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Cellular vimentin content regulates the protein level of hepatitis C virus core protein and the hepatitis C virus production in cultured cells

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ABSTRACT

Hepatitis C virus (HCV) core protein is essential for virus particle formation. Using HCV core-expressing and non-expressing Huh7 cell lines, Uc39-6 and Uc321, respectively, we performed comparative proteomic studies of proteins in the 0.5% Triton X-100-insoluble fractions of cells, and found that core-expressing Uc39-6 cells had much lower vimentin content than Uc321 cells. In experiments using vimentin-overexpressing and vimentin-knocked-down cells, we demonstrated that core protein levels were affected by cellular vimentin content. When vimentin expression was knocked-down, there was no difference in mRNA level of core protein; but proteasome-dependent degradation of the core protein was strongly reduced. These findings suggest that the turnover rate of core protein is regulated by cellular vimentin content. HCV production was also affected by cellular vimentin content. Our findings together suggest that modulation of hepatic vimentin expression might enable the control of HCV production.

Introduction

Hepatitis C virus (HCV) is a major causative agent of chronic hepatitis (Choo et al., 1989; Kuo et al., 1989). Persistent HCV infection, which develops in at least 70% of infected patients, is strongly correlated with the development of severe liver diseases such as fibrosis, steatosis, cirrhosis, and hepatocellular carcinomas (HCC). Since more than 170 million people in the world are currently infected with HCV (Choo et al., 1989) and there is no treatment completely effective in curing HCV, HCV infection is one of the most important global public health issues. Understanding of the life cycle of HCV and the mechanism by which HCV induces serious liver diseases is crucial for the development of novel anti-HCV strategies.

HCV is an RNA virus of the *Flaviviridae* family and possesses a single-stranded, positive-sense RNA genome of ~9.6 kb (Bartenschlager and Lohmann, 2000). The HCV RNA genome encodes a polyprotein of ~3000 amino acids that is processed by host and viral proteases into 10 individual components including 4 structural and 6 nonstructural proteins (reviewed by Reed and Rice, 2000). HCV core protein is crucial for virus particle production as the structural component of the viral nucleocapsid and as a unit required for formation of the active HCV replication/assembly complex in host cells (Boulant et al., 2007; Miyanari et al., 2007). In addition, the core protein plays pivotal roles in the pathogenesis of HCV infection, as suggested by the finding that transgenic mice expressing core protein in the liver tend to develop liver steatosis with subsequent HCC (Moriya et al., 1998; Moriya et al., 1997). A large number of studies have revealed that a variety of host proteins interact with the core protein (Suzuki et al., 2007). Although these interactions can markedly affect various biological functions in host cells, it is not clearly known yet which interactions and molecules play roles in HCV production or its pathogenicity. Recent exhaustive gene-silencing analyses of host factors using RNAi demonstrated that RNA helicase DDX3, one of the proteins that interacts with the core, is required for HCV RNA replication as well as HCV production (Ariumi et al., 2007; Randall et al., 2007).

In host hepatic cells, HCV core protein is distributed preferentially in the detergent-resistant fractions (Matto et al., 2004), and HCV RNA replication also occurs on detergent-resistant membranes (Aizaki et al., 2004; Shi et al., 2003), suggesting that host factors in the detergent-resistant fractions play roles in core protein functions. In this study, we focused on HCV core protein and the detergent-insoluble proteins of host cells, and performed comparative targeted proteomic analysis of the detergent-insoluble proteins in HCV core-expressing and non-expressing hepatic cells. We identified vimentin as a protein the amount of which was reduced in core-expressing cell lines, and demonstrated that cellular vimentin content affects levels of HCV core protein through the proteasome-mediated protein

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degradation system. Since cellular vimentin levels ultimately affected HCV production, vimentin may be a novel target for strategies of anti-HCV treatment.

Results

Proteomic analysis of detergent-insoluble fractions (DISFs) by second-dimensional polyacrylamide gel electrophoresis (2D-PAGE)/MALDI-QIT-TOF MS

DISFs and detergent-soluble fractions (DSFs) were prepared from HCV core-expressing Uc39-6 and non-expressing Uc321 cells by a sucrose density gradient ultracentrifugation method as described in Materials and methods. Proteins in the DISFs and DSFs were analyzed by immunoblotting with antibodies to HCV core protein and various organelle markers (Fig. 1A). A significant amount of HCV core protein (~70%) was distributed in the DISF of Uc39-6 cells. Nuclear proteins such as laminA/C and laminB were concentrated only in the DISFs of both types of cells, whereas other organelle proteins such as annexin II (plasma membrane), fatty acid synthase (cytosol), prohibitin (mitochondria), and calnexin (endoplasmic reticulum) were detected in the DSFs but not DISFs (Fig. 1A), suggesting that the DISFs in both types of cells contain minor (~15%) discrete populations of cellular proteins. Next, we performed 2D-PAGE analysis of the DISFs in Uc321 and Uc39-6 cells. Proteins in the DISFs were separated by isoelectric focusing (IEF) (pH 4–7) and 12% sodium dodecyl sulfate (SDS)-PAGE, and visualized by SYPRO-Ruby staining (Fig. 1B). Intensity of each spot in 2-D images was compared between Uc321 and Uc39-6 cells. The most difference in DISF proteins between Uc321 and Uc39-6 cells (the Uc39-6/Uc321 ratios of intensity normalized with actin: 10.8–28.0) was detected in the spots numbered as 1 in Fig. 1B (MW ~ 57 kDa, *p*I ~ 4.7), in which vimentin alone was identified by mass spectrometric analysis. We therefore focused on the relationship between cellular vimentin and core protein in further investigations.

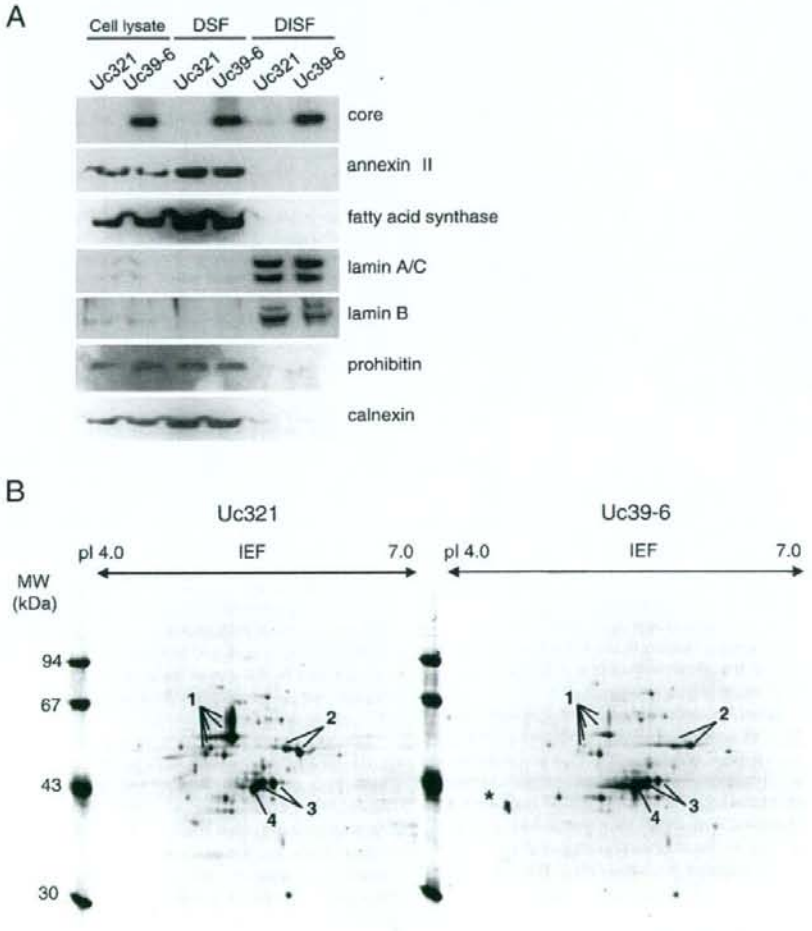


Fig. 1. Immunoblot and 2D-PAGE analysis of DISFs. (A) Total cell lysate fractions (5 μg of protein), DSFs (50 μg of protein), and DISFs (5 μg of protein) from core-expressing Uc39-6 and non-expressing Uc321 cells were analyzed by immunoblotting with antibodies to HCV core protein and various organelle markers as indicated. (B) 2D-PAGE analysis of proteins in DISFs of Uc321 and Uc39-6 cells. Proteins (150 μg) were separated by IEF (pH 4–7), followed by SDS-PAGE on a 12% gel. The gels were stained with SYPRO-Ruby. Major spots, identified as cytoskeletal proteins, are marked: 1, vimentin; 2, cytokeratin 8; 3, cytokeratin 18; 4, actin. *: a non-specific spot.

HCV core-expressing cell lines exhibited reduced vimentin content

To confirm the reduction of vimentin levels in DISFs of HCV core-expressing Uc39-6 cells, immunoblot analysis was performed using anti-vimentin antibody. Uc39-6 cells exhibited lower vimentin contents not only in DISF but also the total cell lysate fraction compared with control Uc321 cells (Fig. 2A). Similar results were obtained in the cell lysate fraction of another independent clone of an HCV core-expressing Huh7 cell line, Uc39-2 (Fig. 2B). Furthermore, a core-expressing hepatic HepG2 cell line, Hep39, also had lower vimentin content than a control cell line, Hepswx (Fig. 2C). These findings exclude the possibility that the reduction of vimentin levels in core-expressing cell lines is a clone- or cell-specific event. Consistent with these findings, levels of vimentin mRNA in Uc39-2 and Uc39-6 cells were also lower than that in Uc321 cells (data not shown). Taken together, these findings demonstrate marked reduction of vimentin expression in HCV core-expressing cell lines.

Cellular vimentin content affects the protein level of HCV core protein

To investigate the relationship between HCV core protein and vimentin, we examined the effect of cellular vimentin content on level of HCV core protein. When the expression of vimentin or control hypoxanthine guanine phosphoribosyltransferase 1 (HPRT) was knocked down in Uc39-6 cells by siRNA treatment, the protein level of HCV core protein in vimentin-knocked-down cells was significantly higher than those in siRNA-untreated and HPRT-knocked-down cells (Fig. 3A). On the other hand, cellular mRNA levels of HCV core protein, corrected for β-actin mRNA content, did not differ substantially among these types of cells (Table 1). These findings revealed that post-translational steps were involved in the increase of HCV core protein level in vimentin-knocked-down cells. Next, we established a vimentin-overexpressing Huh7 cell line, Huh7/vimentin, and compared the level of the core protein in Huh7/vimentin cells with that in control Huh7/hygro cells after transient expression of the core protein with pcEF39neo vector. After 9-day culture with G418 selection, the viabilities of the two types of cells were similar, though the level of expression of the core protein in Huh7/vimentin was significantly lower than that in Huh7/hygro cells (Fig. 3B). These findings demonstrated that level of HCV core protein was inversely correlated with cellular vimentin content, and thus strongly suggested that it was affected by cellular vimentin content.

We further attempted to verify these effects of vimentin using the vimentin-null cell line 1HF5 and the vimentin-expressing control cell line 2CB5, derived from human adrenal carcinoma SW13 cells (Sarria et al., 1990). When 1HF5 and 2CB5 cells were transfected with the core expression vector pcEF39neo and cultured with G418 selection, the viabilities of the two types of cells were similar, though the level of expression of the core protein in 1HF5 cells was much higher than that in 2CB5 cells (Fig. 3C), consistent with the results in Figs. 3A, B. An exogenously vimentin-expressing 1HF5 cell line carrying pcDNA3.1/Hygro/vimentin, 1HF5/vimentin, and a control vimentin-null cell line carrying pcDNA3.1/Hygro, 1HF5/hygro, were then established, and transfected with the green fluorescent protein (GFP)-expressing pcDNA3.1/EGFP vector, the core-coding pcEF39neo vector, or the control pcFE321swxneo vector. After selection under G418 for 9 days, the viabilities of these transfected cells were nearly the same. The levels of expression of GFP were similar in 1HF5/hygro and 1HF5/vimentin cells, while the core protein level in 1HF5/vimentin cells was significantly lower than that in 1HF5/hygro cells (Fig. 3D), consistent with the results in Fig. 3B.

These findings together indicate that cellular vimentin content regulates the level of HCV core protein in post-translational fashion.

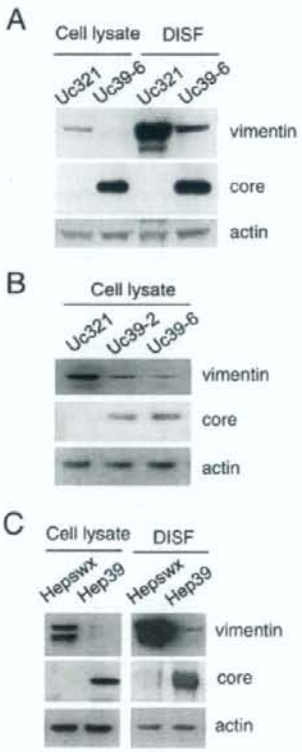


Fig. 2. Immunoblot analysis of vimentin in HCV core-expressing cell lines. Cell lysate fractions and DISFs from various cell lines were analyzed by immunoblotting with antibodies to vimentin, HCV core protein, and β-actin as indicated: cell lysate fractions and DISFs from Uc321 and Uc39-6 cells in (A), cell lysate fractions from Uc321, Uc39-2, and Uc39-6 cells in (B), and cell lysate fractions and DISFs from Hepswx and Hep39 cells in (C). Amounts of protein loaded were 18 μg in (A) and (B), and 5 μg in (C).

Vimentin is involved in proteasomal degradation of core proteins in cells

HCV core proteins are known to be preferentially degraded by the proteasome-dependent pathway (Suzuki et al., 2001). To determine whether cellular vimentin content affects proteasome-dependent degradation of the core protein, we examined the effects of the proteasome inhibitor MG132 on core protein levels in vimentin-knocked-down cells. After Huh7 cells transfected with pCAG/Flag-core (Huh7/Flag-core cells) had been treated with MG132 for 16 h, cellular accumulation of core protein was analyzed by immunoblot (Fig. 4; lanes 3 vs 4), which indicated substantial proteasomal degradation of the core proteins in cultured cells, as described previously (Hope and McLauchlan, 2000; McLauchlan et al., 2002; Suzuki et al., 2001). Huh7/Flag-core cells transfected with control and HPRT siRNA duplexes exhibited similar increases in core protein levels by treatment with MG132 (Fig. 4; lanes 5 vs 6, and 9 vs 10). On the other hand, vimentin-knocked-down Huh7/Flag-core cells that were transfected with vimentin siRNA duplexes exhibited higher content of core protein (lane 7) than the other siRNA-treated cells (lanes 5 and 9), and MG132 treatment resulted in no significant difference in core protein levels in vimentin-knocked-down cells (lanes 7 and 8), indicating that proteasomal degradation of core proteins was markedly inhibited in the vimentin-knocked-down cells. These observations strongly suggested that vimentin plays an important role in the proteasome-mediated proteolytic pathway of the HCV core protein.

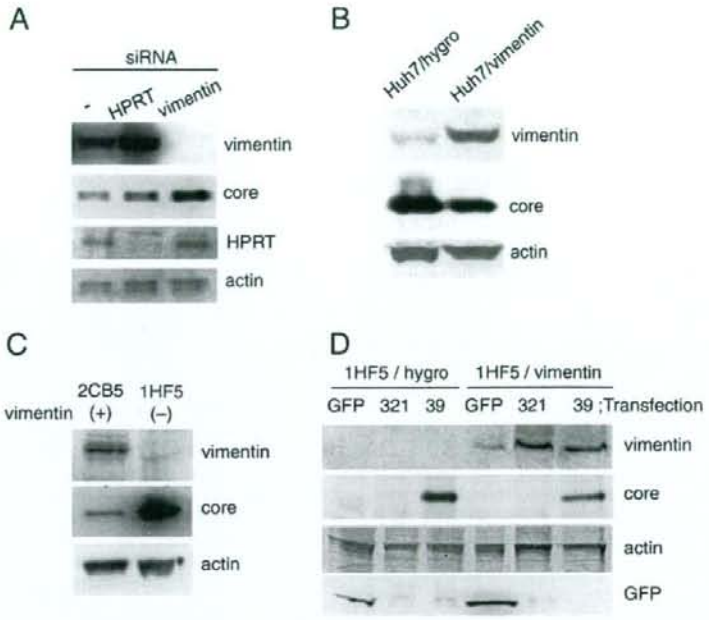


Fig. 3. Effects of cellular vimentin content on the level of expression of HCV core protein. Cellular level of expression of vimentin, HCV core protein, HPRT, β-actin, and GFP were analyzed by immunoblotting of total cell lysates (40 μg of protein) from various cells using specific antibodies. (A) Uc39-6 cells were transfected twice with a 2-day interval without (−) or with vimentin or control HPRT siRNA duplexes. Four days after the first transfection, cell lysates were collected and analyzed. (B) Huh7/hygro and Huh7/vimentin cells were transfected with the core protein-expression vector pcEF39neo, and selected under 1 mg/ml of G418 for 9 days. (C) The 2CB5 (vimentin+) and 1HF5 (vimentin−) lines of SW13 cells were transfected with pcEF39neo, and selected under 1 mg/ml of G418 for a week, followed by additional 2-week culture in normal culture medium. (D) 1HF5/hygro and 1HF5/vimentin cells were transfected with pcDNA3.1/EGFP (GFP), pcFE321swxneo (321), or pcEF39neo (39), and selected under 1 mg/ml of G418 for 9 days.

Under the various siRNA-treated conditions in Fig. 4, we also examined the protein levels of p53, one of the endogeneous host proteins the degradation of which is mainly regulated by proteasomal system (Morimoto et al., 2008). The pattern of p53 protein levels was very similar to that of core protein levels (Fig. 4), suggesting that the vimentin-dependent proteasomal degradation is not specific for the viral core protein. Vimentin-dependent proteasomal degradation system might be generally important for the turnover of endogeneous cellular proteins as well as the viral core protein.

Cellular vimentin contents affect HCV production

Since the level of expression of HCV core protein was affected by cellular vimentin content, we examined whether HCV production was also affected by cellular vimentin content. Infectious HCV (JFH1 strain) particles were used for the following infection assays. HCV production activity was determined by quantification of HCV core protein levels in the infected cells and culture supernatants. We first tested the effect of vimentin knockdown on HCV production. Examination of HCV-infected Huh7 cells treated with vimentin siRNA revealed higher amounts of HCV core protein in both cells and culture medium than examination of non-treated and control HPRT siRNA-treated cells (Fig. 5A). To examine whether the core protein levels in the cell-cultured media reflect the content of infectious HCV particles in them, Huh7 cells were treated with cell-cultured medium containing equal amounts (1.4 fmol) of the core protein collected from each type of cell described in Fig. 5A, and cellular levels of production of the core protein were determined by immunoblot analysis. They were nearly the same among the cells treated with each culture medium (Fig. 5B). These findings indicated that reduction of vimentin expression in Huh7 cells leads to more active HCV production and enhanced release to the supernatant.

We next examined the effects of vimentin overexpression on HCV production. When vimentin-overexpressing Huh7/vimentin and control Huh7/hygro cells were infected with HCV particles, Huh7/vimentin cells exhibited lower amounts of intracellular and extracellular HCV core protein than Huh7/hygro cells (Fig. 5C). Consistent with the results in Fig. 5A, these findings suggested that higher expression of vimentin in host cells resulted in lower HCV production.

We also examined the effect of vimentin knockdown on HCV RNA replication using a JFH1-subgenomic replicon (Fig. 5D). There were no significant differences in replication activities between vimentin-knocked-down cells and the other control cells. These findings indicated that cellular level of vimentin has no effects on HCV non-structural proteins which serve as a unit of RNA replication machinery of HCV. Collectively, these results demonstrated that HCV production activity but not HCV-RNA replication was inversely correlated with cellular vimentin content.

Table 1
mRNA levels of HCV core protein and β-actin in vimentin-knocked-down Uc39-6 cells

	siRNA		
	–	HPRT	vimentin
HCV core ($\times 10^4$ copies/μg total RNA)	2.9±0.3	1.7±0.1	3.0±0.2
β-actin ($\times 10^7$ copies/μg total RNA)	3.0±0.1	1.4±0.1	2.3±0.0
HCV core/β-actin[a]	1	1.3	1.3

Total RNA was isolated from Uc39-6 cells that had been treated twice with a 2-day interval with HPRT siRNA duplexes, with vimentin siRNA duplexes or without (−) either of them, and cultured for 4 days. mRNA levels of HCV core protein and β-actin (a control housekeeping gene) were determined by quantitative real-time PCR. Values are the mean±SD for three determinations.

[a] Numbers represent the relative amounts of HCV core protein mRNA normalized to β-actin level.

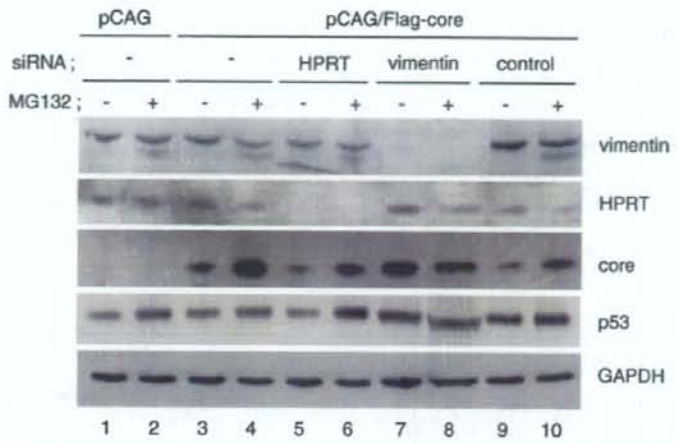


Fig. 4. Effects of the proteasome inhibitor MG132 on level of expression of HCV core protein in Huh7 cells. siRNA duplexes of control, HPRT, or vimentin, together with the core protein-expression vector pCAG/Flag-core, were transfected into Huh7 cells. After 2 days, transfection of these siRNAs was repeated. Cells were further cultured for 2 days and treated with (+) or without (−) MG132 (50 μM) for 16 h. Equivalent amounts of cell lysates were analyzed by immunoblotting with antibodies to vimentin, HPRT, core protein, p53, and GAPDH.

Furthermore, expression of vimentin and HCV core protein in Huh7 cells after HCV infection was observed by immunofluorescence microscopy (Fig. 5E), and the fluorescent intensity of vimentin in core-positive and core-negative Huh7 cells under HCV-infected conditions was determined (Fig. 5F). HCV-infected cells stained with the core-specific antibody always had lower vimentin content (Figs. 5E, F). Moreover, as shown in Fig. 5F, HCV core-negative cells exhibited more variable vimentin levels, whereas the core-positive cells had vimentin levels within a narrow range. These observations, which showed that a Huh7 cell population with lower vimentin content can preferentially produce HCV, were consistent with the results shown in Figs. 5A, C.

Finally, we examined the effects of MG132 on HCV core protein levels in HCV-infected cells in which vimentin was knocked-down or overexpressed. In the presence of MG132, non-treated and control HPRT siRNA-treated cells showed the significant increase of cellular HCV core protein levels, whereas vimentin-knocked-down cells did not (Fig. 5G). These results were consistent with those using HCV core-expressing cells (Fig. 4). HCV core content in vimentin-overexpressing Huh7/vimentin cells was lower than that in control Huh7/hygro cells, but after MG132 treatment Huh7/vimentin and Huh7/hygro cells showed the similar HCV core protein levels (Fig. 5H). Taken together, these results demonstrated the significant involvement of vimentin in proteasome-dependent degradation of HCV core protein in HCV-infected cells (Figs. 5G, H), as well as in HCV core-expressing cells (Fig. 4).

Discussion

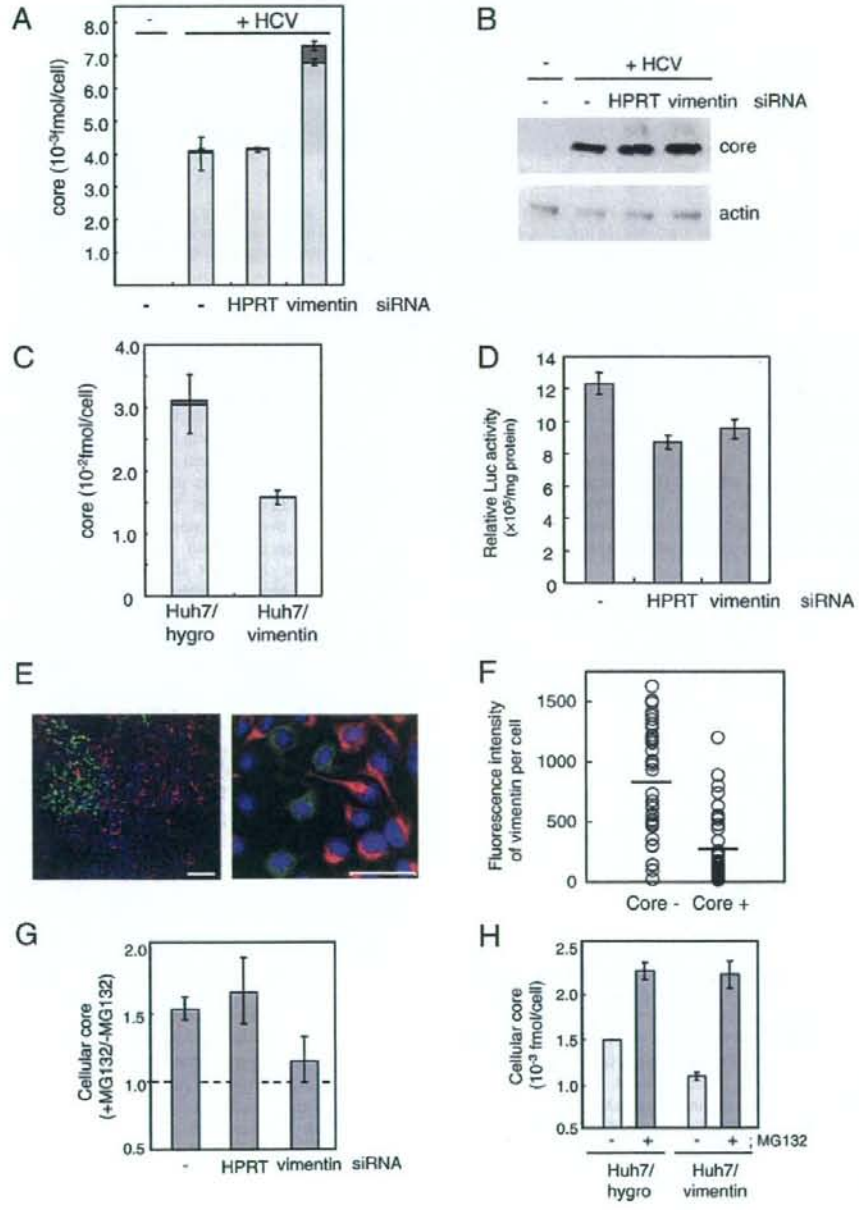
By comparative proteomic analysis of the detergent-insoluble proteins in HCV core-expressing and non-expressing Huh7 cell lines, vimentin, an intermediate filament protein, was identified as the protein with the most dramatic reduction in level in the detergent-insoluble fraction of HCV core-expressing Uc39-6 cells (Figs. 1B and 2). On the other hand, there were no significant differences in the amounts of other major proteins including cytoskeletal components such as actin and cytokeratin 8/18 in the detergent-insoluble fractions between the core-expressing and non-expressing cells (Fig. 1B). These findings, together with similar results for other core-expressing cells (Fig. 2), suggested the existence of a specific relationship between the core protein and cellular vimentin. Consistent with these findings, immunofluorescence microscopic analysis of core-expressing cells (data not shown) and HCV-infected cells (Figs. 5E, F) showed that cells with intrinsic lower amount of vimentin are more permissive for higher HCV core protein content.

Knockdown of vimentin expression by siRNA treatment resulted in an increase in HCV core protein levels (Fig. 3A), while overexpression of vimentin reduced core protein contents (Fig. 3B). Similar results were obtained in the experiments using the vimentin-null cell line 1HF5 derived from SW13 cells (Figs. 3C and D). On the other hand, transient knockdown and overexpression of the core proteins in Uc39-6 and Huh7 cells, respectively, did not result in differences in cellular vimentin content (data not shown). These findings indicated that cellular vimentin level affects the level of expression of the core protein but not vice versa. Although transient expression of the core protein did not affect cellular vimentin content, why do various stable cell lines expressing the core protein have lower vimentin level? Since it was very hard to establish the cells stably expressing the core protein, we speculate that only the minor cell population innately having lower vimentin content was able to maintain the substantial core expression levels and was therefore selected.

We next demonstrated that vimentin affects core protein levels in post-translational fashion (Table 1) and is required for the proteasomal degradation of core protein in core-expressing cells (Fig. 4) and also in HCV-infected cells (Figs. 5G, H). Many studies with proteasome inhibitors have shown that a major pathway of degradation of core protein is mediated by the proteasomal system (Hope and McLauchlan, 2000; McLauchlan et al., 2002; Moriishi et al., 2003; Suzuki et al., 2001). PA28γ, a REG family proteasome activator also known as REGγ and Ki antigen, which is located in the nucleus, was shown to play an important role in the proteasomal degradation of the core protein (Moriishi et al., 2003). It was recently reported that the ubiquitin ligase E6AP, which is distributed in the perinuclear cytoplasm and colocalized with the core protein, is also involved in ubiquitylation and degradation of core protein (Shirakura et al., 2007). Vimentin filaments extend from the nuclear membrane toward the cell periphery. In addition, vimentin is known to colocalize with ubiquitinated protein aggregates and form aggresomes when the capacity of the proteasome is exceeded (Johnston et al., 1998). Pull-down assays against the core protein in core-expressing Huh7 cells indicated that a minor portion of cellular vimentin can interact with HCV core protein (data not shown), as

Kang et al. had reported previously (Kang et al., 2005). Co-staining of cellular vimentin and the core protein on immunofluorescence microscopy also supported the existence of a minor but definite association between them (data not shown). Based on these findings, we speculate that vimentin plays a role in the transport of the core protein to the nucleus, where it is then degraded, although further biochemical studies will be needed to demonstrate this.

HCV core protein is distributed mainly in the ER and lipid droplets in host cells (Barba et al., 1997), and the ER membrane associating the lipid droplets with core protein has been recognized as a site important for HCV production, particularly HCV RNA replication and virus particle assembly (Boulant et al., 2007; Miyanari et al., 2007). Vimentin is also closely associated with lipid droplets (Brasaemle et al., 2004; Lieber and Evans, 1996; Schweitzer and Evans, 1998). Thus, in addition to its degradative modulation of core protein, vimentin might also affect the function of lipid droplets and consequently inhibit HCV production. The effects of vimentin knock-down and overexpression on HCV production were actually stronger at the extracellular core protein level (secretion



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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Characterization of infectious hepatitis C virus from liver-derived cell lines

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ABSTRACT

The efficient production of infectious HCV from the JFH-1 strain is restricted to the Huh7 cell line and its derivatives. However, the factors involved in this restriction are unknown. In this study, we examined the production of infectious HCV from other liver-derived cell lines, and characterized the produced viruses. Clones of the Huh7, HepG2, and IMY-N9, harboring the JFH-1 full-genomic replicon, were obtained. The supernatant of each cell clone exhibited infectivity for naïve Huh7. Each infectious supernatant was then characterized by sucrose density gradient. For all of the cell lines, the main peak of the HCV-core protein and RNA exhibited at approximately 1.15 g/mL of buoyant density. However, the supernatant from the IMY-N9 differed from that of Huh7 in the ratio of core:RNA at 1.15 g/mL and significant peaks were also observed at lower density. The virus particles produced from the different cell lines may have different characteristics.

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. HCV is a human pathogen and HCV infection is a major cause of chronic hepatitis, liver cirrhosis and hepatic carcinoma. The main therapy for HCV is treatment with pegylated-interferon and rivabirin. However, these agents show little effect for patients that have a high titer of HCV-RNA, genotype 1. Thus, it is necessary to develop new, more effective therapies and preventive treatments to counteract HCV infection. It was discovered that a genotype 2a strain of HCV, JFH-1, can efficiently replicate in the Huh7 cell line [1], and an *in vitro* culture model of infectious HCV has also been successfully developed using the JFH-1 genome [2–4]. Recently, it has become possible to produce various chimeric HCV by replacement of the JFH-1 structural protein region with that of other strains. The HCV particles produced from such chimera are expected to lead to the development of a HCV vaccine, and new anti-HCV pharmaceuticals.

The infectious HCV-derived JFH-1 genome was developed using the human hepatoma Huh7 cell line [5]. Although the sub-genomic replicon RNA of JFH-1 can autonomously replicate, not only in Huh7 cells, but in other human liver [6], non-hepatic [7], and mouse [8] cells, infectious HCV production has been restricted to Huh7-derived cells. In this study, we undertook a comparative study of infectious HCV particles produced from different cell lines including Huh7. Infectious HCV particles were successfully produced into the culture media and characterized.

Materials and methods

Cell culture. Huh7, Huh7.5.1 ([3], a generous gift from Dr. Francis V. Chisari), HepG2, and IMY-N9 cells were cultured at 37 °C in 5% CO_2. The HepG2 cells were cultured in modified Eagle's medium containing 10% fetal bovine serum. All of the other cells were cultured in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum, as described previously [6].

Plasmids. The pFGR-JFH1 and pFGR-JFH1/deltaE12 plasmids, encoding the full-genomic replicon, and envelope-deleted replicons, respectively, were generated as previously described [9].

RNA synthesis. RNA synthesis was performed as described previously [2]. Briefly, the pFGR-JFH1 plasmid was digested with XbaI and then treated with Mung Bean nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment was then purified and used as a template for RNA synthesis. HCV-RNA was synthesized *in vitro* using a MEGAscript™ T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

Establishment of replicon cells. Cell lines harboring FGR-JFH1 replicons were produced as described previously [9]. Briefly, trypsinized cells were washed with Opti-MEM I™ reduced-serum medium (Invitrogen, Carlsbad, CA) and resuspended at 7.5×10^6 cells/mL with Cytomix buffer [1]. RNA (10 μg), synthesized from pFGR-JFH1, was mixed with 400 μL of cell suspension and transferred to an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950 μF with the Gene Pulser II™ apparatus (Bio-Rad,

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Hercules, CA). Transfected cells were immediately transferred to 10-cm culture dishes, each containing 8 mL of culture medium. G418 (0.8–1.0 mg/mL) (Nacalai Tesque, Kyoto, Japan) was added to the culture medium at 16–24 h after transfection. Culture medium, supplemented with G418, was replaced twice per week. Three weeks after transfection, sparsely grown G418-resistant colonies were independently isolated using a cloning cylinder (Asahi Techno Glass Co., Tokyo, Japan), and were expanded.

Preparation of supernatants from FGR-JFH1 replicon cells. Culture media was collected from Huh7, IMY-N9, and HepG2 cell lines harboring the FGR-JFH1 replicon and was passed through a 0.45-μm filter. Filtrated culture media was then pooled and concentrated 50-fold using Amicon Ultra-15 (100,000 Molecular weight cut off; Millipore, Bedford, MA), and stored at −80 °C until use.

Assay of infection of naïve Huh7 cells. Infection of naïve Huh7 cells were assayed by immunofluorescence and colony formation assays. For the immunofluorescence assay naïve Huh7.5.1 cells were seeded at 1×10^4 cells/well in an 8-well chamber slide (Becton Dickinson, Franklin Lakes, NJ), cultured overnight and then inoculated with diluted culture media containing infectious HCV particles (1×10^6 HCV-RNA copies). At 72 h after inoculation, the cells were fixed in acetone/methanol (1:1) for 10 min at −20 °C, and the infected foci were visualized by immunofluorescence as follows.

An anti-core HCV protein monoclonal antibody 2H9 [2] was added to the cells at 50 μg/mL in BlockAce (Dainippon Sumitomo Pharma, Osaka, Japan). After incubation for 1 h at room temperature, the cells were washed and incubated with a 1:400 dilution of AlexaFluor 488-conjugated anti-mouse IgG (Molecular Probes, Eugene, OR) diluted in BlockAce. The cells were then washed, treated with DAPI solution (Sigma, Saint Louis, MO) at 0.1 μg/mL and examined by Biozero fluorescence microscopy (Keyence, Osaka, Japan).

Colony formation assays were performed as described previously [9]. Briefly, naïve Huh7 cells were inoculated with culture supernatants from replicon-expressing cell lines for 2 h, and then cultured with complete medium. Inoculated cells were cultured for 3 weeks in medium supplemented with G418 (0.3 mg/mL). Cell survival was assessed by staining with crystal violet.

Titration of infectivity. The infectivity titer of the culture supernatants was determined on Huh7.5.1 cells by end point dilution and immunofluorescence as described above. Briefly, each sample was serially diluted 10-fold in DMEM-10% FBS and 100 μL was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-core antibody and secondary anti-mouse IgG-Alexa 488 conjugated antibodies. Infectious foci were counted and the titer was calculated and expressed as focus forming units per mL (FFU/mL).

Sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer containing 10 mM Tris, pH7.5, 150 mM NaCl, and 0.1 mM EDTA. Gradients were centrifuged in an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100 μL drop from each fraction following a gradient run.

Quantification of HCV-core protein and RNA. The level of the HCV-core protein in culture supernatants or sucrose density gradient fractions, was assayed using an immunoassay as described elsewhere [10]. Viral RNA was isolated from harvested culture media, or sucrose density gradient fractions, using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). The copy number of HCV RNA was determined by real-time detection reverse transcription-polymerase chain reaction (RTD-PCR), using an ABI Prism 7500fast sequence detector system (Applied Biosystems, Tokyo, Japan) [11].

Results

Production of infectious HCV from human liver-derived cell lines

We first determined if it was possible to produce infectious HCV from cell lines other than Huh7. We selected the HepG2 and IMY-N9 cell lines to establish human liver-derived cell lines that enable replication of the JFH-1 genome [6]. Since full-genomic JFH-1 did not transiently replicate in these cells (data not shown), we established FGR-JFH1 replicon cells that stably replicate the JFH-1 genome. In the culture media obtained from these full-genomic replicon cells, HCV-RNA titers were detected by RTD-PCR. The titer of HCV-RNA was highest in the supernatant from an IMY-N9 cell clone and lowest from a HepG2 cell clone (Table 1). When naïve Huh7.5.1 cells were inoculated with culture supernatants from the replicon cells, infected cells could be detected by immunofluorescence using an anti-HCV-core protein antibody (Fig. 1A). These data suggested that HepG2 and IMY-N9 cells are able to produce infectious HCV.

We then compared the specific infectivity of the replicon containing culture supernatants from the different cells. Specific infectivity was calculated by dividing the infectious titer, calculated by immunofluorescence of infectious foci, of the culture media by the titer obtained for HCV-RNA. Using these calculations the culture media from Huh7 and HepG2 cells showed almost the same specific infectivity whereas that from IMY-N9 cell was relatively higher (Table 1). Thus the infectious HCV in the culture media might differ according to the cell line from which it was obtained.

To clarify the differences observed in specific infectivity, we next examined the ability of the various cellular supernatants to induce colony formation. For this assay naïve Huh7 cells were inoculated with culture media of the same HCV-RNA titer as that of the FGR-JFH1 virus and were cultured in G418-containing medium. Cell survival was assayed by staining with crystal violet, and the number of colonies formed was counted. Consistent with the specific infectivity results, the supernatant of the IMY-N9 replicon cell showed higher colony formation compared with that of Huh7 and HepG2 replicon cells (Fig. 1B and C). Thus IMY-N9 cells produce infectious HCV with a relatively higher infectivity than the other cell lines suggesting that the supernatant derived from the different replicon producing cells may differ.

Characterization of the FGR-JFH1 virus from different liver-derived cells

To further characterize potential differences between the viruses produced by the different cell lines we next characterized the FGR-JFH1 virus in the media of the different cell lines by sucrose density gradient analysis. Concentrated cell supernatants were layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Twenty-four fractions were collected and the HCV-core protein and RNA was assayed in each fraction. The peak fraction of the HCV-core protein and that of the RNA coincided at a density of 1.15 g/mL in all supernatants. However, the supernatant of the IMY-N9 cells showed different profiles for both the HCV-core protein and RNA compared to those of Huh7. Thus the IMY-N9 cells had a different ratio of

Table 1
Infectivity of the supernatant of replicon cell lines.

Producing cell	HCV-RNA (copies/mL)	Infectious titer (FFU/mL)	Specific infectivity (FFU/RNA copy)
Huh7	$1.36 \pm 0.02 \times 10^8$	$1.30 \pm 0.32 \times 10^4$	9.56×10^{-5}
IMY-N9	$2.80 \pm 0.04 \times 10^8$	$3.75 \pm 0.38 \times 10^4$	1.34×10^{-4}
HepG2	$8.80 \pm 0.75 \times 10^7$	$7.70 \pm 1.41 \times 10^3$	7.96×10^{-5}

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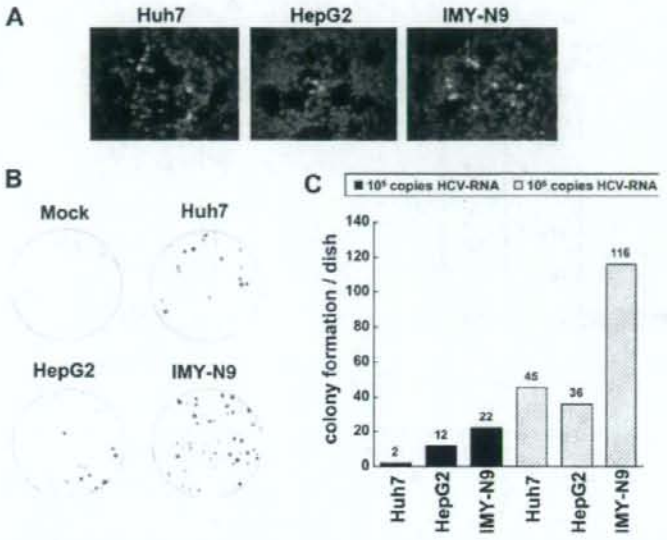

Fig. 1. Naïve Huh7 cell infection assay of JFH-1 full-genomic replicon cell culture supernatants. (A) JFH-1 full-genomic replicon (FGR-JFH1) cells were established in Huh7, HepG2, and IMY-N9 cell lines. Supernatants derived from Huh7 (*left*), HepG2 (*middle*), and IMY-N9 (*right*) cells (1×10^6 HCV-RNA copies) were inoculated into naïve Huh7.5.1 cells (1×10^4) for 48 h, and infected cells were then detected by immunofluorescence using an anti-core antibody (clone 2H9) (green). (B) Naïve Huh7 cells (5×10^5) were inoculated with mock, Huh7, HepG2, and IMY-N9-derived supernatants (10^6 HCV-RNA copies per 10-cm dish) of FGR-JFH1 cells for 2 h. Inoculated cells were cultured for 3 weeks in complete medium supplemented with G418 (0.3 mg/mL), and G418-resistant cells were stained using crystal violet. (C) The number of G418-resistant colonies obtained in (B) was calculated when 10^5 or 10^6 copies of HCV-RNA were tested. Mean values of colony number were indicated in duplicate experiment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

HCV-core protein and RNA at a density of 1.15 g/mL (RNA/Core ratio; Huh7: 511, IMY-N9: 133 copies/fmol) and also showed a secondary peak at lower density (approximately 1.05 g/mL). For all supernatants the peak of infectivity exhibited at a density of 1.10 g/mL that was slightly lower than that of the HCV-core protein and RNA peaks. Furthermore infectivity was barely detectable in the lower density fractions (Fig. 2) suggesting that the HCV-core protein and RNA that was detected at lower density was irrelevant for infectivity of the different supernatants.

We considered the possibility that the core protein and RNA in the lighter fractions may be due to cellular debris containing a replication complex. To determine if this might be the case we therefore analyzed the supernatants from Huh7 and IMY-N9 envelope-deleted replicon cells (FGR-JFH1/deltaE12). The HCV-core protein and RNA were detected in the supernatants of these cells although the titers were very low. These supernatants were not infective for naïve Huh7 cells (data not shown). Furthermore, analysis of the concentrated supernatants of these cell lines by sucrose density gradient analysis detected both the HCV-core protein and RNA, and the major peaks of HCV-RNA were detected in the lower density (approximately 1.10 g/mL) fractions (Fig. 3). However, the profiles of HCV-core protein and RNA did not coincide for either cell line.

Discussion

Infectious HCV can be produced in cell culture by using the JFH-1 genome. This system permits investigation of various aspects of the HCV life cycle such as the steps of entry into cells, replication, and secretion. Infectious HCV derived from JFH-1 is robustly produced in Huh7 cell lines [2,3], and the infectious particles have been characterized. However the difficulty in robustly producing infectious HCV from other cell lines prevents a comparative study of HCV production among different cell lines. In this study, we compared infectious HCV production in Huh7 with that of other cell lines, and characterized the viruses produced.

First, we established Huh7, IMY-N9, and HepG2 FGR-JFH1 replicon cells. These cell lines were able to replicate the JFH-1 subgenomic replicon [6]. The HCV-core protein and RNA were detected in all of the supernatants and all of these supernatants showed infectivity for naïve Huh7. Infectivity was evaluated by transient infection and colony formation assays. These assays indicated that the infectious supernatant from IMY-N9 cell had higher infectivity than the other cell lines for naïve Huh7 cells.

Next, we characterized each supernatant by sucrose density gradient analysis, which revealed both similarities and differences among the infectious supernatants. All samples showed typical peaks at 1.15 g/mL buoyant density for HCV-core protein and RNA, and infectious fractions showed an almost identical buoyant density of 1.10 g/mL. However, the supernatant from the IMY-N9 cells showed a difference in the core/RNA ratio at a density of 1.15 g/mL and higher secondary peak of HCV-core protein and RNA at a lower density (approximately 1.05 g/mL). Since the fractions at lower density did not correlate with infectivity, it is believed that the component at lower density does not contain infectious HCV particles but rather cellular debris that contains HCV proteins, RNA, and lipids [12]. HCV can associate with lipoprotein [13,14], and is secreted with VLDL [15]. Thus, the observed differences in the HCV-producing cells may derive from differences in lipoprotein synthesis. However, it is also possible that the components migrating at lower density contain virus particles. The deletion mutant of FGR-JFH1 (FGR-JFH1/deltaE12) did replicate in Huh7 and IMY-N9 cells, and these replicon cells secreted the HCV-core protein into the culture media, although at low levels. HCV-RNA was also detected in the same culture

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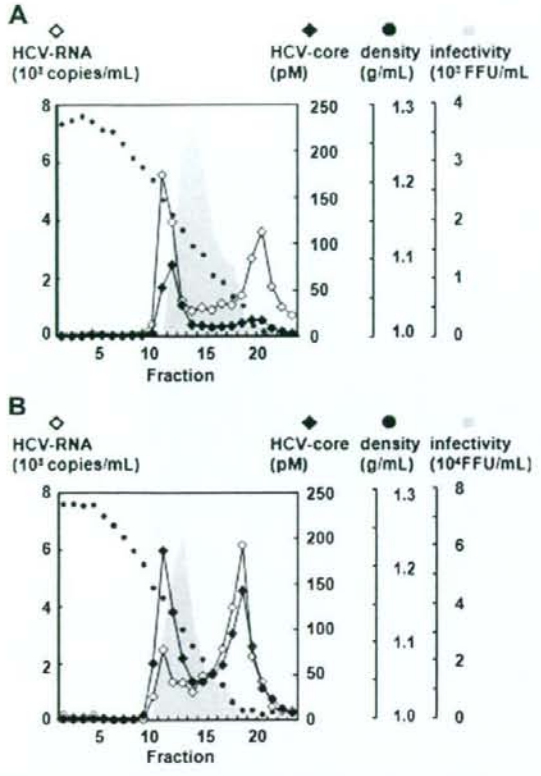

Fig. 2. Density gradient analysis of infectious HCV derived from Huh7 and IMY-N9 cells. Concentrated supernatants of Huh7 cells (A) and IMY-N9 cells (B) were layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged in a SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (400 μL each) were collected from the bottom of the tube. The buoyant density (closed circles), HCV-core protein (closed diamonds), HCV-RNA (open diamonds) and infectivity for naïve Huh7.5.1 cells (shown in gray) was detected in each fraction as described in Materials and methods.

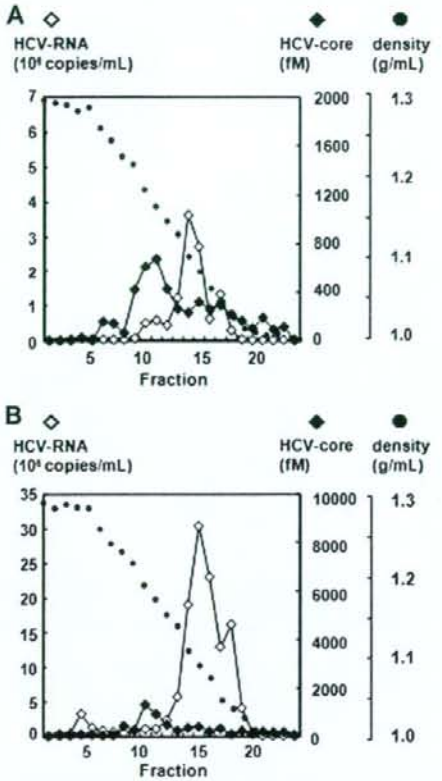

Fig. 3. Density gradient analysis of supernatants derived from Huh7 and IMY-N9 cells transfected with FGR-JFH1/deltaE12 RNA. Concentrated supernatants from Huh7 (A) and IMY-N9 (B) cells were analyzed by sucrose density gradient as described in the legend to Fig. 2. The buoyant density (closed circles), HCV-core protein (closed diamonds) and HCV-RNA (open diamonds) was analyzed in each fraction.

medium, and the profile of this HCV-RNA differed from that of the HCV-core protein in sucrose density gradient analysis. Thus, the peak fractions containing the HCV-core protein and RNA from the supernatant of FGR-JFH1/deltaE12 cells were different from the peak fractions from that of FGR-JFH1 cells. Therefore it is possible that all of the peaks of HCV-core protein and RNA observed in the supernatant of FGR-JFH1 replicon cells may correlate to virus particles with different densities. However, the reason why they centrifuge at different densities is unclear. Interestingly, the supernatants from cells transfected with envelope-deleted replicon RNA exhibit non-identical HCV-core protein and RNA profiles on a sucrose density gradient. Envelope-deleted replicon RNA may have a decreased ability to form nucleocapsids although a detailed examination is necessary to establish this point.

We previously developed a method for infectious HCV production using the FGR-JFH1 [9], and have now succeeded in producing infectious HCV in the supernatant of cultured liver-derived cell lines harboring FGR-JFH1 RNA. Infectious HCV particles are useful for vaccine production and are considered good antigens for the generation of useful antibodies. Selection of an appropriate cell line is important for the production of HCV particles for vaccine development. The technique used in this study seemed to be appropriate for producing infectious HCV in various cell lines [8].

A second advantage of using HepG2 and IMY-N9 cells for the production of virus particles is that these parental cell lines, unlike the Huh7 cell line, do not express the CD81 molecule on the cell surface, however, the expression on cell clones used in this study was not confirmed. This means that the FGR-JFH1 replicon of these cell lines may have a single cycle of HCV production, encompassing replication, assembly, budding and secretion, and do not show HCV permissiveness. These cells should therefore be useful for the discovery of drugs targeted against HCV assembly and secretion.

Acknowledgments

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Short Communication

Virological characterization of the hepatitis C virus JFH-1 strain in lymphocytic cell lines

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While hepatocytes are the major site of hepatitis C virus (HCV) infection, a number of studies have suggested that HCV can replicate in lymphocytes. However, *in vitro* culture systems to investigate replication of HCV in lymphocytic cells are severely limited. Robust HCV culture systems have been established using the HCV JFH-1 strain and Huh-7 cells. To gain more insights into the tissue tropism of HCV, we investigated the infection, replication, internal ribosome entry site (IRES)-dependent translation and polyprotein processing of the HCV JFH-1 strain in nine lymphocytic cell lines. HCV JFH-1 failed to infect lymphocytes and replicate, but exhibited efficient polyprotein processing and IRES-dependent translation in lymphocytes as well as in Huh-7 cells. Our results suggest that lymphocytic cells can support HCV JFH-1 translation and polyprotein processing, but may lack some host factors essential for HCV JFH-1 infection and replication.

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Hepatitis C virus (HCV) is a major cause of chronic hepatitis, liver cirrhosis and hepatocellular carcinoma (Choo *et al.*, 1989; Saito *et al.*, 1990). Infection with HCV is frequently associated with B-cell-related diseases, such as mixed cryoglobulinaemia and non-Hodgkin's lymphoma (Hausfater *et al.*, 2000). A number of studies have suggested that HCV can replicate not only in hepatocytes, but also in lymphocytes (Ducoulombier *et al.*, 2004; Karavattathayyil *et al.*, 2000, Lerat *et al.*, 1998), whereas the determinants of HCV tropism are still unknown. The development of HCV strain JFH-1, which generates infectious HCV in culture, has made an important contribution to the study of the HCV life cycle (Lindenbach *et al.*, 2005; Wakita *et al.*, 2005; Zhong *et al.*, 2005). The HCV life cycle is divided into several steps. After entry into the cell and uncoating, the HCV life cycle leads to translation, polyprotein processing, RNA replication, virion assembly, transport and release. The JFH-1 subgenomic replicon can replicate in non-hepatic cell lines, such as HeLa cells and 293 cells, suggesting that the host factors required for HCV replication are not hepatocyte-specific (Kato *et al.*, 2005b). The SB strain of HCV (genotype 2b strain) was isolated from an HCV-infected non-Hodgkin's B-cell lymphoma and has been reported to infect B and T cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). The virus titres of the SB strain in lymphocytes were, however, lower than those of JFH-1 in Huh-7 cells and the expression of HCV proteins was not confirmed (Kondo *et al.*, 2007). It is unknown whether HCV JFH-1 can infect and replicate in lymphocytes. To gain more insight into the tissue tropism of HCV infection, we investigated the infection, replication, IRES-dependent translation and polyprotein processing of the JFH-1 strain in nine lymphocytic cell lines.

We first sought to determine whether HCV JFH-1 can infect lymphocytic cell lines. We chose nine lymphocytic cell lines derived from Burkitt's lymphoma, the EBV-immortalized human B cell line, lymphoblasts and acute T-cell leukaemia. C1R, IB4, Namalwa, P3HR1 and Raji cells were Epstein–Barr virus (EBV)-positive (Table 1). Infectious HCV was generated from HCV JFH-1 RNA in Huh-7 cells (Shirakura *et al.*, 2007; Wakita *et al.*, 2005) and the calculation of the 50 % tissue culture infectious dose ($TCID_{50}$) was based on methods described previously (Lindenbach *et al.*, 2005). These cell lines (1×10^5 cells per well of a six-well plate) were incubated with 2 ml inoculum (5×10^3 or 5×10^4 $TCID_{50}$ ml^{-1}) for 3 h, washed three times with PBS, and cultured in fresh medium. The culture medium was changed every 2 days. Cells were harvested at 0 (3 h post-infection [p.i.]), 4 and 8 day p.i. HCV core antigen within cells was quantified by immunoassay (Ortho HCV-core ELISA kit; Ortho-Clinical Diagnostics). As shown in Fig. 1(a), increasing the HCV titre of the inoculum resulted in a 7.2-fold increase in the levels of HCV core protein in Huh-7 cells at 3 h p.i. Increasing the HCV titre of the inoculum resulted in a 1.5- to 3.2-fold increase in the levels of the core protein in C1R, BL41,

Table 1. Summary of the virological characterization of HCV JFH-1 in lymphocytes

Name	Source	EBV	Transfection			Concentration of G418 for selection (μg ml^{-1})	HCVcc infection	HCV-RNA replication	Translation*		Polyprotein processing†
			Buffer	Program	Efficiency				HCV-IRES	EMCV-IRES	
Bjab	Burkitt's lymphoma	−	T	T−16	>90 %	600–800	−	−	+	++	+
BL41	Burkitt's lymphoma	−	V	I−10	60–70 %	1000	−	−	+	++	ND
C1R	B lymphoblast	+	V	T−20	70–80 %	100	−	−	++++	++++	+
IB4	Lymphoblastoid	+	V	T−20	80–90 %	1000	−	−	+++	++++	+
Jurkat	Acute T cell leukaemia	−	V	I−10	60–70 %	600	−	−	++	+	ND
Namalwa	Burkitt's lymphoma	+	V	M−13	60–70 %	600–800	−	−	+++	++++	+
P3HR1	Burkitt's lymphoma	+	V	A−23	60–70 %	800	−	−	+++	++++	ND
Raji	Burkitt's lymphoma	+	V	T−27	70–80 %	800	−	−	++	++++	+
Ramos	Burkitt's lymphoma	−	V	M−13	40–60 %	400	−	−	+	+	ND
Huh7	Hepatoma	−	T	T−14	70–80 %	500	+	+	+++	+++	+

*+, <0.25 fold IRES activity of Huh-7; ++, 0.25–0.75 fold; +++, 0.75–1.5-fold; ++++, >1.5-fold.
†ND, Not determined.

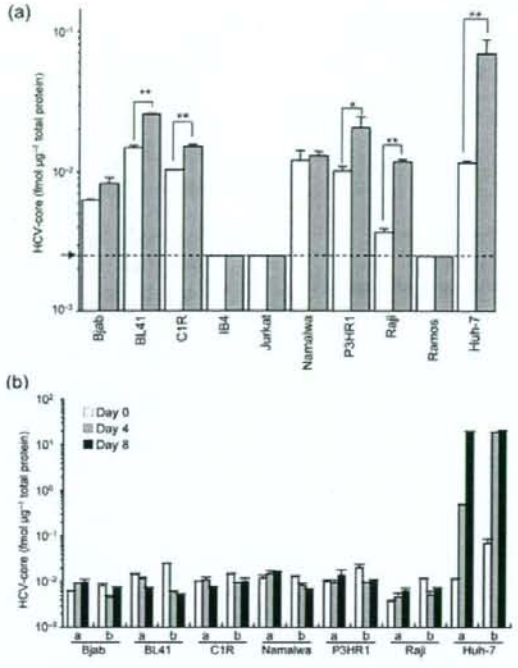

Fig. 1. HCV infection assay. (a) HCV core protein levels 3 h after infection. A total of 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [white bars] or 5×10^4 [grey bars] $TCID_{50}$ ml^{-1}) for 3 h at 37 °C and harvested at 3 h p.i. HCV core protein in cell lysate was quantified by ELISA. The average values with standard deviations from triplicate samples are shown. The cut-off value of the immunoassay is indicated by an arrow and a dotted line. The difference between low m.o.i. (white bars) and high m.o.i. (grey bars) was significant (*, $P<0.05$; **, $P<0.01$, Student's *t*-test). (b) Time-course of HCV core protein levels after infection. In total, 1×10^5 cells were infected with 2 ml of the inoculum (5×10^3 [a] or 5×10^4 [b] $TCID_{50}$ ml^{-1}) for 3 h and harvested at 0, 4 and 8 days p.i. HCV core protein in cell lysate was quantified by ELISA. Average values ± SD from triplicate samples are shown.

P3HR1 and Raji cells, suggesting that HCV can bind to these cell lines (Fig. 1a). In contrast, the levels of HCV core protein in IB4, Jurkat and Ramos cells at 3 h p.i. were below the detection limits and there were no significant differences in the levels of the core protein in Bjab cells and Namalwa cells, suggesting that HCV binding to these cells was very inefficient (Fig. 1a). Moreover, the levels of HCV core protein increased in Huh-7 cells but, in the case of all lymphocytic cell lines, including Raji cells, the core titre did not increase at day 4 and 8 p.i., suggesting that HCV JFH-1 does not infect and/or replicate efficiently in these lymphocytic cell lines (Fig. 1b).

To assess the replication of JFH-1 in our lymphocytic cell lines, we utilized the HCV replicon system. To visualize the

replicating cells, a reporter replicon plasmid was constructed as follows. The gene encoding green fluorescence protein (GFP) was fused to the neomycin resistance gene using an overlap PCR amplification technique and the fusion product was inserted into pSGR-JFH1. The resultant plasmid was pSGR-GFPneo-JFH1. This plasmid was linearized with *Xba*I and used as a template for *in vitro* transcription using an AmpliScribe T7 High Yield Transcription kit (Epicentre Biotechnologies). RNA was transfected with high transfection efficiency and low cytotoxicity using the Nucleofector system (Amaxa Biosystems) (Coughlin *et al.*, 2004; Miyahara *et al.*, 2005; Van De Parre *et al.*, 2005). The transfection efficiencies ranged from 60 to 80 % after optimization of transfection conditions (Table 1). GFP expression was monitored periodically during the selection of HCV-replicon cells by G418 (Table 1). The GFP-expressing cells were detected at day 3 post-transfection (p.t.) in Huh-7, P3HR1, Raji, C1R and Namalwa cells. The rate of GFP expression in Huh-7 cells was more than 50 %. The rate of GFP-expression in lymphocytic cell lines was less than 1 %, despite the high transfection efficiencies. After 3 weeks of G418 selection, SGR-GFPneo-JFH1 replicon cells were established in Huh-7 cells, but not in lymphocytic cells. These data suggest that JFH-1 subgenomic replicon RNA cannot replicate in the lymphocytic cell lines.

To facilitate quantification of replication, we performed luciferase assays using subgenomic replicon RNA (SGR-JFH1/Luc) carrying firefly luciferase as a reporter. SGR-JFH1/Luc RNA was *in vitro*-transcribed using the linearized pSGR-JFH1/Luc (Kato *et al.*, 2005a) as template DNA. Cells were harvested at 4, 24, 48 and 72 h p.t. and luciferase activities were assayed with luciferase assay reagent (Promega). Assays were performed at least in triplicate. There were significant differences in luciferase activities at 4 h p.t. among the cell lines, probably because there were differences in transfection efficiencies and the doubling time of the cell lines. Thus, the replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1 (Fig. 2a). HCV subgenomic replicon RNA efficiently replicated in Huh-7 cells (Fig. 2a). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B served as a negative control in Huh-7 cells. The luciferase activities of replication-deficient subgenomic replicon RNA in lymphocytic cell lines also decreased rapidly (data not shown). As shown in Fig. 2(a), the luciferase activities of HCV subgenomic replicon RNA in lymphocytic cell lines decreased rapidly, suggesting that HCV subgenomic replicon RNA did not replicate efficiently in lymphocytic cell lines. Thus, these two different replicon assays demonstrated that the HCV JFH-1 subgenomic replicon failed to replicate in our lymphocytic cell lines.

To determine which steps of the HCV life cycle are impaired, we further examined translation and polyprotein processing. At first, we assessed HCV IRES-dependent translational efficiencies in the lymphocytic cell lines. Cells were co-transfected with the subgenomic replicon RNA (SGR-JFH1/Luc) and a capped RNA encoding *Renilla* luciferase (cap-luc). Cap-luc RNA was *in vitro*-transcribed using a T7 mMessage mMachine kit (Ambion). The HCV IRES activities in IB4, Namalwa and P3HR1 cells were as high as in Huh-7 cells. The HCV IRES activities in Jurkat and Raji cells were about 50 % of those in Huh-7 cells, and the HCV IRES activities in Bjab, BL41 and Ramos cells were less than 25 % of those in Huh-7 cells. On the other hand, the HCV IRES activity in C1R cells was about twofold higher than in Huh-7 cells (Fig. 2b). Replication-deficient subgenomic replicon RNA encoding a GDD to GND mutation in NS5B showed a luciferase activity level similar to that of the wild-type, suggesting that the luciferase activity at 4 h after transfection reflected translational levels but not replication levels (data not shown). Our data indicate high HCV IRES activities in all cell lines, except in Bjab, BL41 and Ramos.

The HCV polyprotein is translated in subgenomic replicon cells in an encephalomyocarditis virus (EMCV) IRES-dependent manner. To rule out the possibility that the EMCV IRES-dependent translation is impaired in lymphocytic cell lines, we assessed the EMCV IRES-dependent translational efficiencies. We assayed EMCV IRES activity using EMCV IRES-driven luciferase RNA (EMC-luc) and Cap-luc RNA. The EMCV IRES activity was five- to tenfold higher in C1R, Namalwa, IB4 and P3HR1 than in Huh-7 cells (Fig. 2c). From these results, HCV IRES and EMCV IRES exhibited sufficient translational activity in C1R, Namalwa, P3HR1 and Raji cells, suggesting that IRES-dependent translation was not impaired in these lymphocytic cell lines.

To determine whether HCV polyprotein is properly processed in lymphocytes, we examined the processing of HCV non-structural (NS) proteins. The construct pSGR-JFH1/Luc expresses the polyprotein NS3-NS4A-NS4B-NS5A-NS5B. The HCV NS3/4A protease is responsible for proteolytic processing at each cleavage site. We used the eukaryotic transient-expression system based on a recombinant vaccinia virus carrying bacteriophage T7 RNA polymerase (T7vac) (Fuerst *et al.*, 1989). To express the SGR-JFH1/Luc encoding HCV NS proteins, 5×10^6 cells were transfected with 5 μg pSGR-JFH1/Luc and infected with 2.5×10^9 p.f.u. T7vac, harvested at 24 h p.i., and analysed by Western blotting. Completely processed NS3, NS5A and NS5B proteins were detected in Bjab, Raji, IB4 and Namalwa cells as well as in pSGR-JFH1/Luc-transfected Huh-7 cells and HCV-JFH1-infected Huh-7 cells (Fig. 2c). The unprocessed polyprotein was not detected by immunoblotting in these lymphocytic cell lines (data not shown). These results suggest that the HCV polyprotein is efficiently processed in these lymphocytic cells.

In this study, we demonstrated that HCV JFH-1 failed to infect and replicate in nine lymphocytic cell lines. In contrast, HCV IRES-dependent translation and polyprotein processing by NS3/NS4A protease functioned properly

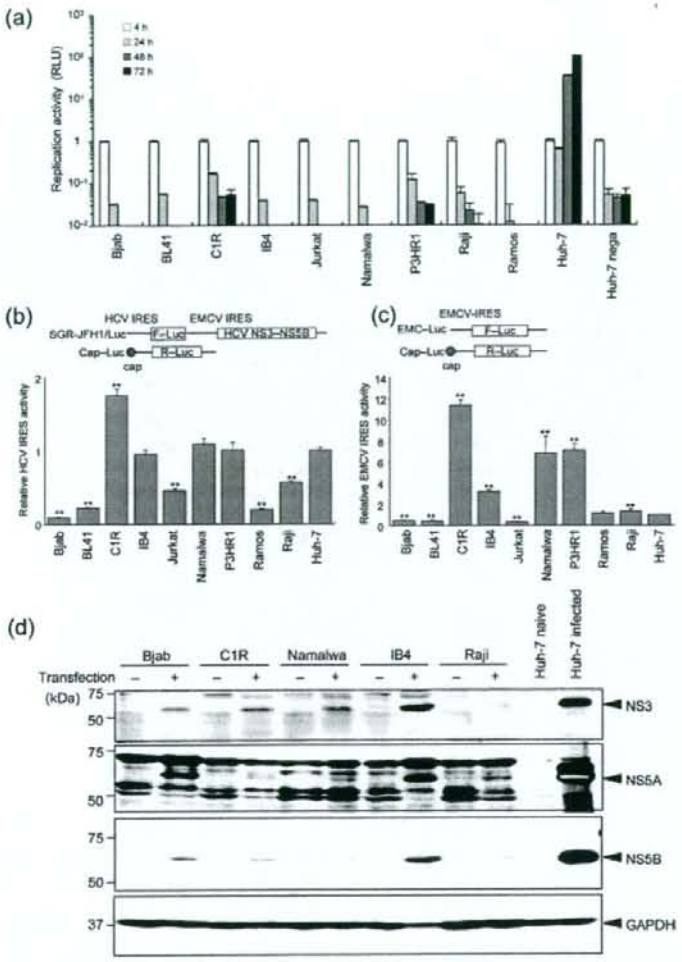

Fig. 2. Replication, HCV IRES-dependent translational efficiencies and polyprotein processing. (a) Subgenomic replicon assay. JFH-1 subgenomic replicon RNA was transfected into several cell lines and harvested at 4, 24, 48 and 72 h p.t. The replication activity was expressed relative to the reporter activity determined 4 h p.t. for each cell line, which was set to 1. RLU, Relative luciferase units; Huh-7 nega, Huh-7 cells transfected with SGR-JFH1/Luc GND, served as a negative control. (b) HCV IRES-dependent translational efficiency. To determine the HCV IRES activities, we co-transfected cells with SGR-JFH1/Luc RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of HCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in HCV IRES activity between Huh-7 cells and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (c) EMCV IRES-dependent translational efficiency. To determine the EMCV IRES activities, we co-transfected cells with EMCV–firefly luciferase RNA and Cap-Renilla luciferase RNA. The IRES activity of each cell line is expressed in relation to Huh-7 IRES activity, that is, as the ratio of EMCV IRES-driven firefly luciferase activity to cap-driven *Renilla* luciferase activity. The difference in EMCV IRES activity between Huh-7 cell and the lymphocytic cell line was significant (**, $P<0.01$, Student's *t*-test). (d) Polyprotein processing by NS3/4A protease in lymphocytic cell lines. pSGR-JFH1/Luc-transfected cells were infected with T7vac and harvested at 24 h p.i. HCV NS proteins, NS3, NS5A and NS5B were detected by using anti-NS3 rabbit polyclonal antibody (PAb), anti-NS5A rabbit PAb and anti-NS5B rabbit PAb. Arrowheads indicate the processed NS3, NS5A and NS5B proteins, respectively.

in these cells. Moreover, subgenomic replicon RNA failed to replicate in these cell lines. Our data suggest that lymphocytic cell lines may lack some host factors required for infection and replication of HCV-JFH1.

Viral entry often requires sequential interactions between viral proteins and several cellular factors. Several molecules (CD81, Claudin-1, Scavenger receptor class B member IR, LDL-receptor and glycosaminoglycans) have been reported to be involved in HCV binding and entry (Barth *et al.*, 2003; Evans *et al.*, 2007; Pileri *et al.*, 1998; Scarselli *et al.*, 2002). Further investigation will be required to clarify HCV binding and entry into lymphocytic cell lines.

HCV IRES and EMCV IRES exhibited sufficient translational activities in C1R, IB4, P3HR1, Namalwa and Raji cells. All these cell lines are EBV-positive. EBV-encoded nuclear antigen (EBNA1) has been reported to support HCV replication (Sugawara *et al.*, 1999). Two small EBV-encoded RNA species (EBERs) bind to the HCV IRES region (Wood *et al.*, 2001). These findings raise the possibility that HCV IRES activities may be modified by the EBV genome.

HCV JFH-1 subgenomic replicon RNA could not replicate in all lymphocytes tested in this study. The HCV SB strain, however, has been reported to infect Raji, Daudi, Molt-4

and Jurkat cells (Kondo *et al.*, 2007; Sung *et al.*, 2003). Still unknown is how hepatotropism and lymphotropism of HCV are determined. The GB virus B (GBV-B) is most closely related to HCV and the GBV-B infection of tamarins has been proposed as a good surrogate model for chronic hepatitis C (Bukh *et al.*, 2001; Jacob *et al.*, 2004; Lanford *et al.*, 2003; Martin *et al.*, 2003). A recent report has shown that GBV can disseminate to not only liver but also a variety of extrahepatic tissues such as haematolymphoid and genital tissues in tamarins (Ishii *et al.*, 2007). Viral RNA cloned from plasma and liver from the tamarins showed no sequence heterogeneity, suggesting that host factors determine the pleiotropism (Ishii *et al.*, 2007). It remains unclear how host factors and/or viral factors determine the tissue tropism of HCV. Further studies will be required to clarify the molecular mechanisms of HCV tissue tropism.

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