particle formation [23], but this is the first time that their importance in replication in HCV-bearing cells has been demonstrated. This finding clearly shows the importance of non-hepatoma cell lines with less genetic abnormalities and mutations for the discovery of new aspects of the life cycle of HCV.

Although, the co-expression of human CD81 and Occludin genes was found to be important for HCVpp entry into murine NIH3T3 cells [3], the expression of hCD81 alone was sufficient for J6JFH1 entry into mouse hepatocytes. This may be explained by the different cell lines used in the different studies. In contrast to NIH3T3 cells, we used immortalized hepatocytes that showed close physiological resemblance to primary mouse hepatocytes and showed the expression of all the mouse counterparts of HCV entry

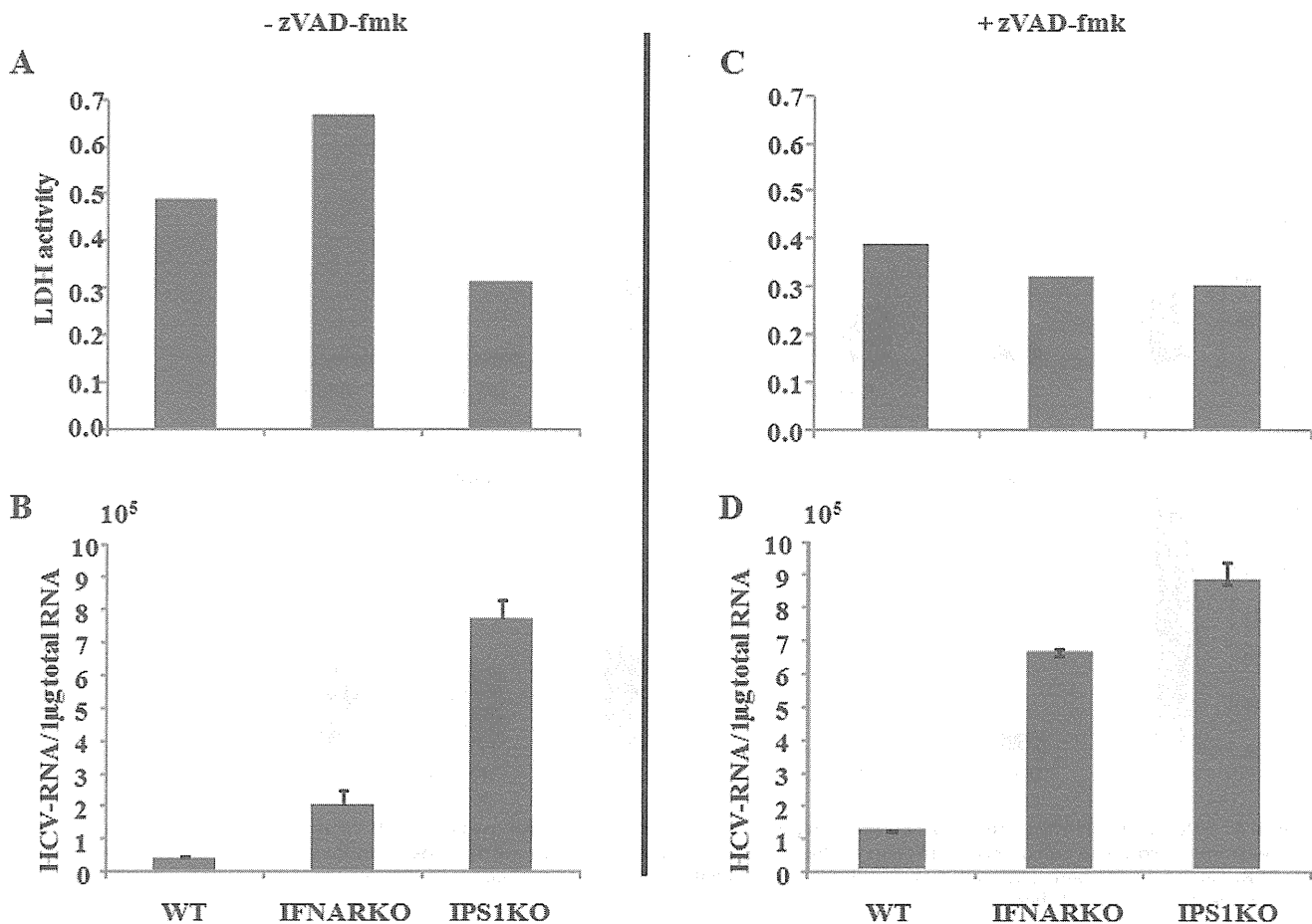


Figure 5. Measurement of J6JFH1 mediated cytopathic effect in wild type, IFNARko, and IPS-1ko mouse hepatocytes. Culture medium were left untreated (A;B) or treated with 20 μ M of zVAD-fmk (C;D) 2 days before and after J6JFH1-RNA transfection. One day after transfection of J6JFH1-RNA, culture medium was discarded and cells were washed with PBS. A new medium was added and cells were cultured for another 24 hours. The LDH activity in the culture medium was measured in 2 different experiments in duplicates and showed similar results, the average levels of a duplicate from a single experiment was plotted (A, C). HCV-RNA titers in the cells were also measured using real-time RT-PCR (B, D), the data shown represent the mean \pm STD of 3 different experiments.
doi:10.1371/journal.pone.0021284.g005

receptors. A study from a different group showed that adaptive mutations in HCV envelope proteins allowing its interaction with murine CD81 is enough for efficient HCVpp entry without the expression of any human entry receptors in murine cells [24]. This report, together with ours, suggest that CD81 is the main human host restriction factor for HCV entry, and that overcoming this problem either by HCV adaptation to murine CD81, or the expression of human CD81 in murine hepatocytes is essential for HCV entry. Although our lentivirus transfection efficiency with CD81 was around 95% in IPK and IRK clones, only 1% of the cells were prone to infection with HCVcc. Also, HCVpp showed lower entry levels in those cells compared to HuH7.5.1 cells (Fig. S6). This suggests that hCD81 expression is the minimum and most crucial requirement for HCV entry into mouse hepatocytes. The discovery and expression of other co-receptors facilitating HCV entry in human cells is still required for efficient and robust HCV infection.

In summary, the suppression of IPS-1 is important for the establishment of HCV infection and replication in mouse hepatocytes through the suppression of both interferon induction and interferon independent J6JFH1-induced cytopathic effect. We have established hepatocytes lines from IPS-1 and IFNARko mice that support HCV replication and infection. These cell lines will be very useful in identifying other species restriction factors and

viral determinants required for further establishment of a robust and efficient HCV life cycle in mouse hepatocytes. Using those cells, we showed for the first time the importance of HCV structural region for viral replication. IRF3ko mouse embryo fibroblasts (MEFs) were previously shown to support HCV replication more efficiently than wild MEFs [25]. Since the knockout of IPS-1 mainly suppresses signaling in response to virus RNA detection, and maintains an intact IFN response to other stimulants, it may result in minimum interference to adaptive immune responses as compared to IRF3 or IFNARko. Therefore, further development of hCD81-transgenic IPS-1ko mice may serve as a good model for the study of immunological responses against HCV infection. This mouse model can be used as a backbone for any further future models supporting robust HCV infectivity for the study of HCV pathogenesis, propagation and vaccine development.

Material and Methods

Cell culture

HuH7.5.1 cells were cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM; Gibco/Invitrogen, Tokyo, Japan) supplemented with 2 mM L-glutamine, 100 U of penicillin/ml, 100 μ g of

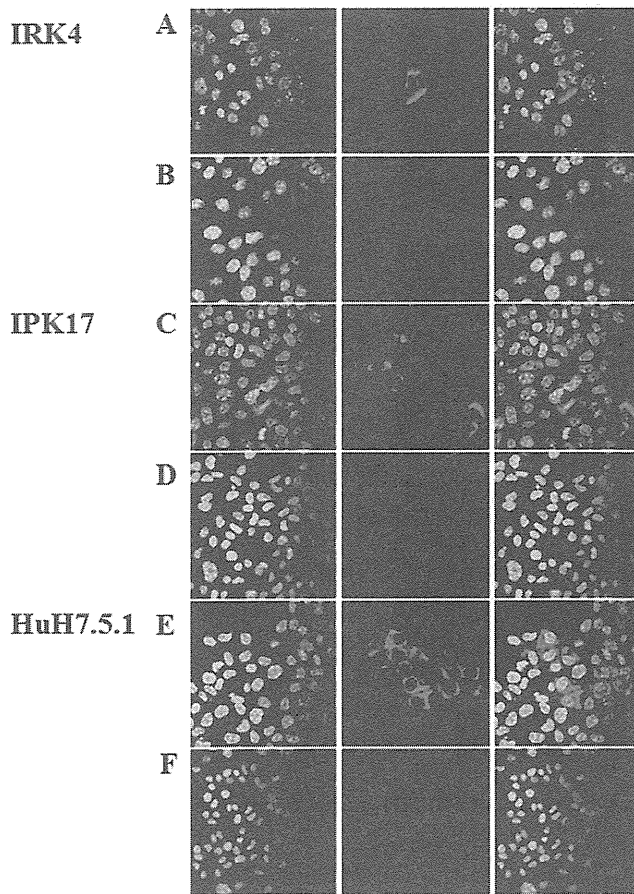


Figure 6. J6JFH1 infection into IRK-4 and IPK17 cells. HCV-NS5A protein detection in mouse IRK4 (A,B) and IPK17 (C,D) and human 7.5.1 cells (E,F). The cells were transduced with lentivirus expressing human CD81 gene at 10 MOI. 48 hours later the cells were infected with 100 times concentrated supernatant medium, collected during 1 week after transfection of HuH7.5.1 cells with J6JFH1-RNA (A, C, and E) or JFH1GND-RNA (B, D, and F). doi:10.1371/journal.pone.0021284.g006

streptomycin/ml and 10% fetal bovine serum. Mouse primary hepatocytes were isolated from the liver using collagenase perfusion through the inferior vena cava (IVC), while clamping the animal's intrathoracic extension. Hepatocyte isolation and perfusion control were performed as previously described [26]. Primary and immortalized hepatocytes were cultured in a similar medium supplemented with: HEPES (Gibco/Invitrogen), 20 mmol/L; L-proline, 30 µg/mL; insulin (Sigma, St. Louis, MO, USA), 0.5 µg/mL; dexamethasone (Wako, Osaka, Japan), 1×10^{-7} mol/L; NaHCO_3 , 44 mmol/L; nicotinamide (Wako), 10 mmol/L; EGF (Wako), 10 ng/mL; L-ascorbic acid 2-phosphate (Wako), 0.2 mmol/L; and MEM-non essential amino acids (Gibco/Invitrogen), 1%.

Gene-disrupted mice

All mice were backcrossed with C57BL/6 mice more than seven times before use. Toll-like receptor adaptor molecule 1 (TICAM-1) ko [27] and IPS-1ko mice [28] were generated in our laboratory (detailed information regarding the IPS-1 mice will be presented elsewhere). All mice were maintained under specific-pathogen-free conditions in the animal facility of the Hokkaido University Graduate School of Medicine (Japan).

RNA extraction, reverse transcriptase polymerase chain reaction (RT-PCR) and real-time RT-PCR

RNA was extracted from cultured cells using Trizol reagent (Invitrogen, San Diego, CA, USA) according to the manufacturer's protocol. Using 1 µg of total RNA as a template, we performed RT-PCR and real-time RT-PCR as previously described [29,30].

In vitro RNA transcription, transfection and preparation of J6JFH1 and Jfh1 viruses

In vitro RNA transcription, transfection into HuH7.5.1 or mouse hepatocytes, and preparation of J6JFH1 and JFH1 viruses, were all performed as previously reported [31]. RNA transfection into human and mouse hepatocytes was performed by electroporation using a Gene Pulser II (Bio-Rad, Berkeley, California) at 260 V and 950 Cap.

HCV infection

J6JFH1 and JFH1 concentrated medium were adjusted to contain a similar RNA copy number by real-time RT-PCR. 2×10^4 cells/well were cultured in 8-well glass chamber slides. After 24 hours, the medium was removed and replaced by concentrated medium containing JFH1 or J6JFH1 viruses. After three hours, the concentrated medium was removed, cells were washed with PBS and incubated in fresh medium for 48 hours, before the detection of infection.

Lentivirus construction, titration and infection

The gene encoding T antigen from simian virus was cloned from plasmid CSII-EF-SVT [32]. The genes encoding human CD81 and occludin were cloned from HuH-7.5.1 cells using the Zero Blunt TOPO PCR Cloning Kit (Invitrogen) according to the manufacturer's protocol. These genes were then inserted into the GFP reporter gene-containing lentiviral expression (pLBIG) vector using the *EcoRI* and *XhoI* restriction sites for SV40T and hCD81, and the *XbaI* and *XhoI* restriction sites for hOccludin. Lentivirus expression vectors were then constructed as previously described [27]. GFP expression was used for the titration of lentivirus vectors, and a multiplicity of infection (MOI) of 10 was used for the infection of mouse cells. Forty-eight hours after the transfection of hCD81 and/or hOccludin, cells were trypsinized and counted. Then, 2×10^4 cells/well were cultured in 8-well glass chamber slides for HCV infection and 5×10^4 cells/well were cultured in 12-well plates, along with 1 ml of medium containing HCVpp, for HCV entry experiments.

HCVpp construction and the detection of luciferase expression

HCVpp containing the E1 and E2 proteins from HCV isolate J6 and expressing the luciferase reporter gene were a kind gift from Dr. Thomas Pietschmann at the TWINCORE Center for Experimental and Clinical Infection Research, Germany. The production of HCVpp and the measurement of luciferase levels were performed as previously described [33].

Indirect immunofluorescence (IF)

IF expression of HCV proteins was detected in the infected cells using antibodies in the serum of chronic HCV patients or rabbit IgG anti-NS5A antibody (Cl-1) (both kind gifts from K. Shimotohno, Chiba Institute of Technology, Japan). Goat anti-human IgG Alexa 594 and goat anti-rabbit Alexa 594 (Invitrogen) were used as secondary antibodies, respectively. Fluorescence

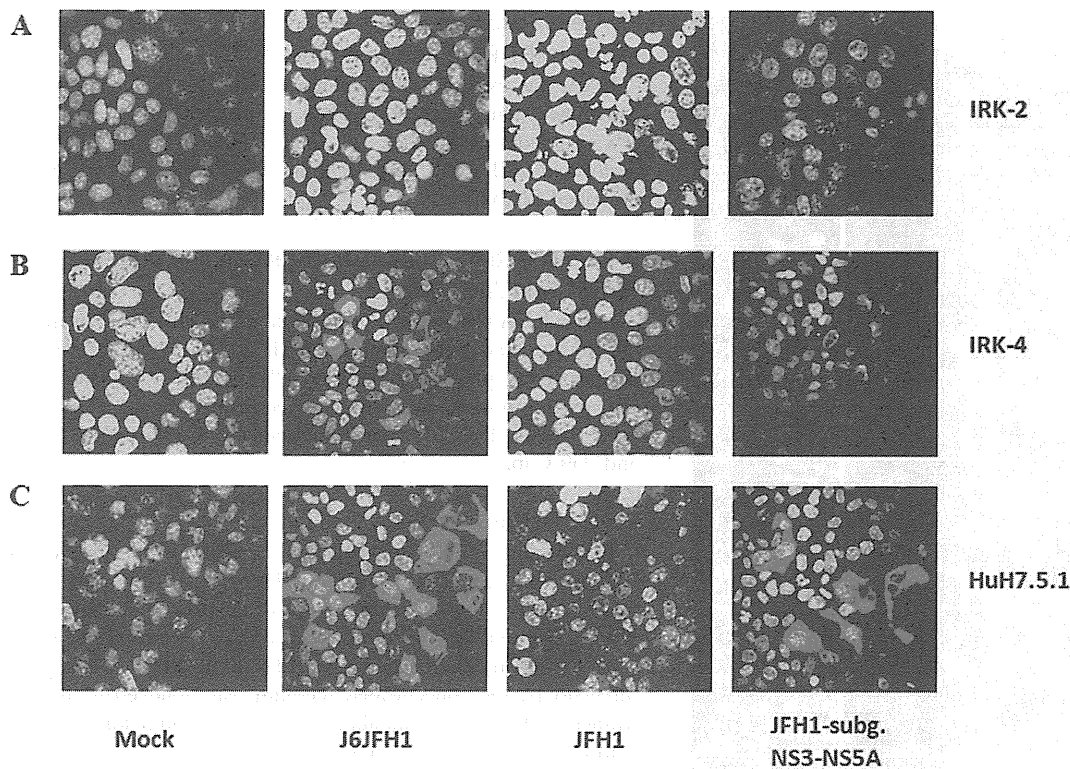


Figure 7. Detection of HCV-NS5A protein in IRK-2 (A), IRK-4 (B) and HuH-7.5 cells (C) by IF 5 days after transfection with J6JFH1, FL-JFH1 or subgenomic JFH1-RNA.
doi:10.1371/journal.pone.0021284.g007

detection was performed on a ZEISS LSM 510 Meta confocal microscope (Zeiss, Jena, Germany).

Detection of cell death

Culture medium was collected from HCV infected and control cells and used for measuring lactate dehydrogenase (LDH) levels using an LDH cytotoxicity detection kit (Takara Biomedicals, Tokyo, Japan). Light absorbance was then measured according to the manufacturer’s protocol.

Ethic Statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments in the Animal Safety Center, Hokkaido University, Japan. All mice were used according to the guidelines of the institutional animal care and use committee of Hokkaido University, who approved this study as ID number: 08-0243, “Analysis of immune modulation by toll-like receptors”.

Supporting Information

Figure S1 RT detection of TLR3, TLR7, RIG-I, and IPS-1 expression in mouse hepatocytes. GAPDH expression was used as internal control, and RNA from CD11c+ splenocytes (dendritic cells) was used as positive control.
(TIF)

Figure S2 Proliferation of HCV in IPS-1, TICAM-1(TRIF) and IFNAR-knockout mouse hepatocytes over time as detected by quantitative real-time RT-PCR analysis of HCV-RNA levels.

JFH1GND transfection into IPS-1 knockout cells was used as a negative control to exclude non replicating HCV RNA. The data plotted represent the average \pm STD of 3 different experiments.
(TIF)

Figure S3 RT detection of CD81, Occludin, Claudin 1, SRB1, and LDL receptor expression in primary, IRK4 and IPK17 mouse hepatocytes. GAPDH expression was used as internal control.
(TIF)

Figure S4 Estimation of the transfection efficiency of lentivirus vector expressing green fluorescent protein (GFP) as a reporter, together with hCD81 or hOccludin. 48 hours after transfection with the lentivirus vector, cells were trypsinized and GFP positive cells were detected by BD FACSCalibur (BD Biosciences).
(TIF)

Figure S5 HCV infection of IRK2 cells transfected with lentivirus expressing hCD81 and/or hOccludin. IRK2 cells were transfected with lentivirus expressing empty vector (A), hCD81 (B), hOccludin (C) or hCD81 and hOccludin (D) at a MOI of 10. After 48 hours, the cells were infected with concentrated J6JFH1 transfected 7.5.1 culture medium. After a further three hours, cells were washed with PBS and incubated in fresh medium. After another 48 hours, HCV infection was examined through the detection of HCV-NS5a protein expression by immunofluorescence staining.
(TIF)

Figure S6 HCVpp entry into mouse cells. A similar number of IPK17 and HuH7.5.1 were cultured in triplicate. IPK17 cells were only transfected with lentivirus expressing hCD81, while HuH7.5.1 cells were transfected with empty vector at a MOI of

10. After 48 hours, the medium was replaced with a new medium containing mock VSVG-pp or HCVpp expressing luciferase. After another 48 hours, pseudoparticles entry was determined by measuring the luciferase activity. In order to compare the HCVpp entry between IPK17 and HuH7.5.1 cells, the luciferase expression from VSV-Gpp entry was used an internal control, while that from HCVpp was plotted relatively. (TIF)

Acknowledgments

We want to thank Dr. Michinori Kohara (Tokyo Metropolitan Institute for Medical Science, Tokyo, Japan); Dr. Tadatsugu Taniguchi (University of

Tokyo, Yokyo, Japan); Dr. Thomas Pietschmann (Division of Experimental Virology, TWINCORE, Hannover, Germany); and Dr. Makoto Hijikata (The Institute for Virus Research, Kyoto University, Japan) for their generous supply of research material. Dr. Hussein H. Aly was supported by a JSPS postdoctoral fellowship from the Japan Society for the Promotion of Science.

Author Contributions

Conceived and designed the experiments: HHA TS. Performed the experiments: HHA HO. Analyzed the data: HHA MM HO HS TS. Contributed reagents/materials/analysis tools: KS TW. Wrote the paper: HHA.

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Future aspects of therapy for hepatitis B virus infection: value of surrogate markers, innovative therapy, and global collaboration

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Maumn Al-Mahtab · Yoichi Hiasa

Received: 16 March 2011 / Accepted: 24 March 2011 / Published online: 28 April 2011
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Abstract Both optimism and frustration exist regarding therapy for patients with chronic hepatitis B virus infection. Due to the recent advent of several drugs with potent antiviral capacities and comparatively low rates of adverse effects, considerable optimism has developed regarding the treatment of these patients. Chronic hepatitis B is now a treatable disease, and suppression of hepatitis B virus replication, normalization of alanine aminotransferase levels, seronegativity/seroconversion of hepatitis B e antigen and hepatitis B surface antigen, and decreased hepatic inflammation and liver fibrosis have been documented in chronic hepatitis B virus-infected patients treated with antiviral therapy. In contrast, many frustrations regarding antiviral therapy for chronic hepatitis B have arisen, because the disease, although treatable, is not curable. The present regimens of antiviral therapy modulate some intermediate parameters or so-called surrogate markers in chronic hepatitis B virus-infected patients, but usually fail to improve all intermediate parameters or ultimate clinical outcomes. In addition, major concerns remain about the applicability and use of antiviral drugs in developing and

resource-constrained countries in which healthcare delivery systems do not support the proper use of antiviral therapy. New and more effective therapeutic regimens for chronic hepatitis B patients are needed that take into account potential surrogate markers of treatment outcomes and allow for effective collaboration between resource-constrained and advanced countries.

Keywords Chronic hepatitis B · Surrogate markers · Developing countries · Immune therapy

Introduction

Hepatitis B virus (HBV), a member of the family *Hepadnaviridae*, is a non-cytopathic DNA virus. Worldwide, nearly 2 billion people have been infected at some point in their lives, and approximately 350–400 million people are chronically infected, a condition that implies ongoing HBV replication in infected hosts. Epidemiologic data indicate that HBV accounts for 0.5–1.2 million deaths annually, and approximately 15–25% of chronic hepatitis B patients will eventually die of HBV-related liver disease [1].

Ideally, all patients with chronic hepatitis B should be treated because they represent a permanent and living reservoir of the virus and can transmit the virus to healthy non-infected subjects. Even with the use of a potent prophylactic vaccine against HBV for the past three decades and the application of different public health measures to contain the spread of HBV, chronic hepatitis B patients are responsible for approximately 50 million new HBV infections each year [2]. In addition, chronic hepatitis B patients often develop complications that may compromise their quality of life and cause early disability and death [3]. However, antiviral therapy is not recommended for all

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chronic HBV-infected subjects, primarily because these drugs are not effective in patients without ongoing host immunity, a fact manifested by evidence of liver inflammation [4]. In addition, all patients with complications of chronic HBV infection should receive antiviral therapy, which aims to delay more severe complications such as hepatic decompensation, cirrhosis of the liver, hepatocellular carcinoma (HCC), and liver-related death. Much optimism has surrounded the use of antiviral therapy because the powerful drugs that have been developed during the past decade can effectively block HBV replication in a considerable number of patients with chronic hepatitis B. Furthermore, antiviral drugs lead to the normalization of alanine aminotransferase (ALT) levels, decrease liver inflammation and hepatic fibrosis, and induce seronegativity and/or seroconversion of hepatitis B e antigen (HBeAg) and hepatitis B surface antigen (HBsAg) in many chronic hepatitis B patients [5–10]. Randomized controlled trials (RCTs) with antiviral drugs in patients with chronic hepatitis B have shown considerable improvements in some intermediate parameters that have long been regarded as surrogate markers of the clinical outcome of HBV infection in these patients. However, improvement in all intermediate parameters of treatment outcome in chronic hepatitis B has not been achieved via the use of antiviral drugs. In addition, there is a lack of consensus regarding improvement of the clinical outcome in chronic hepatitis B patients using antiviral drugs. Some studies have reported some beneficial effects, whereas others have not shown significant improvement of clinical outcomes with antiviral drugs [11–17]. Furthermore, concerns have been raised about the different surrogate markers of outcome of antiviral therapy for chronic hepatitis B that have been used to assess the therapeutic efficacy of the antiviral drugs.

In addition to concerns about the efficacy of antiviral drugs, there are considerable reservations about the practical use of current antiviral regimens in developing and resource-constrained countries. Most clinical trials and RCTs with antiviral drugs in chronic hepatitis B have been conducted in developed, wealthy, and advanced countries. The inherent strength of developed and advanced countries lies in the fact of their advanced healthcare systems, and their socio-economic infrastructure allows for satisfactory drug compliance, proper assessment of patients for treatment, periodic assessment of viral and other markers that herald adverse effects, and adequate tackling of adverse effects [18, 19]. It is unclear whether chronic hepatitis B patients in developing countries can be treated with antiviral drugs according to recommendations based on the data from RCTs conducted in advanced countries [18–20].

Considering these facts, it appears that there are two major challenges for the therapy of chronic hepatitis B.

One is a matter of science and is related to the development of potent and evidence-based therapeutic regimens against HBV. The second is a complex issue that is related to the social, economic, and healthcare delivery systems of developing countries that cannot be altered quickly [18–20]. We assume that these issues can be addressed in a scientific manner by creating real collaboration between developed and developing countries. On the one hand, scientific developments along with basic and clinical trials in advanced countries would elucidate designs for innovative and alternative therapeutic approaches for chronic hepatitis B. However, considerable numbers of chronic hepatitis B patients in advanced countries have already been treated with antiviral drugs. In addition, more powerful antiviral drugs would be used for treatment for chronic hepatitis B in advanced and wealthy countries. This might mean that there will be few cases of treatment-naïve chronic hepatitis B patients in developed countries in whom to conduct clinical trials with new drugs or therapeutic approaches. In contrast, millions of treatment-naïve chronic hepatitis B patients are present in developing countries. The real efficacy of the innovative and alternative therapies developed in advanced countries can only be properly assessed in treatment-naïve chronic hepatitis B patients in developing countries after the proper assessment of safety and efficacy in advanced countries. In addition, the future of therapy for chronic HBV infection may depend on the identification of surrogate markers of treatment outcome for antiviral drugs and a proper understanding of the pathogenesis of chronic HBV infection.

Need for globally applicable therapeutic recommendations for future treatment of chronic hepatitis B

Patients with chronic hepatitis B are treated according to therapeutic recommendations provided by different international and national liver organizations. The American Association for the Study of the Liver (AASLD) [21], the European Association for the Study of Liver Diseases (EASL) [22], and the Asia-Pacific Association for the Study of the Liver (APASL) [23] represent three major liver-related professional organizations that provide therapeutic guidelines for treating chronic hepatitis B patients in international arenas. In addition, different national liver organizations also produce their own recommendations for treating chronic hepatitis B patients, based on local scientific and social realities. The liver organizations update their recommendations from time to time on the basis of scientific developments and transmit these to physicians, policy makers, and patients. The scenario for therapeutic recommendations in most developing and resource-constrained

countries is different from that in advanced countries. Most developing countries that harbor millions of HBV carriers lack viable professional liver organizations. They rarely develop therapeutic recommendations based on local realities and constraints. In fact, recommendations prepared by the AASLD, EASL, and APASL are partially followed by physicians in developing countries [20]. In fact, almost all types of drugs are available in these countries and are used in chronic hepatitis B patients as soon as professional associations, like the AASLD, EASL or APASL, recommend them. If this trend continues, it will be difficult to develop logical and rational treatment approaches that take local factors into account in developing countries. Therapeutic recommendations provided by the AASLD, EASL, and APASL have provided detailed criteria about viral load, and the biochemical and histological aspects required to select patients for antiviral therapy. However, there is a paucity of information about criteria for starting therapy in developing countries. Considering the present realities, it seems that therapeutic recommendations should include a note of caution indicating that for patients who would not be able to continue therapy due to economic and social factors, antiviral drugs for chronic hepatitis B should be prescribed cautiously. Physicians in developing countries should carefully counsel their patients about their economic status before prescribing antiviral drugs against HBV infection.

Ongoing regimens of antiviral therapy against chronic hepatitis B

Standard interferon (IFN) and its pegylated form (Peg IFN) have been used in chronic hepatitis B patients for the past three decades. At present, hepatitis B treatments also include nucleos(t)ide analogs (NAs) categorized as L-nucleosides (lamivudine, emtricitabine, telbivudine, and clevudine), acyclic phosphatases (adevovir and tenofovir), and cyclopentanes (entecavir). These drugs are used as monotherapy or in combination. IFN is usually given for a finite duration. On the other hand, a long-term suppressive course is used for NAs, with drugs being given for years. Different regimens of antiviral therapy with different combinations of drugs have been used in chronic hepatitis B patients. The rationale for these different approaches is to maximize therapeutic benefits while minimizing side effects. The effect of antiviral drugs in chronic hepatitis B patients is determined by the on-treatment effect or a sustained response. Different RCTs have shown that antiviral treatment leads to the improvement of one or more intermediate parameters, also regarded as surrogate markers or prognostic markers, in variable numbers of chronic hepatitis B patients; these parameters include decreased levels

of HBV DNA, normalization of ALT, seronegativity of HBeAg, development of anti-HBe, clearance of HBsAg, expression of anti-HBs, decreased liver inflammation, and containment of liver fibrosis [5–10]. However, a conclusive positive impact of antiviral drugs on all intermediate parameters has not been shown. In addition, there is a lack of consensus about the modulation of clinical outcomes by antiviral therapy in chronic hepatitis B [11–17]. The Minnesota Evidence-based Practice Center (EPC) for a National Institutes of Health (NIH) Consensus Conference related to the management of chronic hepatitis B analyzed different RCTs of antiviral therapy in chronic hepatitis B [24]. Their data show that antiviral drugs did not reduce death, liver failure, or HCC in any credible study that they included in the analyses [25, 26]. They also reported that no single treatment improved all intermediate outcomes. Furthermore, they indicated that there was insufficient evidence to determine whether biochemical, viral, or histological measures are valid surrogates of treatment effects on mortality, liver failure, or HCC.

The apparent discrepancies regarding outcomes of antiviral therapy in chronic hepatitis B patients in individual RCTs may have been influenced by the nature of the study, the number and type of patients, the drugs used, and the method of assessment. On the other hand, the apparent discrepancies among the outcomes and conclusions of different meta-analyses may be attributable to: (1) the number of studies included, (2) the number of studies excluded, (3) the study size, (4) the quality of the studies, (5) criteria for inclusion, (6) criteria for exclusion, and (7) variability of follow-up duration. We are not going to provide a detailed description of these meta-analyses and will not comment on the quality of individual studies. However, the importance of RCTs and meta-analyses is immense in a global context because these studies provide a rationale for designing therapeutic approaches in different countries. The rest of this review focuses on two main issues that seem to be vital for the development of future therapy for HBV infection: (1) the use of surrogate markers for determining the efficacy of antiviral treatment for chronic hepatitis B and (2) the significance of HBV reduction due to antiviral therapy versus that occurring during the natural course of HBV infection, a factor that would validate the use of an alternative therapeutic regimen, such as immune therapy, in chronic hepatitis B.

Use of surrogate markers for determining efficacy of antiviral treatment for chronic hepatitis B

Antiviral drugs, especially NAs, cause a reduction of HBV DNA. In addition, ALT normalization, HBeAg seronegativity, anti-HBe seroconversion, HBsAg seronegativity, anti-HBs seroconversion, and improvement of liver