of the virus) than at the intracellular core protein level (Figs. 5A, C), suggesting additional activity of vimentin in the processes of HCV particle release.

Since the level of expression of vimentin in carcinomas is correlated with parameters of malignant potential such as tumor grade and tumor invasion, vimentin has been used as a marker of malignant tumors (Bannasch et al., 1982). It has indeed been reported that some HCV-infected patients with hepatocellular carcinoma exhibited up-regulation of vimentin expression in tumor tissue (Kim et al., 2003) although further statistical studies are required to clearly demonstrate this. Tanaka et al. noted that in livers of HCV-infected patients with hepatocelllular carcinoma the virus existed predominantly in non-cancerous tissue, at levels 10- to 100-fold higher than in cancerous tissue (Tanaka et al., 2004). These observations in human liver samples suggest that the reduction in HCV levels in hepatic tumor can be explained by the increase of vimentin expression in tumor, consistent with our findings for cultured cells.

In this study we demonstrated that cellular vimentin expression enhanced the proteasomal degradation of core protein and eventually restricted HCV production. Vimentin itself and sites of vimentin/core interaction may thus be novel targets of treatment using anti-HCV strategies.

Materials and methods

Antibodies

Mouse monoclonal antibodies to annexin II, fatty acid synthase, calnexin, lamin A/C, and GFP were purchased from BD Transduction Laboratories. Mouse monoclonal antibodies to HCV core protein, prohibitin, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were from Anogen, Lab Vision, and Abcam, respectively. Rabbit polyclonal antibodies to vimentin, lamin B1, p53, and HPRT were from Santa Cruz Biotechnology Inc., while those to actin were from Biomedical Technologies Inc.

Plasmids

The mammalian expression vector of HCV core protein, pcEF39neo (Ruggieri et al., 1997), and the empty vector pcEF321swxneo (Harada et al., 1995) were described previously. The mammalian expression vector of Flag-tagged HCV core protein, pCAG/Flag-core, and the empty vector, pCAG, were described previously (Moriishi et al., 2003). For construction of a mammalian expression vector of vimentin, pcDNA3.1/Hygro/vimentin, vimentin fragment was amplified by PCR using the reverse-transcribed cDNAs of Huh7 cells as a template. The PCR primer pairs used were 5′-GCCATGTCCACCAGGTCCGTGTCC-3′ and 5′-TTATTATTCAAGGTCATCGTGATG-3′. The PCR products were inserted into the EcoRV site of pBluescript SKII(+). pBluescript SK(+)/vimentin was digested with Hind III and Xba I, and the vimentin fragment was inserted into pcDNA3.1/Hygro (Invitrogen), which had been digested with Hind III and Xba I. For construction of pcDNA3.1/EGFP, EGFP fragment was prepared by digestion of pEGFP-N1 (Clontech Laboratories, Inc.) with Nhe I and Hind III and inserted into pcDNA3.1/Hygro, which had been digested with Nhe I and Hind III. The subgenomic replicon constructs, pSGR-JFH1/Luc (wild type) and pSGR-JFH1/Luc-GND (GND mutation in the NS5B sequence), with the firefly luciferase reporter gene were described previously (Kato et al., 2005).

Cell lines

All hepatic cells used in this study were plated on collagen-coated dishes (Asahi Techno Glass, Japan). Human hepatic Huh7 and Huh7.5.1 cells were grown in normal culture medium [Dulbecco's modified Eagle's medium (DMEM) (KOJIN BIO, Japan) supplemented with 10% fetal bovine serum (FBS), 100 U/ml Penicillin G, and 100 mg/ml streptomycin sulfate] containing 0.1 mM non-essential amino acids (GIBCO) under a 5% CO_2 atmosphere at 37 °C. We used human hepatic cell lines constitutively expressing HCV core protein, including Hep39 from HepG2 cells (Harada et al., 1995; Ruggieri et al., 1997) and Uc39-2 and Uc39-6 from Huh7 cells (Fukasawa et al., 2006; Sato et al., 2006). Huh7 and HepG2 cell lines carrying the empty vector, Hepswx and Uc321, respectively, were used as a mock control. All of these stable transfectants were maintained in normal culture medium containing 1 mg/ml G418 (Sigma). The human adrenal carcinoma cell line SW13, the subtypes 2CB5 and 1HF5 of which do or do not express vimentin, respectively (Sarria et al., 1990), was maintained in normal culture medium. When the pcDNA3.1/EGFP vector was transfected into 2CB5 and 1HF5 cells, the percentage of GFP-positive cells was 56.3% and 53.6%, respectively, 2 days after transfection ($n=3$), indicating that there was no difference in the transfection efficiency between these cells. To establish vimentin-overexpressing cells, pcDNA3.1/Hygro/vimentin was transfected into 1HF5 and Huh7 cells using FuGENE 6 transfection regent (Roche). The vimentin-overexpressing Huh7 and 1HF5 cells were selected under hygromycin for 2 weeks and cloned to obtain Huh7/vimentin cells and 1HF5/vimentin cells, respectively. Huh7 and 1HF5 cells carrying the empty vector pcDNA/Hygro were also established, as Huh7/hygro cells and 1HF5/hygro cells, respectively.

Preparation of DISFs

Confluent monolayers of Uc321 and Uc39 cells in four culture dishes (150 mm inner diameter) were harvested by trypsinization, and 1.5×10^7 cells of each were pelleted by centrifugation (218 ×g for 5 min at 4 °C). After washing with PBS three times, each cell pellet was resuspended in 1 ml of lysis buffer [10 mM HEPES-HCl, pH 7.5, 10 mM NaCl, 140 mM KCl, 0.5 mM DTT, 0.5% Triton X-100 (Pierce Biotechnology), 10 mM NaF, Complete™ EDTA-free (Roche)] (i.e. a 20% cell suspension). The cell suspension was lysed with a ball-bearing homogenizer (Hope et al., 2002). The soluble fraction (designated the detergent-soluble fraction, DSF) containing ~85% of the total cellular proteins was collected by centrifugation of the cell

Fig. 5. HCV production in vimentin-knockdown and vimentin-overexpressing Huh7 cells. (A) Huh7 cells (5×10^4 cells) in 48-well plates were incubated with or without HCV particles (including 8.0 fmol of core protein) for 6 h, and then treated twice with a 3-day interval without (−) or with siRNA duplexes of HPRT or vimentin. After 7-day culture, the amounts of HCV core protein per cell in cells (light gray bar) and culture medium (dark gray bar) were determined. $n=3$. (B) Culture medium was collected at day 6 in the infection experiment described above in (A). The concentration of HCV core protein in these samples of medium was adjusted to 2.7 fmol/ml with fresh medium. Cells were infected with these samples of medium containing 1.4 fmol of HCV core protein for 2 days, and harvested after 7-day incubation. Infectivity was analyzed by the immunoblotting of cell lysates with antibodies to HCV core protein and β-actin. (C) Vimentin-overexpressing Huh7/vimentin and control Huh7/hygro cells infected with HCV were harvested after 7-day incubation. The amounts of HCV core protein per cell in cells (light gray bar) and culture medium (dark gray bar) were determined. (D) Huh7 cells harboring the HCV subgenomic replicon containing a luciferase reporter gene were transfected without (−) or with siRNA duplexes of HPRT or vimentin. After 2.5-day culture, luciferase activity in cell extracts was determined. $n=3$. (E) Immunofluorescence microscopic analysis of HCV-infected Huh7 cells. After infection with HCV, Huh7 cells were cultured for 6 days. HCV core protein (green) and vimentin (red) were then detected with specific antibodies. Nuclei (blue) were stained with DAPI. Two views showing low and high magnifications are displayed. Bars, 100 μm in the left panel; 50 μm in the right panel. (F) Under the HCV-infected conditions in panel E, fluorescence intensity of vimentin in core-positive and core-negative Huh7 cells was determined by line profile analysis. $n=40$. Statistical significance of differences in fluorescence intensity of vimentin between core-positive and core-negative cells was evaluated using Student's t test, showing $p<10^{-8}$. (G) As in (A), Huh7 cells were incubated with HCV particles, and then treated twice with a 2-day interval without (−) or with siRNA duplexes of HPRT or vimentin. After 4.5-day culture, cells were treated with (+) or without (−) MG132 (50 μM) for 16 h. In each culture condition, the ratio of HCV core protein level in the MG132-treated cells to that in MG132-untreated cells was determined. $n=3$. (H) Huh7/vimentin and Huh7/hygro cells infected with HCV were cultured for 4 days and treated with (+) or without (−) MG132 (50 μM) for 16 h. The amounts of cellular core protein per cell were determined. $n=3$.

lysate performed twice at 218 ×g for 5 min at 4 °C. The insoluble pellet was suspended in 2 ml of lysis buffer containing 1.62 M sucrose and then centrifuged at 10,000 ×g for 1 h at 4 °C. The pellet was resuspended in 1 ml of lysis buffer containing 1.0 M sucrose and layered over 2 ml of lysis buffer containing 2.0 M sucrose. After centrifugation at 50,000 ×g for 2 h at 4 °C, the precipitated fraction containing ~15% of total cellular proteins was collected and resuspended in lysis buffer containing 0.25 M sucrose at a concentration of 3 mg protein/ml (designated the detergent-insoluble fraction, DISF). Each fraction was stored at −80 °C until use. The protein concentrations in these preparations were determined with BCA protein assay reagents (Pierce Biotechnology) using BSA as a standard.

2D-PAGE/MALDI-QIT-TOF MS analysis

The DISF (0.15 mg protein) of each cell line was cleaned using a PlusOne™ 2-D Clean Up kit (GE Healthcare) and resuspended in rehydration solution containing 9 M urea, 4% CHAPS, 65 mM dithioerythritol, and 0.5% ampholyte. The first-dimensional IEF was performed with an Immobiline Dry Strip pH 4–7 according to the manufacturer's instruction (GE Healthcare). The second-dimensional electrophoresis was carried out on 12% SDS-polyacrylamide gel, and the gel was stained with SYPRO-Ruby (Bio-Rad). Spot detection and comparison in 2D images were accomplished with PDQuest™ 2-D analysis software ver. 7.3 (Bio-Rad). The protein bands were excised from the gel and subjected to in-gel trypsin digestion. The tryptic peptide mixtures were analyzed by MALDI-QIT-TOF MS (AXIMA-QIT, Shimazu Biotech, Japan) as described previously (Sato et al., 2006; Shevchenko et al., 1996). Mascot software (Matrix Science) was used for protein identification.

Immunoblot analysis

The proteins were separated by electrophoresis in precast NuPAGE 10% or 12% Bis–Tris gels (Invitrogen), and then transferred to a polyvinylidene difluoride membrane. The membranes were blocked overnight at 4 °C or for 60 min at room temperature in Tris-buffered saline containing 0.1% Tween 20 and 5% skim milk. The blots were probed with the first antibodies at 1:1000 dilution for 60 min at room temperature and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG (Bio-Rad) or HRP-conjugated goat anti-mouse IgG (GE Healthcare) at 1:2000 dilution for 45 min. Detection of immunoreactive proteins was performed using an ECL system (GE Healthcare).

Quantitative real-time PCR analysis

Cellular total RNAs were prepared with an RNeasy kit (Qiagen). The total RNA fraction (1 μg) was processed directly to cDNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche). Of the total 20 μl cDNA solution, an aliquot of 0.5–2 μl was used for each real-time PCR assay. The PCR primers used for HCV core protein were: forward, 5′-AGGAAGACTTCCGAGCG-3′, and reverse, 5′-GGGTGACAGGAGCCATC-3′. The PCR primers for actin were obtained from the LightCycler™-Primer Set (Roche). Quantitative real-time PCR was carried out in a LightCycler (Roche) using LightCycler-FastStart DNA Master SYBR Green I (Roche).

Transfection of siRNA

Subconfluent cells cultured in a 48-well plate were transfected twice at a 2- or 3-day interval with 30 nM of vimentin-specific, HPRT-specific, or negative control (Invitrogen) siRNA duplexes using Lipofectamine RNAiMAX (Invitrogen) following the manufacturer's instructions. The siRNA target sequences were as follows: vimentin (sense), 5′-ACCTTGAACGCAAAGTGGAATCTTT-3′; HPRT-S1 (sense), 5′-AAGCCAGACUUUGUUGGAUUUGAAA-3′.

Infection of Huh7 cells with HCV

Infectious HCV (JFH1 strain) particles were produced in Huh7.5.1 cells as described previously (Wakita et al., 2005). Culture supernatant containing infectious HCV particles was collected and stored at −80 °C until use. Subconfluent naïve Huh7, Huh7/hygro, or Huh7/vimentin cells in 24-well or 48-well plates were exposed to normal culture medium containing HCV particles (1.4–8 fmol core protein/well, corresponding to moi=0.0175–0.1) for 6 h at 37 °C. Cells were then washed and maintained in 500 μl (24-well) or 250 μl (48-well) of normal culture medium for 6–7 days at 37 °C. To determine HCV production activity, the amounts of HCV core protein in the culture medium and cell lysates were quantified with an enzyme-linked immunosorbent assay (ELISA) (Ortho® HCV antigen ELISA test, Ortho-Clinical Diagnostics, Japan).

Assay for activity of HCV genomic RNA replication

The RNAs (30 μg) transcribed from pSGR-JFH1/Luc and pSGR-JFH1/Luc-GND (Kato et al., 2005) were transfected into Huh7 cells (1.6×10^6 cells) by electroporation. Transfected cells in normal culture medium were immediately seeded into 48-well plates at 9.0×10^4 cells/well. Four hours after transfection, siRNAs were also transfected into these cells. After incubation for 2.5 days, cells were harvested and the luciferase activity in cell lysates was determined with the Luciferase Assay System (Promega). Since the luciferase activities of the JFH1/Luc replicon were ~400-fold higher than those of the JFH1/Luc-GND mutant replicon, background luciferase activity, which is independent of replication activity, was very low in our experimental conditions.

Immunofluorescence microscopy

Cells cultured on glass cover slips (in 24-well plates) were fixed in 1% formaldehyde-PBS for 1 h at 4 °C, permeabilized in PBS containing 0.1% Triton X-100 for 5 min, and washed twice with PBS. The cell monolayers were incubated with rabbit anti-vimentin antibodies (1:100) and mouse anti-HCV core protein antibodies (1:100) for 60 min at room temperature. After washing with PBS, the cells were incubated with Alexa488-conjugated anti-mouse IgG, Alexa594-conjugated anti-rabbit IgG, and DAPI (4′, 6′-diamidino-2-phenylindole) (Invitrogen) for 60 min at 4 °C. Coverslips were washed with PBS and mounted on glass slides. Immunofluorescence was visualized and quantitated with a confocal laser-scanning microscope (Axiovert 100M, Carl Zeiss) equipped with a LSM510 system (Carl Zeiss).

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Clinical Brief Report

Knockdown of autophagy-related gene decreases the production of infectious hepatitis C virus particles

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Abbreviations: Atg, autophagy related genes and their products; Beclin 1, autophagy-related bcl2-interacting Atg6 homologue; BECN1, *beclin 1* gene and mRNA; HCV, hepatitis C virus; IFNA1, interferon alpha1 gene; IFNA2, interferon alpha2 gene; IFNB1, interferon beta1 gene; NS, nonstructural protein; LC3, wild-type human microtubule-associated protein 1 light chain 3; LC3-I, soluble unlipidated form of LC3; LC3-II, LC3-phospholipid conjugate

Key words: autophagy, Atg7, LC3, Beclin 1, Hepatitis C virus, infectious HCV particles

Hepatitis C virus (HCV) is a positive-strand RNA virus, and classified within the Flaviridae family. Atg7-knockdown decreases the amount of HCV replicon RNA, when HCV JFH1 RNA and HCV subgenomic replicon are transfected into Huh7.5 cells. However, when infectious naive HCV particles are directly infected into Huh7.5.1 cells, it is still unclear whether Atg7-knockdown decreases the production of intracellular HCV-related proteins, HCV mRNA and infectious HCV particles. When Atg7 protein in HCV-infected Huh7.5.1 cells was knocked down by RNA-interference, the levels of intracellular HCV core, NS3, NS5A proteins, HCV mRNA and secreted albumin remained unchanged compared with those in the control HCV-infected cells. However, the level of infectious HCV particles released in the medium was decreased by the Atg7-knockdown. Similar results were obtained when Beclin 1 was knocked down by RNA-interference. The colocalization of endogenous LC3-puncta with HCV core, HS5A proteins and lipid droplets was also investigated. However, little endogenous LC3-puncta colocalized with HCV core, NS5A proteins or lipid droplets. These results suggested that autophagy contributed to the effective production of HCV particles, but little to the intracellular production of HCV-related proteins, HCV mRNA and the secretory pathway, in a naive HCV particles-infection system.

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Introduction

Hepatitis C virus (HCV) is a positive-strand RNA virus with a genome size of 9.6 kb, and is classified within the Flaviridae family.[1] HCV is spread by blood-to-blood contact, and infection can lead to liver cirrhosis and heaptocellular carcinoma. More than 150 million people in the world are infected with HCV. The HCV genome encodes polyproteins that are processed into structural proteins (core protein and envelope glycoproteins) and nonstructural (NS) proteins (NS1-NS5A including a protease and RNA helicase).[2] HCV replicates in association with intracellular membrane structures called "the membranous web."[3] HCV-infected cells accumulate lipid droplets, and the lipid droplets play an important role in the assembly of HCV particles.[4] HCV core protein recruits NS proteins and replication complexes to lipid droplets-associated membranes. This recruitment is critical for producing infectious viruses.

Recently, when HCV JFH1 RNA and HCV subgenomic replicon were transfected into Huh7.5 cells, HCV infection leads to incomplete autophagic flux, and Atg7-knockdown decreases the amount of HCV replicon RNA.[5-7] Interestingly, endogenous LC3 can associate with cytoplasmic lipid droplets in uninfected Huh7.5.1 cells.[8] Considering that HCV core and NS5A proteins associate with lipid droplets for the assembly of HCV particles, it is possible that Atg7-knockdown will result in a decreased production of these particles. Therefore, the contribution of Atg7 to the production of intracellular HCV-related proteins and the release of infectious HCV particles was investigated. In addition, the authors investigated whether endogenous LC3 colocalizes to HCV lipid droplets, HCV core and NS5A proteins.

Results

Human *ATG7* and *BECN1* RNAi resulted in decreased release of HCV particles in the medium without a decrease of the intracellular production of HCV-related proteins and HCV

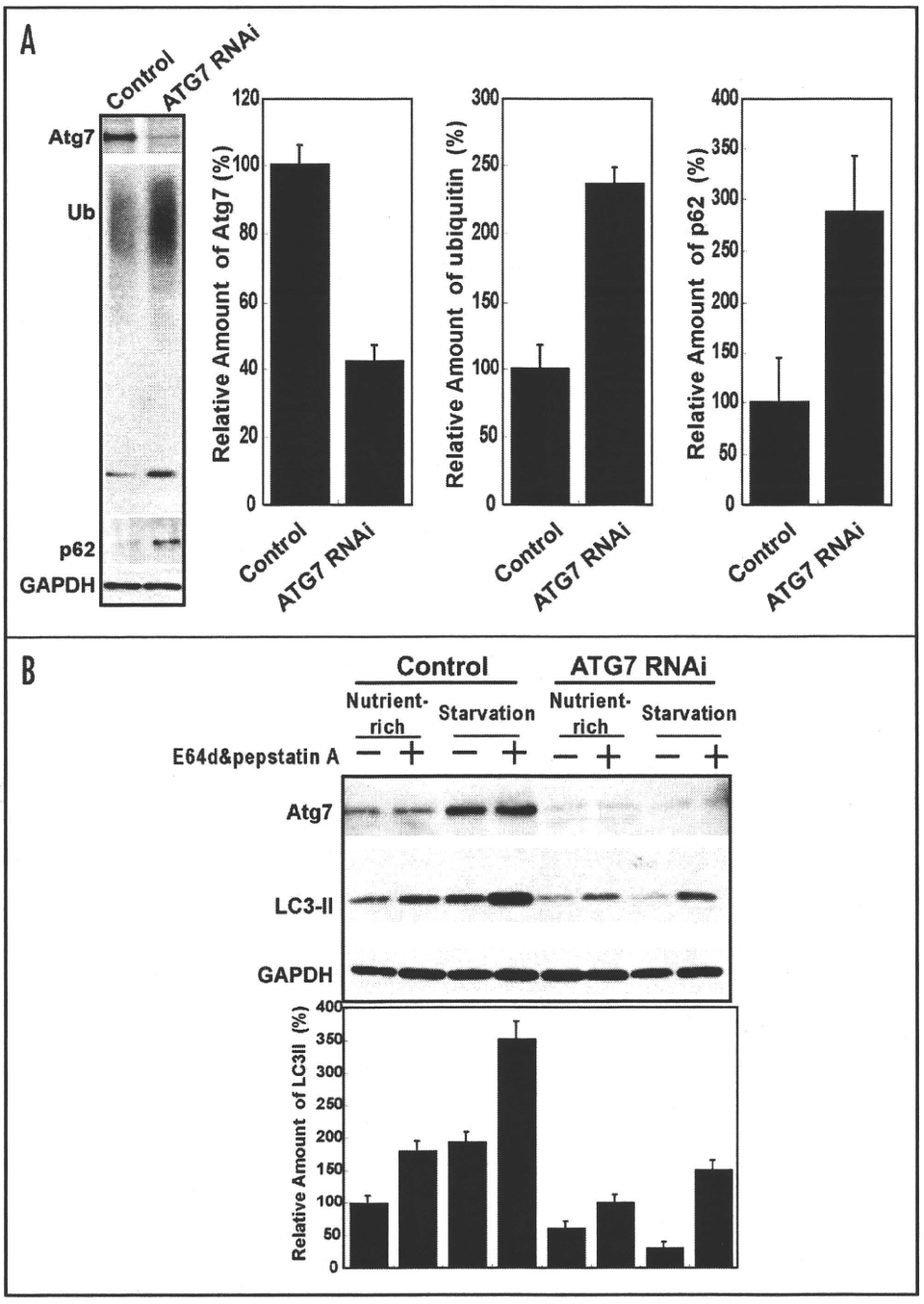

Figure 1. Effect of *ATG7*-RNAi on p62, ubiquitinated proteins and lysosomal turnover of LC3-II. (A) Increase of p62 and ubiquitinated proteins in Huh7 cells by *ATG7*-RNAi. dsRNA for *ATG7*-RNAi was transfected into Huh7.5.1 cells (ATG7 RNAi). As a negative control, scrambled dsRNA was employed (Control). Cells were harvested after incubation at 37°C for 48 h, and total proteins (20 μg) were separated on SDS-PAGE. Atg7, p62 and ubiquitinated proteins (ubiquitin) in the lysate were recognized by immunoblotting. GAPDH was employed as a loading control. Right: indicated the relative levels of intensity of each band of three independent experiments estimated by densitometry. Error bars indicate standard errors. (B) Effect of *ATG7*-RNAi on lysosomal turnover of LC3-II. Atg7-knockdown was performed as described in (A), and cells were cultured for 48 h. For Nutrient-rich conditions, cells were cultured in DMEM medium containing 10% FCS. Where indicated, cells were treated with the protease inhibitors, E64d (10 μg/ml) and pepstatin A (10 μg/ml) (E64d & pepstatin A +) for 4 h, or, as a negative control (Inhibitors -), with the solvent, dimethylsulfoxide. For Starvation conditions, cells were incubated for 4 hrs in Krebs-Ringer medium (KRB) in the presence (+) or absence (-) of the protease inhibitors. The cells were lysed, total proteins (10 μg per lane) were separated by SDS-PAGE, and endogenous LC3 and Atg7 in the lysates was recognized by immunoblotting. LC3-II, membrane-bound form of LC3. Lower: indicated the relative levels of intensity of each band estimated by densitometry. Error bars indicate standard errors.

mRNA. The initial investigation was on whether Atg7-knockdown leads to a decreased HCV core, NS3 and NS5A proteins in an in vitro naive HCV particle-infection system. Atg7 in Huh7.5.1 cells was knocked down by double-stranded RNAs for *ATG7* RNAi compared with scrambled double-stranded RNAs (Fig. 1A). The Atg7-knockdown in Huh7.5.1 cells resulted in an increase of p62 and ubiquitinated proteins (Fig. 1A), and led to an insufficiency in lysosomal turnover of LC3-II under starvation conditions (Fig. 1B).[14-18] HCV was infected into Huh7.5.1 cells, and the *ATG7* RNAi was performed at day 1 and day 3 post-infection. At day 5 post-infection, cells were harvested, and Atg7 and HCV core, NS3 and NS5A proteins in the cell lysate were analyzed by SDS-PAGE and immunoblotting. Under these conditions, the Atg7 protein was significantly decreased in HCV-infected Huh7.5.1 cells (Fig. 2A). However, the levels of HCV core, NS3 and NS5A proteins in Atg7-knockdown HCV-infected cells remained unchanged as compared with control HCV-infected cells (Fig. 2A). In addition, the levels of HCV mRNA in Atg7-knockdown HCV-infected cells also remained unchanged as compared with control HCV-infected cells (Fig. 2B). These results indicated that the *ATG7* RNAi has little effect on the intracellular production of HCV-related proteins and HCV mRNA.

The next investigation was on whether Atg7-knockdown affects the release of HCV particles. The amount of HCV core protein in the medium at day 5 post-infection was estimated by an ELISA for HCV core antigen. Interestingly, the amount of released HCV core protein derived from Atg7-knockdown cells decreased by about 40 ± 8% compared with control scrambled RNA-treated cells at day 5 post-infection ($p < 0.03$) (Fig. 2E). Under these conditions,

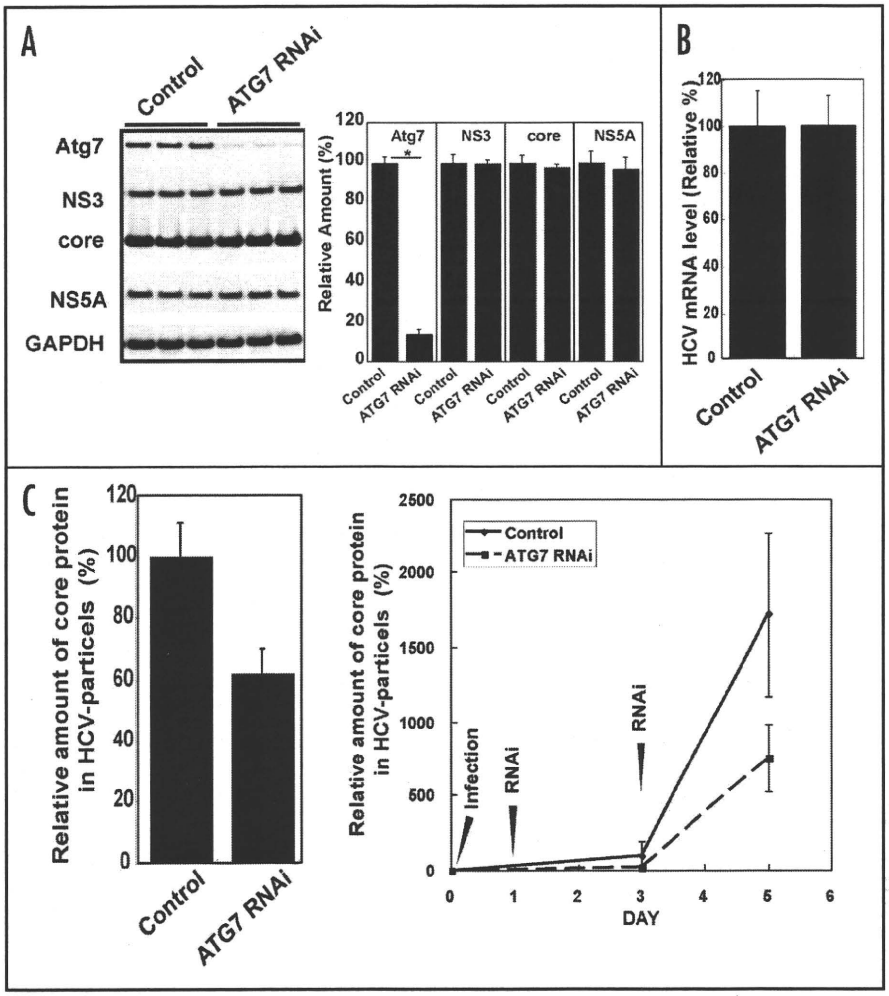

Figure 2. Decrease of infectious HCV particles in the medium by *ATG7*-RNAi. (A) Minor effect of *ATG7* RNAi on the intracellular production of HCV core, NS3 and NS5A proteins. HCV particles were infected into Huh7.5.1 cells. After incubation at 37°C for 2 h, cells were washed and cultured in the DMEM containing 10% fetal calf serum. At day 1 and 3 after HCV infection, dsRNA for *ATG7*-RNAi was transfected into the cells (ATG7 RNAi). As a negative control, scrambled dsRNA was employed (Control). Cells were harvested on day 5 after HCV infection, total proteins (10 μg) were separated by SDS-PAGE, and Atg7, HCV core, NS3 and NS5A proteins in the lysate were recognized by immunoblotting. Three independent experiments are shown. Note that HCV-related proteins remained unchanged, whereas Atg7 protein decreased by the RNAi treatment. Right: indicated the relative levels of intensity of each band estimated by densitometry. Asterisks indicated that the p-value of a Student's t-test is <0.03. The average intensity of "control" in each protein was regarded as 100%. Error bars indicate standard errors. (B) Relative amount of HCV mRNA in Atg7-knockdown cells and control cells. 140 ng of total RNA was used for quantitative RT-PCR. The level of HCV mRNA was normalized by the levels of GAPDH mRNA. Error bars indicate standard errors. (C) Decrease in the amount of the core protein of HCV particle released in the medium by *ATG7*-RNAi. Atg7-knockdown was performed as described in (A). The amount of HCV core protein in HCV particles released into the medium was estimated by a HCV core ELISA kit. The data are from three independent experiments. Relative amount of HCV core protein in Atg7-knockdown cells compared to scrambled RNA infected cells is shown, and the p-value of a Student's t-test is <0.03. Error bars indicate standard errors. Right: indicates the time course of the relative amount of HCV particles. The average amount of HCV particles from control HCV-infected cells at day 3 post-infection was set as 100%.

there was little difference in viability between Atg7-knockdown cells and control cells (Fig. 3A). The amount of HCV mRNA in Huh7.5.1 cells after the reinfection of HCV particles derived from Atg7-knockdown and control cells increased in a time-dependent manner, indicating that the HCV particles derived from Atg7-knockdown cells have a reinfectivity (Fig. 3B). It would be possible that Atg7-knockdown could inhibit the secretory pathway in addition to the release of HCV particles. However, the levels of secreted human albumin, one of the major secreted proteins of hepatocytes, in the medium of Atg7-knockdown cells remained unchanged as compared with that in the medium of control cells, suggesting that there was little, if any, effect on the secretory pathway in HCV-infected cells by the RNAi (Fig. 3C).

To clarify whether or not the decrease of released HCV particles in the medium by *ATG7* RNAi is caused by a defect in autophagy, we investigated the effect of Beclin 1-knockdown on the production of naive HCV-particles (Figs. 3B and C; and 4). Beclin 1, a yeast Atg6 homologue, was isolated as a Bcl-2-interacting protein,[12] and is a subunit of the class III PtdIns 3-kinase lipid-kinase complex essential for autophagy.[19,20] HCV was infected into Huh7.5.1 cells, and *BECN1* RNAi was performed at day 1 and day 3 post-infection. At day 5 post-infection, Beclin 1 protein in HCV-infected Huh7.5.1 cells was knocked down by *BECN1* RNAi compared with scrambled double-stranded RNAs (Fig. 4A). The levels of HCV core, NS3 and NS5A proteins in Beclin 1-knockdown HCV-infected cells remained unchanged as compared with control HCV-infected cells (Fig. 4A). The levels of HCV mRNA in Beclin 1-knockdown HCV-infected cells also remained unchanged as compared with control HCV-infected cells (Fig. 4B). Under these conditions, the amount of released HCV core protein derived from Beclin 1-knockdown cells decreased by about 60 ± 9% compared with control scrambled RNA-treated cells at day 5 post-infection ($p < 0.03$) (Fig. 4C). The HCV particles released from Beclin 1-knockdown cells have reinfectivity (Fig. 3B). There was little difference in viability between Beclin 1-knockdown cells and control cells (Fig. 4D). The levels of secreted human albumin in the medium of Beclin 1-knockdown cells remained unchanged as compared with that in the medium of control cells (Fig. 3C). These results suggested that autophagy contributes to an effective production of infectious HCV particles from the cells without a decrease of the intracellular levels of HCV core, NS3, NS5A or HCV mRNA.

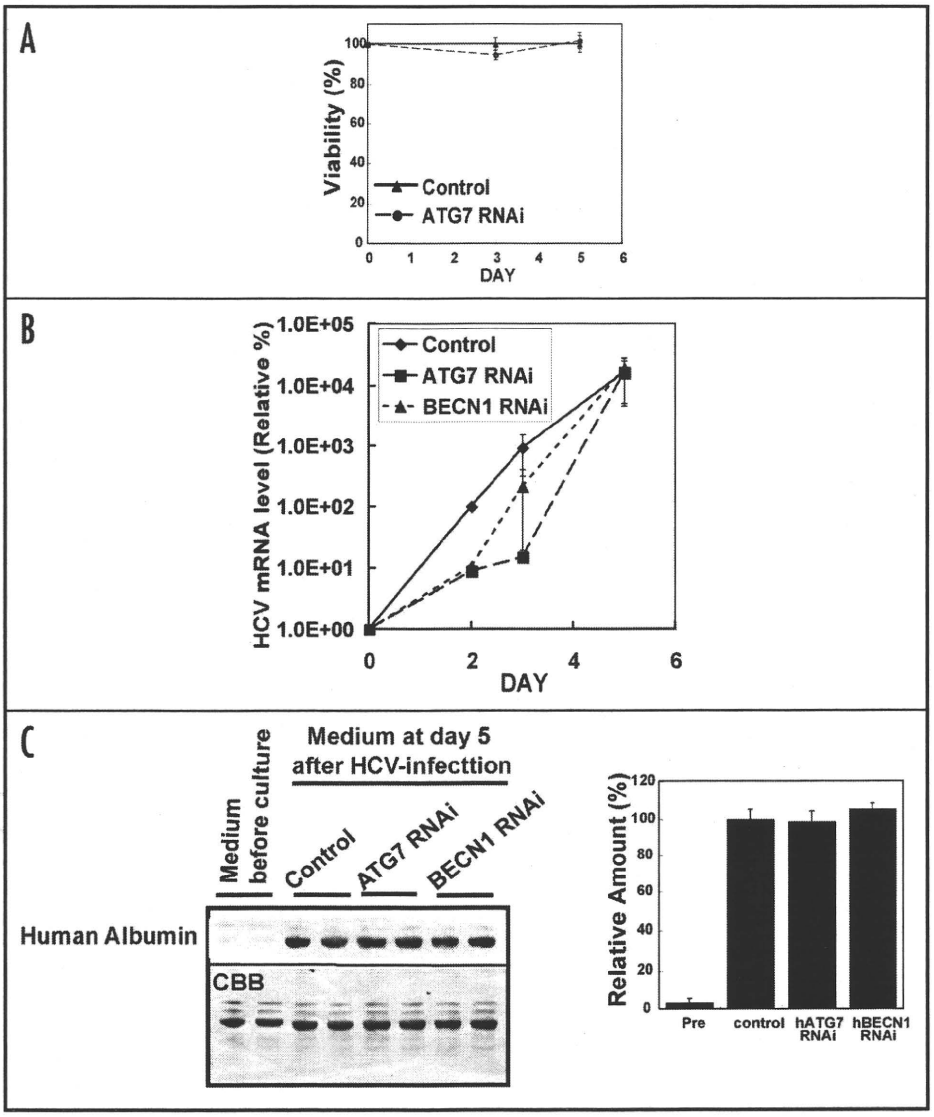

Figure 3. Effects of Atg7-knockdown on cell viability, reinfectivity of HCV particles, and secretion of human albumin. (A) Cell viability of Atg7-knockdown HCV-infected cells. As a negative control for *ATG7* RNAi, scrambled RNA was employed. Error bars indicate standard errors. (B) Reinfectivity of HCV particles derived from Atg7-knockdown HCV-infected cells. HCV-infection, *ATG7*-RNAi and *BECN1*-RNAi were performed as described in Figure 2. At day 5 post-infection, the medium containing HCV particles was harvested. The same volume of the medium containing HCV particles was added to the medium, in which Huh7.5.1 cells were cultured to semiconfluency. After incubation at 37°C for 2 h, cells were washed twice with culture medium, and incubated at 37°C for 2, 3 and 5 days. After RNA-preparation, 140 ng of total RNA was used for quantitative RT-PCR. The level of HCV mRNA was normalized by the levels of GAPDH mRNA. Error bars indicate standard errors. The average amount of HCV mRNA in the control HCV-infected cells at day 2 post-infection was set to 100%. (C) Minor effect of Atg7- and Beclin1-knockdowns on secretion of human albumin in HCV-infected cells. Media from cultured Atg7-(ATG7 RNAi) and beclin1-(BECN1 RNAi) knockdown HCV-infected cells was harvested. As a negative control for RNAi, scrambled RNA was employed (Control). The medium before cell culture was used as a non-incubation control (Medium before culture). Total proteins in the medium were separated by SDS-PAGE, and human albumin in the medium was recognized by immunoblotting with anti-human albumin IgG. To avoid cross-reactivity of the antibody against bovine albumin, the IgG was pre-absorbed with bovine albumin. As a loading control, the membrane was stained with Coomassie Brilliant Blue (CBB). Right: indicates the average relative levels of each bands estimated by densitometry.

Endogenous LC3-puncta displayed little colocalization with HCV core, NS5A or lipid droplets. Intracellular colocalization of endogenous LC3-puncta with HCV core, NS5A and lipid droplets in the HCV-infected cells was investigated next. GFP-LC3-puncta increase in HCV-infected cells.[5-7] However, there are some problems concerning the estimation of autophagy using GFP-LC3.[21-23] Therefore, using affinity purified anti-LC3 IgG, intracellular distribution of endogenous LC3 in the HCV-infected Huh7.5.1 cells was investigated (Figs. 5–9). As positive controls, endogenous LC3 in uninfected Huh7.5.1 cells were stained under starvation conditions in the absence and presence of E64d and pepstatin A (Fig. 6A–F). When uninfected Huh7.5.1 cells were cultured under nutrient-rich conditions, few puncta of LC3 were recognized (Fig. 6A and B). When the uninfected Huh7.5.1 cells were incubated under starvation conditions for 2 h, fluorescence of LC3-positive puncta was increased at a perinuclear region (Fig. 6C and D). The LC3-positive puncta increased in the presence of E64d and pepstatin A under starvation conditions (Fig. 6E and F). In HCV-infected Huh7.5.1 cells, the LC3-positive puncta increased significantly at day 5 post-infection (Figs. 5A vs. 6A, C and E). Deconvolution of high-magnification images (Fig. 8) indicated that LC3-positive puncta in HCV-infected cells were larger than those in starved cells even in the presence of these inhibitors (Fig. 8C and D vs. B). Quantitative analyses of the intensity of the fluorescence of LC3-puncta in each cell indicated that the total intensity of fluorescence of LC3-puncta per cell in HCV-infected cells was about 4.5-fold higher than in uninfected cell under starvation conditions in the presence of E64d and pepstatin A (Fig. 9). Further investigation was made on whether endogenous LC3-puncta colocalized with HCV core, NS5A and lipid droplets. However, there was little colocalization between LC3 and these structures (Figs. 7 and 8).

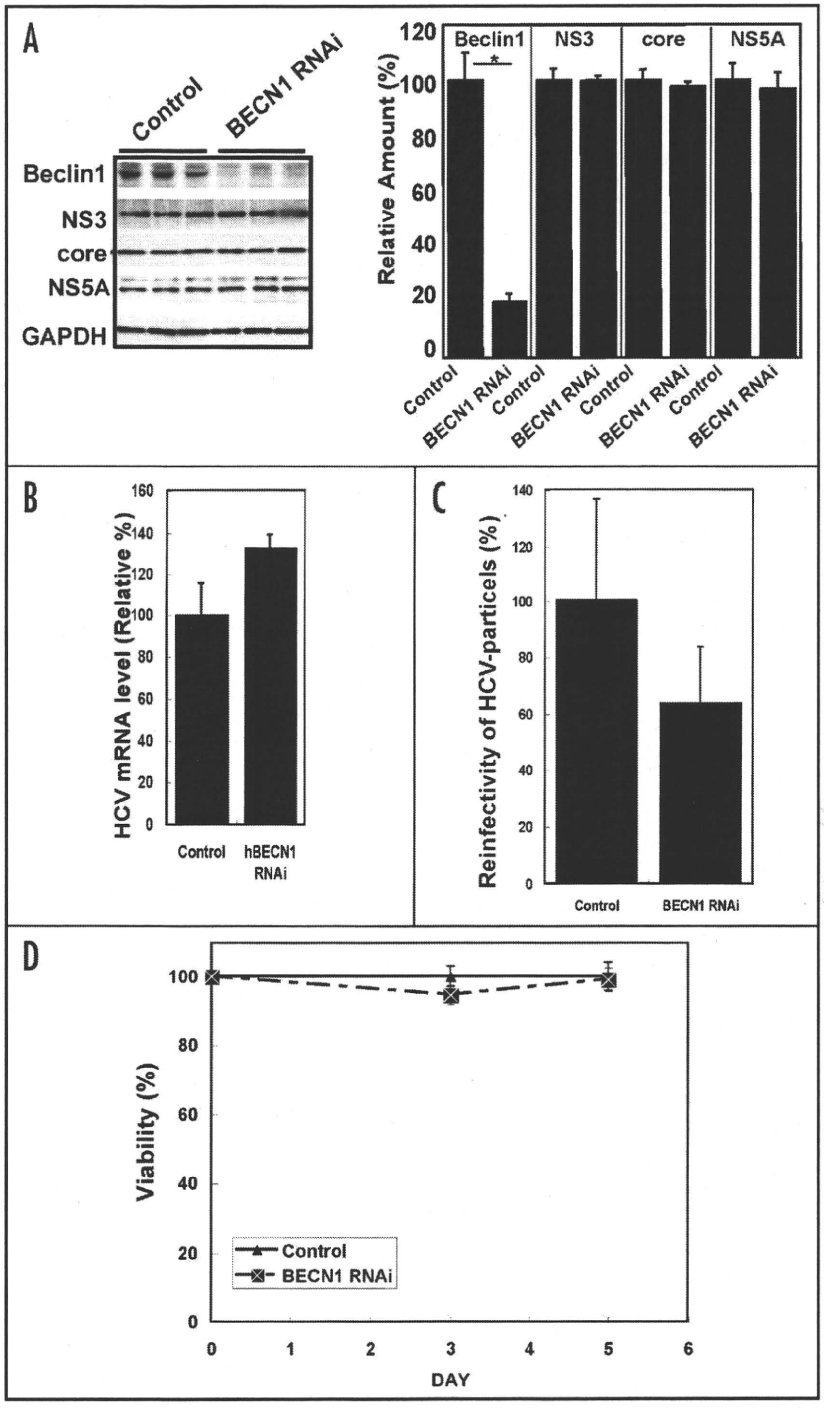

Figure 4. Decrease of infectious HCV particles in the medium by Beclin1 knockdown. (A) Minor effect of Beclin1-knockdown on the intracellular production of HCV core, NS3 and NS5A proteins. HCV-infection, *BECN1* RNAi and immunological analyses were performed as described in Figure 2. BECN1 RNAi, Beclin1-knockdown; Control, scrambled dsRNA. Right: indicates the relative levels of intensity of each band estimated by densitometry. Asterisks indicated that the p-value of a Student's t-test is <0.03. The average intensity of "control" in each protein was set to 100%. Error bars indicate standard errors. (B) Relative amount of HCV mRNA in Beclin1-knockdown cells and control cells. 140 ng of toal RNA was used for quantitative RT-PCR. The level of HCV mRNA was normalized by the levels of ACTB mRNA. Error bars indicate standard errors. (C) Decrease in the amount of the core protein of HCV particle released in the medium by Beclin 1-knockdown. Beclin 1-knockdown-dependent estimation of core protein in HCV particles was performed as described above. The data are from three independent experiments. Relative amount of HCV core protein in Beclin1-knockdown cells compared to scrambled RNA infected cells is shown, and the p-value of a Student's t-test is <0.03. Error bars indicate standard errors. (D) Cell viability of Beclin 1-knockdown HCV-infected cells. As a negative control for *BECN1* RNAi, scrambled RNA was employed. Error bars indicate standard errors.

Discussion

Results of this study showed that Atg7- and Beclin 1-knockdowns in HCV-infected cells resulted in a decrease in the production of infectious HCV particles in the medium, whereas the intracellular production of HCV mRNA and HCV proteins examined remained unchanged. Few endogenous LC3-puncta in HCV-infected cells were colocalized to lipid droplets, core and NS5A proteins.

Using transfection of HCV JFH1 mRNA into Huh7.5 cells, Atg7-knockdown decreases the amount of HCV replicon RNA.[5-7] However, using an in vitro naive HCV JFH1 particle-infection system, Atg7-knockdown decreased the level of infectious particles in the medium by about 40%, whereas intracellular HCV mRNA and HCV proteins remained unchanged. This discrepancy results from the difference between the naive HCV particle-infection system and the transfection of HCV mRNA.

Against some pathogens, autophagy plays a role in intracellular immunity. Initially, it was hypothesized that if autophagy plays a protective role against HCV infection, Atg7- and Beclin 1-knockdowns could lead to an increase of HCV particles and/or intracellular HCV-related proteins. However, contrary to the hypothesis, these knockdowns decreased the production of HCV particles. Atg7 is a key enzyme essential for formation of autophagosomes, and Beclin 1 is a subunit of the class III PtdIns 3-kinase lipid-kinase complex that induces autophagy. Considering that Atg7 and Beclin 1 have different functions in autophagy, these results indicated that autophagy contributes to the effective production of HCV particles in the medium, but little to the intracellular levels of HCV mRNA and HCV-related proteins. It is possible that this knockdown would inhibit the secretory pathway. However, there was little difference in the level of secreted albumin in the medium between autophagy-knockdown cells and control cells. In yeast, no *atg* mutants have defects in the secretory pathway, and there is no report that

ATG gene-knockout influences the secretory pathway in animals and plants. Therefore, it is unlikely that these knockdowns inhibit the secretory pathway. Considering these results, autophagy will contribute to the release and/or assembly of HCV particles, but little to the intracellular production of HCV mRNA and HCV-related proteins.

LC3-II can associate with lipid droplets,[24] and lipid droplets are induced by HCV infection.[4] However, little endogenous LC3-puncta associated with lipid droplets in the HCV-infected Huh7.5.1 cells. These results show that LC3-II cannot always associate with lipid droplets. There is a certain mechanism that allows LC3-II to associate with lipid droplets, which will be the subject of future research.

Materials and Methods

Cells, media, materials and antibodies. Huh7.5.1 cells derived from the Huh7 cell line (ATCC CCL-185) were cultured in Dulbecco's modified Eagle medium (DMEM; Wako, 045-30285) containing 10% fetal calf serum (JRH biosciences/SIGMA, 12603C) and 1% nonessential amino acids (Invitrogen, 11140050). Polyclonal antibodies against Atg7 and LC3 were described previously.[9-11] For the preparation of antiserum against human Beclin 1, rabbits were immunized with a glutathione S-transferase-human Beclin 1 fusion protein. The anti-Beclin 1 IgG was affinity-purified using recombinant human Beclin 1-conjugated Sepharose. The monoclonal antibody against HCV core protein was purchased from Anogen (MO-I40015B), the monoclonal antibody against HCV NS5A protein (HCM-131-5) was from Austral, the monoclonal antibodies against GAPDH (ab8245) and HCV NS3 proteins (ab18664) were from Abcam, and the polyclonal antibody against human albumin (126584) was from Calbiochem. For RNA-interference for human *ATG7* (*ATG7* RNAi) and *BECN1* (*BECN1* RNAi),[12] lipofectamine RNAi MAX, and Stealth™ select RNAi sets (Invitrogen, 1299003), respectively, and Stealth™ RNAi negative control (Invitrogen, 935300) were used. Little interferon mRNAs (IFNA1, IFNA2 and IFNB1) were activated by the transfection of these double-stranded RNAs (Table 1). Protein concentrations were determined using the bicinchoninic acid (BCA) protein assay reagent (Pierce, 23225). E64d (4321-v) and pepstatin A (4397-v) were purchased from Peptide Institute.

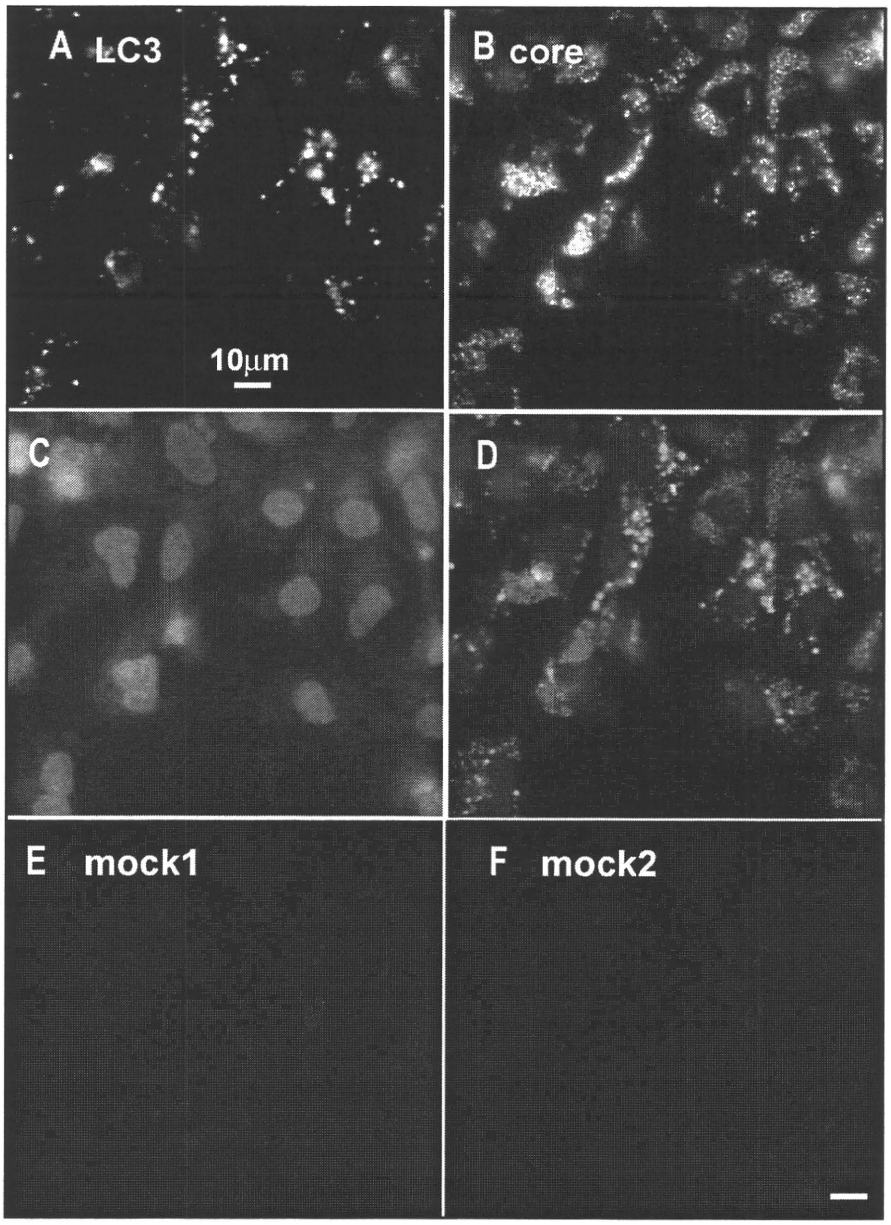

Figure 5. Intracellular distribution of endogenous LC3 in HCV-infected Huh7.5.1 cells. Cells were fixed in 4% paraformaldehyde/PBS at day 5 post-infection (A–F), and permeabilized in 50 μg/ml of digitonin. Endogenous LC3 in the cells was recognized with rabbit anti-LC3 IgG and Alexa488-conjugated goat anti-rabbit IgG (A). Intracellular HCV core protein was recognized with mouse anti-HCV core IgG and Alexa594-conjugated goat ant-mouse IgG (B). DAPI staining was in (C). As a negative control for LC3-staining, cells were stained with normal rabbit IgG and Alexa488-conjugated goat anti-rabbit IgG (E mock 1). As a negative control for HCV core-staining, cells were stained with normal mouse IgG and Alexa594-conjugated goat anti-mouse IgG (F mock 2). Fluorescence of Alexa488, Alexa594 and DAPI was monitored by fluorescence microscopy. Merged pseudo color images (LC3, green: HCV core, red: and DAPI, blue) was shown in (D). Bars indicate 10 μm.

Infection of Huh7.5.1 cells with HCV. Infectious HCV (JFH1 strain) particles were produced in Huh7.5.1 cells as described in an earlier study.[13] Culture supernatant containing infectious HCV particles was collected and stored at -80°C until use. Subconfluent Huh7.5.1 cells in 24-well or 48-well plates were exposed to

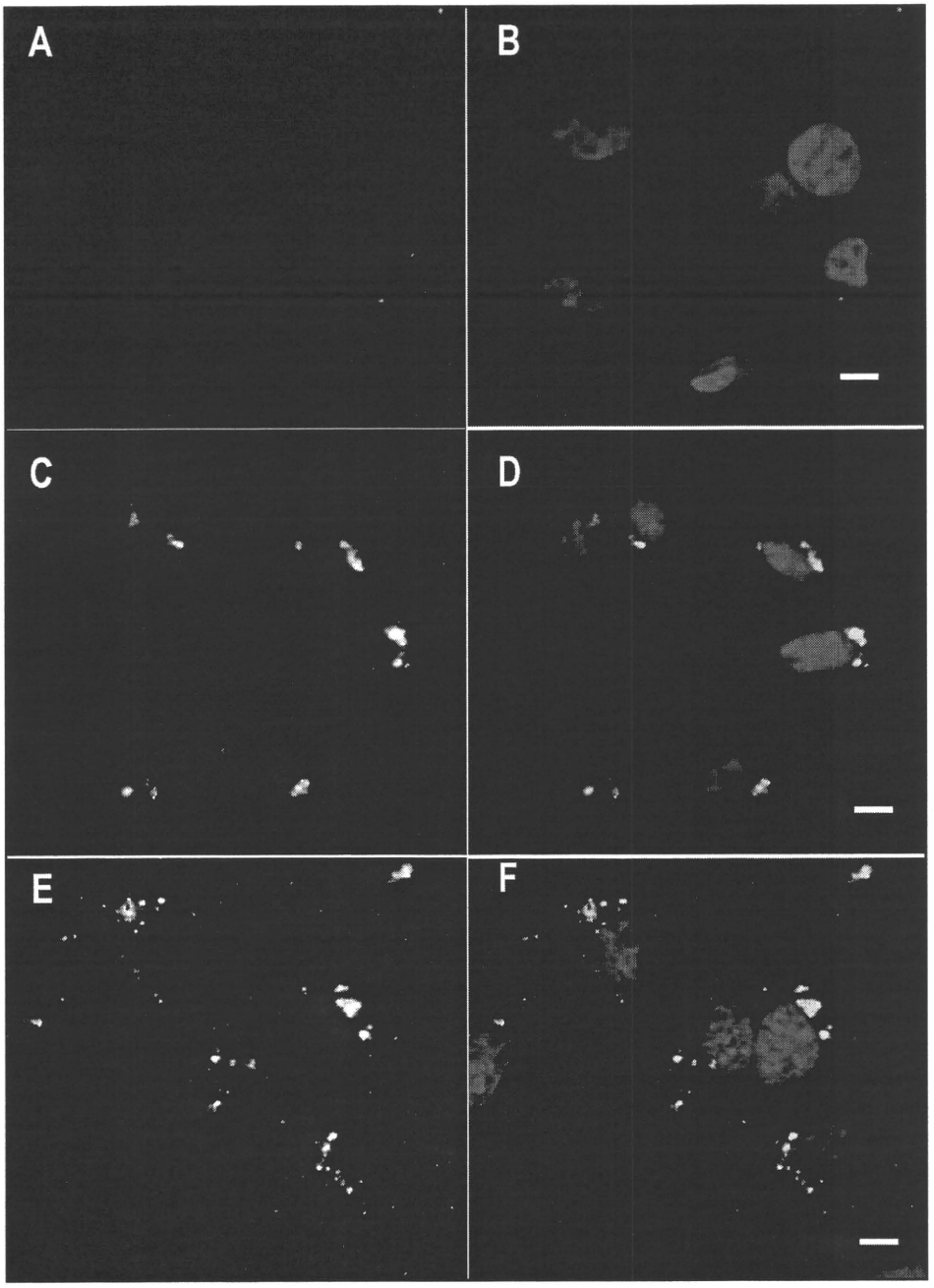

Figure 6. Intracellular distribution of endogenous LC3 in starved Huh7.5.1 cells. Huh7.5.1 cells were cultured in the nutrient-rich medium (A and B). For starvation-induced autophagosomes and autolysosomes, cells were incubated in the Krebs-Ringer buffered medium for 4 h in the absence (C and D) or presence (E and F) of E64d and pepstatin A. Endogenous LC3 in the cells was recognized with rabbit anti-LC3 IgG and Alexa488-conjugated goat anti-rabbit IgG (A, C and E). Merged pseudo color images (LC3, green: and DAPI, blue) were shown in (B, D and E). Bars indicate 10 μm.

normal culture medium containing HCV particles (8 fmoles of core protein/well, corresponding to moi = 0.1) for 6 h at 37°C. Cells were then washed and maintained in 500 μl (24-well) or 250 μl (48-well) of normal culture medium for 6–7 days at 37°C. To determine HCV production activity, the amounts of HCV core protein in the culture medium was quantified with an enzyme-linked immunosorbent assay (ELISA) (Ortho® HCV antigen ELISA test, Ortho-Clinical Diagnostics, 601002).

Immunoblotting analyses. After HCV infection, cells were washed twice in phosphate-buffered saline, lysed in lysis buffer (10 mM sodium phosphate, pH 7.2, 150 mM NaCl, and 1% sodium dodecyl sulfate) containing a Complete® protease-inhibitor cocktail (Roche Diagnostics, 1697498). Proteins (10 μg) of the lysate were separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (4–12% Bis-Tris, Invitrogen, NP0322BOX). After transferring the proteins to a polyvinylidine difluoride membrane using a Trans-Blot SD transfer cell (Bio-Rad, 170-3940), HCV core protein, HCV NS3 protein, HCV NS5A protein, and Atg7 in the lysate were recognized with appropriate antibodies. A chemiluminescent method was carried out according to standard protocols with SuperSignal West Dura Extended Duration Substrate (Pierce, 34075) or SuperSignal West Pico Chemiluminescent Substrate (Pierce, 34077).

Immunofluorescence analyses. Indirect immunofluoresence analysis was basically performed as described in the literature.[11,14] Briefly, cells were fixed in a fixation solution (phosphate buffered saline containing 4% paraformaldehyde) at room temperature for 5 min, and permeabilized in phosphate-buffered saline containing 1% digitonin. Rabbit polyclonal anti-LC3 antibody (10 μg/ml), mouse monoclonal anti-HCV core antibody (10 μg/ml), and mouse monoclonal anti-HCV NS5A antibody were used for recognizing LC3, HCV core protein, and HCV NS5A protein, respectively. As secondary antibodies, Alexa488-conjugated goat anti-rabbit IgG (Invitrogen, A11008) and Alexa594-conjugated goat anti-mouse IgG (Invitrogen, A11005) were used. Nile Red (Invitrogen, N1142) was used for lipid droplet staining. Fluorescence of Alexa488 and Alexa594 was monitored with Biozero BZ-8000 (KEYENCE, Tokyo, Japan).

Other techniques. Densitometric analyses of images were performed with an ImageJ program (http://rsbweb.nih.gov/ij/) on a PowerMac G4 computer. Cell viability was measured with a CellTiter 96 nonradioactive cell proliferation assay kit (Promega,

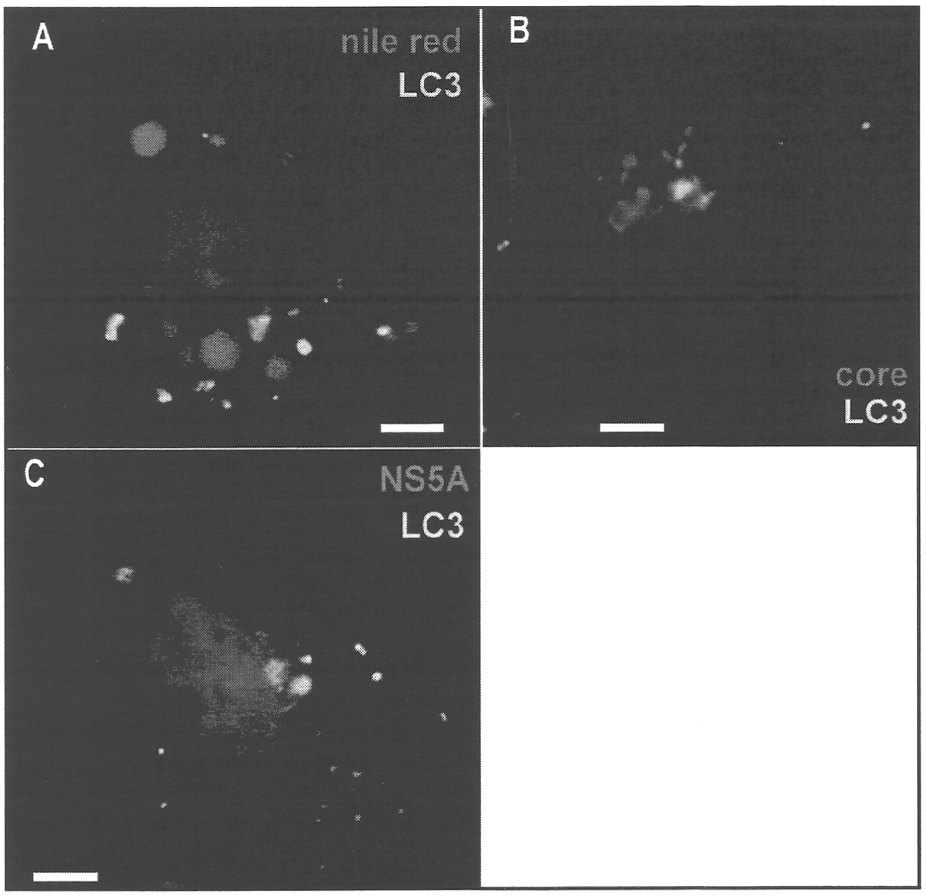

Figure 7. Minor colocalization of LC3 with lipid droplets, HCV core or HCV NS5A proteins. Lipid droplets in HCV-infected cells were stained nile red (A, pseudo color is red), HCV core proteins were stained with mouse anti-HCV core antibody and Alexa594-conjugated goat anti-mouse IgG (B, pseudo color is red), and HCV NS5A proteins were stained with mouse anti-HCV NS5A antibody and Alexa594-conjugated goat anti-mouse IgG (C, pseudo color is red). Pseudo color of LC3 is green in (A–C). Merged images are shown. Bars indicate 5 μm.

G4000). Total RNA was prepared by a RNeasy plus mini kit (Qiagen, 74134). Quantitative RT-PCR of mRNA was performed with a Lightcycler480 using a Lightcycler RNA Master SYBR Green I kit (Roche, 3064760).

Acknowledgements

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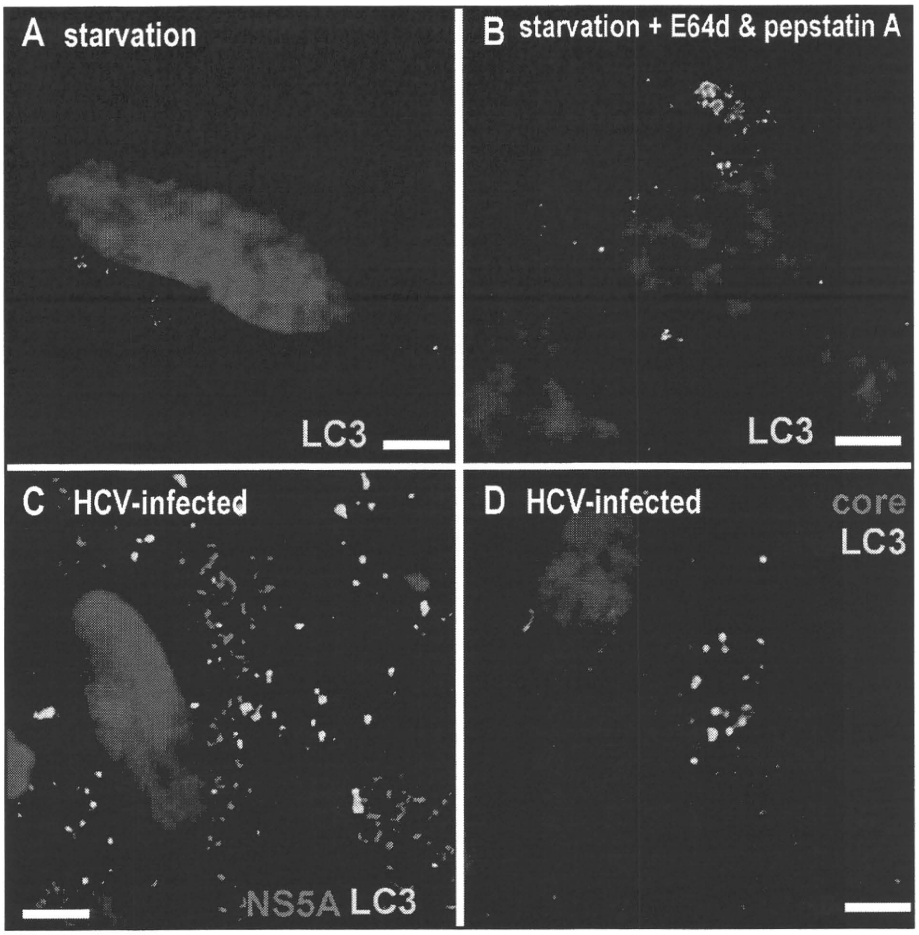

Figure 8. Deconvoluted images of LC3, core and NS5A in HCV-infected Huh7.5.1 cells. The fluorescence images of endogenous LC3 (A–D, pseudo color is green), NS5A (C, pseudo color is red), and core (D, pseudo color is red) were obtained by fluorescent microscopy. The images were deconvoluted with ImageJ program (http://rsbweb.nih.gov/ij/) and a plug-in for iterative deconvolution (http://www.optinav.com/Iterative-Deconvolution.htm). Pseudo color for DAPI staining is blue. Merged images are shown. Note that the size of LC3-puncta in HCV-infected cells (C and D) were larger than under starvation conditions in the absence (A) or presence (B) of E64d and pepstatin A.

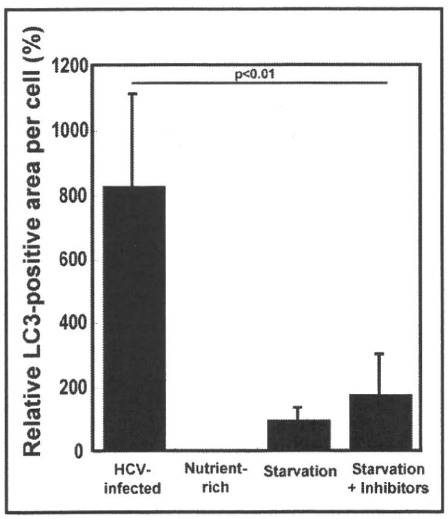

Figure 9. Increase of the fluorescence of LC3 in HCV-infected cells. The images of fluorescence of LC3 were analyzed with an ImageJ program (http://rsbweb.nih.gov/ij/) and plug-ins for iterative deconvolution (http://www.optinav.com/Iterative-Deconvolution.htm) and tophat-filter (http://rsb.info.nih.gov/ij/plugins/lipschitz/). The fluorescent images of over 200 cells were analyzed, and relative intensity was calculated; the average intensity of LC3 in Huh7.5.1 cell under starvation conditions (starvation) in the absence of E64d and pepstatin A was set to 100%.

Table 1 **Little mRNAs of interferons were activated by *ATG7*- and *BECN1*-RNAi**

	IFNA1	*IFNA2*	*IFNB1*
	(copies of mRNA/100 ng total RNA)		
Uninfected Huh7	5.6 ± 1.5	0.8 ± 0.1	0.4 ± 0.1
HCV-infected/scrambled RNA	5.8 ± 5.0	1.8 ± 0.1	4.9 ± 3.6
HCV-infected/*ATG7* RNAi	0.6 ± 0.1	1.3 ± 0.3	0.9 ± 0.5
HCV-infected/*BECN1* RNAi	0.3 ± 0.1	1.1 ± 0.2	2.1 ± 1.3

HCV was infected into Huh7.5.1 cells, and RNA interference was performed at day 1 and day 3 post-infection (HCV-infected). At day 5 post-infection, total RNA was prepared with a RNeasy plus kit. As a negative control for RNAi, scrambled RNA was employed. As a negative control for HCV-infection, Huh7 cells were incubated for 5 days (Uninfected Huh7), and total RNA was prepared. Quantitative RT-PCR of mRNA was performed with a Lightcycler480 (Roche) with appropriate primer-sets for each genes.

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ORIGINAL ARTICLE

17β-estradiol inhibits the production of infectious particles of hepatitis C virus

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ABSTRACT

Persistent infection with hepatitis C virus causes serious liver diseases, such as chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma. The male gender is one of the critical factors in progression of hepatic fibrosis due to chronic HCV infection; thus female hormones may play a role in delaying the progression of hepatic fibrosis. It has also been reported that women are more likely than men to clear HCV in the acute phase of infection. These observations lead the present authors to the question: do female hormones inhibit HCV infection? In this study using HCV J6/JFH1 and Huh-7.5 cells, the possible inhibitory effect(s) of female hormones such as 17β-estradiol (the most potent physiological estrogen) and progesterone on HCV RNA replication, HCV protein synthesis and production of HCV infectious particles (virions) were analyzed. It was found that E_2, but not P_4, significantly inhibited production of the HCV virion without inhibiting HCV RNA replication or HCV protein synthesis. E_2–mediated inhibition of HCV virion production was abolished by a nuclear estrogen receptor (ER) antagonist ICI182780. Moreover, treatment with the ERα-selective agonist 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol (PPT), but not with the ERβ-selective agonist 2, 3-bis (4-hydroxyphenyl)-propionitrile (DPN) or the G protein-coupled receptor 30 (GPR30)-selective agonist 1-(4-[6-bromobenzo 1, 3 dioxol-5-yl]-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta [c] quinolin-8-yl)-ethanone (G-1), significantly inhibited HCV virion production. Taken together, the present results suggest that the most potent physiological estrogen, E_2, inhibits the production of HCV infectious particles in an ERα–dependent manner.

Key words 17β-estradiol, estrogen receptor, hepatitis C virus, sex difference.

HCV, an enveloped RNA virus which belongs to the genus *Hepacivirus* within the family *Flaviviridae*, prevails in most parts of the world with an estimated number of about 170 million carriers; hence HCV infection is a major global health-care problem (1). Persistent infection with HCV causes serious liver diseases, such as chronic hepatitis, hepatic cirrhosis and hepatocellular carcinoma (2, 3). In the USA, the prevalence of anti-HCV antibodies is twice as high in men as in women (4). The male gender is thought to be one of the critical factors in progression of hepatic fibrosis in chronic HCV infection (5, 6). It has also been reported that progression of hepatic fibrosis is faster in postmenopausal than in premenopausal women, and that hormone replacement therapy with estrogen and

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List of Abbreviations: DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; DPN, 2, 3-bis (4-hydroxyphenyl)-propionitrile; E_2, 17β-estradiol; ER, estrogen receptor; G-1, 1-(4-[6-bromobenzo 1, 3 dioxol-5-yl]-3a, 4, 5, 9b-tetrahydro-3H-cyclopenta [c] quinolin-8-yl)-ethanone; GPR30, G protein-coupled receptor 30; HCV, hepatitis C virus; P_4, progesterone; PPT, 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl)trisphenol; SEM, standard error of the mean.

progesterone significantly delays progression of hepatic fibrosis in postmenopausal women (6, 7). This potential innate resistance of premenopausal women to hepatic fibrosis may be attributed to female hormones, such as estrogens and progesterone. In fact, E_2, the most potent physiological estrogen, has been reported to suppress the progression of liver fibrosis and hepatocarcinogenesis (8, 9). Moreover, women are more likely than men to clear HCV in the acute phase of infection, even within a few months after infection (10). These observations imply the possibility that female hormones inhibit HCV infection, either at the level(s) of virus attachment/entry, virus RNA replication, virus protein synthesis or production of infectious virus particles (virions).

Estrogens utilize three kinds of ER; ERα, ERβ and GPR30 (11–15). Specific agonists and antagonists of ER are available and widely used to examine the roles of estrogens. In the present study, we examined the possible effects of female hormones, especially E_2 and P_4, on HCV RNA replication, protein synthesis and virion production in cultured cells.

MATERIALS AND METHODS

Cell culture and virus infection

A human hepatoma-derived cell line, Huh-7.5, which is highly permissive to HCV RNA replication (16), was kindly provided by Dr. C. M. Rice (The Rockefeller University, New York, NY, USA). The cells were maintained in phenol red-free DMEM (Sigma–Aldrich, St Louis, MO, USA) supplemented with 10% heat-inactivated and charcoal-stripped FBS (Israel Beit Haemek, Haemek, Israel), 0.1 mM non-essential amino acids (Invitrogen, Carlsbad, CA, USA), 100 IU/mL penicillin and 100 μg/mL streptomycin (Invitrogen).

The pFL-J6/JFH1 plasmid that encodes the entire viral genome of a chimeric strain of HCV-2a, J6/JFH1 (17) was kindly provided by Dr. C. M. Rice. A cell culture-adapted mutant derived from J6/JFH1 (P-47 strain) (18, 19) was used for infection experiments. The virus was inoculated into Huh-7.5 cells at a multiplicity of infection of 1.0 and incubated for 2 hr. After the residual virus had been removed by washing, the cells were cultured in the presence or absence of female hormones, and agonists and an antagonist of estrogen receptors (see below). Culture supernatants were collected at 0, 1, 2 and 3 days postinfection and virus titers were determined, as described below.

Virus titration

Culture supernatants containing HCV were serially diluted 10-fold in DMEM and inoculated into Huh-7.5 cells (2×10^5 cells per well in a 24-well plate). After incubation at 37°C for 6 hr, the cells were fed with fresh DMEM. At 24 hr postinfection, the cells were fixed with ice-cold methanol, blocked with 5% goat serum in PBS and subjected to immunofluorescence analysis using mouse monoclonal antibody against the HCV core protein (2H9) and Alexa Fluor 488-conjugated goat anti-mouse IgG (H+L, Molecular Probes, Eugene, OR, USA). Hoechst 33342 (Molecular Probes) was used for counterstaining of the nuclei. HCV-positive foci were counted under a fluorescent microscope (BX51; Olympus, Tokyo, Japan) and virus titers were expressed as focus-forming units per ml, as reported previously (18, 19).

Chemicals

E_2 and P_4 were purchased from Sigma–Aldrich (St Louis, MO, USA). ICI182780 (an antagonist of ERα and ERβ), PPT (an ERα-selective agonist) (20) and DPN (an ERβ-selective agonist) (21) were purchased from Tocris Bioscience (Bristol, UK). G-1 (a GPR30-selective agonist) (22) was purchased from Calbiochem (Darmstadt, Germany). DMSO, which was used as a solvent, was obtained from Wako Pure Chemical Industries (Osaka, Japan). The concentrations of E_2 and P_4 used in this study were 0.4 μM and 3 μM, respectively, which correspond to the estimated highest concentrations in the sera of pregnant women. ICI182780 was used at a concentration of 1 μM, PPT and DPN at 0.1, 1 and 10 μM, and G-1 at 0.1 and 1 μM. As G-1 has been reported to lose its GPR30-binding specificity at concentrations over 1 μM, a concentration of 10 μM for G-1 was not tested. The final concentration of DMSO as a control never exceeded 0.01%.

Cell viability assay

Cells plated on 96-well microtiter plates (2.0×10^4 cells/well) were inoculated with HCV and treated with E_2, P_4 or DMSO. The cell viability in each well was determined by WST-1 assay (Roche Diagnostics, Mannheim, Germany) until 3 days postinfection.

Real-time quantitative RT-PCR

Total cellular RNA was isolated using the RNAiso reagent (Takara Bio, Kyoto, Japan) and cDNA was generated using the QuantiTect Reverse Transcription system (Qiagen, Valencia, CA, USA). Real-time quantitative PCR was performed on a SYBR *Premix Ex Taq* (Takara Bio) using SYBR green chemistry in ABI PRISM 7000 (Applied Biosystems, Foster, CA, USA). Primer sets used in this study are shown below: HCV NS5B, 5′-ACCAAGCTCAAACTCACTCCA-3′ and 5′-AGCGGGGTCGGGCAC GAGACA-3′ (23);

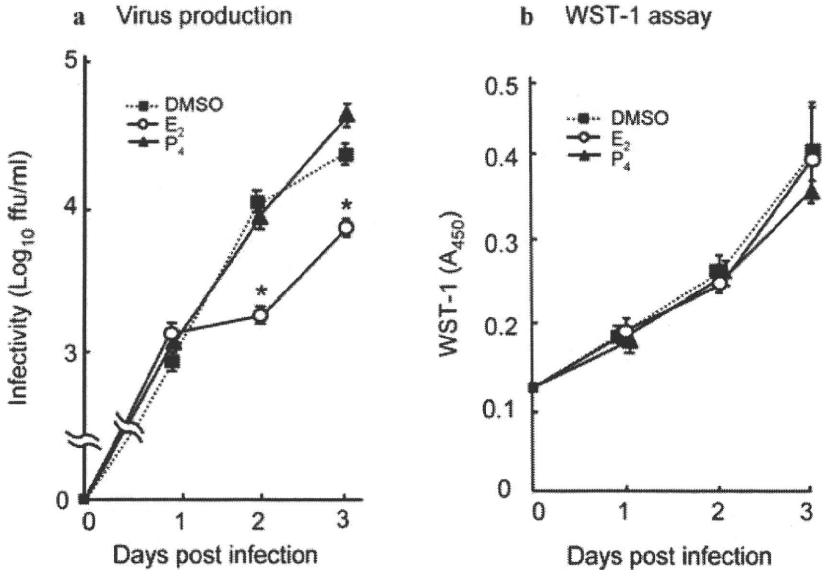

Fig. 1. Effects of E_2 and P_4 on HCV virion production and cell growth. (a) HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM), P_4 (3 μM) or DMSO (control) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for virus infectivity. Data are shown as mean ± SEM. (b) Cell growth. HCV-infected cells were treated with E_2, P_4 or DMSO (control) from 2 hr to 3 days postinfection. Cell growth in each culture was determined by WST-1 assay. Data are shown as mean ± SEM.

β-actin, 5′-GCGGGAAATCGTGCGTGACATT-3′ and 5′-GATGGAGTTGAAGGTAGTTTCGTG-3′.

Immunoblotting

Cells were solubilized in lysis buffer as reported previously (18, 19). The cell lysates were subjected to 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were incubated with mouse monoclonal antibodies against HCV NS3 (Chemicon International, Temecula, CA, USA), followed by incubation with peroxidase-conjugated goat anti-mouse IgG (Medical & Biological Laboratories Co. Ltd., Nagoya, Japan). The positive bands were visualized by using ECL detection system (GE Healthcare UK, Buckinghamshire, UK).

Statistical analysis

Results were expressed as mean ± SEM. Statistical significance was evaluated by one-way analyses of variances.

RESULTS

E_2 inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis

We first examined the effect of E_2 or P_4 treatment on HCV virion production. At 2 hr after virus inoculation, the HCV-infected Huh-7.5 cells were treated with E_2 (0.4 μM) or P_4 (3 μM) for 3 days. Culture supernatants were collected every day and titrated for viral infectivity. As shown in Figure 1a, E_2 treatment significantly suppressed HCV virion production at 2 and 3 days postinfection, whereas treatment with P_4 did not. The same treatment (E_2 or P_4) did not exert significant cytotoxicity (Fig. 1b). Next, we examined the effect of E_2 on HCV RNA replication and HCV protein synthesis under the same experimental conditions. We found that HCV RNA replication and HCV protein synthesis in both HCV-infected cells and HCV RNA replicon-harboring cells (23) were all unaffected by treatment with E_2 or P_4 (Fig. 2a–c). Moreover, treatment of the cells with E2 either prior to, or during, virus inoculation did not significantly inhibit HCV virion production (Fig. 3a). These results collectively suggest that E_2 inhibits HCV virion production, but not at the level of virus entry, RNA replication or protein synthesis. We also observed that E_2 –mediated inhibition of HCV virion production occurs in a dose-dependent manner (Fig. 3b).

A nuclear estrogen receptor antagonist, ICI182780, abolishes E_2-mediated inhibition of HCV virion production

We hypothesized that E_2 signaling through nuclear ER (ERα and ERβ) was involved in the E_2–mediated inhibition of HCV virion production. To test this possibility, we used ICI182780 (1 μM), an antagonist of ERα and ERβ. The results clearly demonstrated that treatment of cells with ICI182780 abolished E_2–mediated inhibition of HCV virion production (Fig. 4).

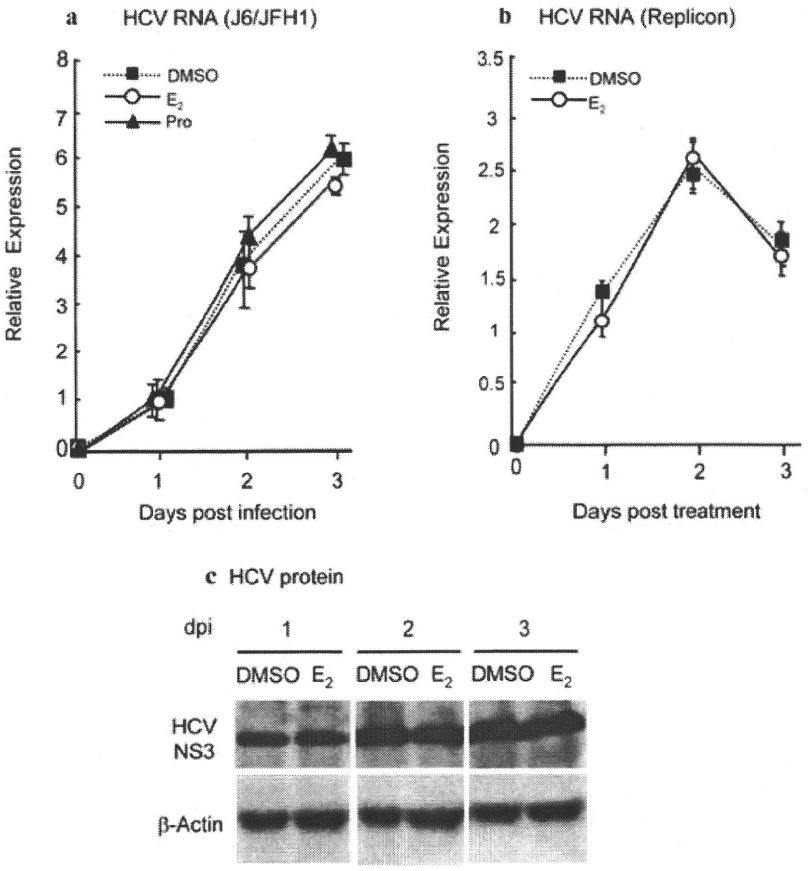

Fig. 2. Effects of E_2 and P_4 on HCV RNA replication and HCV protein synthesis. (a) HCV RNA replication. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM) or DMSO (control) from 2 hr to sampling time (days 1, 2 and 3). HCV RNA replication levels were determined by real-time quantitative RT-PCR and normalized with β-actin mRNA levels. Data are shown as mean ± SEM. (b) Huh-7.5 cells harboring a full-genomic HCV RNA replicon (23) were treated with E_2 (0.4 μM) or DMSO, and HCV RNA replication levels determined as in (a). (c) HCV protein synthesis. HCV-infected cells were treated with E2 or DMSO as in (a) and the amount of HCV protein synthesis determined by immunoblot analysis using anti-NS3 antibody. The degree of β-actin expression as determined by anti-β-actin antibody served as a control. dpi, days postinfection.

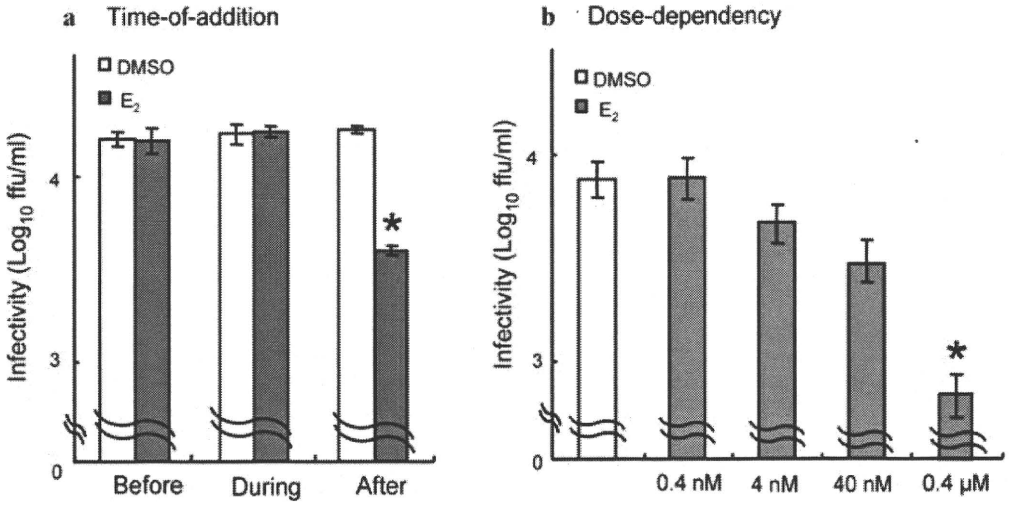

Fig. 3. Kinetic analysis of E_2–mediated inhibition of HCV virion production. (a) Time-of-addition experiment. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured up to 2 days after virus infection. Treatment of the cells with E_2 (0.4 μM) was performed before or during virus inoculation for 2 hr, or after virus inoculation until sampling time (day 2). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control. (b) Dose–dependency experiment. Huh-7.5 cells were inoculated with HCV as in (a). The HCV-infected cells were treated with various concentrations of E_2 (0.4 nM to 0.4 μM]) from 2 hr postinfection to sampling time (day 2). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

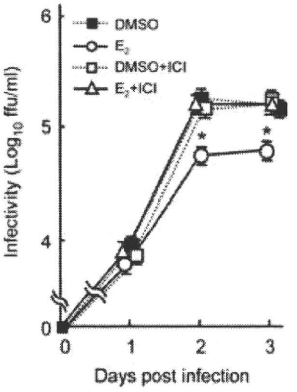

Fig. 4. Effects of ER antagonist, ICI182780, on HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with E_2 (0.4 μM) and/or ICI182780 (1 μM) or DMSO (control) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for virus infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

Estrogen receptor-α-selective agonist 4, 4′, 4″- (4-propyl-[1H]-pyrazole-1, 3, 5-triyl) trisphenol inhibits HCV virion production

To determine which estrogen receptor(s) is/are involved in the E_2–mediated down-regulation of HCV virion production, we used receptor-specific agonists, such as PPT (an ERα-selective agonist) (20), DPN (an ERβ-selective agonist) (21) and G-1 (a GPR30-selective agonist) (22). Treatment of cells with PPT (10 μM), but not with DPN (10 μM) or G-1 (1 μM), significantly inhibited HCV virion production (Fig. 5). PPT treatment at a concentration of 1 μM also brought about a weak, but significant, inhibition of HCV virion production at 2 days postinfection. On the other hand, PPT did not mediate significant cytotoxicity at the concentrations tested (data not shown).

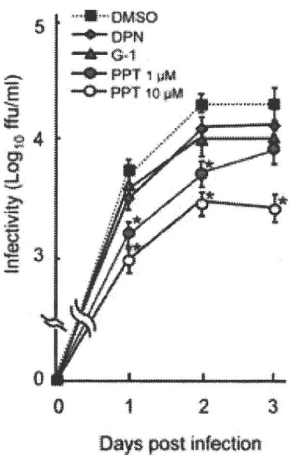

Fig. 5. Effects of ER-specific agonists on HCV virion production. Huh-7.5 cells were inoculated with HCV at a multiplicity of infection of 1.0, incubated for 2 hr, and cultured for 0, 1, 2 and 3 days after virus infection. The HCV-infected cells were treated with PPT (ERα-selective agonist; 1 and 10 μM), DPN (ERβ-selective agonist; 10 μM) or G-1 (GPR30-selective agonist; 1 μM) from 2 hr postinfection to sampling time (days 1, 2 and 3). The culture supernatants of HCV-infected cells were assayed for viral infectivity. Data are shown as mean ± SEM. *$P < 0.05$, compared with DMSO control.

DISCUSSION

We have demonstrated in the present study that treatment of Huh-7.5 cells with E_2 inhibits HCV virion production, but not HCV RNA replication or HCV protein synthesis (Figs 1 and 2). Treatment of the cells with E_2 either prior to, or during, virus inoculation did not significantly suppress HCV virion production (Fig. 3a). These results collectively suggest that E_2 inhibits HCV infection at the virion assembly/secretion level, but not at the level of virus attachment/entry, virus RNA replication or virus protein synthesis. E_2 has been reported to possess antioxidant and anti-apoptotic activities in fibrotic liver and cultured hepatocytes (24, 25). It should be noted, however, that E_2 did not exert anti-apoptotic or cytotoxic (pro-apoptotic) effect under our experimental conditions (Fig. 1b). In contrast to E_2, another female hormone, P_4, did not significantly affect HCV virion production (Fig. 1a).

E_2-mediated inhibition of HCV virion production was abolished by a nuclear ER (ERα and ERβ) antagonist, ICI182780 (Fig. 4), this result suggesting that suppression of HCV virion production may be induced by ER signal transduction. Three types of ER have been reported so far; ERα, ERβ and GPR30 (11–15). To determine which ER is involved in the suppression of HCV virion production, we used ER-specific agonists, PPT (for ERα) (20), DPN (for ERβ) (21) and G-1 (for GPR30) (22). We found that PPT, but not DPN or G-1, inhibits the production of HCV infectious particles (Fig. 5), suggesting that ERα plays an important role in the inhibition of HCV virion production. It has been reported that, in hepatocytes, ERα constitutes a minor proportion of the total ER, and that an estrogen-mediated anti-apoptotic effect is mediated principally through ERβ (26). However, the importance of ERα–mediated signal transduction should not be ignored. The rationale for this assertion is that ERα is known to be involved in lipid metabolism (27), that certain lipid metabolism disorder(s) possibly result(s) in abnormal accumulation of lipid droplets, and that such an accumulation is required for HCV virion maturation in virus-infected cells (27), that certain lipid metabolism disorder(s) possibly result(s) in abnormal accumulation of

lipid droplets, and that such an accumulation is required for HCV virion maturation in virus-infected cells (28). Also, we should not yet exclude the possible importance of ERβ and GPR30, because they may not be expressed at a sufficient level in the Huh7.5 cell line maintained in our laboratory.

Other relevant observations are that ERα interacts with HCV NS5B, the viral RNA polymerase, and promotes association of NS5B with the replication complex in human hepatoma-derived Huh-7 cells, and that tamoxifen, a competitive inhibitor of estrogens, suppresses the ERα–mediated association of NS5B with the replication complex, thereby inhibiting HCV RNA replication (29). Similarly, E_2 binding to ERα may abrogate its interaction with NS5B. However, in our experiments we did not observe E_2–mediated inhibition of HCV RNA replication (Fig. 2a,b). We therefore assume that E_2 inhibits HCV virion production through a mechanism other than E_2–ERα–NS5B interactions. Further study is needed to elucidate this issue.

In conclusion, the most potent physiological estrogen, E_2, inhibits production of HCV infectious particles in Huh-7.5 cell cultures in an ERα–dependent manner. This may explain, at least in part, why the incidence of HCV-associated liver disease is lower in premenopausal women than in postmenopausal women and men.

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The Hepatitis C Virus Core Protein Contains a BH3 Domain That Regulates Apoptosis through Specific Interaction with Human Mcl-1▽†

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The hepatitis C virus (HCV) core protein is known to modulate apoptosis and contribute to viral replication and pathogenesis. In this study, we have identified a Bcl-2 homology 3 (BH3) domain in the core protein that is essential for its proapoptotic property. Coimmunoprecipitation experiments showed that the core protein interacts specifically with the human myeloid cell factor 1 (Mcl-1), a prosurvival member of the Bcl-2 family, but not with other prosurvival members (Bcl-X_L and Bcl-w). Moreover, the overexpression of Mcl-1 protects against core-induced apoptosis. By using peptide mimetics, core was found to release cytochrome *c* from isolated mitochondria when complemented with Bad. Thus, core is a bona fide BH3-only protein having properties similar to those of Noxa, a BH3-only member of the Bcl-2 family that binds preferentially to Mcl-1. There are three critical hydrophobic residues in the BH3 domain of the core protein, and they are essential for the proapoptotic property of the core protein. Furthermore, the genotype 1b core protein is more effective than the genotype 2a core protein in inducing apoptosis due to a single-amino-acid difference at one of these hydrophobic residues (residue 119). Replacing this residue in the J6/JFH-1 infectious clone (genotype 2a) with the corresponding amino acid in the genotype 1b core protein produced a mutant virus, J6/JFH-1(V119L), which induced significantly higher levels of apoptosis in the infected cells than the parental J6/JFH-1 virus. Furthermore, the core protein of J6/JFH-1(V119L), but not that of J6/JFH-1, interacted with Mcl-1 in virus-infected cells. Taken together, the core protein is a novel BH3-only viral homologue that contributes to the induction of apoptosis during HCV infection.

Hepatitis C virus (HCV), a positive-stranded RNA virus of the family *Flaviviridae*, is the major cause of non-A, non-B hepatitis worldwide. The HCV genome encodes a precursor polyprotein of ~3,000 amino acids (aa) that is processed cotranslationally and posttranslationally to give rise to viral structural and nonstructural proteins (2). The core protein is encoded by the N-terminal portion of the HCV precursor polyprotein and cleaved from the polyprotein by cellular signal peptidase to give the immature form of the core protein (aa 1 to 191). This then is further cleaved by membrane-associated signal peptide peptidase to give the mature core protein, whose C terminus is not precisely known but lies between residues 170 and 179 (see reviews in references 33, 42, and 52). The mature core protein is thought to constitute the HCV capsid and is the predominant form detected in virus particles purified from the sera of patients with chronic HCV infection (42, 74). A recent paper also reported that the maturation of the core protein is required for the production of HCV using the JFH-1 infectious clone (65).

Besides its role in the encapsidation of viral RNA, the core protein has been found to interfere with many cellular pathways, including cell signaling, transcriptional activation, lipid metabolism, carcinogenesis, and apoptosis (see reviews in references 33, 42, and 52). As the regulation of apoptosis during viral infection is an important determinant in the struggle between virus and host for survival, many viruses encode viral proteins that can regulate apoptosis in the infected host cells and manipulate this process to their advantage. In the case of HCV, the mechanisms by which the virus maintains viral persistence and promotes hepatocellular carcinoma are not well understood, but several HCV proteins have the ability to modulate apoptosis (see recent reviews in references 20 and 28). In particular, the core protein has been shown to modulate apoptosis, and it seems that the core protein can inhibit as well as promote apoptosis, depending on the death stimuli and types of cells used (3, 9, 13, 25, 36, 40, 49, 53, 54, 57, 60, 76).

In this study, we characterized one of the mechanisms by which the mature form of the core protein from a genotype 1b strain induces apoptosis in Huh7 cells. Following the experimental designs used in previous studies (29, 38, 40, 55, 72), the mature form of the core protein is assumed to be constituted by residues 1 to 173 of the HCV precursor polyprotein, and

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