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Scavenger Receptor Class B Type I Is a Key Host Factor for Hepatitis C Virus Infection Required for an Entry Step Closely Linked to CD81

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis worldwide. Scavenger receptor class B type I (SR-BI) has been shown to bind HCV envelope glycoprotein E2, participate in entry of HCV pseudotype particles, and modulate HCV infection. However, the functional role of SR-BI for productive HCV infection remains unclear. In this study, we investigated the role of SR-BI as an entry factor for infection of human hepatoma cells using cell culture–derived HCV (HCVcc). Anti-SR-BI antibodies directed against epitopes of the human SR-BI extracellular loop specifically inhibited HCVcc infection in a dose-dependent manner. Down-regulation of SR-BI expression by SR-BI-specific short interfering RNAs (siRNAs) markedly reduced the susceptibility of human hepatoma cells to HCVcc infection. Kinetic studies demonstrated that SR-BI acts predominately after binding of HCV at an entry step occurring at a similar time point as CD81–HCV interaction. Although the addition of high-density lipoprotein (HDL) enhanced the efficiency of HCVcc infection, anti-SR-BI antibodies and SR-BI-specific siRNA efficiently inhibited HCV infection independent of lipoprotein. **Conclusion:** Our data suggest that SR-BI (i) represents a key host factor for HCV entry, (ii) is implicated in the same HCV entry pathway as CD81, and (iii) targets an entry step closely linked to HCV–CD81 interaction. (HEPATOLOGY 2007;46: 1722–1731.)

Abbreviations: cDNA, complementary DNA; CHO, Chinese hamster ovary; HCV, hepatitis C virus; HCVcc, cell culture–derived HCV; HCVpp, HCV pseudotype particles; HDL, high-density lipoprotein; HRP, horseradish peroxidase; IgG, immunoglobulin G; LDL, low-density lipoprotein; LPDS, lipoprotein-deficient human serum; siRNA, small interfering RNA; SR-BI, scavenger receptor class B type I.

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With an estimated 170 million infected individuals, hepatitis C virus (HCV) has a major impact on public health.¹ The liver is the primary target organ of HCV, and the hepatocyte is its primary target cell. Attachment of the virus to the cell surface followed by viral entry is the first step in a cascade of interactions between the virus and the target cell that is required for successful entry into the cell and initiation of infection.² Using recombinant HCV envelope glycoproteins³ and HCV pseudotype particles (HCVpp),^{4,5} several cell surface molecules have been identified interacting with HCV during viral binding and entry. These include the tetraspanins CD81³ and claudin-1,⁶ highly sulfated heparan sulfate,⁷ the low-density lipoprotein (LDL) receptor,⁸ and scavenger receptor class B type I (SR-BI).⁹

SR-BI is a 509–amino acid glycoprotein with a large extracellular loop anchored to the plasma membrane at both the N- and C- termini by transmembrane domains with short extensions into the cytoplasm.¹⁰ SR-BI is involved in bidirectional cholesterol transport at the cell membrane and can bind both native high-density lipoprotein (HDL) and LDL as well as modified lipoproteins such as oxidized LDL. SR-BI is highly expressed in liver and steroidogenic tissues¹⁰ as well as antigen-presenting cells.¹¹ Furthermore, SR-BI and its splicing variant SR-BII, have been found to mediate binding and uptake of a broad range of bacteria into human epithelial cells overexpressing SR-BI and SR-BII,^{12,13} suggesting that class B scavenger receptors may serve as pattern recognition receptors for bacteria.

Cross-linking studies using recombinant C-terminally truncated HCV envelope glycoprotein E2 isolated SR-BI as a cellular protein binding envelope glycoprotein E2.⁹ Antibodies directed against cell surface expressed SR-BI partially inhibited cellular binding of recombinant envelope glycoproteins¹⁴ as well as HCVpp entry.^{15–17} Moreover, it has been shown that physiological SR-BI ligands, such as HDL or oxidized LDL, can modulate HCV infection either by enhancing or by inhibiting HCVpp entry, respectively.^{18–20}

Recently, several laboratories succeeded in establishing a model for the efficient production of infectious HCV particles in cell culture (HCVcc),^{21–23} now allowing determining of the role of cell surface molecules involved in HCV infection. Recent evidence suggests that SR-BI and CD81 may act in a cooperative manner for the initiation of HCVcc infection²⁴ and that overexpression of SR-BI can modulate HCVcc infection.²⁵ However, the functional role of SR-BI in productive HCV infection still remains elusive. In particular, it is unclear whether the impact of SR-BI for HCV entry is of key importance or optional, whether SR-BI and CD81 are involved in the

same pathways of HCV entry, and which HCV entry step is targeted by SR-BI.

Therefore, in this study, we used the HCVcc system to analyze the functional role of SR-BI for productive HCV infection of human hepatoma cells. Using novel anti-SR-BI antibodies and SR-BI-specific short interfering RNAs (siRNAs), we demonstrate that SR-BI (i) represents a key host factor for HCV entry, (ii) is most likely implicated in the same HCV entry pathway as CD81, and (iii) mediates an entry step occurring postbinding and closely linked to HCV–CD81 interaction.

Materials and Methods

Cells. Human embryonic kidney cells 293T, Chinese hamster ovary cells CHO, and Huh7.5 have been described.^{4,7,26,27} Primary human hepatocytes were isolated and cultured as described.²⁸

Antibodies. Antibodies directed against the extracellular loop of SR-BI were raised by genetic immunization of Wistar rats and Balb/c mice using a pcDNA-expression vector containing the full-length human SR-BI complementary DNA (cDNA) (pcDNA SR-BI/CLA-1) (Genovac GmbH, Freiburg, Germany).²⁹ In brief, animals received 4 applications of 50 μ g pcDNA SR-BI intradermally using a GeneGun (BioRad) at 2-week intervals. Pre-immune control serum was collected from the same animal bled before immunization. To analyze specificity of the produced anti-SR-BI polyclonal serum, CHO cells were transfected with pcDNA (control vector) or pcDNA SR-BI using liposome-mediated gene transfer (Lipofectamine; Invitrogen, Karlsruhe, Germany) according to the manufacturer's protocol. CHO cells were then incubated with anti-SR-BI polyclonal serum or pre-immune control serum and analyzed for cell surface SR-BI expression by flow cytometry as described.¹⁴ R-phycoerythrin-conjugated goat anti-rat immunoglobulin G (IgG) antibody was purchased from Jackson ImmunoResearch Laboratories (West Grove, PA). Because of the small amounts of pre-immune sera from individual animals used for immunization, commercially available nonimmune rat serum (PAN Biotech) was used as an additional negative control serum for experiments. Rabbit anti-SR-BI antibody (NB 400-104) was obtained from Novus Biologicals (Littleton, CO). This antibody is directed against an epitope within the SR-BI cytoplasmic C-terminal domain (CSPAAGKGTVLQEAKL, corresponding to amino acids 496 through 509). Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG and HRP-conjugated anti-mouse IgG antibodies were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden), mouse anti-

β -actin antibody was from Sigma and anti-CD81 (JS-81) from BD Biosciences (Heidelberg, Germany).

RNA Interference Assay. Commercially available siRNA pools targeting SR-BI, CD81, and CD13 as well as control nontargeting siRNAs were purchased from Dharmacon (Pierce) and transfected into Huh7.5 cells using DharmaFect solution (Pierce) following the manufacturer's protocol. Silencing of SR-BI expression was assessed by western blot and flow cytometry 72 hours after transfection as described previously.¹⁴ Seventy-two hours after transfection, cells were incubated with HCVcc, and HCV infection was assessed as described in the following sections.

Production of Viral Stocks and Infection Assays. Plasmids pJFH1, pFK-Jc1, and pFK-Luc-Jc1 have been described^{21,30,31} and encode the full-length HCV Japanese fulminant hepatitis (JFH) cDNA or the chimeric HCV genome designated Jc1, which consists of J6CF and JFH1 segments. The latter construct (pFK-Luc-Jc1) represents a bicistronic reporter virus carrying a firefly-luciferase reporter gene.³⁰ *In vitro* HCV RNA synthesis³⁰ and RNA transfection was performed as described.^{21,29} To study the effect of HDL on HCVcc infection, JFH1 HCVcc were also generated in lipoprotein-deficient human serum (LPDS), and HDL (30 μ g/mL)³³ was added extemporaneously for infection experiments. Culture supernatants from transfected cells were cleared and concentrated as previously described using Amicon Ultra 15 (Millipore, Billerica, MA)²¹ and used directly or stored at 4°C or -80°C. Viruses were titered by using the limiting dilution assay on Huh7.5 cells with a few minor modifications, and 50% tissue culture infective dose was calculated based on the method described.²² siRNA expressing cells and naïve cells were seeded 24 hours before infection experiments in 12-well tissue culture plates at a density of 5×10^4 cells/well. Cells were preincubated in the presence or absence of anti-SR-BI serum or control serum for 1 hour at 37°C and then infected at 37°C for 3 hours with JFH1 HCVcc challenge virus titers ranging from 1×10^7 to 5×10^9 copies/mL or 4 hours with Luc-Jc1 HCVcc at low multiplicity of infection. Alternatively, Huh7.5 were inoculated with a high-titer Luc-Jc1 stock for 1 hour at 4°C in the presence or absence of anti-SR-BI serum or control serum, heparin, anti-CD81 monoclonal antibodies, or concanamycin A at concentrations indicated in the text. Subsequently, cells were washed 3 times with ice-cold phosphate-buffered saline, supplied with fresh culture fluid prewarmed to 37°C and supplemented with the respective inhibitors and shifted to 37°C. Finally, 4 hours later, cells were washed with prewarmed phosphate-buffered saline, supplied with fresh culture fluid without inhibitors, and cultured an additional 48 hours at 37°C. Depending on the experiment, cells were then washed

with ice-cold phosphate-buffered saline and RNA extracted using RNeasy Mini kit (Qiagen, Hilden, Germany). Alternatively, cells were lysed for luciferase assay as previously described.³⁰ HCV RNA was quantitated using VERSANT HCV-RNA 3.0 Assay (bDNA) (Bayer Corporation Diagnostic, Tarrytown, NY) or TaqMan real-time polymerase chain reaction as described.³⁴

Results

Production of Antibodies Directed Against the Extracellular Loop of SR-BI Expressed on Human Hepatocytes. To assess the functional role of SR-BI for initiation of HCV infection, we first generated polyclonal anti-SR-BI sera directed against the extracellular loop of SR-BI by genetic immunization. After completion of immunization, antibodies were selected for their ability to bind to human SR-BI expressed on the cell surface of nonpermeabilized transfected CHO cells. As shown in Fig. 1, incubation of CHO cells expressing human SR-BI with rat polyclonal anti-SR-BI antibodies resulted in a specific interaction of this serum with the extracellular ectodomain of SR-BI (Fig. 1). In contrast, no interaction was present in CHO cells transfected with the pcDNA3 control vector and incubated with rat anti-SR-BI serum or in CHO cells transfected with human SR-BI cDNA and incubated with rat preimmune serum

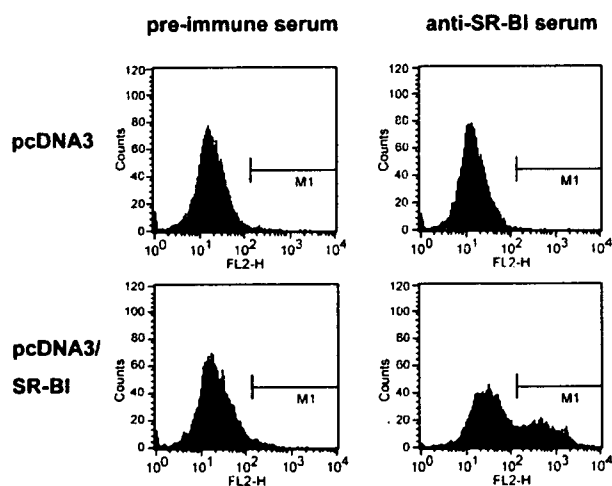


Fig. 1. Production of antibodies directed against the human SR-BI ectodomain by genetic immunization. Specific binding of rat anti-human SR-BI serum to SR-BI expressed in CHO cells. Anti-SR-BI polyclonal serum directed against the SR-BI ectodomain loop was raised by genetic immunization of Wistar rats using a plasmid harboring human SR-BI cDNA. CHO cells were transfected with pcDNA-SR-BI (pCDhSR-BI) or control vector (pcDNA). Flow cytometry of SR-BI or control transfected nonpermeabilized CHO cells incubated with rat anti-human SR-BI polyclonal serum and phycoerythrin-conjugated anti-rat IgG demonstrated specific interaction of anti-SR-BI antibodies with human SR-BI. In contrast, no interaction was present in CHO cells transfected with control vector and incubated with anti-SR-BI serum.

(Fig. 1). To study whether anti-human SR-BI recognizes SR-BI on cells susceptible to HCV infection, human hepatocytes and Huh7.5 hepatoma cells were incubated with the sera and analyzed by flow cytometry. As shown in Fig. 2, incubation of human Huh7.5 cells (Fig. 2A) and human hepatocytes (Fig. 2B) with rat polyclonal anti-SR-BI antibody demonstrated that the antibody recognized SR-BI expressed on HCV target cells, including human hepatocytes. In contrast, no interaction could be detected in the mouse

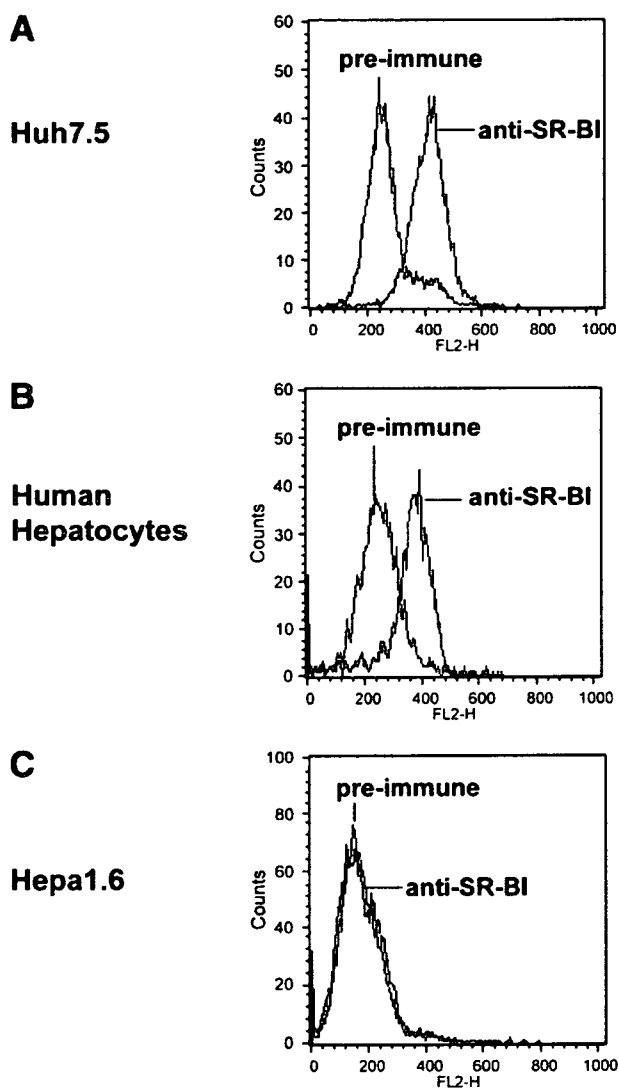


Fig. 2. Interaction of anti-SR-BI antibodies with the SR-BI ectodomain on human hepatocytes and Huh7.5 hepatoma cells. Cell surface expression of SR-BI was determined by flow cytometry using rat anti-human SR-BI serum or control pre-immune serum as described in Fig. 1. Histograms corresponding to cell surface expression of the respective cell surface molecules (open curves) are overlaid with histograms of cells incubated with the appropriate isotype control (gray shaded curves). In contrast to absent interaction on murine Hepa1.6 hepatoma cells, rat anti-human SR-BI serum specifically detected SR-BI on the cell surface of human hepatoma Huh7.5 cells and human primary hepatocytes.

cell line Hepa1.6 (Fig. 2C), confirming the species specificity of the antibody. Similar results were obtained for anti-SR-BI antibodies raised in Balb/c mice (data not shown). Taken together, these data demonstrate that anti-SR-BI sera produced by genetic immunization specifically binds to the ectodomain of human SR-BI expressed on hepatocytes.

Inhibition of HCV Infection of Different Isolates by Anti-SR-BI Antibodies. To assess the role of SR-BI for HCV infection, we studied JFH1 HCVcc infection of Huh7.5 cells in the presence of anti-SR-BI antibodies directed against epitopes of the SR-BI extracellular loop. Anti-SR-BI polyclonal antibodies markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a dose-dependent manner (Fig. 3A). Fig. 3A shows that anti-SR-BI serum (rat 4) inhibited JFH1 HCVcc infection by more than 70% (Fig. 3A). In contrast, the control pre-immune serum had no inhibitory effect on JFH1 HCVcc infection (Figs. 5, 6). Moreover, mouse anti-SR-BI antibodies generated by genetic immunization of Balb/c mice but not mouse control pre-immune serum were able to reduce JFH1 HCVcc infection of Huh7.5 in a similar manner (data not shown). Taken together, the data demonstrate that antibodies directed against the SR-BI ectodomain efficiently inhibit HCV infection.

To confirm that inhibition of JFH1 HCVcc infection was indeed mediated by anti-SR-BI antibodies, we purified IgG from both rat anti-SRBI (rat 4) and control serum. As shown in Fig. 3B, anti-SR-BI IgG (100 μ g/mL) markedly inhibited JFH1 HCVcc infection of Huh7.5 cells in a similar manner as anti-SR-BI serum (Fig. 3B). In contrast, control IgG (100 μ g/mL) purified from pre-immune serum did not inhibit JFH1 HCVcc infection (Fig. 3B). These data clearly demonstrate that the inhibitory effect of anti-SR-BI serum is mediated by anti-SR-BI antibodies and not by other substances present in the serum (such as oxidized lipoproteins potentially interfering with SR-BI function).

To study whether anti-SR-BI-mediated inhibition of HCV infection applies also to isolates other than JFH1, we performed similar experiments using chimeric J6/CF-JFH1 firefly luciferase reporter virus Luc-Jc1.³⁰ Figure 3C shows that, similar as for JFH1 HCVcc, both rat (rat 4) and mouse anti-SR-BI antibodies (data not shown) reduced the chimeric reporter virus infectivity in a dose-dependent manner (Fig. 3C), whereas the control pre-immune sera had no inhibitory effect (Fig. 3C and data not shown). Interestingly, we observed variations between the inhibitory effect of anti-SR-BI sera from different rats ranging from 70%-90%. Strongest inhibition was obtained with anti-SR-BI serum from rat 5 (Figs. 5 and 6), demonstrating greater than 90% inhibition of HCVcc infection, with both JFH1 isolate and Luc-Jc1 chimera.

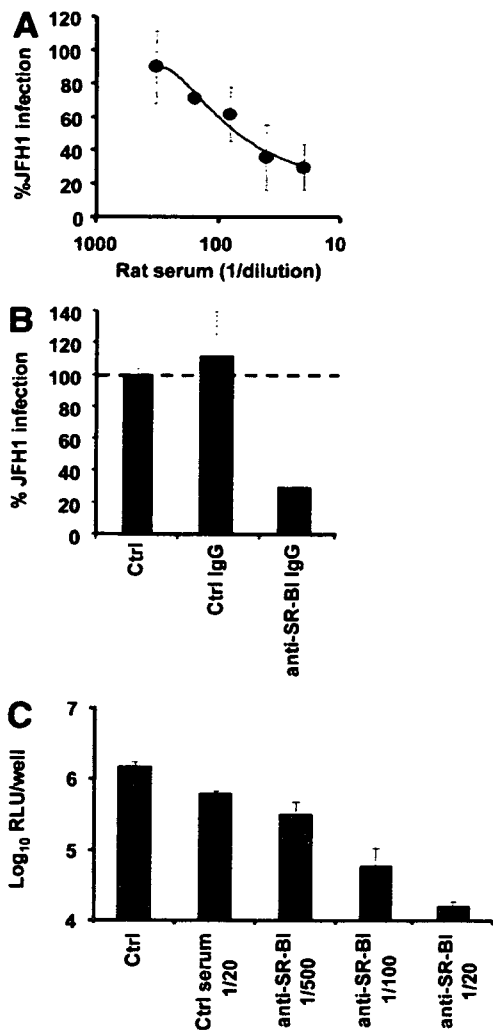


Fig. 3. Inhibition of HCV infection by anti-SR-BI antibodies. (A) Inhibition of JFH1 HCVcc infection by rat polyclonal anti-SR-BI antiserum. Huh7.5 cells were preincubated for 1 hour at 37°C with various dilutions of rat anti-SR-BI or control serum before infection with JFH1 HCVcc for 3 hours at 37°C. HCV infection was assessed by HCV RNA quantitation in lysates of infected Huh7.5 cells 72 hours post-infection. Total RNA was isolated and HCV RNA was quantified as described in Materials and Methods. Results are expressed as mean percentage HCVcc infectivity in the absence of antibody (mean \pm SD; n = 4). (B) Inhibition of JFH1 HCVcc infection by purified rat anti-SR-BI IgG. Huh7.5 cells were preincubated for 1 hour at 37°C with 100 μ g/mL IgG isolated from rat anti-SR-BI or control serum before infection with JFH1 HCVcc. Results are expressed as percent HCVcc infectivity in the absence of antibody (mean \pm SD; n = 4). (C) Inhibition of Luc-Jc1 HCVcc infection by anti-SR-BI. Huh7.5 cells were preincubated for 1 hour at 37°C with various dilutions of rat anti-SR-BI serum or control serum before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed by measurement of luciferase activity 48 hours after infection in lysates of infected cells. Results are expressed as mean Log₁₀ RLU/well (mean \pm SD; n = 4).

Silencing of SR-BI Expression Results in Markedly Reduced Susceptibility to HCV Infection. To further investigate the role of SR-BI in HCVcc infection, we silenced SR-BI expression in Huh7.5 cells using siRNAs tar-

geting SR-BI expression. Immunoblot analysis of transfected cells shows that SR-BI-specific siRNA reproducibly down-regulates SR-BI expression, whereas β -actin expression was not affected (Fig. 4A). In contrast, a pool of negative control siRNA as well as siRNA targeting CD81 or CD13 did not significantly modulate SR-BI expression, confirming the specificity of the siRNA used (Fig. 4A). Down-regulation of SR-BI expressed on the cell surface of Huh7.5 cells by SR-BI-specific siRNA was also confirmed by flow cytometry (difference in mean fluorescence intensity (Δ MFI) of SR-BI siRNA-treated cells = 13.98 versus Δ MFI of naïve cells = 148.31). Importantly, down-regulation of SR-BI cell surface expression strongly reduced the susceptibility of human hepatoma cells to infection with HCV (Fig. 4B). As shown in Fig. 4B, siRNA targeting SR-BI or CD81 markedly inhibited JFH1 HCVcc infection of Huh7.5 cells as compared with cells without silenced cell surface molecules (Fig. 4B).

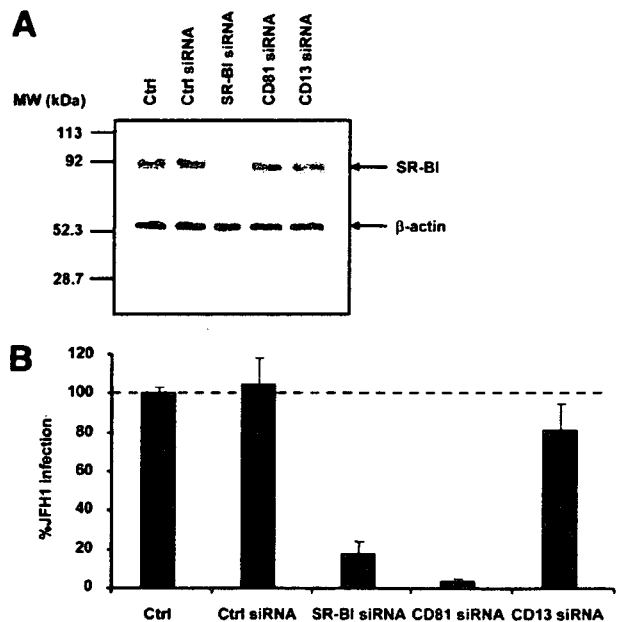


Fig. 4. Silencing of SR-BI expression results in reduced susceptibility to HCV infection. (A) Western blot analysis of siRNA mediated down-regulation of SR-BI expression in Huh7.5 cells. Lysates of control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis. Immunoblotting was performed using rabbit anti-SR-BI antibodies (1/4000) and HRP-conjugated anti-rabbit antibodies (1/1000) or mouse anti- β -actin monoclonal antibody (1/5000) and HRP-conjugated anti-mouse antibodies (1/1000). The presence or absence of SR-BI and β -actin is indicated on the right, and molecular weight (MW) markers (kDa) are indicated on the left. (B) Susceptibility to HCVcc infection is reduced in SR-BI specific siRNA expressing Huh7.5 cells. Control naïve Huh7.5 (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were incubated with JFH1 HCVcc. Total RNA was extracted 72 hours after infection, and HCV RNA was quantified. Data are expressed as percent HCVcc infectivity of naïve control cells (mean \pm SD; n = 4).

In contrast, pools of negative control siRNA as well as CD13-specific siRNA did not significantly reduce JFH1 HCVcc infection (Fig. 4B). Taken together, reduced susceptibility to HCV infection by specific silencing of SR-BI expression clearly demonstrates that SR-BI plays a key role for the establishment of HCV infection of human hepatoma cells.

Impact of Lipoproteins for SR-BI-Mediated HCV Infection. To investigate the impact of lipoproteins on SR-BI-mediated HCV infection, we determined the ability of anti-SR-BI antibodies to inhibit JFH1 HCVcc infection in the absence of HDL, a physiological SR-BI ligand that has been shown to enhance HCVcc infection of human hepatoma cells.³⁵ To study the role of HDL during inhibition experiments, HCVcc were generated in medium supplemented with LPDS, and HDL was added extemporaneously for infection experiments. Fig. 5A shows that rat anti-SR-BI serum (rat 5) inhibited JFH1 HCVcc infection of Huh7.5 cells in the absence of HDL (Fig. 5A). Interestingly, whereas HDL was able to enhance JFH1 HCVcc infection in control cells and control serum preincubated cells, no such effect was observed in the presence of anti-SR-BI antibodies in concentrations blocking HCVcc infection (Fig. 5A). These results suggest that these antibodies may block both HCV interaction with SR-BI and HDL-mediated enhancing effect on HCVcc infection. To study whether HDL-dependent enhancement of HCVcc infection was dependent on the level of input virus, we repeated experiments using different JFH1 HCVcc preparations with challenge virus titers ranging from 1×10^7 copies/mL to 5×10^9 copies/mL, resulting in similar observations (data not shown). Furthermore, the effects of HDL on HCVcc infection were confirmed by titration experiments using anti-SR-BI antibodies: as shown in Fig. 5A, the enhancing effect of HDL on HCVcc infection appeared to be restored when anti-SR-BI antibodies were used at decreasing concentrations (Fig. 5A). In addition, the role of HDL on JFH1 HCVcc infection was also studied in siRNA-transfected Huh7.5 cells. As shown in Fig. 5B, a minor enhancing effect of HDL was detected in Huh7.5 cells transfected with siRNA targeting SR-BI, suggesting that a low level of SR-BI may still be available for HCV/HDL interplay on these cells. In cells with silenced CD81 expression, no marked enhancing effect of HDL on JFH1 HCVcc infection was observed (Fig. 5B).

SR-BI Mediates an HCV Entry Step Occurring Postbinding and Closely Linked to CD81. Kinetic studies using chimeric JFH1 firefly luciferase reporter virus have demonstrated that glycosaminoglycans predominantly act at the stage of HCV attachment to target cells, whereas CD81 mediates HCV infection at a step post-binding.³¹ To map the step targeted by SR-BI during HCV entry, we investigated the inhibitory capacity of

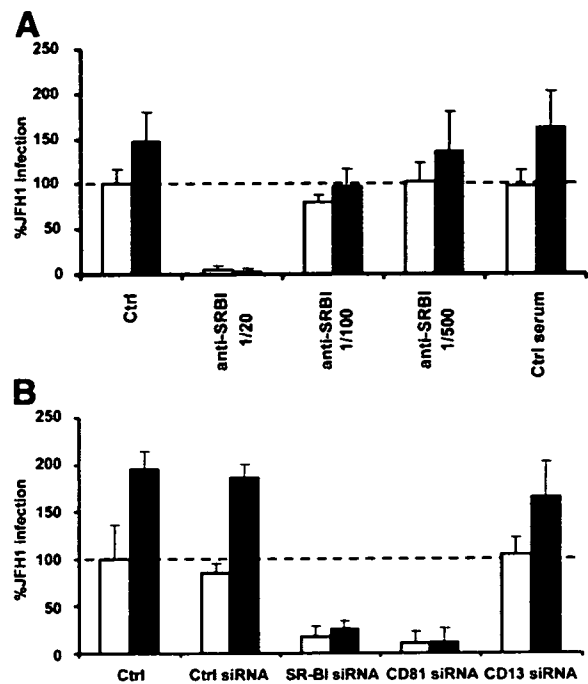


Fig. 5. SR-BI-mediated HCV infection is not dependent on the presence of lipoproteins. (A) Anti-SR-BI inhibits HCVcc infection in the absence of lipoproteins. Huh7.5 cells were preincubated for 1 hour at 37°C with rat anti-SR-BI serum or control serum (diluted 1/20, 1/100, and 1/500) before infection with JFH1 HCVcc generated in LPDS-medium in the presence (black bars) or absence (open bars) of HDL (30 μ g/mL). Total RNA was isolated 72 hours after infection, and HCV RNA was quantified. Results are expressed as percent HCVcc infectivity in the absence of antibody (mean \pm SD; n = 4). (B) Reduced susceptibility to HCVcc infection in SR-BI-specific siRNA expressing Huh7.5 cells is independent of lipoproteins. Control naïve Huh7.5 cells (Ctrl), Huh7.5 cells expressing control siRNA (Ctrl siRNA), or siRNA targeting SR-BI, CD81, or CD13 were incubated with JFH1 HCVcc generated in LPDS medium in the presence (black bars) or absence (open bars) of HDL (30 μ g/mL). Total RNA was extracted 72 hours after infection, and HCV RNA was quantified. Data are expressed as mean percent HCVcc infectivity of naïve control cells (mean \pm SD; n = 4).

anti-SR-BI serum (rat 5) and corresponding purified IgG when administered during or after virus binding in side-by-side experiments using heparin—a structural and functional homolog of highly sulfated heparan sulfate and anti-CD81 antibody. Luc-Jc1 HCVcc binding to Huh7.5 cells was performed for 1 hour at 4°C in the presence or absence of inhibitors. Under these conditions, virus attaches to the cells but does not efficiently enter, thus permitting synchronous infection when the inoculum is removed and cells are shifted to 37°C. Therefore, subsequent to virus attachment, unbound virus was washed away, cells were shifted to 37°C to allow entry to proceed, and inhibitors or control medium were added for 4 hours (Fig. 6A). Figure 6B shows that rat anti-SR-BI serum as well as purified anti-SR-BI IgG were able to inhibit Luc-Jc1 HCVcc infection when added following

binding of the virus to the target cell (Fig. 6B). The control serum only had no significant effect on Luc-Jc1 HCVcc infection (Fig. 6B). In contrast, heparin—a homolog of highly sulfated heparan sulfate, inhibited Luc-Jc1 HCVcc infection only when it was present during virus binding but not when added postbinding (Fig. 6B). To further characterize the entry step mediated by SR-BI, anti-SR-BI and anti-CD81 antibodies were added in side-by-side experiments every 20 minutes for up to 120 minutes after viral binding (Fig. 7A). Rat anti-SR-BI serum was able to inhibit Luc-Jc1 HCVcc infection even when added up to 60 minutes after HCVcc binding (Fig. 7B). These data clearly indicate that SR-BI is involved in an entry step occurring after binding. Because almost identical kinetics of inhibition of HCV infection was observed for anti-CD81 antibody assessed in side-by-side

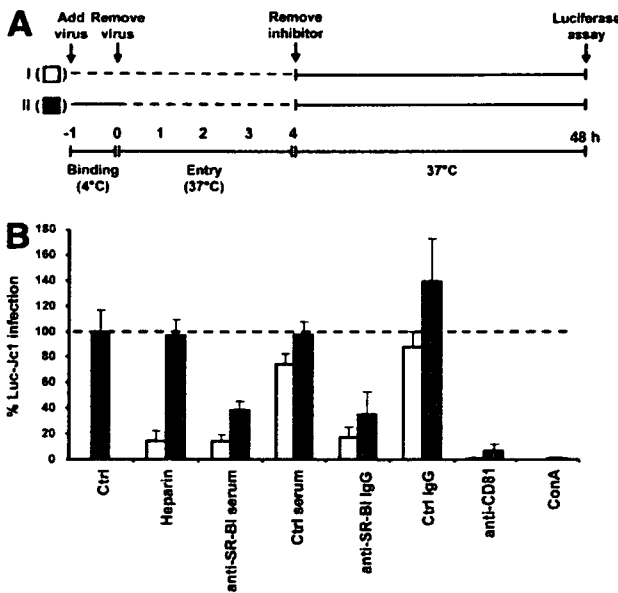


Fig. 6. SR-BI mediates an HCV entry step occurring postbinding of virions. (A) Schematic drawing of the experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5 cells by rat anti-SR-BI serum or control serum (1/200), anti-SR-BI, or control IgG (100 μ g/mL), anti-CD81 monoclonal antibody (10 μ g/mL), heparin (250 μ g/mL), or concanamycin A (25 nM) was compared using 2 different protocols. Virus binding to target cells was performed in the presence (protocol I) or absence (protocol II) of compounds. Subsequently, in both protocols, cells were washed, supplemented with fresh medium containing the given inhibitors, and shifted to 37°C to allow entry to proceed. Four hours later, cells were again washed and supplied with medium without inhibitors or antibodies. Dashed lines indicate the time intervals where inhibitors or antibodies were present. Luciferase activity was determined 48 hours later and is expressed relative to control infections performed in the same way but without addition of inhibitor. (B) Kinetics of HCVcc entry into human hepatoma cells. The efficiency of infection using the protocols depicted in panel A (protocol I: open bars; protocol II: black bars) was measured as described in (A). Results are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of inhibitory compound or antibody (mean \pm SD; n = 4).

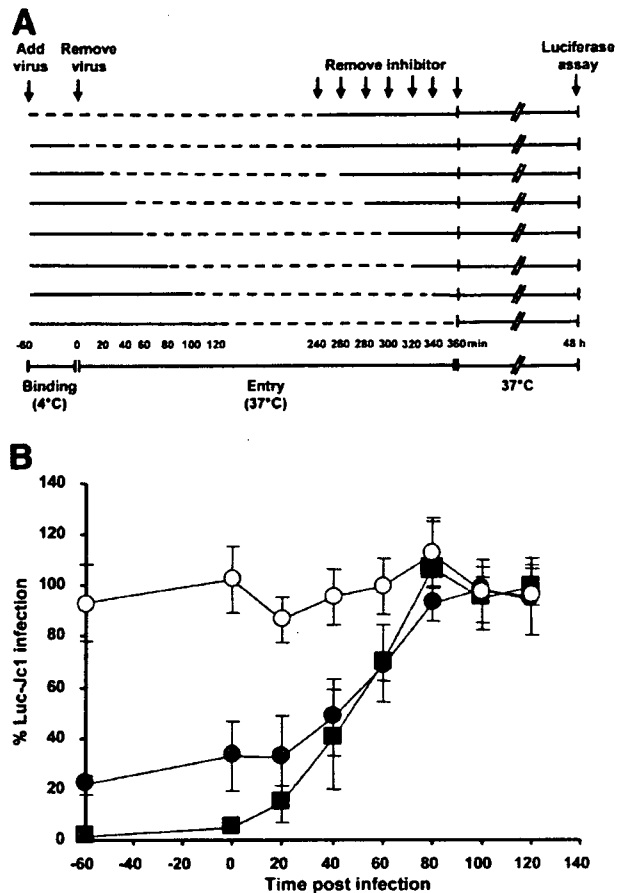


Fig. 7. SR-BI mediates an HCV entry step closely linked to CD81. (A) Schematic drawing of the experimental setup. Inhibition of Luc-Jc1 HCVcc entry into Huh7.5 cells by rat anti-SR-BI serum or control serum (1/200) as well as anti-CD81 monoclonal antibody (10 μ g/mL) was performed as described in the legend to Fig. 6 but inhibitors were added every 20 minutes for 120 minutes after viral binding. Dashed lines indicate the time intervals where inhibitors are present. Luciferase activity was determined 48 hours later and is expressed relative to control infections performed in the same way but without addition of inhibitor. (B) Kinetics of HCVcc entry into human hepatoma cells. The efficiency of infection using rat anti-SR-BI serum (black circle), control serum (white circle), or anti-CD81 antibody (black square) was measured by luciferase assay 48 hours later. Results are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of antibody (mean \pm SD; n = 4).

experiments (Fig. 7B), it is likely that the entry steps mediated by SR-BI and CD81 occur during a similar time point and are closely linked to each other. To further address the contribution and interplay of CD81 and SR-BI in HCV entry, we added anti-CD81 and anti-SR-BI IgG simultaneously before Luc-Jc1 HCVcc infection. Figure 8B shows that blocking both CD81 and SR-BI inhibited Luc-Jc1 HCVcc infection more potently than blocking of each receptor alone (Fig. 8A,B). This effect was not observed when control IgG were used in combination with anti-CD81 monoclonal antibody (Fig.

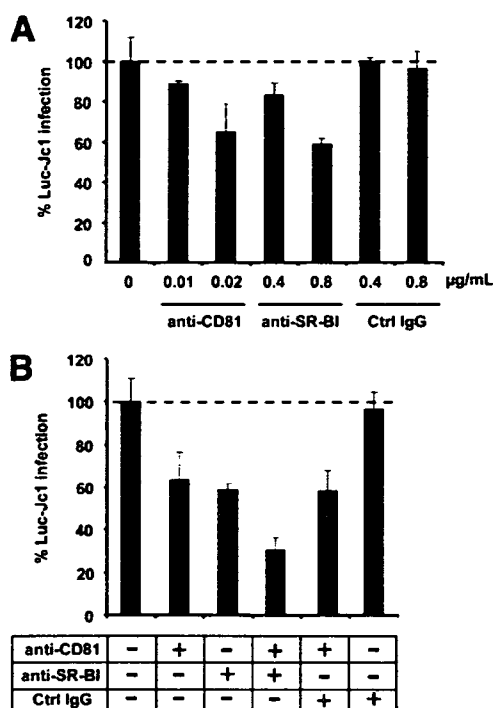


Fig. 8. SR-BI and CD81 act in concert to mediate HCV entry. (A) Dose-dependent inhibition of Luc-Jc1 HCVcc infectivity by anti-SR-BI and anti-CD81 antibodies. Huh7.5 cells were preincubated for 1 hour at 37°C with anti-CD81 monoclonal antibody (0.01 and 0.02 μg/mL), rat anti-SR-BI IgG (0.4 and 0.8 μg/mL) or control IgG (0.4 and 0.8 μg/mL) before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed by measurement of luciferase activity 48 hours after infection. Data are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4). (B) Additive effect of anti-SR-BI and anti-CD81 antibodies in inhibition of HCVcc entry. Huh7.5 cells were preincubated for 1 hour at 37°C with rat anti-SR-BI IgG (0.8 μg/mL) or control IgG (0.8 μg/mL) either alone or in combination with anti-CD81 monoclonal antibody (0.02 μg/mL) before infection with Luc-Jc1 HCVcc for 4 hours at 37°C. HCV infection was assessed as described in (A). Data are expressed as percent Luc-Jc1 HCVcc infectivity in the absence of antibody (mean ± SD; n = 4).

8B). Taken together, our results suggest that SR-BI and CD81 may act in concert to mediate HCV entry.

Discussion

Using an infectious HCV tissue culture system, we demonstrate that SR-BI (1) represents a key host factor for HCV entry, (2) is implicated in the same HCV entry pathway as CD81, and (3) targets an entry step occurring after binding closely linked to CD81.

SR-BI delivers HDL cholesteryl ester to the liver and steroidogenic tissues by a process termed the "selective uptake pathway."³⁶⁻³⁹ This process differs markedly from that of the classic clathrin coated pit-mediated LDL receptor endocytic pathway, in which the entire lipoprotein is internalized and degraded.⁴⁰ In the selective uptake

pathway, SR-BI binds HDL and the core cholesteryl ester are delivered to the plasma membrane without the endocytosis of the entire HDL particle. SR-BI-mediated selective HDL cholesteryl ester uptake is a 2-step process: the first step involves lipoprotein binding to the extracellular domain of SR-BI and the second step consists in the selective transfer of lipid to the plasma membrane.^{41,42}

The marked inhibition of HCV infection of 2 different isolates (JFH1 and Jc1) by anti-SR-BI antibodies and siRNAs suggests that SR-BI plays a key role for establishment of HCV infection. These results extend recently obtained evidence suggesting that SR-BIs modulate HCV infection.^{24,25} Extending previous studies, we demonstrate that inhibition of HCVcc infection by anti-SR-BI antibodies or SR-BI-specific siRNA was not dependent on the presence of lipoproteins in the tissue culture medium, suggesting that SR-BI can mediate HCV entry independent from an interaction of HCV or SR-BI with HDL. Lavillette et al.¹⁷ demonstrated that silencing SR-BI expression markedly reduced HCVpp entry independent of HDL, whereas Voisset et al.¹⁹ demonstrated that SR-BI silencing only reduced the HDL-mediated enhancement of HCVpp entry. Using the HCVcc model system and transfected siRNAs, we now demonstrate that silencing of SR-BI expression resulted in a marked down-regulation of susceptibility to HCV infection independent of the presence of lipoproteins, although HDL was able to enhance HCV infection. In our hands, the use of an optimal siRNA delivery system was crucial for the study of HCV infection. Whereas recombinant lentiviral vectors were characterized by interference with HCV infection unrelated to the expressed siRNA (data not shown), the transfection of *in vitro* transcribed SR-BI siRNAs specifically resulted in down-regulation of HCV infection. The specific effect of SR-BI siRNAs is demonstrated by the lack of inhibitory effects of various control siRNAs including siRNAs targeting another protein expressed on the cell surface of hepatoma cells (CD13).

Because the presence of HDL did not inhibit but rather enhanced HCV infection, it is unlikely that HCV and HDL compete for the SR-BI HDL binding domain. The highly reproducible enhancement of HCV infection by HDL may point to a more efficient interaction of SR-BI with HCV, for example, as a result of a conformational change induced by HDL. These findings are in line with a previous study demonstrating that HDL is a serum factor that attenuates neutralization by antiviral antibodies of HCVpp or HCVcc.³³ The authors hypothesized that HDL may stimulate cell entry of viral particles by accelerating their endocytosis.³⁵ In contrast to results of ectodomain blocking by anti-SR-BI, HDL appeared to slightly enhance HCVcc infection in cells with silenced SR-BI. This may not be un-

expected because down-regulation by siRNA, in contrast to ectodomain blocking, most likely leaves some SR-BI accessible for HDL/HCV interplay.

Furthermore, we demonstrate that in target cells with silenced CD81 expression, HDL appeared not to markedly enhance HCVcc infection (Fig. 5B). These findings strongly suggest that the HDL/SR-BI-HCV interaction acts in concert with CD81 within the same entry pathway and does not represent another or redundant route of cell entry. This conclusion is further supported by an additive inhibitory effect of anti-SR-BI and anti-CD81 antibodies on Luc-Jc1 HCVcc infection (Fig. 8B), confirming previous results obtained for JFH1.²⁴

Using an HCVcc-based kinetic entry assay (Figs. 6 and 7), we mapped the HCV entry step targeted by SR-BI. As shown in Figs. 6 and 7, anti-SR-BI IgG markedly inhibited HCVcc infection when added up to 60 minutes post-binding of attached virus. These data for the first time directly demonstrate that SR-BI predominantly mediates an HCV entry step occurring after binding of HCV to the hepatocyte cell surface membrane. These findings confirm the hypothesis raised by von Hahn and colleagues²⁰ based on experiments using oxidized lipoproteins as SR-BI ligands. In contrast to anti-SR-BI and anti-CD81 antibodies, heparin—a homolog of highly sulfated heparan sulfate—was able to inhibit HCVcc infection only when added before HCV binding. Taken together, these data suggest that glycosaminoglycans such as highly sulfated heparan sulfate act predominantly at the stage of viral attachment, whereas SR-BI and CD81 mediate entry steps occurring postbinding. Subsequent steps in HCV entry are most likely mediated by claudin-1, a recently discovered co-host factor for HCV infection.⁶

Kinetic studies using anti-SR-BI and CD81 antibodies in side-by-side experiments demonstrated that SR-BI is required for an entry step occurring at a similar time point as CD81-HCV interaction. Although the magnitude of antibody-mediated inhibition of HCVcc infection was different, the kinetics of inhibition of HCV infection by anti-SR-BI and anti-CD81 antibodies was remarkably similar (Fig. 6). Both anti-SR-BI and anti-CD81 antibodies were able to inhibit HCV infection when added up to 60 minutes after binding and lost their ability to inhibit HCV infection when added 80 minutes after binding. The rate of loss of CD81 antibody HCVcc inhibition overtime in our study appeared to be different from that observed for HCVpp in previously published studies.^{6,43} This either may be due to experimental differences or may be a difference between the behaviors of HCVpp and HCVcc. Taken together, our results demonstrate that the entry steps mediated by SR-BI and CD81 occur during a similar time frame and are closely linked to each other.

SR-BI and its splicing variant SR-BII contain an identical extracellular domain. SR-BII is encoded by an alternatively spliced messenger RNA from the SR-BI gene and differs from SR-BI only in the carboxy-terminal cytoplasmic tail, which, as shown previously, must contain a signal that confers predominant intracellular expression and rapid endocytosis of HDL.⁴⁴ Scavenger receptor BII, which is expressed at low levels in the liver compared with SR-BI,⁴⁵ mediates rapid HDL endocytosis through a clathrin-dependent, caveolae-independent pathway,⁴⁴ but is inefficient compared with SR-BI in HDL cholesteryl ester selective uptake.⁴⁶ Because our tools (anti-SR-BI antibody and SR-BI siRNAs) also may target SR-BII, we cannot completely exclude a role for SR-BII in HCV infection as most recently shown by other investigators.²⁵

In conclusion, our results demonstrate that SR-BI plays a key role for the establishment of HCV infection mediating HCV infection during an entry step occurring postbinding closely linked to the interaction of HCV with CD81. The functional mapping of SR-BI-HCV interaction and its impact for HCV entry has important implications for the understanding of the very first steps of HCV infection and the development of novel antiviral strategies targeting HCV entry.

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Development of plaque assays for hepatitis C virus-JFH1 strain and isolation of mutants with enhanced cytopathogenicity and replication capacity

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Abstract

HCV culture *in vitro* results in massive cell death, which suggests the presence of HCV-induced cytopathic effects. Therefore, we investigated its mechanisms and viral nucleotide sequences involved in this effect using HCV-JFH1 cell culture and a newly developed HCV plaque assay technique. The plaque assay developed cytopathic plaques, depending on the titer of the inoculum. In the virus-infected cells, the ER stress markers, GRP78 and phosphorylated eIF2- α , were overexpressed. Cells in the plaques were strongly positive for an apoptosis marker, annexin V. Isolated virus subclones from individual plaque showed greater replication efficiency and cytopathogenicity than the parental virus. The plaque-purified virus had 9 amino acid substitutions, of which 5 were clustered in the C terminal of the NS5B region. Taken together, the cytopathic effect of HCV infection involves ER-stress-induced apoptotic cell death. Certain HCV genomic structures may determine the viral replication capacity and cytopathogenicity.

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Keywords: HCV-JFH1; HCV cell culture; Plaque assay; ER stress; Unfolded protein responses; Apoptosis; NS5B RNA-dependent RNA polymerase

Introduction

Molecular analyses of the HCV life cycle, virus–host interactions, and mechanisms of liver cell damage by the virus are not understood completely, mainly because of the lack of cell culture systems. These problems have been partly overcome by the development of the HCV subgenomic replicon (Lohmann

et al., 1999) and HCV cell culture systems (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). The HCV-JFH1 strain, which is a genotype 2a clone derived from a Japanese fulminant hepatitis patient that can replicate efficiently in Huh7 cells (Kato et al., 2003; Kato et al., 2001), has contributed to the establishment of the HCV cell culture system. Furthermore, the Huh7-derived cell lines, Huh-7.5 cells, Huh-7.5.1, and Lunet cells allow production of higher viral titers and have a higher permissiveness for HCV (Koutsoudakis et al., 2007; Lindenbach et al., 2005; Zhong et al., 2005). The HCV-JFH1 cell culture system now allows us to study the complete HCV life cycle: virus–cell entry, translation, protein processing, RNA replication, virion assembly, and virus release.

HCV belongs to the family *Flaviviridae*. One of the characteristics of the *Flaviviridae* is that they cause cytopathic effects (CPE). The viruses have positive strand RNA genomes of ~10 kilobases that encode a polyprotein of ~3000 amino acids.

Abbreviations: HCV, hepatitis C virus; IFN, interferon; CPE, cytopathic effect; ER, endoplasmic reticulum; UPR, unfolded protein response; PFU, plaque-forming unit; FFU, focus-forming unit; RdRp, RNA-dependent RNA polymerase.

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The protein is post-translationally processed by cellular and viral proteases into at least 10 mature proteins. The viral nonstructural proteins accumulate in the ER and direct genomic replication and viral protein synthesis (Bartenschlager and Lohmann, 2000; Jordan et al., 2002; Mottola et al., 2002). It has been reported that Japanese encephalitis virus (JEV), bovine viral diarrhoea virus (BVDV), and dengue viruses (DEN) cause apoptotic cell death (Despres et al., 1996; He, 2006; Jordan et al., 2002; Su et al., 2002). In addition, certain amino acid substitutions in the viral structural or nonstructural proteins affect the replication and cytopathogenicity of these viruses substantially (Blight et al., 2000; Maekawa et al., 2004; Mendez et al., 1998). It has been recently reported that HCV-JFH1-transfected Huh-7.5.1 cells died when all of the cells were infected and intracellular HCV-RNA reached maximum levels (Zhong et al., 2006). These findings suggest HCV-induced cytopathogenicity. However, the mechanisms have not been well documented.

In the present study, we investigated the cellular effects of HCV infection and replication using the HCV-JFH1 cell culture system. Here, we report that HCV-JFH1-transfected and infected cells show substantial CPE that are characterized by massive apoptotic cell death with the expression of several ER stress-induced proteins. Taking advantage of the CPE, we developed a plaque assay for HCV in cell culture and isolated subclones of HCV that showed enhanced replication and cytopathogenicity. We have demonstrated that these viral characters were determined by mutations at certain positions in the structural and nonstructural regions of the HCV genome, especially the NS5B region.

Results

Production of infectious HCV-JFH1 by JFH1-RNA transfected cells

After transfection of HCV-JFH1 RNA into Huh-7.5.1 cells, intracellular HCV RNA and HCV antigen were continuously detectable in the cell culture (Fig. 1A). Furthermore, the culture supernatant from the transfected cells was positive for core protein, which reached maximum levels at 14 days post-transfection and was continuously detectable during the cell culture (Fig. 1A, black bar). The culture supernatant was readily infectable to naive Huh-7.5.1 cells (data not shown). Immunofluorescence assay showed that 48% of the JFH1-RNA-transfected cells and 42% of the virus-infected cells were positive for HCV core protein. These results demonstrate that the transcript of HCV-JFH1 clone replicates efficiently and produces infectious virus particles in cells, as reported previously (Wakita et al., 2005; Zhong et al., 2005).

Hepatitis C virus infection induced cytopathic effects in vitro

By the seventh day post-transfection, the production of virus decreased concomitant with massive cell death and then cell growth gradually recovered. At 14–16 days post-transfection, the levels of HCV-RNA and core antigen reached maximum (Fig. 1). In the JFH1 mutants JFH1/GND and JFH1/ Δ E1-E2-RNA-transfected Huh-7.5.1 cells, the viral replication and host cell death were not observed. The massive cell death after HCV-

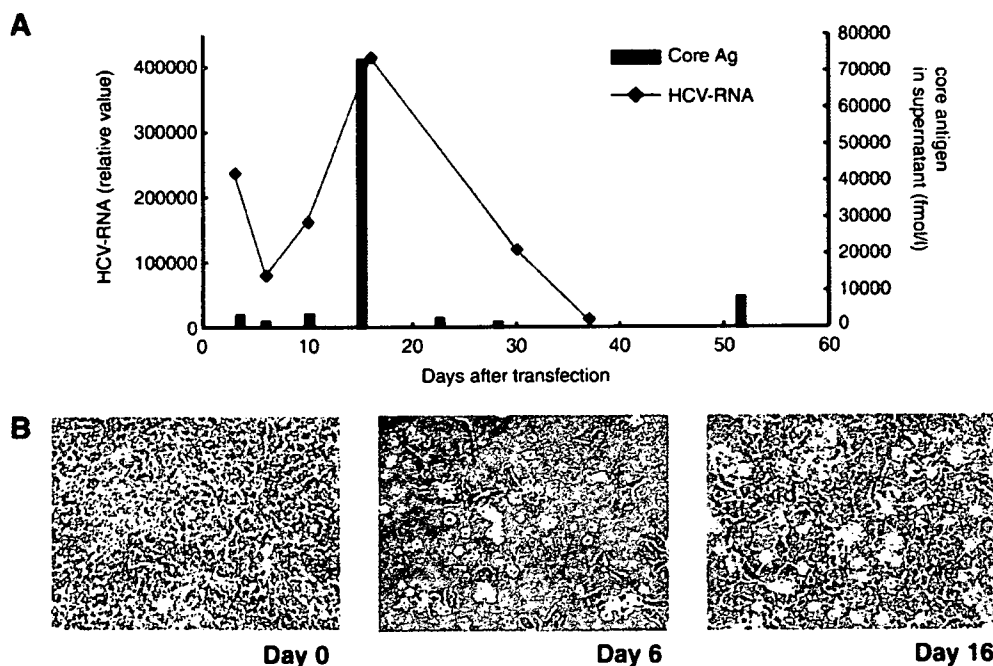


Fig. 1. Replication of HCV-JFH1 RNA in JFH1-transfected and infected Huh-7.5.1 cells. (A) Levels of HCV-RNA in JFH1 RNA-transfected cells. After transfection of the *in vitro* transcribed JFH1-RNA into Huh-7.5.1 cells, total cellular RNA was isolated on indicated days and quantified by real-time RT-PCR. Furthermore, the culture supernatant of JFH1-RNA transfected Huh-7.5.1 cells was collected on the days indicated and the levels of core antigen in the culture supernatant were measured (black bar). (B) HCV-JFH1-transfected Huh-7.5.1 cells (the left panel, day 0; the middle panel, day 6; the right panel, day 16).

JFH1 transfection led us to suspect the occurrence of CPE, produced in host cells by HCV infection and replication. A plaque assay was performed (see Materials and methods) to

investigate the morphological CPE following HCV-JFH1 infection. Culture supernatants from JFH1-transfected cells were diluted serially and inoculated onto uninfected Huh-7.5.1

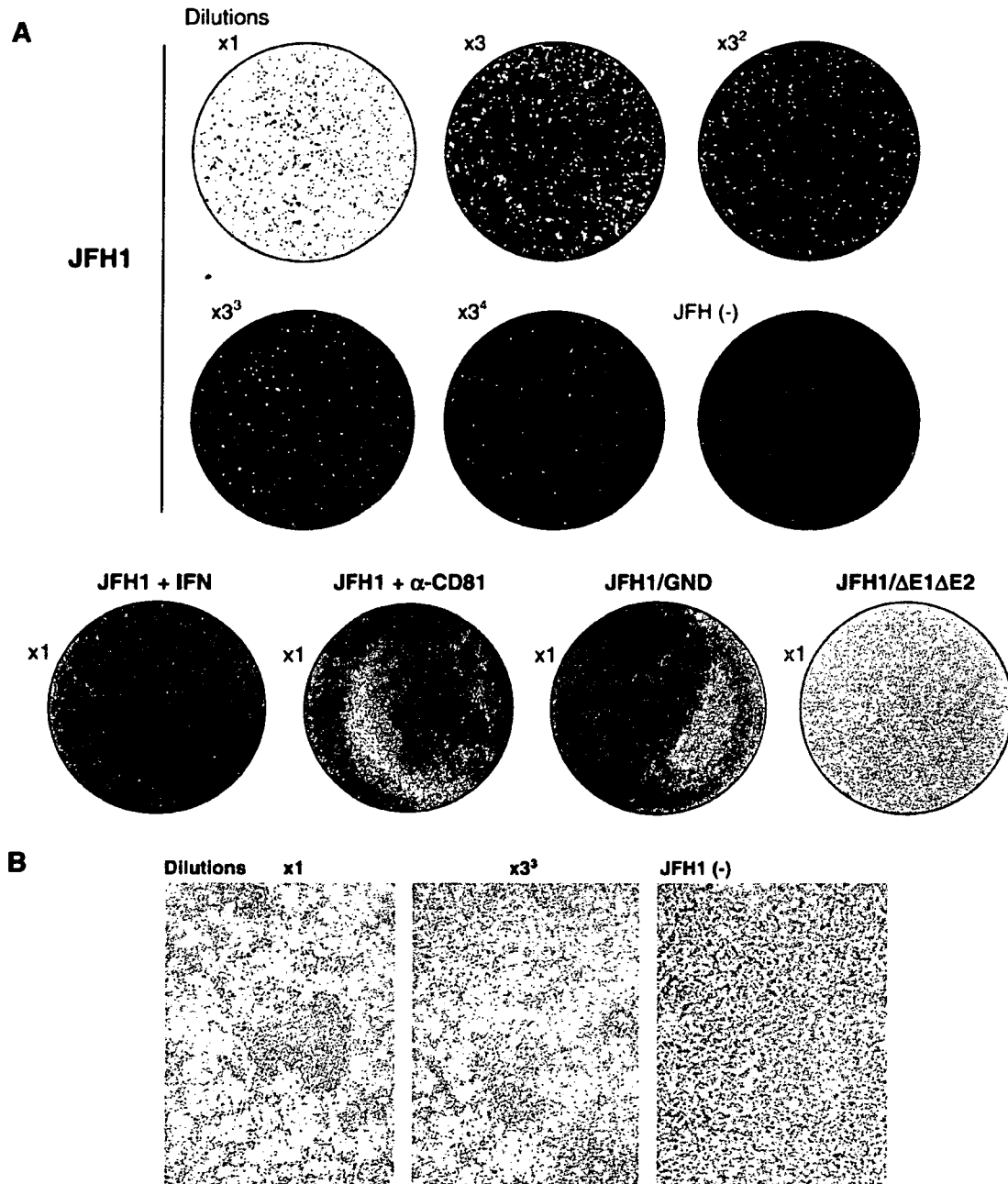


Fig. 2. The cytopathic effects of HCV-JFH1 *in vitro*. (A) Plaque assay. Upper panel, Huh-7.5.1 cells were seeded in collagen-coated 60-mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO₂ (as described above). After overnight incubation, HCV-infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~ 5 h of incubation, the inocula were removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methylcellulose and incubated under normal conditions. After 7 days culture, formation of cytopathic plaque was visualized by staining with 0.08% crystal violet solution. Lower panel, JFH1 + IFN; after infection of the virus supernatant, the cells were cultured in the presence of 50 U/ml interferon-alpha. JFH1 + α-CD81, Huh-7.5.1 cells were pretreated with 10 μg/plate of anti-CD81 antibody. After incubation at 37 °C for 30 min, anti-CD81 was removed, the cells were washed with PBS, and the HCV-JFH1 culture supernatant was transferred. After ~ 5 h incubation, the supernatant was removed and the infected cells were overlaid with 8 ml of culture medium containing 0.8% methylcellulose and controls for the plaque assay were also performed with the JFH1/GND or JFH1/ΔE1-E2 culture supernatant. (B) The cytopathic plaques were observed by phase-contrast microscopy at day 7 after HCV-JFH1 infection.

cells. The cells were subsequently cultured in medium containing agarose. Almost 10 days after the inoculation, viable cells were stained and plaques were visualized (Fig. 2A, upper panel). HCV-inoculated cell cultures developed plaques as unstained areas that were accompanied by round cells in the periphery (Fig. 2B). The formation of cytopathic plaques was not observed in a parental Huh7 cell line (data not shown). Immunocytochemistry of the foci revealed the presence of HCV core-positive cells surrounding the cytopathic plaques (Fig. 3A). Culture of the HCV-inoculated cells in the presence of interferon-alpha (50 U/ml) completely abolished the formation of plaques (Fig. 2A, lower panel). Uninfected Huh-7.5.1 cells (Fig. 2A, upper panel), Huh-7.5.1 cells treated with anti-CD81 antibody before HCV-JFH1 infection and JFH1/GND or JFH1/ Δ E1-E2-transfected cell cultures did not develop plaques (Fig. 2A, lower panel). These findings suggest that HCV-infected cells develop cytopathic plaques depending on the quantity of the inoculums and that HCV replication, viral protein expression and the propagation of viral particles were the features of these plaques.

HCV-JFH1 infection induced host-cell apoptosis

We next determined whether the cytopathic effects of HCV-JFH1 replication include process of apoptotic cell death. Cells including plaques were double-stained with annexin V-FITC and PI. The ligand of annexin V, phosphatidylserine, is normally confined to the cytoplasmic leaflets of the plasma membrane. In the early phase of apoptosis, phosphatidylserine is exposed on the outer surface of the plasma membrane, which enables detection of FITC-labeled annexin V. As shown in Fig. 4, the fluorescence of annexin V was observed in the cells around the plaques. Foci of apoptotic cells were scattered in the plaques. On the other hand, the expression of annexin V was slightly detectable in the subgenomic replicon-harboring cells, though they were at the same level as the uninfected Huh-7.5.1 cells and the cell death was not observed. Therefore, the cells that express HCV subgenomic replicons did not induce apoptotic cell death. These findings demonstrate that the cytopathic effects of HCV replication and the particle formation induce apoptotic cell death.

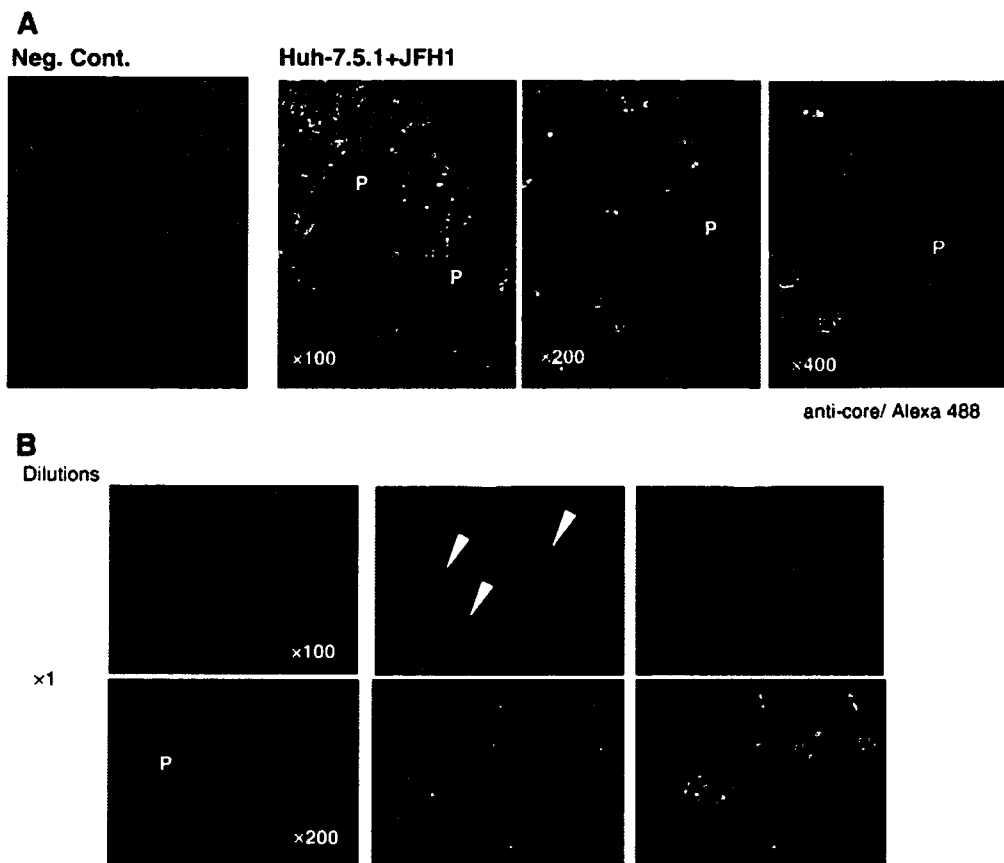


Fig. 3. Immunofluorescence detection of HCV core protein in cytopathic plaques. (A) The HCV-JFH1 culture supernatant was transferred onto uninfected Huh-7.5.1 cells, plated on 22 mm-round micro cover glasses in 60-mm-diameter plates at density of 2×10^5 cells per plate. After ~5 h incubation, the supernatant was replaced with medium containing 0.8% methylcellulose. Immunocytochemistry was performed 12 days after infection. A 'P' indicates a cytopathic plaque. (B) Immunofluorescence detection of HCV-positive foci and cytopathic plaques. The HCV-JFH1 culture supernatant was transferred at various dilutions onto uninfected Huh-7.5.1 cells. After ~5 h incubation, the supernatant was removed and the infected cells were cultured in 60-mm-diameter plate with medium containing 0.8% methylcellulose. Immunocytochemistry was performed 5 days after infection using mouse anti-core antibody. The infectivity and cytotoxicity were quantified by counting HCV-positive foci (FFU/ml) and cytopathic plaque (PFU/ml) respectively. White arrowheads indicate HCV-positive foci.

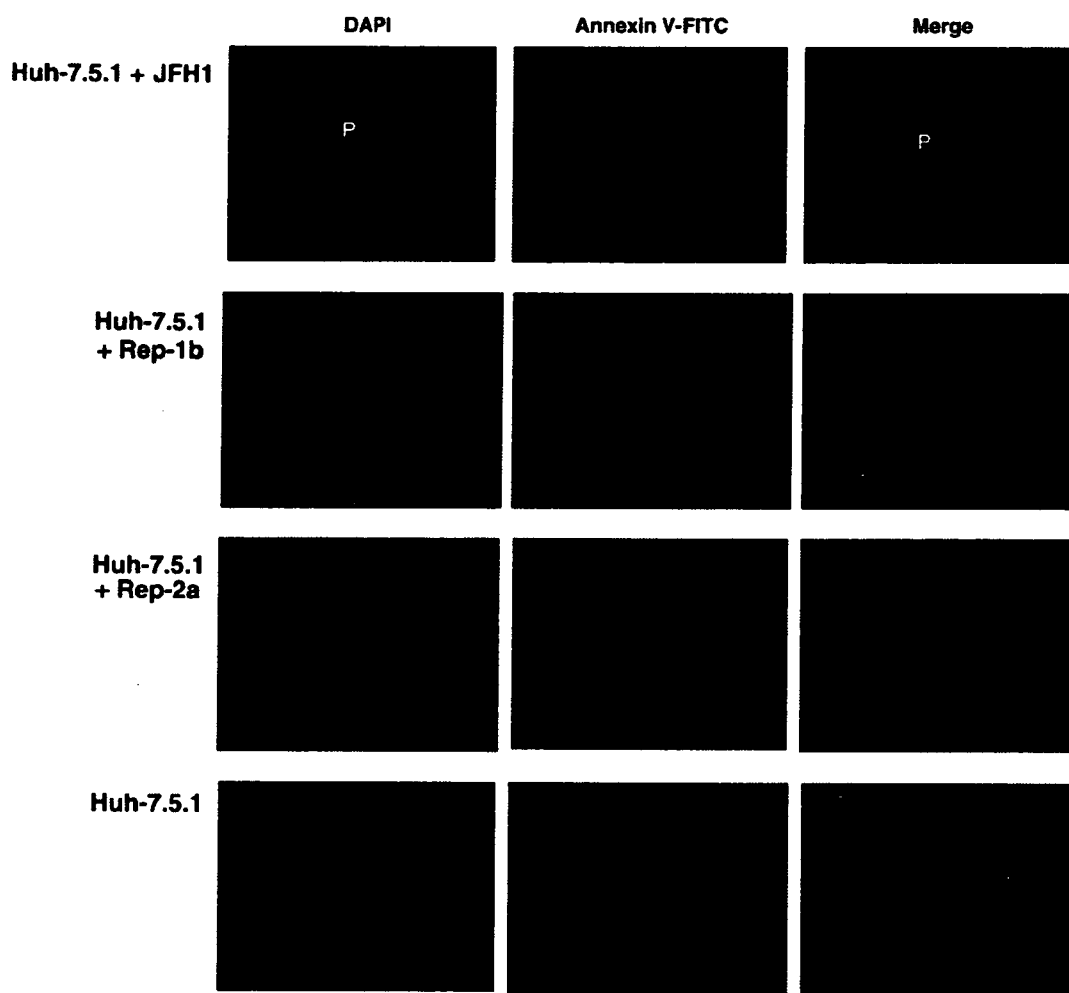


Fig. 4. HCV-JFH1 infection induces apoptosis and leads to plaque formation. The HCV-JFH1 culture supernatant was transferred onto uninfected Huh-7.5.1 cells plated on 22-mm round micro cover glasses in 60-mm-diameter plates at density of 2×10^5 cells per plate. After ~ 5 h incubation, the supernatant was replaced with medium containing 0.8% methylcellulose. Thirteen days after infection, cover glasses were incubated with 100 μ l of staining solution containing Annexin V-FITC at room temperature for 10 to 15 min. The cells that express HCV subgenomic replicons were also incubated and stained with Annexin V-FITC. Rep 1b, Rep-Feo; Rep 2a, SGR-JFH1 (see Materials and methods).

JFH1 replication activates expression of ER stress-related proteins

Cellular stresses such as virus infections prevent protein folding and maturation in the endoplasmic reticulum (ER) and result in the accumulation of misfolded proteins (ER stress) (Kaufman, 1999; Pahl, 1999), triggering the unfolded protein response (UPR). The UPR leads to global shut-off of protein translation and to apoptotic cell death (Ferri and Kroemer, 2001; Mori, 2000; Munro and Pelham, 1986). We and other groups have previously reported that subgenomic or genomic HCV replication induces ER stress and triggers UPR (Nakagawa et al., 2005; Tardif et al., 2002). Therefore, we next studied expression of the ER stress-related proteins, GRP78 and phosphorylated eIF2-alpha, in JFH1-infected cells (Fig. 5). GRP78 is one of the ER chaperones whose expression is induced by ER stress through cleavage and nuclear translocation of ATF6. The eIF2-alpha is phosphorylated by PER-like

ER kinase (PERK) on ER stress, causing direct global inhibition of initiation of protein translation (Harding et al., 1999). Huh-7.5.1 cells were infected with HCV-JFH1 supernatant and harvested on the fourth and seventh days post-infection (Fig. 5). As the expression of HCV core protein increased, expression levels of GRP78 and phosphorylated eIF2-alpha also increased substantially. Suppression of virus replication by interferon-alpha treatment led to a decrease of cellular GRP78 and phosphorylated eIF2-alpha. Interferon-alpha treatment did not eliminate the expression of HCV completely, though the levels of core and phosphorylated eIF2-alpha expression apparently decreased compared with the JFH-1 infected Huh-7.5.1 cells at seventh days post-infection. These findings demonstrated that HCV-JFH1 infection induced ER stress.

Persistence of ER stress activates apoptosis signaling pathways, including the induction of C/EBP homologous protein (CHOP) and activation of JNK kinase and caspase12, leading to cell death (Ferri and Kroemer, 2001). As shown in Fig. 5, the

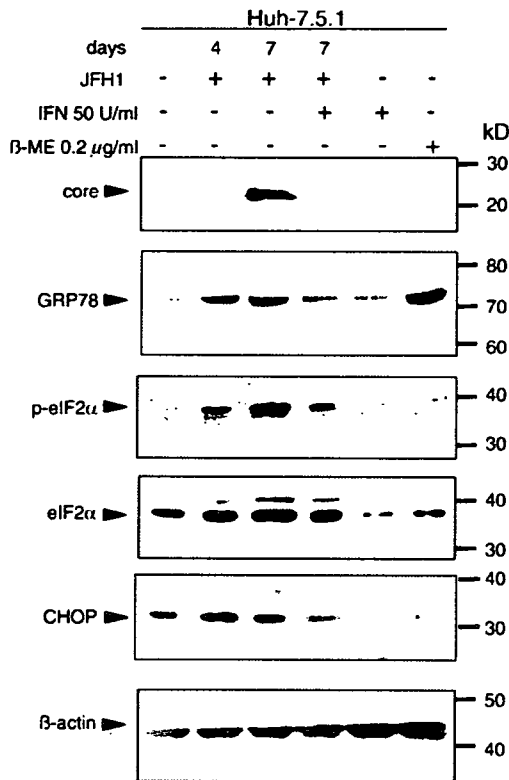


Fig. 5. Expression of ER stress-related proteins in HCV-JFH1 infected cells. The supernatant of JFH1-transfected Huh-7.5.1 cells was transferred onto uninfected Huh-7.5.1 cells. The cells were harvested at 4 and 7 days after infection. The JFH1-infected cells were also cultured with interferon (50 U/ml) or 2-mercaptoethanol (0.2 μ g/ml) and harvested after 48 h after treatment. 2-Mercaptoethanol was used as a positive control to induce ER stress (Nakagawa et al., 2005). Western blotting was performed using anti-core, anti-GRP78, anti-phospho-eIF2- α (p-eIF2 α), anti-eIF2- α , anti-GADD153/CHOP, and anti-beta-actin antibodies. β -ME, 2-mercaptoethanol.

level of CHOP expression was apparently increased in JFH1-infected Huh-7.5.1 cells.

To determine whether ER stress contributes to the formation of cytopathic plaques, JFH1-infected cells were incubated in methylcellulose-containing medium and double immunofluorescence staining of the plaques was performed. As shown in Fig. 6, overexpression of GRP78 was colocalized with HCV-core-positive cells with and without CPE. Together with the result shown in Fig. 4, these findings suggest that ER stress is induced in the HCV-JFH1-infected cells and these responses may be involved in development of apoptosis and the formation of cytopathic plaques.

A cytopathic clone could be isolated and this had acquired a high infection efficiency and increased cytopathogenicity

The plaque assay enabled differential quantification of viral infectivity and cytopathogenicity by the immunofluorescence detection of HCV core protein in JFH1-infected, plaque-developed cultures. The number of plaques, as well as infectious foci, was linearly proportional to the dilution of an inoculum (Fig. 7B). It was revealed that only a few populations

of HCV-positive foci developed cytopathic plaques (Fig. 3B and Table 1). The infectious focus-forming units and plaque-forming units were 5.6×10^3 FFU/ml and 9.7×10^2 PFU/ml, respectively (Table 1).

To determine whether the difference between the cytopathic and noncytopathic HCV-JFH1 replication might be attributable to viral factors, we isolated clones from each cytopathic plaque. JFH1-infected Huh-7.5.1 cells were incubated in DMEM containing methylcellulose. Cytopathic plaques became visible at ~ 1 week after inoculation. We isolated cells from each plaque using a cloning cylinder, subcultured, and transferred supernatant onto uninfected Huh-7.5.1 cells. To our surprise, infection of naive cells with plaque-derived supernatants led to massive cell death at 10 days post-infection (Fig. 8A). The supernatant of these cells was transferred again onto uninfected Huh-7.5.1 cells again. Immunofluorescence assay revealed that almost 100% of the cells were positive for HCV core protein (Fig. 8B). The infectivity and cytopathogenicity of this isolated plaque (PI #1) were 4.9×10^3 FFU/ml and 3.0×10^3 PFU/ml respectively (Table 1), much higher than the parental JFH1 clone. Moreover, the ratio of PFU to FFU in a plaque-isolated clone (PI #1) was significantly higher than that of parental JFH1 clone (0.58 and 0.17 respectively) (Table 1 and Figs. 7B and C). We next performed an infection experiment of the parental JFH1 and a plaque-derived clone by adjusting infectious titers of the inocula by HCV core antigen levels. As shown in Fig. 8C, virus from cytopathic plaque (PI #1, #2, #3) showed significantly higher elevation of core antigen levels in supernatants than the parental JFH1 in every time point. The second round isolation of plaques from the PI #1 subclone (PI #1-1, #1-2 and #1-3 in the Table 3) showed consistently higher replication efficiency and cytopathogenicity. These results indicated that JFH1 subclones isolated from cytopathic plaques showed significantly higher infection efficiency and greater cytopathic effects than the original JFH1.

The isolated plaque had amino acid substitutions clustered in the NS5B region

To determine whether there are viral mutations in the cytopathic JFH1 subclone (PI #1), we performed sequence analyses. As shown in Table 2, 11 nucleotide changes were found in the cytopathic plaque, and 9 of these were non-synonymous mutations (81.8%). In particular, 6 of the 11 mutations (9153, 9232, 9293, 9295, 9353, and 9355) were clustered in the C terminal half of the NS5B region. We also performed sequence analyses of the PI #1-isolated subclones, PI #1-1, #1-2, and #1-3, and other clones that had been independently isolated from different plaques, PI #2, #3, and #4 (Table 3). Those subclones showed similar mutations within NS5B region. The C2438S, P2934S, and S3001N substitutions were redundantly appeared in the 4 plaque-isolated clones and in all three PI #1-derived subclones. In contrast, no mutations were found in the virus from infectious foci without plaque formation. These results showed an evidence that certain amino acid mutations were directly associated with the viral replication efficiency and cytopathogenicity.

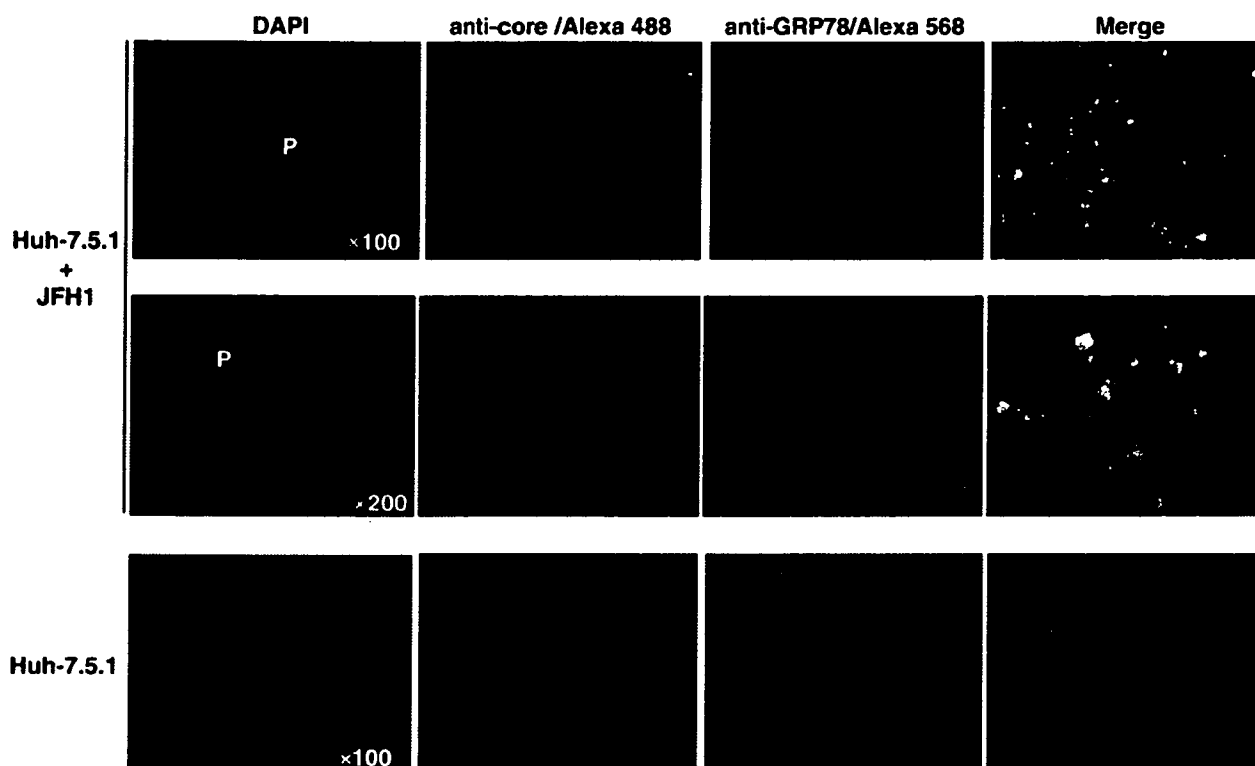


Fig. 6. Co-expression of HCV core and GRP78 in the cytopathic plaque. The HCV-JFH1 culture supernatant was transferred onto uninfected Huh-7.5.1 cells plated on 22-mm round micro cover glasses in 60-mm-diameter plates at density of 4×10^5 cells per plate. After ~ 5 h incubation, the supernatant was replaced with medium containing 0.8% methylcellulose. Double immunofluorescence was performed 10 days after infection using mouse anti-core antibody and goat-anti-GRP78 antibody.

Introduction of NS5B mutations in JFH1 clone showed higher replication efficiency and cytopathogenicity

We finally investigated on phenotypes of the amino acid mutations identified in the isolated cytopathic subclones. We constructed mutant clones from the wild type JFH1 plasmid, in which three amino acid mutations in NS5B region were individually introduced; T7662A, C9153T, and G9295C (see Tables 2 and 3). Transfection of the mutant HCV-RNAs showed that all mutants developed massive cell death on 10 days after transfection and that their extents of the CPE were apparently greater than the wild type JFH1 clone (Fig. 9A). The levels of core antigen in the culture medium were significantly higher in the mutant clones than in the wild type (Fig. 9B). Furthermore, the expression levels of cellular HCV core protein were significantly higher in the mutant clones than in the wild type with the order of T7662>C9153>>G9295C>JFH1 (Fig. 9C).

Discussions

Our results show that replication of HCV-JFH1 resulted in morphologic changes to the host cells, which are characterized by massive cell death (Figs. 1–3). These observations suggested that HCV infection and replication could cause CPE on the host cells. The development of the CPE involved virus protein-induced ER stress and subsequent apoptotic cell death (Figs. 4–6). The JFH1/ Δ E1–E2 with deletion of the HCV

envelope proteins-infected Huh-7.5.1 cells did not induce the CPE (Fig. 2A), which indicates that the key factors of plaque formation are not only viral replication but also the propagation of virus particles and re-infection. We took advantage of the HCV-induced CPE and developed a plaque assay using highly permissive Huh-7.5.1 cells. The assay revealed that the HCV-induced cytopathogenicity varied between infectious foci with cytopathic and noncytopathic infection (Fig. 3B). Interestingly, isolated JFH1 subclones from the plaques showed significantly increased infectivity and cytopathogenicity (Table 1 and Fig. 8). Viral genetic analyses showed nine amino acid substitutions; among them five were clustered in the C terminal half of the NS5B region, which might contribute to virus replication efficiency and cytopathogenicity (Table 2).

Cytopathic effects are key characteristics of the *Flaviviridae* that include Japanese encephalitis virus (JEV) (Vaughn and Hoke, 1992), West Nile Virus (Borisevich et al., 2006), yellow fever virus (Quaresma et al., 2006), dengue virus (DEN) (Despres et al., 1993), and bovine viral diarrhea virus (BVDV) (Mendez et al., 1998) and also of viruses such as adenovirus (Shinoura et al., 1999), Epstein–Barr virus (Sato et al., 1989), poliovirus (Yanagiya et al., 2005), and influenza virus (Hinshaw et al., 1994). The *Flaviviridae* utilizes the ER as the primary site for genomic replication and protein synthesis (Jordan et al., 2002; Su et al., 2002; Tardif et al., 2004). It has been reported that apoptotic cell death mediated by virus-induced ER stress contributes to the cytotoxicity of JEV, BVDV, and DEN-2

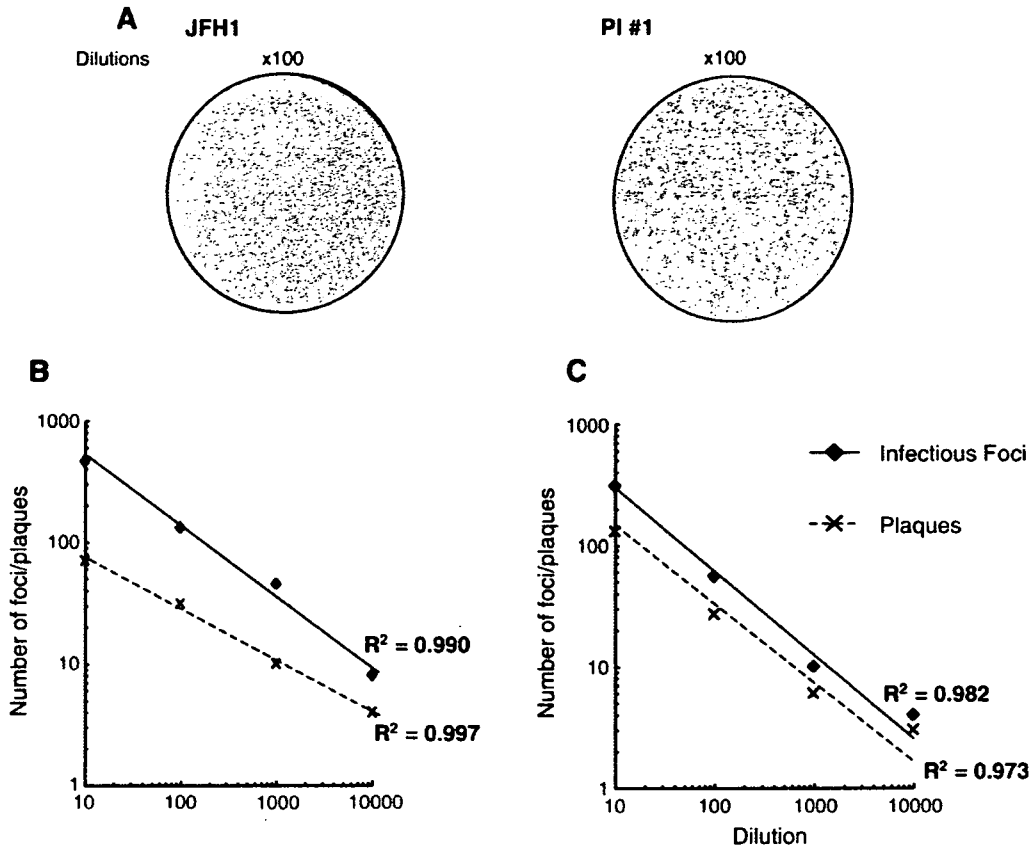


Fig. 7. Correlation of infectious foci or plaques with dilution of an inoculum. (A) Plaque assay. Huh-7.5.1 cells were seeded in collagen-coated 60-mm-diameter plates at density of 4×10^5 cells per plates and were incubated at 37 °C under 5.0% CO₂. After overnight incubation, HCV-JFH1 (left panel) or plaque-purified clone (PI #1) (right panel) infected culture supernatants were serially diluted in a final volume of 2 ml per plates and transferred onto the cell monolayers. After ~5 h of incubation, the inocula were removed, and the cell monolayers were overlaid with 8 ml of culture medium containing 0.8% methylcellulose. After 7 days of culture under normal conditions, formation of cytopathic plaque was visualized with 0.08% crystal violet. (B and C) The PFU-adjusted culture supernatant of parental HCV-JFH1 (B) or plaque-purified clone (PI #1) (C) was transferred at various dilutions onto uninfected Huh-7.5.1 cells, and the plaque assay and immunocytochemistry were performed (described above). The infectivity and cytotoxicity were quantified by counting HCV-positive foci and cytopathic plaque respectively. The horizontal axis showed dilutions of the viral supernatant and the vertical axis showed the number of infectious foci or plaques.

(Jordan et al., 2002; Su et al., 2002; Yu et al., 2006). In DEN-2-infected cells, the NS2B-3 protein causes XBP1 splicing and induces ER stress (Yu et al., 2006). These findings are consistent with our results for HCV in that the JFH1 infection induced ER stress and unfolded protein responses and led to apoptotic cell death and formation of plaques.

The ER is closely associated with viral replication and assembly. Most of the HCV structural and nonstructural proteins accumulate in the ER membrane and form a membranous web that is characterized by a convoluted ER structure (Gosert et al., 2003). Moreover, the folding and assembly of HCV

proteins require interaction with ER chaperone proteins such as calreticulin, BiP/GRP78, and heat shock protein-90 (HSP90) (Choukhi et al., 1998; Waxman et al., 2001). The ER stress, which is induced by virus replication, involves three different mechanisms (Tardif et al., 2002): transcriptional induction, translational attenuation, and protein degradation. In our study, both GRP78 and phosphorylated eIF2- α proteins were induced as viral proteins increased in concentration in HCV-JFH1 infected cells, and the GRP78 or annexin V and HCV core proteins co-localize in cytopathic plaques, showing that HCV infection and replication induce UPR and that ER stress-mediated apoptosis causes the viral cytopathic effects on host cells.

Several HCV structural and nonstructural proteins are involved in the ER stress. The structural glycoproteins, E1 and E2, interact with ER chaperones (Choukhi et al., 1998; Liberman et al., 1999), HCV NS4B induces UPR through ATF6 or the IRE1-XBP1 pathway (Zheng et al., 2005), and HCV core triggers apoptosis by inducing ER stress and ER calcium depletion both *in vitro* and *in vivo* (Benali-Furet et al., 2005).

Table 1
Cytopathogenicity and infectivity of JFH1 clones

| | PFU/ml ^a | FFU/ml ^b | PFU/FFU |
|-------|----------------------------------------|---------------------------|-----------------|
| JFH1 | $9.7 \pm 3.8 \times 10^2$ ^c | $5.6 \pm 0.9 \times 10^3$ | 0.17 ± 0.05 |
| PI #1 | $3.0 \pm 1.9 \times 10^3$ | $4.9 \pm 1.6 \times 10^3$ | 0.58 ± 0.21 |

^a PFU, plaque-forming unit.

^b FFU, focus-forming unit.

^c Values are displayed as mean \pm S.D.

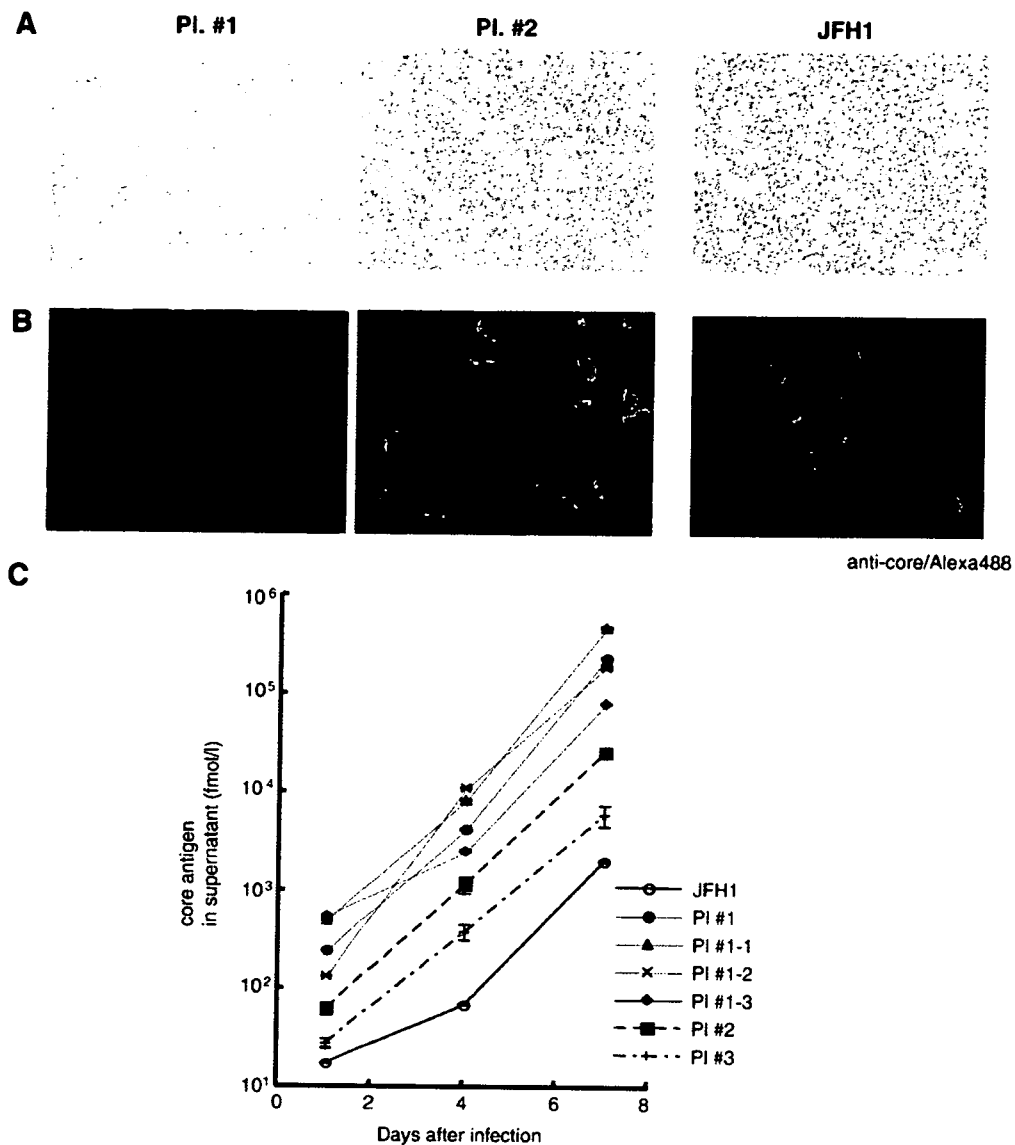


Fig. 8. The isolation of cytopathic plaques. The HCV-JFH1 culture supernatant was transferred at various dilutions onto uninfected Huh-7.5.1 cells. After ~5 h incubation, the supernatant was removed then infected cells were cultured in 0.8% methylcellulose-containing medium in 60-mm-diameter plates. Cytopathic plaques were detectable at 8 days after infection. Cells from each plaque were isolated using a cloning cylinder, subcultured, and transferred onto uninfected Huh-7.5.1 cells. (A) Observation by phase-contrast microscopy at 10 days of culture. (B) After 15 days of culture, the supernatant was transferred onto uninfected Huh-7.5.1 cells and an immunofluorescence assay was performed 5 days after infection using anti-core antibody. (C) Supernatants from parental JFH1, plaque-derived viruses (PI #1, #2, and #3) and the second round isolation of plaques from the PI #1 subclones (PI #1-1, #1-2, and #1-3) were inoculated onto Huh-7.5.1 cells with PFU-adjusted doses, respectively. HCV core antigen levels in culture medium were measured on the days indicated. Inoculation and the assays were done in triplicate. The S.D.s were within 4% in each plot.

HCV E2 induces ER stress at lower levels but binds to PERK and inhibits phosphorylation of eIF2- α at high levels of expression (Pavio et al., 2003). These reports have shown that HCV may induce ER stress and regulate subsequent intracellular responses to promote its survival in hepatocytes. Consistently with these reports, our findings that HCV-JFH1 induces the expression of an ER chaperon protein and phosphorylation of eIF2- α indicates that robust replication of HCV-JFH1 produces unfolded proteins in the ER, leading to activation of ATF6 and stimulation of the transcription of ER chaperon proteins to promote protein folding. HCV-JFH1-induced un-

folded proteins also activate PERK, which phosphorylates eIF2- α to inhibit the protein translation. Furthermore, the severe ER stress finally activates apoptosis signaling pathways at the early stage of viral infection. Although which HCV-JFH1 gene product is involved in ER stress-mediated apoptosis is not identified in our study, such proteins may contribute to the regulation of ER stress signaling in the host cell that leads to viral survival or cell death.

The plaque assay is often used to quantify virus infectious titers by visualizing the viral-induced CPE. However, due to the noncytopathic nature of HCV and the lack of highly permissive