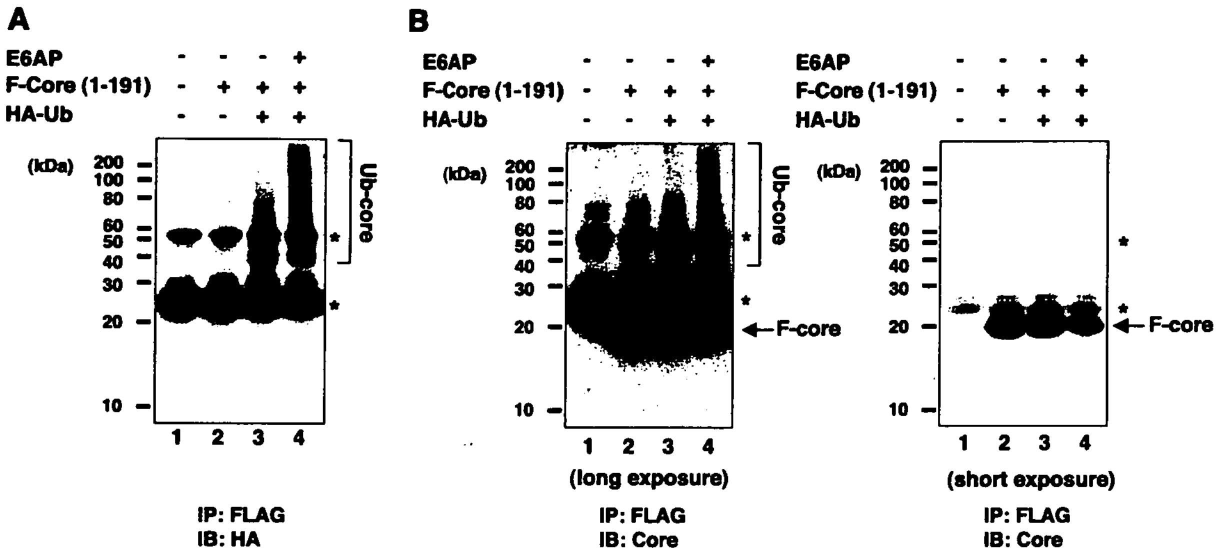

FIG. 7. E6AP-dependent ubiquitylation of HCV core protein in vivo. 293T cells (1×10^6 cells/10-cm dish) were transfected with 1 μg of pCAG FLAG-core (1–191) together with 2 μg of plasmid encoding E6AP as indicated. Each transfection also included 2 μg of plasmid encoding HA-ubiquitin. The cell lysates were immunoprecipitated with FLAG beads and analyzed by immunoblotting with anti-HA PAb (A) or anticore PAb (B). A shorter exposure of the core blot shows immunoprecipitated FLAG-core protein (B, right panel). A longer exposure of the core blot shows the presence of a ubiquitin smear (B, left panel). Asterisks indicate cross-reacting immunoglobulin light chain or heavy chain. Arrows indicate FLAG-core. IB, immunoblot; IP, immunoprecipitation.

jugated with HA-ubiquitin were indeed ubiquitylated forms of the core protein (Fig. 7B, lanes 3 and 4, long exposure).

E6AP mediates ubiquitylation of HCV core protein in vitro. To rule out the possibility that E6AP contributes to core protein degradation by inducing degradation of inhibitors of core turnover, we determined whether E6AP functions directly as a ubiquitin ligase by testing the ability of purified MEF-E6AP to mediate in vitro ubiquitylation of the purified recombinant HCV core protein. HCV core protein was expressed as a fusion protein containing N-terminal GST tag and C-terminal His tag and purified as described in Materials and Methods. GST-C173HT (aa 1–173) and GST-C152HT (aa 1–152) (see Materials and Methods) were used to determine whether the mature core protein and the C-terminally truncated core protein are targeted for ubiquitylation in vitro. The validity of this assay was established by demonstrating that E6AP but not E6AP C-A induced ATP-dependent ubiquitylation of GST-core protein. When in vitro ubiquitylation reactions were carried out either in the absence of MEF-E6AP or in the presence of MEF-E6AP C-A, no ubiquitylation signal was detected (Fig. 8A, lanes 4 and 5). However, inclusion of purified MEF-E6AP in the reaction mixture resulted in marked ubiquitylation of GST-C173HT (Fig. 8A, lane 6), while no ubiquitylation was observed in the absence of ATP (Fig. 8A, lane 7). No signal was detected when GST-HT was used as a substrate (Fig. 8A, lane 8). The higher-molecular-weight species of GST-core proteins were reactive with both anti-ubiquitin MAb (Fig. 8B, right panel, lanes 2 and 4) and anti-GST MAb (Fig. 8B, left panel, lanes 2 and 4). Both GST-C152HT and GST-C173HT were polyubiquitylated by E6AP in vitro (Fig. 8B), indicating that both the C-terminally truncated core and the mature core are polyubiquitylated by E6AP in vitro. These results revealed that E6AP directly mediated ubiquitylation of HCV core proteins in an ATP-dependent manner.

Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. We used a recently developed system for the production of infectious HCV particles using the HCV JFH1 strain (28, 56, 61) to examine whether E6AP can promote degradation of HCV core protein expressed from infectious HCV. E6AP-dependent core degradation was assessed in Huh-7 cells inoculated with the culture supernatant containing HCV JFH1. Levels of HCV core protein were detectable at day 3 postinfection and increased with time. Immunofluorescence staining for the core protein indicated that the percentage of HCV core-positive cells in the Huh-7 cells was almost 100 at day 7 postinfection. Transfection efficiency was 50 to 60% as measured with GFP-expressing plasmid. At day 7 postinfection, exogenous expression of E6AP reduced the intracellular core protein level by about 60% compared to the empty plasmid-transfected control cells (Fig. 9A). Inactive E6AP had little effect on the core protein levels. Total protein levels in the cells (Fig. 9B) and intracellular HCV RNA levels (Fig. 9C) did not change after transfection of wild-type E6AP or inactive E6AP. The immunofluorescence study revealed that HCV core protein was variably detected and the intensity of core staining was reduced in the cells staining positive for wild-type E6AP compared with neighboring cells staining negative for E6AP (Fig. 9E). Using inactive E6AP revealed colocalization of the core protein and E6AP in the perinuclear region (Fig. 9F) of HCV-infected cells. These results suggest that E6AP enhanced degradation of HCV core protein expressed from infectious HCV. Then we titrated HCV infectivity in the culture supernatant at day 7 postinfection by limiting

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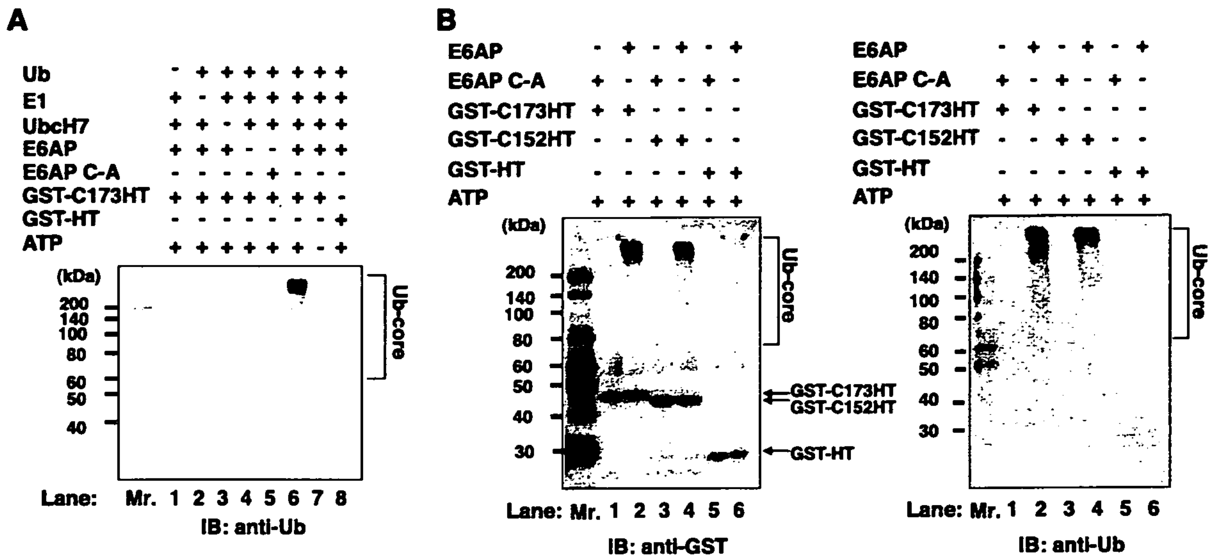

FIG. 8. In vitro ubiquitylation of HCV core protein by recombinant E6AP. For in vitro ubiquitylation of HCV core protein, purified GST-C173HT and GST-C152HT were used as substrates. Purified GST-HT was used as a negative control. Assays were done in 40-μl volumes containing each component as indicated. The reaction mixture is described in Materials and Methods. The reaction was carried out at 37°C for 120 min followed by purification with glutathione-Sepharose beads and analysis by immunoblotting with the indicated antibodies. Arrows indicate GST-C173HT, GST-C152HT, and GST-HT, respectively. Ubiquitylated species of GST-core proteins are marked by brackets. IB, immunoblot.

dilution assays. Exogenous expression of E6AP reduced the supernatant infectivity titer, whereas inactive E6AP had no effect on its infectivity titer (Fig. 9D), suggesting that the E6AP-dependent ubiquitin proteasome pathway affects the production of HCV particles through downregulation of the core protein.

E6AP silencing increases the levels of intracellular HCV core protein and supernatant infectivity titers in HCV-infected Huh-7 cells. Finally, to further validate the role of E6AP in HCV production, expression of endogenous E6AP was knocked down by siRNA and the HCV infectivity titers released from HCV JFH1-infected cells were examined. Knockdown of E6AP by siRNA led to an increase in intracellular core protein levels (Fig. 10A) and supernatant HCV infectivity titers (Fig. 10B). Taken together, our results suggest that E6AP mediates ubiquitylation and degradation of HCV core protein in HCV-infected cells, thereby affecting the production of HCV particles.

DISCUSSION

HCV core protein is a major component of viral nucleocapsid, plays a central role in viral assembly (25, 40), and contributes to viral pathogenesis and hepatocarcinogenesis (9). Therefore, it is important to clarify the molecular mechanisms that govern the cellular stability of this viral protein. We have previously reported that processing at the C-terminal hydrophobic domain of the core protein leads to efficient polyubiquitylation of the core protein (52). In this study, we identified E6AP as an HCV core-binding protein and showed that HCV core protein interacts with E6AP in vivo and in vitro, that E6AP enhances ubiquitylation and degradation of the mature core protein as well as the C-terminally truncated core protein, and that HCV core protein expressed from infectious HCV is degraded via E6AP-dependent proteolysis. HCV core protein and E6AP were found to colocalize in the cytoplasm, especially in the perinuclear region. Moreover, exogenous expression of E6AP reduces intracellular core protein levels and supernatant HCV infectivity titers in HCV-infected Huh-7 cells. Knockdown of endogenous E6AP by siRNA increases intracellular core protein levels and supernatant infectivity titers in HCV-infected cells. These findings suggest that E6AP mediates ubiquitylation and degradation of HCV core protein, thereby affecting the production of HCV particles.

HCV core protein interacts with E6AP through the region of the core protein between aa 58 and aa 71. These 14 amino acids are highly conserved, with the first nine amino acids (PRGRRQPIP) present in the core protein of all the HCV genotypes (3). This result suggests that E6AP-dependent degradation of HCV core protein is common to all HCV genotypes and plays an important role in the HCV life cycle or viral pathogenesis. Our data indicated that HCV core proteins of genotypes 1b and 2a are subjected to proteolysis through an E6AP-mediated degradation pathway. We are currently examining whether E6AP promotes degradation of HCV core proteins of other genotypes.

Studies in addition to ours have reported that other HCV proteins, such as NS5B (8), the unglycosylated cytosolic form of E2 (39), NS2 (7), and F protein (58), are degraded through the ubiquitin-proteasome pathway. These studies suggest that the ubiquitin-proteasome pathway plays a role in the HCV life cycle or viral pathogenesis. To our knowledge, the present study is the first to demonstrate that the ubiquitin-proteasome pathway affects the HCV life cycle.

PA28γ was found to interact with HCV core protein in hepatocytes and promote proteasomal degradation of HCV core protein (30). PA28γ, however, has been shown to function

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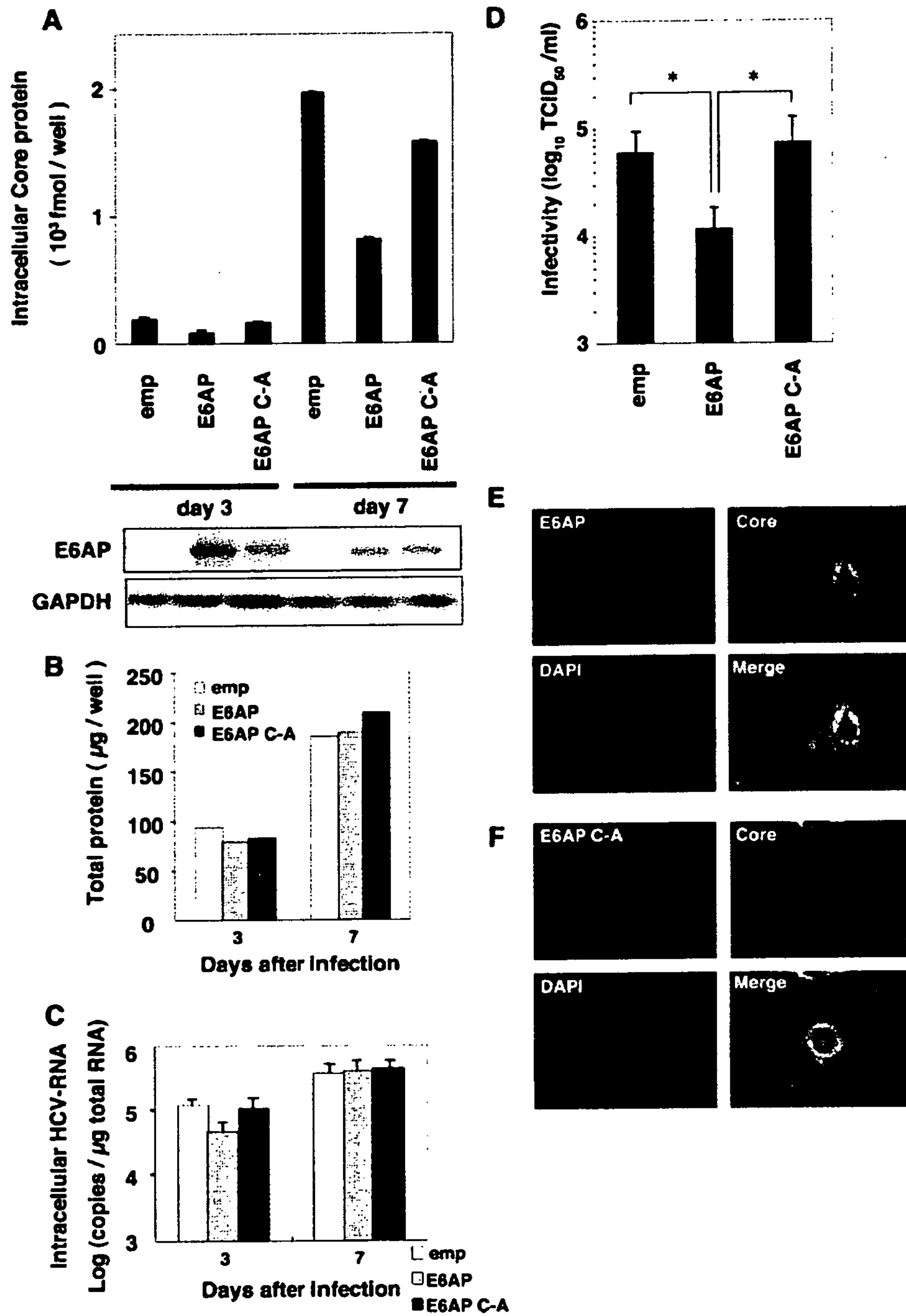

FIG. 9. Exogenous expression of E6AP reduces intracellular HCV core protein levels and supernatant infectivity titers in HCV-infected Huh-7 cells. Naïve Huh-7 cells were seeded as described in Materials and Methods; inoculated with 2.5 ml of the inoculum including infectious HCV JFH1 (6.5×10^3 $TCID_{50}$/ml); and transfected with 6 µg of empty plasmid, pCAG-HA-E6AP, or pCAG-HA-E6AP C-A. The culture supernatant and the cells were collected at days 3 and 7 postinfection. (A) Intracellular HCV core protein levels. (B) Levels of total protein. (C) Levels of intracellular HCV RNA in HCV-infected Huh-7 cells. Data represent the averages of three experiments with error bars. (D) Supernatant infectivity titers. At day 7 postinfection, culture supernatants were collected and assayed for $TCID_{50}$ determinations. The difference between empty vector and E6AP or between E6AP and E6AP C-A was significant (*, $P < 0.05$, Student's t test). (E and F) HCV JFH1-infected Huh-7 cells were transfected with either MEF-E6AP plasmid or MEF-E6AP C-A plasmid, grown on coverslips, fixed, and processed for double-label immunofluorescence for HCV core and MEF-E6AP (E) or MEF-E6AP C-A (F). Anticore MAb (2H9) and anti-FLAG PAb were used as primary antibodies. Nuclei were visualized by staining the cells with DAPI. All the samples were examined with a BZ-8000 microscope. Representative images of individual cells are shown with merge images. emp, empty vector.

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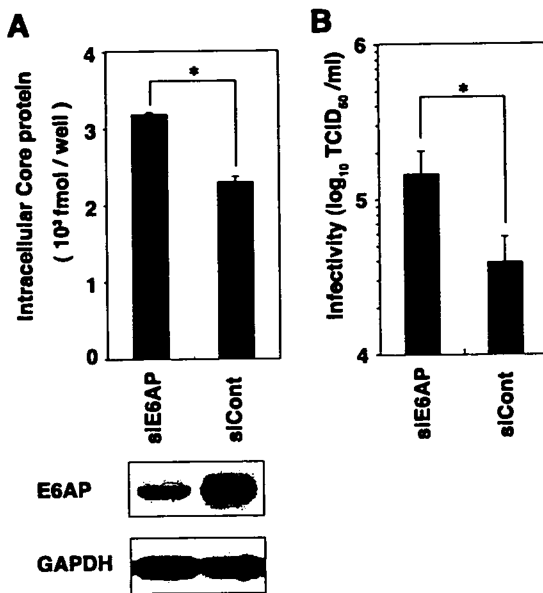

FIG. 10. E6AP silencing leads to an increase in the level of intracellular HCV core protein and supernatant infectivity titer in HCV-infected Huh-7 cells. (A) HCV JFH1-infected cells were replated in a six-well plate at 3×10^5 cells/well and transfected with 40 pmol of E6AP siRNA or control siRNA. The culture medium was changed at 24 h after transfection. The cells were harvested at day 2 after transfection, and the intracellular core protein levels were quantitated using the HCV core antigen ELISA. Equivalent amounts of the whole-cell lysates were separated by SDS-PAGE and analyzed by immunoblotting with anti-E6AP MAb or anti-GAPDH MAb. (B) Culture supernatants were collected at day 2 after transfection and assayed for $TCID_{50}$ determinations. For both panels, the difference between E6AP siRNA and control siRNA was significant (*, $P < 0.05$, Student's t test).

in a ubiquitin-independent, ATP-independent, and 20S proteasome-dependent pathway (27). There have been reports that several cellular factors, such as p53 (2), p73 (2), and RPN4 (18), are degraded through two alternative pathways, the ubiquitin-dependent 26S proteasome-dependent pathway and the ubiquitin-independent 20S proteasome-dependent pathway. Here we provide evidence that E6AP mediates ubiquitylation of HCV core protein. Still unclear is whether the PA28γ-dependent pathway requires polyubiquitylation of HCV core protein. HCV core protein is predominantly localized in the cytoplasm, especially at the endoplasmic reticulum membrane, on the surface of lipid droplets, and on mitochondria and mitochondrion-associated membranes (51). In HCV JFH1-infected cells, HCV core was found to localize in the cytoplasm and frequently to accumulate in the perinuclear region and the lipid droplets (44). Our results indicated that E6AP colocalized with HCV core protein especially in the perinuclear region. PA28γ was found to colocalize with HCV core protein in the nucleus. Functional differences may exist between the E6AP-dependent pathway and the PA28γ-dependent pathway in the stability control of HCV core protein. The functional role of the E6AP-dependent pathway and the PA28γ-dependent pathway remains to be elucidated.

The HCV core-binding region of E6AP was mapped to the region between aa 418 and aa 517. The multicopy maintenance protein 7, Mcm7, interacts with E6AP through a short motif, termed the L2G box (aa 412 to 414), that lies within the E6 binding site of E6AP (23). Our data indicated that the E6 binding region containing the L2G motif is not required for interaction between HCV core protein and E6AP (Fig. 2C, lane M).

We propose here that E6AP may affect the production of HCV particles through controlling the amounts of HCV core protein. This mechanism may contribute to persistent infection. The E6AP binding domain of the core protein resides in the RNA-binding domain and binding domains for many host factors (40). These factors may affect the binding between E6AP and HCV core protein, resulting in control of E6AP-dependent core degradation. Another possibility is that HCV core protein may affect the normal function of E6AP, thereby contributing to pathogenesis. It will be intriguing to investigate whether HCV core protein has any effect on E6AP-dependent degradation of host factors. The other intriguing possibility is that HCV core-E6AP complex may function as an E3 ligase-like E6-E6AP complex to target host factors for proteasomal degradation and contribute to viral pathogenesis.

In conclusion, we have demonstrated that E6AP interacts with HCV core protein in vitro and in vivo and mediates ubiquitin-dependent degradation of the core protein, leading to downregulation of HCV particles. We propose that the E6AP-mediated ubiquitin-proteasome pathway may play a role in affecting the production of HCV particles through controlling the amounts of viral nucleocapsid protein. Identification of the specific E3 ubiquitin ligase may contribute to gaining a better understanding of the biology of the HCV life cycle as well as molecular details of the ubiquitin-dependent degradation of HCV core protein.

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Brief Review

Binding activity of norovirus and sapovirus to histo-blood group antigens

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Summary

Noroviruses (NoVs) and sapoviruses (SaVs) are causative agents of human gastroenteritis. There is increasing evidence that certain human NoV strains bind to histo-blood group antigens (HBGAs). We found that several NoV virus-like particles (VLPs) showed binding activity to HBGAs, while neither SaV genogroup I (GI) VLP nor SaV GV VLP showed such activity.

*

Human noroviruses (NoVs) and human sapoviruses (SaVs) are etiological agents of human gastroenteritis. Human NoV strains can be grouped into two genogroups (GI and GII), and at least 14 GI and 17 GII genotypes (GI/1–14 and GII/1–17) [11]. SaV strains can be divided into five genogroups (GI–GV), of which the GI, GII, GIV and GV strains infect humans, while the GIII strains infect porcine species [1]. Human NoV and SaV strains are noncultivable, but the expression of the recombinant capsid protein VP1 (rVP1) in insect cells results in the self-assembly of virus-like particles (VLPs) that are antigenically similar to native viruses [2, 9]. In the past several years, increasing evidence has emerged that human NoVs bind to histo-blood group antigens (HBGAs) [8, 12]. These carbohydrate epitopes are present in mucosal secretions and throughout many tissues of the human body, including the small intestine, which may be specifically targeted by certain NoV strains. To the best of our knowledge, the relationship between human SaVs and HBGAs has not yet been reported.

In the present study, we examined the binding activities of human NoV and SaV VLPs to HBGAs present in human saliva and to synthetic carbohydrates. Four NoV strains belonging to different genotypes were examined: the GI/1 124 strain (accession number AB031013), the GI/2 258 strain (AB078335), the GII/4 104 strain (AB078336), and the GII/1 Hawaii strain (U07611). Hawaii VLPs were used as a negative control [5]. Two SaV strains belonging to two different genogroups were also examined: the SaV GI Mc114 strain (AY237422) and the SaV GV NK24 strain (AY646856). Saliva samples were collected from 29 healthy donors. The amounts of Lewis a (Le^a), Lewis b (Le^b), H, A and B antigens in the saliva samples were determined semi-quantitatively by hemagglutination inhibition, and 12 saliva samples with relatively high amounts

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Table 1. Semiquantitation of soluble ABH and Lewis antigens

Donor no.	Interpretation of saliva testing	Grouping	Hemagglutination inhibition titer				
			H	A	B	Lewis-a	Lewis-b
1	Secretor/O/Lewis-positive	H^{high}/Le-b^{high}	>256	0	0	32	32
2	Secretor/O/Lewis-positive		>256	0	0	32	32
3	Secretor/O/Lewis-positive		128	0	0	32	64
4	Secretor/AB/Lewis-positive	A^{high}	16	>256	32	8	2
5	Secretor/AB/Lewis-positive		8	>256	8	8	4
6	Secretor/AB/Lewis-positive		16	128	8	4	4
7	Secretor/B/Lewis-negative	B^{high}	16	0	>256	0	0
8	Secretor/B/Lewis-positive		16	0	128	16	4
9	Secretor/B/Lewis-positive		16	0	128	2	4
10	Nonsecretor	Le-a^{high}	4	8	0	>256	8
11	Nonsecretor		4	1	0	>256	4
12	Nonsecretor		0	1	0	>256	4

of antigens were selected for saliva-VLP binding assay (Table 1). We then used 2 enzyme-linked immunosorbent assay (ELISA)-based assays, a saliva-VLP binding assay and a carbohydrate-VLP binding assay to examine the binding activities of the NoV and SaV VLPs to HBGAs.

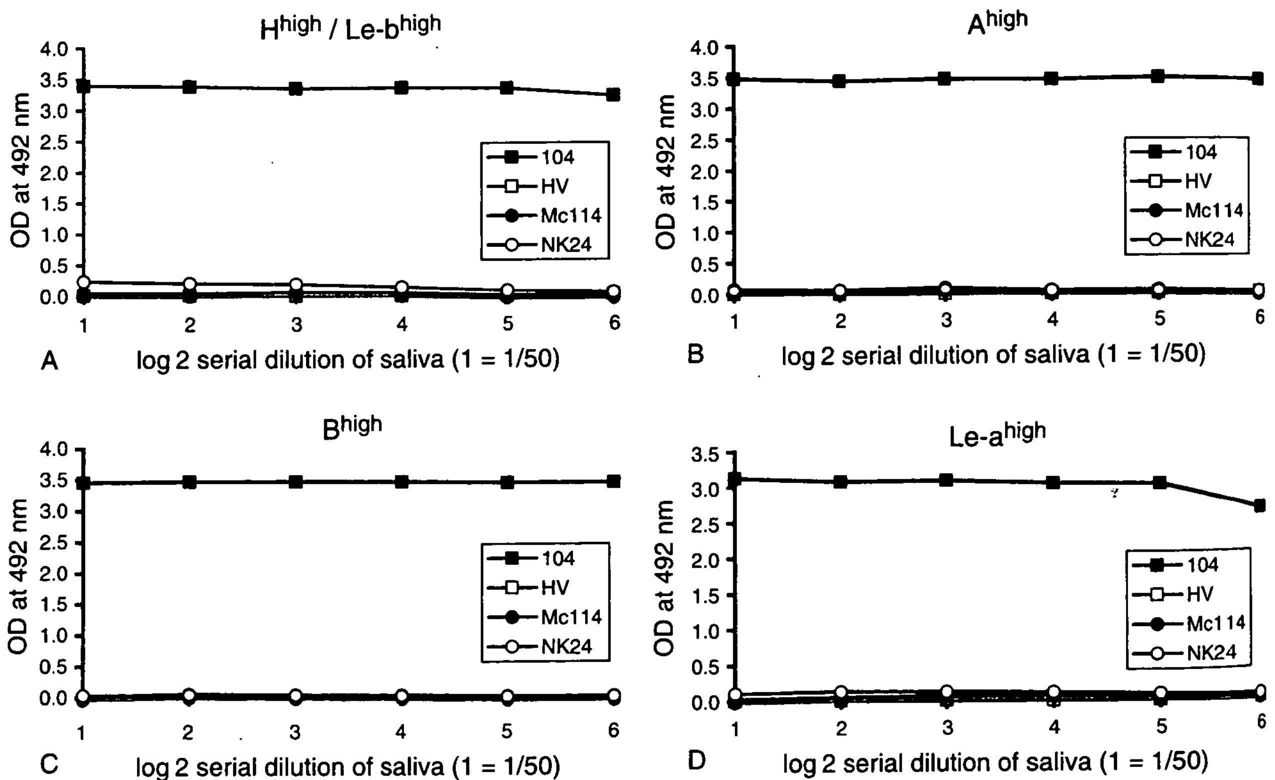

Fig. 1. NoV and SaV VLP binding activity to the saliva samples. Optical densities were measured at 492 nm and were plotted against the serial diluted saliva samples. Each experiment was performed with three donors from each HBGA group (Table 1) and repeated twice. Three samples from the same group produced similar results (data not shown). The results from the donors 1 (**A**), 4 (**B**), 7 (**C**) and 10 (**D**) are shown

In the saliva-VLP binding assay, we examined the possibility that NoV and SaV VLPs may bind to saliva samples. Briefly, 100 µl of serially diluted saliva samples dissolved in carbonate/bicarbonate buffer (50 mmol/L, pH 9.6) were added to wells and incubated at 37 °C overnight. The wells were washed 3–6 times with 300 µl of phosphate-buffered saline containing 0.05% Tween 20 (PBS-T) and washed again after each of the following steps. The wells were blocked with 200 µl of PBS containing 5% skim milk (SM/PBS) for 1 h at room temperature. The purified VLPs, dissolved in 1% SM/PBS-T (final 1 µg/mL), were added (100 µl) to the wells and incubated for 1 h at 37 °C. Next, 100 µl of rabbit anti-rSaV or NoV VLP antiserum (1:2000) in 1% SM/PBS-T was added, and incubated for 1 h at 37 °C. Horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (100 µl; Zymed Laboratories Inc., San Francisco, CA, USA) in 1% SM/PBS-T was then added and incubated for 1 h at 37 °C. One hundred microliter of O-phenylenediamine (Sigma, St. Louis, MO, USA) was added as substrate, and incubated at room temperature for 30 min, at which point 50 µl of 4N H_2SO_4 was added to stop the reaction, and the optical density (OD) at 492 nm was measured. The wells incubated with carbonate/bicarbonate buffer instead of serially diluted saliva samples were used as plate blank (Fig. 1). The Hawaii VLPs have been reported to show no binding to HBGAs [5]; under the present experimental conditions, Hawaii VLPs also showed no binding activity (OD values less than 0.01) at all to HBGAs in saliva. The VLPs of the NoV 104 strain, which resembles Camberwell virus (AF145896) and is classified into GII/4, showed strong binding activity to the diluted saliva at all dilutions of all HBGA samples (OD values greater than 2.7), while SaV Mc114 VLPs showed little binding activity at all dilutions of all samples (OD less than 0.09), as did SaV NK24 VLPs (OD values less than 0.24). These results indicate that the SaV Mc114 and NK24 VLPs have no binding activity with saliva antigens.

In the carbohydrate-VLP binding assay, we examined the possibility that NoV and SaV VLPs may bind to different synthetic carbohydrates, such as H-1 (trisaccharides), A (trisaccharides), B (trisaccharides), Le^a (trisaccharides) and Le^b (tetrasaccharides). Briefly, multivalent carbohydrate-biotin reagents conjugated to polyacrylamide (CHO-PAA-biotin; GlycoTech, Rockville, MD, USA) were resuspended in 0.3 M sodium phosphate buffer at 1 mg/ml, diluted to 20 µg/ml in Tris-buffered saline, and serially diluted twofold, after which 100 µl was added per well to streptavidin-precoated plates (Thermo Labsystems, Basingstoke, United Kingdom) and incubated for 2 h at 37 °C. The wells were washed 3–6 times with PBS-T and were washed again after each of the following steps. The plates were blocked with 300 µl of 5% SM/PBS overnight at 4 °C. The VLPs (1 µg/ml in 100 µl of 5% SM/PBS) were added to each well and incubated for 4 h at 37 °C. Next, 100 µl of rabbit anti-rNoV VLPs antiserum (1:2000 in 5% SM/PBS) was added and incubated for 2 h at 37 °C. One hundred microliter of HRP-conjugated anti-rabbit IgG in 5% SM/PBS was then added and incubated for 1 h at 37 °C. The plates were processed as described above. The wells incubated with Tris-buffered saline and 5% SM/PBS instead of serially diluted synthetic carbohydrates and VLPs were used as plate blank.

The NoV 104 VLPs were found to show strong binding activity to three of five synthetic carbohydrates: A, B and Le^b (Fig. 2B, C and E). Although 104 VLPs showed strong binding activity to the saliva samples containing relatively high amounts of H antigen (Fig. 1A) and Le^a antigen (Fig. 1D), they showed only moderate binding activity to H synthetic carbohydrates and no binding activity to Le^a synthetic carbohydrates (Fig. 2A and D). Differences in the reactivity between saliva samples and synthetic carbohydrates may be due to differences between synthetic products and the authentic antigens found in vivo, which are thought to be present on mucin or mucin-like molecules [7]. Therefore, we included two additional NoV VLPs, the 124 and 258 VLPs, as positive controls for the H and Le^a synthetic carbohydrates (Fig. 2A and D), respectively, which showed strong binding activity to the H-high and Le^a-high saliva samples (data not shown). The 124 strain is genetically close to the GI/1 prototype Norwalk virus (NV/68; M87661), and the binding properties of recombinant NV/68

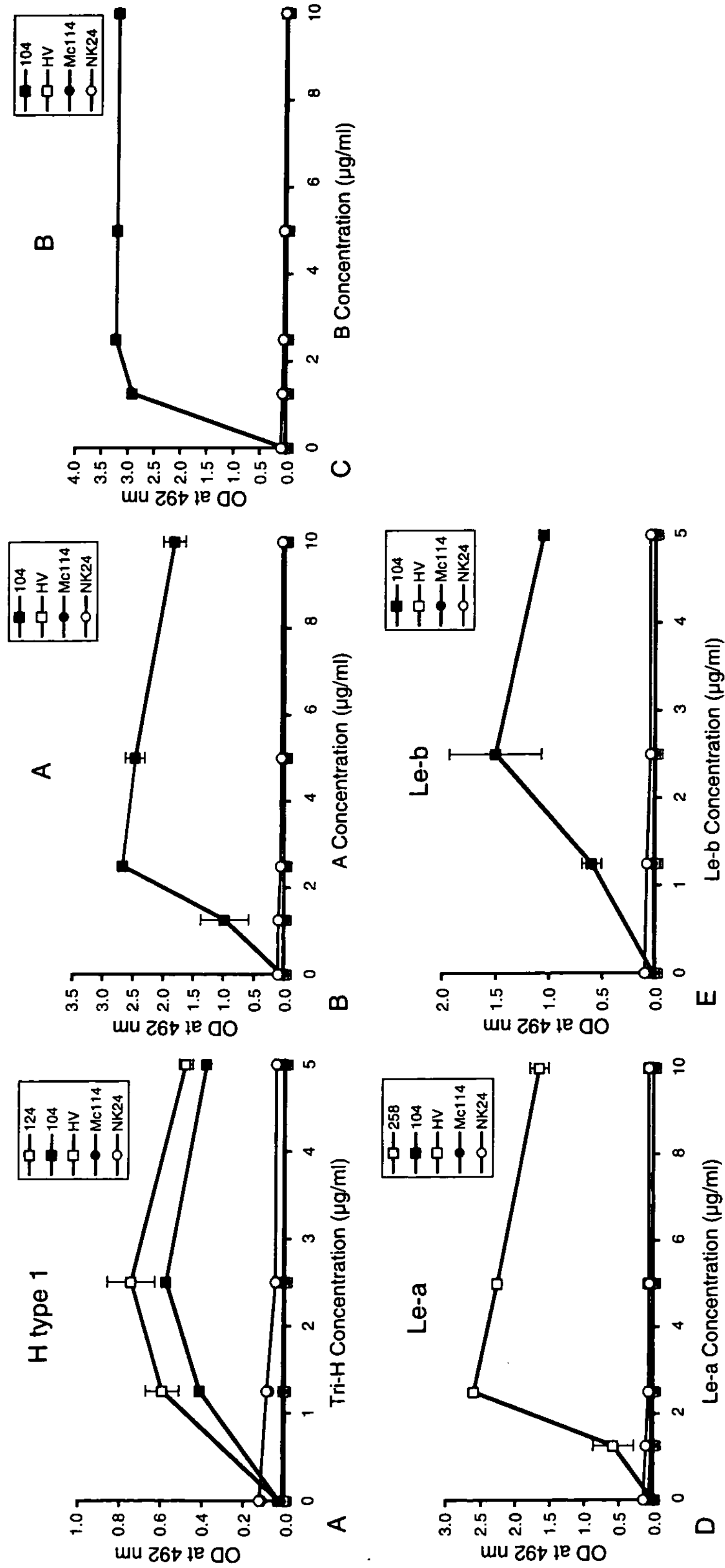

Fig. 2. NoV and SaV VLP binding activity to the synthetic carbohydrates. The optical densities at 492 nm are plotted against the dilutions. Each experiment was performed in duplicate and repeated twice

to H antigen have been well characterized [5, 13]. The 124 strain showed 99% amino acid identity with NV/68 in the P2 domain. There are known to be two amino acid differences at residues 370 and 376 which are not related to HBGA binding [14]. The 258 strain resembles Southampton virus (L07418) and is classified into GI/2. The NoV Hawaii VLPs showed no binding activity to any of these synthetic carbohydrates (Fig. 2). The SaV Mc114 and NK24 VLPs also showed no binding activity to any of the synthetic carbohydrates (Fig. 2).

A number studies have found that different NoV strains exhibit different binding patterns to HBGAs [5–7]. In the present study, we found that NoV GII/4 104 VLPs showed binding activity to HBGAs, while SaV GI and GV VLPs showed no such binding activity. Human SaVs are becoming an increasingly important cause of gastroenteritis worldwide [3, 4, 10]. Further studies are needed to examine the possibility that other human SaV genogroups have binding activity.

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Dynamic behavior of hepatitis C virus quasispecies in a long-term culture of the three-dimensional radial-flow bioreactor system

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Abstract

Hepatitis C virus (HCV) exists in infected individuals as quasispecies, usually consisting of a dominant viral isolate and a variable mixture of related, yet genetically distinct, variants. A prior HCV infection system was developed using human hepatocellular carcinoma cells cultured in the three-dimensional radial-flow bioreactor (RFB), in which the cells retain morphological appearance and their differentiated hepatocyte functions for an extended period of time. This report studies the selection and alteration of the viral quasispecies in the RFB system inoculated with pooled serum derived from HCV carriers. Monitoring the viral RNA and core protein in the culture supernatants, together with nucleotide sequencing of hypervariable region 1 of the HCV genome, demonstrated that (1) the virus production intermittently fluctuated in the cultures, (2) the viral genetic diversity was markedly reduced 3 days post-infection (p.i.), and (3) dominant species changed on days 19–33 p.i., suggesting that the virus populations can be selected according to susceptibility to the viral infection and replication. A therapeutic effect of interferon-α also demonstrated the inhibition of HCV expression. Thus, this HCV infection model in the RFB system should be useful for investigating the dynamic behavior of HCV quasispecies in cultured cells and evaluating anti-HCV compounds.
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Keywords: Hepatitis C virus; Three-dimensional culture; Radial-flow bioreactor; Dynamics; Quasispecies

1. Introduction

Hepatitis C virus (HCV) is a major cause of chronic liver diseases (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990) and has been estimated to infect more than 170 million people throughout the world (Poynard et al., 2003). Symptoms of persistent HCV infection extend from chronic hepatitis to cirrhosis and ultimately hepatocellular carcinoma (Choo et al., 1989; Kuo et al., 1989; Saito et al., 1990). HCV belongs to the genus *Hepacivirus*, included in the family of Flaviviridae, and possesses a viral genome of a single, positive-stranded RNA with a nucleotide (nt) length of approximately 9.6 kb (Choo et al., 1991; Grakoui et al., 1993; Hijikata et al., 1991). It has been shown that HCV, like many other RNA viruses, circulates within infected individuals as a diverse population and closely related variants are referred to as quasispecies (Martell et al., 1992). This quasispecies model of mixed virus populations may imply a significant survival advantage because the simultaneous presence of multiple variant genomes and/or high rate of generation of new variants allow rapid selection of the mutants are better suited to new environmental conditions (Pawlotsky, 2006).

Studies on HCV replication and development of selective antiviral drugs have been hampered primarily by the lack of efficient cell culture systems. Establishment of selectable dicistronic HCV RNAs that are capable of autonomous replication to high levels in human hepatoma Huh-7 cells was a

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significant breakthrough in HCV research; however, virus production has not been observed in the conventional monolayer cultures (Blight et al., 2000; Lohmann et al., 1999). Recently, it has been described that infectious HCV particles are efficiently produced from a genotype 2a isolate JFH-1 in Huh-7 cells (Blight et al., 2000; Wakita et al., 2005; Zhong et al., 2005). This JFH-1 based HCV culture system is an invaluable achievement permitting a variety of studies on the complete HCV life cycle. However, HCV infection systems with human sera or plasmas containing intact virions are still limited because of low levels of propagation in the cultures. Reverse transcription (RT)-PCR was typically used to detect the viral RNA in cell extracts; however, synthesized viral proteins were not observed in these systems (Ikeda et al., 1998; Tagawa et al., 1995).

There are reports of differentiated human hepatoma FLC4 (functional liver cell 4) cells grown in a three-dimensional (3D) radial-flow bioreactor (RFB) that can be infected by HCV-positive serum and support viral replication (Aizaki et al., 2003). Furthermore, production and release of infectious HCV has been observed in the RFB system following transfection of FLC4 cells with *in vitro* transcribed HCV genomic RNA, as well as in a 3D system using Huh-7 cells harboring genome-length dicistronic RNAs (Murakami et al., 2006). The RFB system, in which the bioreactor column consists of a cylindrical matrix with porous bead microcarriers extended vertically, was aimed initially at developing artificial liver tissues and allows liver-derived cells to maintain morphological appearance as well as their physiological functions, such as the ability to synthesize albumin and drug-metabolizing activity mediated by cytochrome P450 (Iwahori et al., 2003). The radial-flow configuration permits full contact between culture medium and cells at a physiologic perfusion flow rate, and prevents excessive shear stresses and buildup of waste products, thus ensuring the long-term viability of 3D cell culture.

The aim of the present study was to characterize HCV dynamics in the RFB system during long-term cultures inoculated with pooled serum obtained from HCV carriers, and to examine the therapeutic effects of interferon-alpha (IFN-α) in this HCV infection model.

2. Materials and methods

2.1. Cell cultures

FLC4 cells (Aoki et al., 1998), which were derived from human hepatocellular carcinoma cells and negative for HCV RNA and HBV DNA, were maintained in serum-free ASF104 medium (Ajinomoto, Japan) supplemented with 4 g/L D-glucose on the collagen-coated dishes before inoculating into the RFB column. The RFB system (ABLE, Japan) was manipulated as described previously (Aizaki et al., 2003) with minor modifications. Briefly, RFB columns, which have bed volumes of 30 or 4 mL and are filled with porous glass microcarriers (diameter 0.6 mm, vacant capacity 50%, pore size <120 μm) (Hongo et al., 2005), were seeded with FLC4 cells, which subsequently attached to the surface and inside of porous glass beads. ASF104 medium containing 2% fetal calf serum was added at a flow rate of 50 mL/day, and the culture condition was automatically controlled by monitoring temperature, pH value and oxygen levels in the vessel throughout the duration of the study.

2.2. Infection of HCV-positive sera

HCV antibody-positive sera used in this study were blood donor samples supplied by The Japanese Red Cross Center, Tokyo, Japan. HCV RNA loads in the sera were as follows: serum A, 2.4×10^6 copies/mL; serum B, 8.6×10^6 copies/mL; serum C, 5.9×10^6 copies/mL; serum D, 2.5×10^6 copies/mL; serum E, 1.0×10^7 copies/mL; serum F, 1.4×10^7 copies/mL (Table 1). In the first experiment (Fig. 3), aliquots of each serum containing 2×10^6 copies of HCV RNA were mixed and pooled serum sample with 1.2×10^7 copies was prepared as an inoculum. The pooled serum (2.5 mL) was added to the 3D cultured-FLC4 cells in the 30-mL RFB column, and the culture medium was changed after 12 h of incubation. At various times during the culture period, culture medium (50 mL) was collected to determine HCV RNA and the core protein. Collected culture media were passed through a 0.20-μm filter to remove the debris, and stored at −80 °C. In the second experiment to evaluate a therapeutic effect of anti-HCV drug (Fig. 4), 4-mL RFB columns were used. IFN-α (Sumiferon 300; Sumitomo Pharmaceuticals, Japan) was added to one of two columns at a final concentration of 100 IU/mL after the infection. Culture medium was periodically collected for determination of HCV RNA, the core protein and transaminases, and was replaced with the same volume of fresh medium with or without IFN-α.

2.3. Quantitation of HCV RNA and core protein

HCV RNA was extracted from 140 μL of each serum or culture medium using QIAamp Viral RNA Mini spin column (QIAGEN); RNA was eluted in 60 μL of water and stored at −80 °C. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems), as described previously (Aizaki et al., 2003; Suzuki et al., 2005). The viral core antigen in the culture medium was quantified by immunoassay (Ortho HCV-Core ELISA Kit; Ortho-Clinical Diagnotics), according to the manufacturer's instruction (Murakami et al., 2006).

2.4. PCR amplification and nucleotide sequencing of HVR1 domain and its flanking region

Five microliters of RNA samples prepared as above were reverse transcribed using SuperScript II (Invitrogen) and a specific primer 5′-CATCCATGTGCAGCCGAACC-3′ (corresponding to nucleotides [nt] 2006–1987 of HCV NIHJ1) (Aizaki et al., 1998). For the nested PCR, a genotype-independent set of primers specific for hypervariable region 1 (HVR1). The first round of PCR was performed with the outer sense primer 5′-GCATGGCTTGGGATATGATG-3′ (nt 1291–1310) and with the reverse transcription primer described above as the outer antisense primer. After the initial 3.5-min denaturation step at 94 °C, 35 PCR cycles, with each cycle

Table 1
HCV-positive sera used in this study

Serum	Clone	HCV HVR1 sequence	% in the serum	genotype
A	A1	KVLIVMLSFAGVDGSTRTIGGRTAHTTQGSASLFSSGPAQKIQLINTNGS	75	1
	A2	-------L------N-H-V--AV-SS---FT---KL----------S---	12.5	
	A3	-------L------N-YAS---AGLL-R-V--I-TA----------S---	12.5	
B	B1	KVVVILLLAAGVDAGTNTIGGSAAQTTSGFTGLFRSGARQNIQLINTNGS	50	2
	B2	---R	12.5	
	B3	--S---------	12.5	
	B4	--L-V---F-----E-HVT--N-GR--A-LV--LTP--K-----------	12.5	
	B5	--I---	12.5	
C	C1	KVLIVMLLFAGVDGDTHVSGGTQGRAAYGLASLFALGPTQKIQLVNTNGS	83.3	1
	C2	--------------------------------------A-----------	16.7	
D	D1	KVLIVMLLFAGVDGVTHTSGAAAGHNARSLSGLFSLGSAQKLQLINTNGS	40	1
	D2	--------------A-Y---GT--Y-TKTFT-F--R-PS--I--------	20	
	D3	---------------------T--Y---T-T----P--------V-----	10	
	D4	--------------------------V---T----P--------V-----	10	
	D5	--V-----	10	
	D6	------------------------Y-T--FT----S-----I--V-----	10	
E	E1	KVLIVMLLFAGVDGSTRVSGGQAGRVTKSLASFFSPGPQQKIQLVNSNGS	40	1
	E2	---------------------------HGFT-L--A-S------------	30	
	E3	---------------------------QGFT-L--A-S------------	10	
	E4	---------------------------S-FT-L-TV--------------	10	
	E5	--------------N-Y---------AH--T-L--A-S------------	10	
F	F1	KVLIVMLLFAGVDGETNVMGGRAGHTTNTFTSLFSVGPAQKIQLVNSNGS	37	1
	F2	--------------D-K----------S-L----N---S-----------	27	
	F3	----------------K----Q-----S-L----N---S-----------	18	
	F4	-----------------------A-------A--TK---------D----	9	
	F5	--------------G--------A--A--L----TR--S-----------	9	

consisting of 1 min at 94 °C, 2 min at 45 °C, and 3 min at 72 °C, were carried out, followed by a 10-min extension step at 72 °C. The second round was performed with the inner sense primer 5′-GGTAAGCTTTCCATGGTGGGGAACTGGGC-3′ (nt 1419–1447) and the inner antisense primer 5′-CTGGAATTCGCAGTCCTGTTGATGTGCCA-3′ (nt 1627–1599). The amplified products were cloned into the pGEM-T vector (Promega), and at least 8 independent clones were sequenced with an automatic DNA sequencer (ABI PRISM 310, PE Applied Biosystems).

3. Results

3.1. The outline of the RFB system

The RFB system was initially aimed at developing artificial liver tissues and allows liver-derived cells to maintain morphological appearance as well as their physiological functions, such as the ability to synthesize albumin and drug-metabolizing activity mediated by cytochrome P450 (Iwahori et al., 2003). Fig. 1 shows the outline of the RFB system. The bioreactor column consists of a vertically extended cylindrical matrix with porous glass microcarriers, which were most suitable for FLC4 culture as described in Section 2. The conditioning vessel is connected to a circulation system including tanks either for supplying fresh medium or for recovering sample aliquots. Oxygen consumption, temperature and pH of the culture medium are monitored continuously and conditioned in the vessel by computer and mass flow controller throughout the culture. Thus, the radial-flow configuration permits full contact between culture medium and cells at a physiologic perfusion flow rate, and prevents excessive shear stresses and a buildup of waste products, thus ensuring the long-term viability of 3D culture. For the long-term culture up to 110 days, temperature in the vessel gradually decreased from 37 to 30 °C as shown in Fig. 2A. The oxygen consumption, which indicates the cell growth condition, increased slowly from days 0 to 80 post-inoculation of the cells, and maintained a constant level afterwards. Under this condition, the production rate of albumin was found to be stable from days 15 to 105. The following experiments of HCV infection were done in such a stable phase of the cell condition after 3 weeks of pre-culture. Cell grown in the RFB column reached confluence at the end of culture (day 110) since the cells were observed outside the matrix bed (Fig. 2B).

3.2. Infection of HCV-positive sera to RFB cultured FLC4 cells

Previously, HCV RNA could be detected in FLC4 cells grown in the RFB up to 4 weeks of culture following inoculation with an HCV carrier plasmid (Aizaki et al., 2003). Establishment of a long-term stable culture system of human liver-derived cells

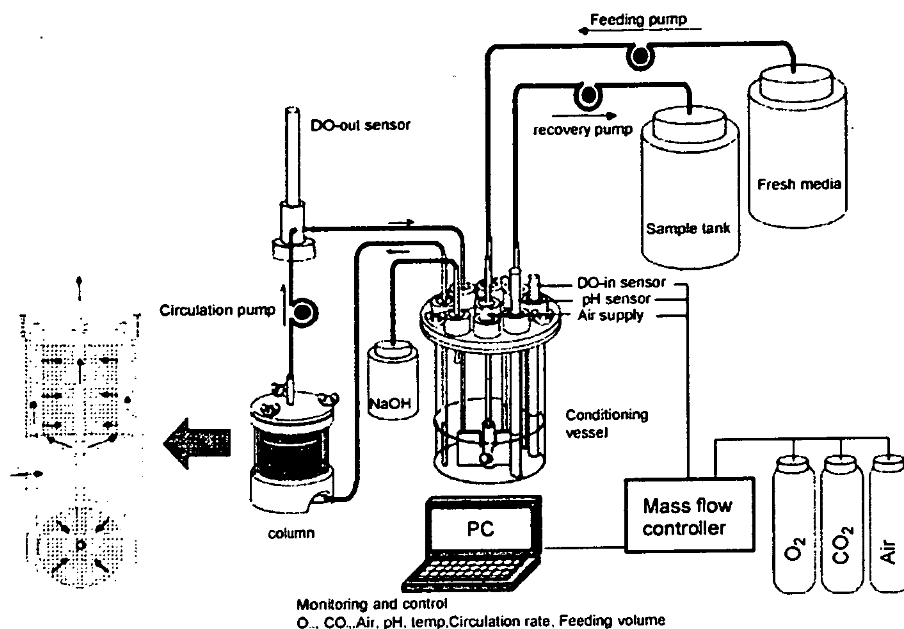


Fig. 1. Outline of the RFB system. RFB system consists of vessel, column and PC monitoring system. Culture condition was automatically controlled: oxgen concentration, temperature, pH, and oxgen level in the conditioning vessel are continuously monitored by PC and conditioned by mass flow controller.

retaining their differentiated hepatocyte function, as described above, enables evaluations of dynamic analysis of HCV replication and selection of viral variability and quasispecies. The potential of this culture system for screening HCV-positive sera was well suited for the viral infection.

Table 1 shows the serum samples (A–F) from six HCV carriers. The nucleotide complexity of HCV in serum samples was determined by sequencing the 1449–1598 nt region of the HCV genome, which includes HVR1 located at the N-terminal region of E2. Each serum was a mixture of a dominant HCV clone and related but distinct viral populations. The dominant species in sera A, C, D, E, and F were found to be genotype 1, and that in serum B was genotype 2. Viral loads in A–F, respectively, were 2.4×10^6, 8.6×10^6, 5.9×10^6, 2.5×10^6, 1.0×10^7 and 1.4×10^7 copies/mL, which were determined by real-time RT-PCR, as previously described (Aizaki et al., 2003; Suzuki et al., 2005). HCV loads of 2×10^6 copies from each serum sample were mixed to prepare a pooled serum sample containing 1.2×10^7 copies of HCV RNA. After FLC4 cells were inoculated into the RFB and subjected to 2 weeks of pre-culture for the preparation of 3D culture, the cells were infected with the pooled serum. Cell number at infection was about 10^8 in the 30-

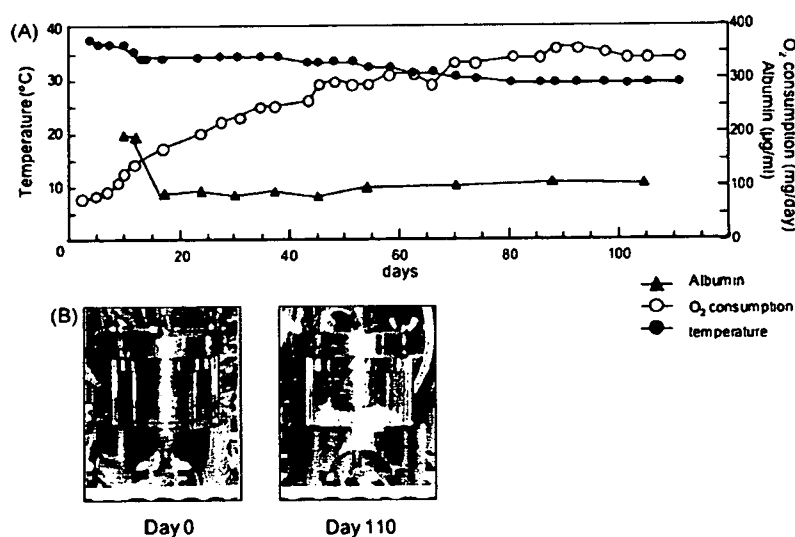


Fig. 2. Long-term culture of FLC4 cells in the RFB system. (A) Long-term culture of FLC4 cells in the RFB system. Temperature (closed circles) was gradually decreased from 37 to 30 °C. Oxygen consumption (open circles) was gradually increased from days 0 to 80 and reached the steady-state level. Albumin concentration (closed triangles) was constant from days 15 to 105. (B) The appearance of the RFB column at the beginning (day 0) and at the end (day 110) of culture.

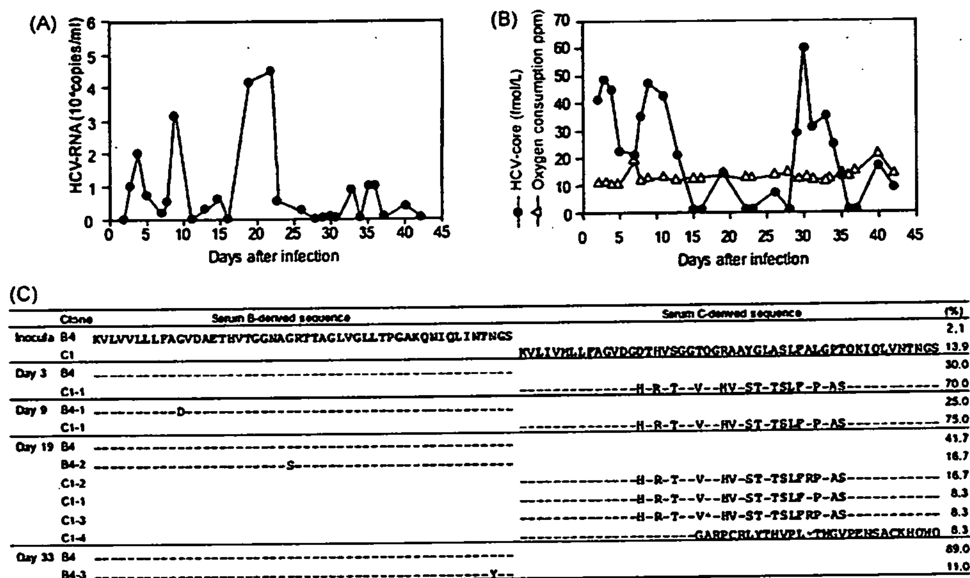


Fig. 3. HCV propagation in FLC4 cells cultured in the RFB system following inoculation with pooled sera obtained from HCV carriers. The 3D-cultured FLC4 cells were incubated with a pooled serum sample for 12 h, followed by changing the culture medium to fresh one. Culture medium was periodically collected for 42 days after inoculation, and HCV RNA and the viral core protein were quantified, respectively, by real-time RT-PCR and ELISA. (A) HCV RNA level in culture supernatant. (B) HCV-core protein (closed circles) and oxygen consumption (open triangles) levels in culture supernatant. (C) Changes in the viral quasispecies distribution after the inoculation. Percentages in the inoculum or in the culture medium at each time point (day 3, 9, 19, or 33 p.i.) are indicated at the right side. *, termination codon.

mL RFB column, as estimated from the glucose consumption (Kawada et al., 1998). Culture medium in the RFB was replaced with fresh medium 12 h post-infection (p.i.) and periodically sampled for 42 days.

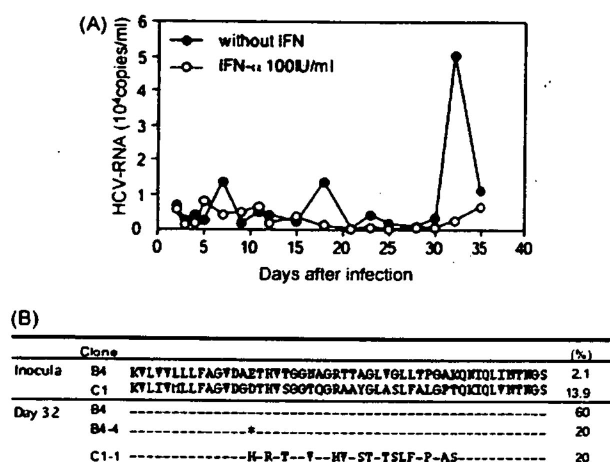
Fig. 3A and B shows the levels of HCV RNA and viral core protein in the culture medium, respectively. HCV RNA was not observed on the first 2 days following infection, but was detectable from day 3 p.i. Viral RNA levels fluctuated, with peaks on days 3, 9, 19–21 and 33–36 p.i. At days 19–21 p.i., the average amount of HCV RNA detected in the culture supernatant was approximately 3×10^6 copies/day. Intermittent peaks were observed in HCV core protein levels in the culture supernatant, and the peak pattern of the core protein was largely consistent with that of viral RNA. During the infection experiment, the level of oxygen consumption was constant at approximately 12 ppm, thus suggesting that the desired conditions (constant or very gradually increasing cell number) were maintained.

3.3. Quasispecies analysis in RFB culture

The above results suggest that, although the environment was consistent in the pooled serum infection, there were periods in which the viruses actively replicated and released from the cells and periods in which they poorly replicated. The pooled serum used for the infection exhibited HCV populations had at least 26 distinct quasispecies (Table 1). To investigate whether the quasispecies distribution was altered due to infection, and whether HCV populations are selected during long-term culture in the RFB, total RNA was extracted from the culture supernatant samples collected on days 3, 9, 19 and 33 p.i., and the nucleotide sequence of the region containing HVR1 was determined, as described above. As shown in Fig. 3C, it is of interest that only two HCV species were detected in the sample at day 3 p.i.; the dominant clone C1-1, comprising approximately 70% of the viral population, and clone B4, comprising 30%. Although clone C1-1 was not detected in the sequence of the inoculum shown in Table 1, it was most similar to clone C1, a dominant clone in plasma C, among the HCV population observed in the inoculum; thus, it is possible that clone C1-1 is one of the minor species in serum C. Clone B4 was found to be derived from serum B. An almost identical HCV population was observed in the sample at day 9 p.i. In this sample, the dominant clone C1-1 and clone B4-1, which differs from clone B4 by only one amino acid, were detected. In contrast, more significant variation in quasispecies structure of the HCV species was observed in the sample at day 19 p.i. than that at day 9 p.i. With B4 as the dominant clone, the serum B-derived HCV species, clones B4 and B4-2, which differs from clone B4 by one amino acid, comprised 58% of the total population. Four types of HCV sequences derived from serum C were detected. Two of these (clones C1-3 and C1-4) contained lethal mutations. It was also found that the HCV species detected in the sample at day 33 p.i. included only two clones (clones B4 and B4-3), derived from serum B. The dominant clone, B4, was found to comprise 89% of the total population.

3.4. Potential use of the RFB system for evaluation of anti-HCV compounds

An experiment was carried out to determine whether this HCV infection experiment system was useful for the evaluation of anti-HCV drugs (Fig. 4). For this purpose, a small,



	Clone		(%)
Inocula	B4	KVLVVLLLFAGVDAETHVTGGNAGRTTAGLVGLLTPGAKQNIQLINTNGS	2.1
	C1	KVLIVMLLFAGVDGDTHVSGGTQGRAAYGLASLFALGPTQKIQLVNTNGS	13.9
Day 32	B4	--	60
	B4-4	-------------*------------------------------------	20
	C1-1	--------------H-R-T--V--HV-ST-TSLF-P-AS-----------	20

Fig. 4. A therapeutic effect of IFN in HCV infection model in the RFB cultures. HCV-infected FLC4 cells were treated with or without 100 IU/mL IFN-α. (A) Culture media were periodically collected, and HCV RNA levels were determined. Closed circles: without IFN treatment, open circles: treatment with IFN. (B) Changes in the viral quasispecies distribution in the cells without IFN treatment. Percentages in the inoculum or in the culture medium on day 32 p.i. are indicated at the right side. *, termination codon.

4-mL RFB column was adopted and a pair of RFB cultures infected with the HCV-positive pooled plasma (Table 1) was prepared. IFN-α was added to one culture at a final concentration of 100 IU/mL at 12 h p.i. No cytotoxicity was observed in FLC4 cells under these conditions (data not shown). Culture media from two cultures (12.5 mL each) were sampled periodically for 35 days and replaced by the same volume of fresh medium in the presence or absence of IFN-α. HCV RNA in the collected media was quantified by real-time RT-PCR, as described above. As shown in Fig. 4A, in the no-treatment culture, fluctuations in the viral RNA levels with the peaks on days 7, 18, and 32 p.i. (1.5–5 × 10^4 copies/mL) were observed. However, while HCV RNA at 0.5–0.8 × 10^4 copies/mL was detected in the IFN-treated culture at days 5–11 p.i., no HCV RNA was detected at days 12–30 p.i. Serum levels of hepatic transaminases such as ALT and AST are known to be markers of liver damage. In the HCV-infection model with FLC4 cells cultured in RFB, the AST levels in the culture medium, which ranged from 5 to 10 IU/L without HCV infection, increased to 20–50 IU/L according to the viral infection (data not shown). Such increased AST levels were found to fall by the IFN treatment to lower than 10 IU/L at day 28 p.i. As reported previously, the ALT levels in the culture medium were constantly low; its levels were less than 10 IU/mL, with or without HCV infection (Aizaki et al., 2003). The viral nucleotide sequence in the no-treatment culture medium at day 32 p.i. was determined. It was found that serum B-derived clone B4 was dominant, and serum C-derived clone C1 was present as a minor clone (Fig. 4B); thus, the results corresponded well with those demonstrated in Fig. 3. An increase in viral RNA in the IFN-treated culture after day 32 p.i. was observed; although the degree of increase was only slight (Fig. 4A). It will be interesting to test whether HCV species grown in the IFN-treated culture is a variant resistant to IFN-α.

4. Discussion

At present an important limitation of the *in vitro* HCV infection system is that the only established culture system is based on genotype 2a, JFH-1 isolate, and Huh-7-derived cell lines. The development of alternate infection systems in which other HCV strains and host cells are available has been needed for the study of HCV dynamics and virus–host interactions, and for testing antivirals. This paper demonstrates that a long-term culture of the 3D RFB system is a useful tool for investigating HCV dynamics. The present results revealed that the viral quasispecies distribution altered in the HCV infection system in the RFB system. The change probably occurs in the following two-stage process. The first change was observed on day 3 p.i.; thus, it is possible that the HCV species were selected according to infectivity in FLC4 cells. It has been reported that HCV particle populations in chronic hepatitis C patients consist of low-density virions and higher-density immune complex forms (Hijikata et al., 1993; Kanto et al., 1994). Inoculation of cultured cells with HCV has demonstrated that the immune complex forms were less infective than the antibody-unbound virions (Shimizu et al., 1994). Therefore, another hypothesis may be that a large number of HCV populations in sera A, D, E, and F are immune complex forms; thus, these sera are less susceptible to the cells than sera B and C. The second change was observed on days 19–33 p.i. While the serum C-derived clone was dominant in the early stages after infection, the serum B-derived HCV clone became dominant over time. In the absence of immunological selection pressure, viral nucleotide mutations at random positions are accumulated during viral replication, and the newly generated variant species are selected principally, if not solely, based on the intrinsic replicative advantages or disadvantages that these mutations confer. Thus, these results suggest that the use of pooled serum sample allowed for screening of infectious materials compatible for the RFB culture.

Evaluation methods for anti-HCV drugs using monolayer culture systems with various culture cells, such as the replicon system and the JFH-1 based virion production system, have been reported (Bartenschlager et al., 2003; Blight et al., 2000; Boriskin et al., 2006; Lanford et al., 2003; Lindenbach et al., 2005; Lohmann et al., 1999; Wakita et al., 2005; Zhong et al., 2005). These methods utilize viral markers, such as HCV RNA and antigens, as indicators of treatment efficacy. However, the utility of long-term cell culture systems for anti-HCV drug evaluation based on infection with human sera is still limited. The use of a chimpanzee model, the only non-human host for HCV infection, is restricted due to several reasons such as problematic availability and ethical consideration. Given intensive efforts to reduce and replace animal testing in the course of development of new therapies worldwide, the RFB-based HCV infection model is a potential alternative to animal models such chimpanzee for assessing anti-HCV compounds. According to the studies with regards to mathematical modeling of HCV kinetics (Dahari et al., 2005; Dixit et al., 2004; Layden et al., 2003; Layden-Almer et al., 2006; Perelson et al., 2005), IFN therapy against HCV infection generally generates a biphasic decline in viral load; there is a rapid decrease in the serum HCV RNA level over the

first 1 day of treatment, followed by the second phase, which is slower than the first-phase viral decline. To date, there were no such observable viral kinetics in the IFN treatment under such experimental settings. Further detailed kinetic analyses of the use of varying doses of IFN and of very early time points to evaluate the antiviral effect are in progress.

In summary, by investigating the dynamics of HCV populations in the RFB culture system, it was demonstrated that HCV was intermittently detected in the culture supernatants of long-term culture, and that changes in viral quasispecies appear to be related to this fluctuation in the virus level. It was also shown that an HCV-infection model using the RFB system is useful for evaluating potential antivirals. Further investigation on the infection and growth of various HCV-positive sera is currently being conducted in order to obtain an adaptive clone with higher replication efficiency in this culture system.

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Efficient regulation of viral replication by siRNA in a non-human primate surrogate model for hepatitis C

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Abstract

RNA interference (RNAi) represents a new technology which could offer potential applications for the therapeutics of human diseases. RNAi-mediated therapy has recently been shown to be effective toward infectious diseases in *in vitro* and rodent models, however, it remains unclear whether RNAi therapy with systemic application could be effective in primates. In this study, we examined if RNAi therapy could be effective toward infectious diseases by using a non-human primate surrogate model for hepatitis C. Administration into marmosets of cationic liposome-encapsulated siRNA (CL-siRNA) for GB virus B (GBV-B), which is most closely related to hepatitis C virus, repressed GBV-B replication in a dose-dependent manner. Especially, 5 mg/kg of the CL-siRNA completely inhibited the viral replication. Since the serum interferons (IFNs) were induced by CL-siRNA *in vivo*, inhibition of viral regulation by anti-GBV-B CL-siRNA may include an antiviral effect of IFN. However, contribution of induced IFN may be partial, since the control CL-siRNA which induced a stronger IFN response than GBV-B CL-siRNA could only delay the viral replication. Our results suggest the feasibility of systemic administration of CL-siRNA as an antiviral strategy.
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Keywords: siRNA; Hepatitis C; Marmoset; Interferon; GB virus B

RNA interference is a powerful tool for silencing gene expression and has spurred considerable interest in its experimental and therapeutic potential. RNAi has been characterized as a cellular process of post-transcriptional gene silencing. An RNaseIII-like enzyme, called Dicer, cleaves double stranded RNA (dsRNA) in to 21–23 nucleotide RNA duplex, termed small interfering RNAs (siRNAs). siRNAs are unwound in the RNA-induced-silencing-complex (RISC), and single-stranded siRNAs then act as a guide to substrate selection, leading to the cleavage of a homologous target RNA molecule [1].

Hepatitis C virus (HCV) infection contributes significantly to human morbidity and mortality worldwide. It is estimated that 40–60% of infected individuals progress to chronic liver disease, and many of these patients develop liver cirrhosis and hepatocellular carcinoma [2]. Currently,

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