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Original Article

Transcriptomic Comparison of Human Hepatoma Huh-7 Cell Clones with Different Hepatitis C Virus Replication Efficiencies

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SUMMARY: Hepatitis C virus (HCV) infection represents a major public health problem throughout the world. The establishment of viral replicons has enhanced our understanding of the mechanism underlying HCV replication. However, the specific virus-host cell interactions involved in HCV RNA replication are not well understood. In the present study, we isolated several human hepatoma Huh-7-derived subclones with a range of HCV RNA replication efficiencies by end-point dilution. Of these, the clones HuhTe4 and HuhTe6 were observed to proliferate at the same rate; however, HuhTe6 supported a significantly greater degree of viral RNA replication. Using cDNA microarray analysis, a total of 36 genes (0.4%) demonstrated variable expression, with a ≧2-fold difference in expression noted between HuhTe4 and HuhTe6. Among genes that are implicated in a variety of functional categories, a subset of these differentially-expressed genes has a role in signal transduction and cell communication, including thioredoxin-interacting protein, Rab6B, sorting nexin 16 and UDP-galactose:ceramide glycosyltransferase. The genes identified in this study should be examined further to determine their roles in HCV RNA replication. The Huh-7 subclones identified in this study provide a tool for identifying novel host factors involved in viral replication.

INTRODUCTION

Hepatitis C virus (HCV) is a positive-strand RNA virus that belongs to the family *Flaviviridae*, which causes acute and chronic hepatitis, as well as hepatocellular carcinoma (1,2). The HCV genome encodes a long polyprotein precursor of approximately 3,000 amino acids that is processed into at least 10 proteins: core, envelope 1 (E1), E2, p7, nonstructural protein 2 (NS2), NS3, NS4A, NS4B, NS5A, and NS5B (3, 4). The nonstructural proteins are processed by two viral proteases; the junction of NS2 and NS3 is cleaved by NS2-3 protease, which spans NS2 and the N-terminal domain of NS3, while four other junctions are cleaved by a serine protease located at the N-terminal 180 residues of NS3. Helicase and nucleotide triphosphatase, which are essential for HCV replication, reside in the C-terminal 500 residues of NS3 in addition to N-terminal protease. NS4A functions as a cofactor for NS3 serine protease and is required for efficient polyprotein processing. Although the replicative functions of NS4B and NS5A have yet to be identified, both are thought to play a role in viral replication. NS4B, a hydrophobic and membrane-associated protein, may contribute to the formation of the HCV RNA replication complex. NS5A is a phosphorylated protein, and most cell culture-adaptive mutations are located within the NS5A region. NS5B is a RNA-dependent RNA polymerase of HCV. A recently established system, which uses a JFH-1 clone isolated from a Japanese patient with fulminant hepatitis C in order to produce infectious HCV particles in cell culture (5-7), is very useful for examining the HCV life cycle. Our understanding of HCV RNA replication has also been enhanced by the establishment of a HCV replicon system (8). Subgenomic and genome-length HCV RNA replicates efficiently and stably under selective pressure in the human hepatoma cell line, Huh-7. The nonstructural proteins NS3 through NS5B are necessary and sufficient for HCV RNA replication (8,9). A relationship between viral replication and physiological status of the host cell, in particular its stage of cell proliferation, is known to exist. HCV RNA replicates efficiently in the early logarithmic growth phase, while RNA levels promptly decline when cells reach the stationary phase (10-12). A number of studies have identified host-cell factors involved in HCV RNA replication (13-21). However, the molecular mechanisms underlying the regulation of viral RNA replication through virus-host interactions remain unclear.

To gain further insight into the various host factors involved in the regulation of HCV replication, we established Huh-7 subclones with different HCV replication efficiencies by limiting the dilution and used cDNA microarray analysis to identify differentially-expressing genes among cell clones with different rates of viral RNA replication.

MATERIALS AND METHODS

Cell culture and single cell cloning: Human hepatoma Huh-7 cells were cultured in Dulbecco's modified Eagle medium (DMEM) (Sigma-Aldrich, St. Louis, Mo., USA) supplemented with 50 IU/ml penicillin, 50 μg/ml streptomycin (Invitrogen, Carlsbad, Calif., USA), and 10% (vol/vol) fetal bovine serum (FBS) (JRH Biosciences, Lenexa, Kans., USA). To obtain Huh7-derived subclones, the cells were diluted to 0.5 cells/well in 96-well plates and grown in complete DMEM as above. Stable cells were selected and four

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clones identified: HuhTe3, HuhTe4, HuhTe6, and HuhTe7.

In vitro transcription, RNA transfection, and selection of G418-resistant cells: The replicon constructs used in this study were kindly provided by Ralf Bartenschlager, University of Heidelberg, Germany. Linearized pFKI389neo/NS3-3'/NK5.1 (22,23) and pFKI389Luci/NS3-3'/NK5.1 (22) with *Sca*I were used as the template DNA for in vitro RNA transcription (AmpliScribe™ T7 High Yield Transcription Kits; EPICENTRE Biotechnologies, Madison, Wis., USA). The concentrations were determined by measuring the optical density at 260 nm, and RNA integrity was confirmed by agarose gel electrophoresis. Parental and subcloned Huh-7 cells (10^7) were electroporated with 50 μg of RNA in K-PBS (30 mM NaCl, 120 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, and 5 mM $MgCl_2$, pH 7.9). The electroporation conditions were 975 μFD and 290 mV using a Gene Pulser system (Bio-Rad Laboratories, Hercules, Calif., USA) and a cuvette with a gap width of 0.4 cm (Bio-Rad Laboratories). For the selection of G418-resistant cells, the transfectants were immediately transferred to 10 ml of complete DMEM and seeded into a 10 cm-diameter cell culture dish. After 24 to 48 h, the medium was replaced by complete DMEM supplemented with 0.5 mg/ml of G418.

Luciferase reporter assay: After Huh-7 cells were transfected with the luciferase-replicon, DMEM with 10% FBS was added, and the cell suspension was seeded into 24-well plates. At the time-points specified in the Results section, the cells were washed once with phosphate-buffered saline and then lysed with 400 μl of cell culture lysis reagent (Promega, Madison, Wis., USA). Aliquots of the lysate samples were mixed with luciferase assay reagent (Promega), after which measurements were performed with a luminometer, LUMAT LB9501 (Berthold Technologies, Bad Wilbad, Germany). Assays were performed at least in triplicate.

Determination of cell growth: To examine cell growth, 10^4 cells per well were seeded into 24-well culture plates and harvested daily. Cells from triplicate wells were lysed with 100 μl of cell culture lysis reagent, and viable cell numbers were measured using Celltitre Glo Luminescent Cell Viability Assay (Promega).

Quantitation of HCV RNA: Total RNA was extracted from cells using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. Real-time RT-PCR was performed using TaqMan EZ RT-PCR Core Reagents (PE Applied Biosystems, Foster City, Calif., USA), as described previously (24-26).

Analysis of gene expression by microarray: Total RNA was isolated from cells using TRIzol reagent and purified using the RNeasy mini kit (Qiagen, Valencia, Calif., USA). The integrity of the RNA was assessed qualitatively by electrophoresis and spectrophotometry using a ratio of A260/A280. Antisense biotinylated cRNA target probes were synthesized from total RNA using GeneChip One-Cycle Target Labeling and Control Reagents (Affymetrix, Santa Clara, Calif., USA), according to the manufacturer's instructions. Probes were individually hybridized to the Human Genome Focus Array (Affymetrix) representing ~8,500 characterized human genes. Arrays were washed and stained with streptavidin-phycoerytrin in Fluidic Station 400 (Affymetrix), and scanned for fluorescence using the GeneChip Scanner 3000 (Affymetrix). Microarray data were processed using GeneChip Operating Software (GCOS; Affymetrix).

RESULTS AND DISCUSSION

Most experiments to date have used Huh-7 cells to examine the expression of HCV replicons. However, it is accepted that the Huh-7 cell line is not homogeneous but rather heterogeneous, as it is inconsistent with regard to the level of HCV RNA replication among the cells maintained in laboratories. To determine whether the Huh-7 cells used in the present experiment also exhibit heterogeneity in terms of HCV replication, a pFKI389neo/NS3-3'/NK5.1 transcript, which is a Con1-derived subgenomic HCV replicon with adaptive mutations, was introduced into Huh-7 cells, after which the transfected cells were grown for 2 weeks under G418 selection. We then isolated 20 of the drug-resistant colonies and quantified HCV RNA in each clone by real-time RT-PCR. As shown in Fig. 1A, the levels of viral RNA varied over a wide range, from 1.7×10^4 to 2.2×10^7 copies/μg total RNA, in cells supporting HCV replication, suggesting that the Huh-7 cells comprised a variety of cell populations with different HCV replication efficiencies. Adaptive mutations of the replicon RNA were unlikely to occur during culture since a highly adapted replicon was used.

To further characterize the diversity of HCV permissiveness among cells, we isolated Huh-7 cell clones using an end-point dilution technique and stabilized four clones: HuhTe3, HuhTe4, HuhTe6, and HuhTe7. These clones were transfected with the replicon RNA as described above and cultured in the presence of G418. As shown in Fig. 1B, HCV RNA levels were comparable among the four clones one day after transfection, indicating that similar amounts of viral RNA were introduced into each cell clone. Fourteen days after transfection, approximately 10^6 copies of HCV RNA/μg total RNA were found among the HuhTe6 and HuhTe7 transfectants, while less than 10^5 copies of HCV RNA/μg total RNA were observed among HuhTe3- and HuhTe4-derived cells, a relatively small amount compared to parental

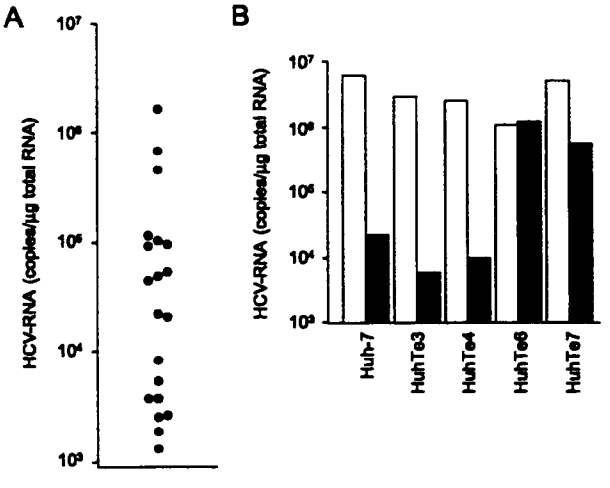

Fig. 1. Copy numbers of HCV RNA after transfection of Huh-7 cells and Huh-7 subclones with subgenomic HCV replicon RNA derived from pFKI389neo/NS3-3'/NK5.1. (A) Transfected Huh-7 cells were subjected to G418 selection. Eighteen colonies were isolated after transfection and cultured in a 96-well plate for 1 week, followed by extraction of total cellular RNA. Dots indicate the copy numbers of HCV RNA per total cellular RNA among individual cell clones, as quantified by real-time RT-PCR. (B) HCV RNA levels in transfected cells (HuhTe3, HuhTe4, HuhTe6, HuhTe8 and parental Huh-7 cells) were determined as described above on day 1 (open bars) and day 14 (closed bars) post-transfection.

Huh-7 cells.

Because the cell growth conditions of HuhTe4 and HuhTe6 were more stable than those of the other two clones after many passages and reproducible results were obtained using HuhTe4 and HuhTe6 cells in the above experiments, the differences in HCV RNA replication efficiencies among HuhTe4 and HuhTe6 cells were further assessed using a luciferase reporter-based transient replication assay enabling sensitive and precise quantification of HCV replication (Fig. 2A). HuhTe4 and HuhTe6 cells were transfected with replicon RNA encoding firefly luciferase as a reporter gene (22) by electroporation. The cells were harvested daily for up to 72 h post-transfection, and luciferase activity was monitored to examine the replication kinetics of replicon RNA in HuhTe4 and HuhTe6 cells. Replicon RNA replicated at low levels in HuhTe4 cells during this time. Conversely, a steady increase in replication was observed in HuhTe6 cells with ~10-fold greater luciferase activity than observed in HuhTe4 cells at 72 h post-transfection. Luciferase activity measured 4 h after transfection was used to verify transfection efficiencies. Thus, two

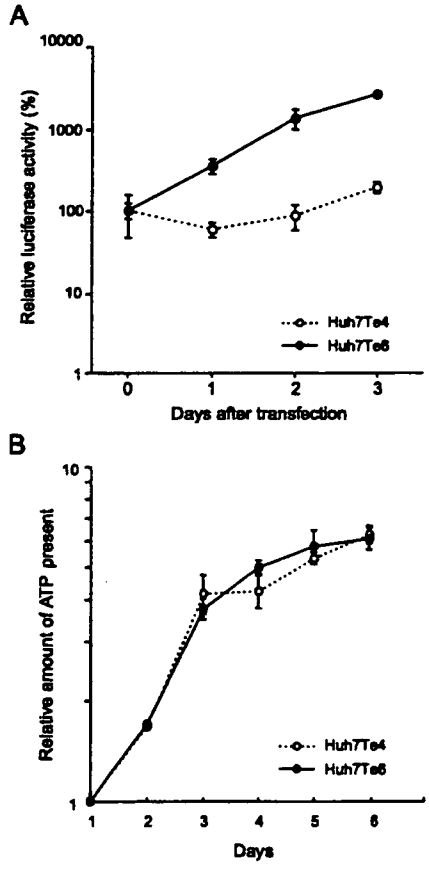

Fig. 2. Cell growth of HuhTe4 and HuhTe6 cells, and transient replication of the HCV replicon. (A) Transient replication of the HCV replicon carrying a luciferase gene in HuhTe4 (open circles) and HuhTe6 (closed circles) cells. Replicon RNA was transfected into the cells by electroporation. Luciferase activity within the cell lysate samples was determined and expressed as a percentage of the relative light units measured at 24, 48, and 72 h, compared to 4 h post-transfection. Each data point indicates the mean and standard deviation of triplicate results. (B) Cell growth of HuhTe4 (open circles) and HuhTe6 (closed circles) cells. Cell growth rates were determined at the indicated time points and are presented as relative values compared to day 1. Each data point indicates the mean and standard deviation of triplicate results.

different replication assays were used to demonstrate that the efficiency of HCV RNA replication in HuhTe6 cells is greater than that in HuhTe4 cells.

HCV replication is related to the physiological state of the host cells, specifically the stage of cell growth (10-12). For example, Huh-7 cells containing the HCV genome carry numerous copies of viral RNA in the early logarithmic growth phase, while RNA levels decline significantly when cells reach the stationary phase. Flow cytometry has shown that synthesis of HCV RNA is specifically stimulated during the S phase of the cell cycle in replicon cells (11). Therefore, HCV replication efficiency might be influenced by the stage of cell growth of Huh7 subclones. We therefore compared the cell growth properties of HuhTe4 and HuhTe6 cells using a cell-based luminescence assay, as described in the Materials and Methods section. As shown in Fig. 2B, both cell clones exhibited similar growth curves with a doubling time of 37-40 h during the exponential growth phase, as well as similar saturation densities under the specified culture conditions, indicating little to no difference between the cell growth of HuhTe4 and HuhTe6 cells. Thus, the differences in their ability to support HCV RNA replication cannot be due to differences in their cell growth conditions.

To further understand the inherent diversity of these clones and to identify any factor(s) which might play a role in the permissiveness of HCV replication, we looked for cellular genes with different transcript levels in HuhTe6 and HuhTe4 cells using a cDNA microarray system of approximately 8,500 known genes. Hybridization image analysis showed enhanced expression of 17 genes (0.2%) and reduced expression of 19 genes (0.2%) in HuhTe6 cells compared with HuhTe4 cells, when the 2-fold change in the signal intensity with statistical significance is considered to be a difference limit for RNA expression. The genes that are up-regulated in HuhTe6 cells are listed in Table 1. Among the genes implicated in a variety of functional categories, some of the genes related to signal transduction and/or cell communication may be fascinating. Thioredoxin-interacting protein is an endogeneous inhibitor of thioredoxin. The thioredoxin system is a ubiquitous thiol oxidoreductase system that regulates cellular reduction/oxidation status. Thioredoxin-interacting protein negatively regulates thioredoxin activity and affects cellular redox status (27). A complex relationship between HCV replication and redox signaling has been revealed. HCV infection is associated with elevated circulating reactive oxygen species (ROS) in patients (28,29), while viral gene expression in cultured cells increases ROS levels through calcium signaling (30). Conversely, biologically relevant concentrations of ROS may suppress HCV RNA replication in Huh-7 cells (31). Rab6B is a member of the Rab subfamily of small GTPases, which plays an important role in the regulation of intracellular transport routes. Rab6B, which mainly localizes at the Golgi apparatus and at ERGIC-53-positive vesicles, may enable retrograde membrane traffic at the level of the Golgi complex (32). Golgi complex-derived lipid raft or membranous webs are known to contain the HCV replication complex (33-35). This raises the possibility that Rab6B-associated intracellular transport might be involved in the assembly and formation of the HCV replication complex. Transglutaminase 2 is a multifunctional protein involved in a range of cellular processes. It has two well-characterized activities: GTP-mediated receptor-stimulated signaling, and calcium-activated transamidation or cross-linking, which is inhibited by GTP (36). Transglutaminase influences the HCV life cycle through

Table 1. Genes with increased levels of expression in HuhTe6 cells compared to HuhTe4 cells

Functional category/Gene name	Accession no.	Fold change
Signal transduction/cell communication		
sema domain, immunoglobulin domain, short basic domain, secreted, 3G	NM_020163	8.6
thioredoxin interacting protein	NM_006472	8.0
stathmin-like 4	NM_030795	5.7
RAB6B, member RAS oncogene family	AW118072	4.0
erythropoietin receptor	X97671	2.6
Metabolism		
sulfotransferase family 1E, estrogen-preferring, member 1	NM_005420	13.9
NADPH oxidase 1	NM_007052	6.1
transglutaminase 2	AL031651	2.1
Transcription		
v-rel reticuloendotheliosis viral oncogene homolog	NM_002908	9.8
Zic family member 3 heterotaxy 1	NM_003413	9.2
neurogenic differentiation 2	AB021742	4.3
Transport		
solute carrier family 10, member 1	NM_003049	2.0
solute carrier organic anion transporter family, member 1C1	NM_017435	2.0
Apoptosis		
phorbol-12-myristate-13-acetate-induced protein 1	NM_021127	16.0
Immune response		
triggering receptor expressed on myeloid cells 1	NM_018643	2.1
Unknown		
similar to RNA binding motif protein, Y chromosome, family 2 member B	NM_005405	9.2
similar to chymotrypsinogen B precursor	NM_001906	4.0

Table 2. Genes with decreased levels of expression in HuhTe6 cells compared to HuhTe4 cells

Functional category/Gene name	Accession no.	Fold change
Signal transduction/cell comminication		
purinergic receptor P2Y, G-protein coupled, 2	BC012104	7.5
activin A receptor type II-like 1	BC042637	4.9
RAB3B, member RAS oncogene family	NM_002867	3.2
adenomatosis polyposis coli	BC111591	2.6
HUS1 checkpoint homolog (S. pombe)	BT019482	2.3
progesterone receptor membrane component 2	DQ496105	2.0
Metabolism		
UDP glycosyltransferase 8 (UDP-galactose ceramide galactosyltransferase)	NM_003360	4.9
bone morphogenetic protein 1	BC101765	2.3
Transcription		
general transcription factor IIH, polypeptide 3, 34 kDa	NM_001516	2.8
Translation		
eukaryotic translation initiation factor 5A2	NM_020390	4.0
Transport		
Rh-associated glycoprotein	NM_000324	7.5
sorting nexin 16	BC0336301	7.5
solute carrier family 7, member 2	BC10490	2.0
Immune response		
GLI pathogenesis-related 1	NM_006851	7.5
Unkown		
neuronal thread protein AD7c-NTP	AF10144	7.5
elastase 2B	BC069412	6.5
NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4-like 2	NM_020142	3.5
ring finger protein 17	BC064847	2.8
hypothetical protein LOC440345	XR_015786	2.3

post-translational modification of the viral core protein (37), and induction of hepatic fibrosis as a result of HCV infection (38).

Genes down-regulated in HuhTe6 cells, compared to HuhTe4 cells, are listed in Table 2. Of interest is the differential expression of sorting nexin 16 and UDP-galactose:ceramide glycosyltransferase among the two cell lines. Sorting nexins are a family of cytoplasmic and membrane-associated pro-

teins that mediate the intracellular trafficking of plasma membrane receptors, such as the epidermal growth factor (EGF) receptor. Sorting nexin 16 is associated with the EGF receptor and accelerates EGF-induced EGF receptor down-regulation (39). It has been shown that HCV NS5A protein, as well as the viral replicon, inhibit EGF-stimulated activation of the Ras-ERK activated protein kinase pathway (40-42). Since signaling pathways downstream from the EGF receptor are known to regulate a variety of cellular processes, thereby influencing cell survival, cytoskeletal rearrangement, vesicular trafficking, and protein synthesis, any perturbation of the events prior to the activation of the EGF receptor may influence the cellular environment to favor HCV replication and persistence. UDP-galactose:ceramide glycosyltransferase mediates galactosylation of glycosphingolipids associated with the Golgi UDP-galactose transporter. Recent studies based on the chemical biological approach have demonstrated the physiological significance of the lipid raft and sphingolipid biosynthesis pathways in HCV RNA replication (14,24,43,44). Altered processing of glycosphingolipids may directly or indirectly affect the HCV life cycle, presumably through modulation of lipid-rich microdomains containing the viral replication complex.

In the present study, we isolated four subclones derived from parental Huh-7 cells with a range of HCV RNA replication efficiencies. Among the four subclones, HuhTe4 and HuhTe6 demonstrated similar cell growth, however the efficiency of HCV RNA replication in HuhTe6 cells was significantly greater than that in HuhTe4 cells. The fact that these subclones share a common origin enables us to explore the differences that result in their different HCV replication efficiencies. cDNA microarray analysis showed significant differences in the transcript levels of 36 genes among HuhTe4 and HuhTe6 cells. Detailed analysis of the correlation between the expression of the candidate genes identified and HCV replication as well as studies to determine the role of these genes in HCV RNA replication are underway. This approach will enable us to expand our research, thereby improving our understanding of the regulatory mechanisms underlying HCV replication.

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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.

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

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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

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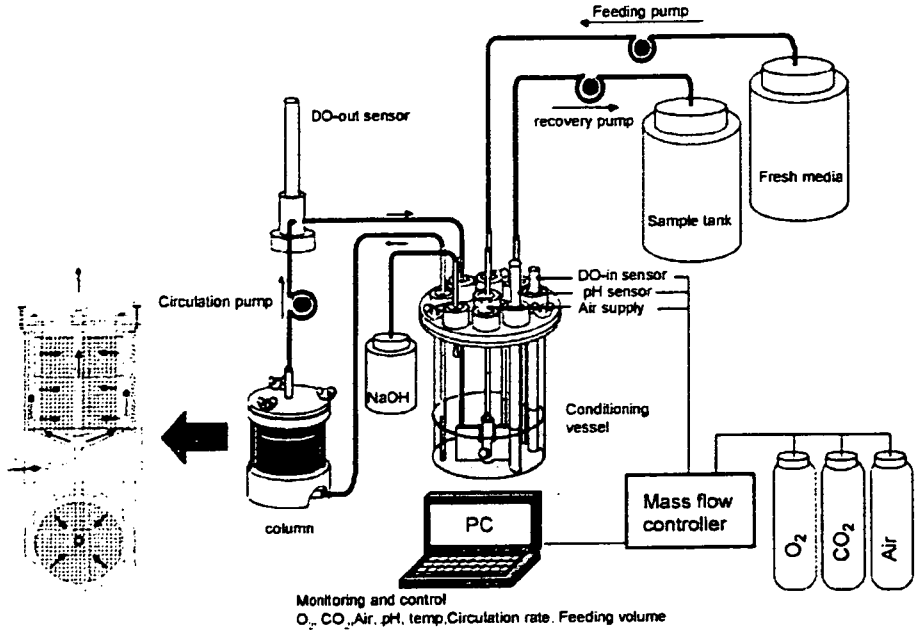

Fig. 1. Outline of the RFB system. RFB system consists of vessel, column and PC monitoring system. Culture condition was automatically controlled: oxgen concentration, temperature, pH, and oxgen level in the conditioning vessel are continuously monitored by PC and conditioned by mass flow controller.

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-

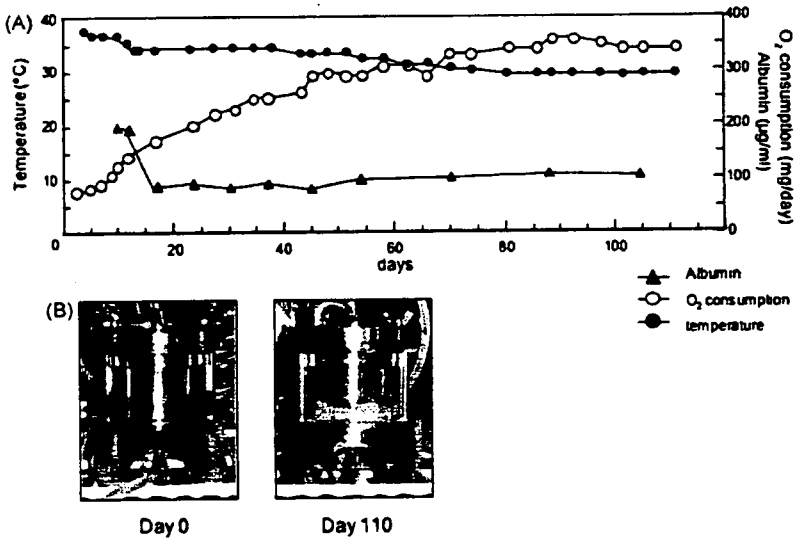

Fig. 2. Long-term culture of FLC4 cells in the RFB system. (A) Long-term culture of FLC4 cells in the RFB system. Temperature (closed circles) was gradually decreased from 37 to 30 °C. Oxygen consumption (open circles) was gradually increased from days 0 to 80 and reached the steady-state level. Albumin 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.

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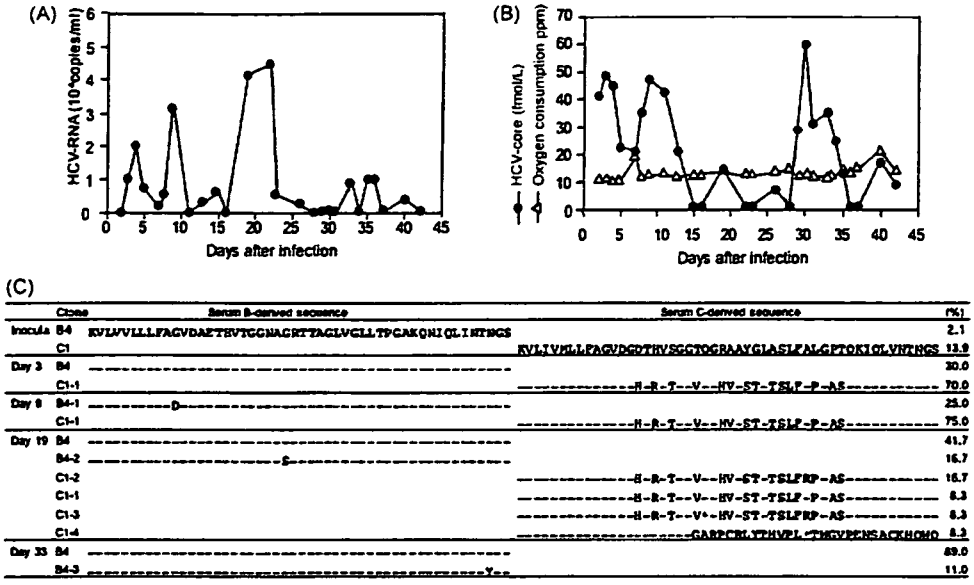


Fig. 3. HCV propagation in FLC4 cells cultured in the RFB system following inoculation with pooled sera obtained from HCV carriers. The 3D-cultured FLC4 cells were incubated with a pooled serum sample for 12 h, followed by changing the culture medium to fresh one. Culture medium was periodically collected 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.

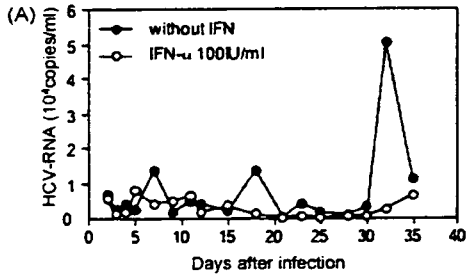
Fig. 3A and B shows the levels of HCV RNA and viral core protein in the culture medium, respectively. HCV RNA was not observed on the first 2 days following infection, but was detectable from day 3 p.i. Viral RNA levels fluctuated, with peaks on days 3, 9, 19–21 and 33–36 p.i. At days 19–21 p.i., the 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,



(B)

	Clone		(%)
Inocula	B4	KVLVVLLLFAGVDAETHVTGGSAGRTTAGLVGLLTPGAKQNIQLINTNGS	2.1
	C1	KVLIVGLLFAGVDGDTHVSGGTGGRAATGLASLFALGPTQKIQLVNTNGS	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. ($1.5–5 \times 10^4$ copies/mL) were observed. However, while HCV RNA at $0.5–0.8 \times 10^4$ 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

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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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Prevalence of Low-Level Hepatitis B Viremia in Patients with HBV Surface Antigen-Negative Hepatocellular Carcinoma with and without Hepatitis C Virus Infection in Japan: Analysis by COBAS TaqMan Real-Time PCR

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Key Words
Hepatocellular carcinoma · HBV surface antigen-negative · COBAS TaqMan HBV test · HBV DNA, circulating low-level

Abstract
Objectives: The effect of circulating low-level hepatitis B virus (HBV), defined as one of the states of 'occult HBV infection', on the development of hepatocellular carcinoma (HCC) in HBV surface antigen (HBsAg)-negative patients is controversial. In addition, the prevalence of occult HBV infection strongly depends on the sensitivity of the HBV detection method. We investigated the prevalence of low-level HBV in the serum of HBsAg-negative patients with HCC using a newly developed, sensitive method based on real-time polymerase chain reaction. ***Methods:*** Serum was examined for HBV DNA in 132 patients with HBsAg-negative HCC (95 with hepatitis C virus [HCV] infection and 37 without detectable hepatitis virus infection) with the COBAS TaqMan HBV test, of which the 95% hit rate is 35 copies/ml (6.7 IU/ml). ***Results:*** Low-level HBV DNA was detected in 2 of 95 (2.1%) patients with HCV-related HCC and 1 of 37 (2.7%) patients with non-viral HCC. ***Conclusion:*** The prevalence of the detection of circulating low-level HBV was low in both HBsAg-negative HCC patients with HCV infection and those without detectable hepatitis virus, even with the use of the most sensitive method for the detection of HBV. Circulating low-level HBV does not appear to play an important role in hepatocarcinogenesis in HBsAg-negative HCC.

Introduction

Occult hepatitis B virus (HBV) infection is a state of persistent HBV infection that was identified after the development of sensitive polymerase chain reaction (PCR) assay. In such a state, a low level of HBV DNA is detected in serum samples and/or liver tissue of individuals who are HBV surface antigen (HBsAg)-negative. Occult HBV infection has been documented in a number of patient subgroups including those with hepatitis C virus (HCV) infection, human immunodeficiency virus (HIV) infection, and cryptogenic liver disease, and the clinical implications of occult HBV infection have been reported with respect to disease progression, treatment efficacy, and the development of hepatocellular carcinoma (HCC) in patients with HBV marker-negative chronic liver disease [1–3].

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Several researchers have reported the effect of occult HBV infection on the development of HCC in HBV virus marker-negative patients with HCV infection and in those without hepatitis virus infection, but the results are controversial [1–4]. Some studies investigated the presence or absence of HBV DNA in liver tissue (HCC and/or noncancerous part), whereas other studies investigated this in serum samples. In addition, the detection methods differed and the sensitivities varied, resulting in differences in the prevalence found even within the same patient population. Increasing the sensitivity of HBV detection will contribute to the precise determination of the prevalence of occult HBV infection in patients with HCC.

In the present study, we investigated the prevalence of circulating low-level HBV in HCC patients in whom the HBV surface antigen (HBsAg) was negative by means of the COBAS TaqMan HBV test. This recently developed test is based on real-time PCR and has high sensitivity for the detection of HBV and can detect HBV of 50 copies/ml [5]. We analyzed two subgroups of patients with HCC, patients with HCV infection and those without hepatitis virus infection.

Table 1. Patient background

	HCV-related HCC (n = 95)	Non-viral HCC (n = 37)
Age, years	65.9 ± 6.7	68.1 ± 7.9
Sex, female/male, n	36/59	14/23
Anti-HBs (+/–), n	27/68	9/28
Anti-HBc (+/–), n	49/46	19/18
Cirrhosis, present/absent, n	77/18	29/8
Total bilirubin, mg/dl	0.88 ± 0.57	1.01 ± 0.61
Albumin, g/dl	3.51 ± 0.53	3.48 ± 0.58
15-min retention of ICG, %	21.3 ± 11.9	27.4 ± 15.1
Prothrombin time, %	85.5 ± 16.4	78.6 ± 19.9
Platelet counts, × 10,000/μl	10.6 ± 5.3	12.8 ± 6.7
Child-Pugh class, A/B/C, n	59/32/4	22/13/2
Tumor size, cm	1.80 ± 0.66	2.62 ± 2.30
Number of tumors	1.36 ± 1.21	1.89 ± 1.29
α-Fetoprotein, ng/ml[a]	20.0 (2.5–2,987)	8.0 (1.0–261)
Tumor stage, I/II/III, n	54/33/8	14/13/10

Unless otherwise indicated the values are give as the mean ± SD. Anti-HBs = Hepatitis B virus surface antibody; Anti-HBc = hepatitis B virus core antibody; ICG = Indocyanine-green test.

[a] Median (range).

Patients and Methods

HCC was diagnosed in a total of 1,083 patients at Ogaki Municipal Hospital between 1992 and 2004. HBsAg was positive in 182 of 1,083 (16.8%) patients and it was negative in the remaining 901 patients (83.2%, HBsAg-negative HCC). Stored serum samples for the measurement of HBV DNA was obtained from 132 patients with HBsAg-negative HCC and these samples were tested. Diagnosis was on the basis of histologic examination of tumor tissue taken from resected or biopsy specimens or on the basis of typical imaging findings including a mosaic pattern with a halo on B-mode ultrasonographic images, hypervascularity on angiographic images, and a high-density mass on arterial phase dynamic computed tomography (CT) images with a low-density mass on portal phase dynamic CT images obtained with a helical or multidetector row CT scanner. For all patients, the serum samples that were analyzed in the study were obtained at the time of diagnosis of HCC and stored at –80°C until analysis.

Patients comprised 82 men and 50 women aged 66.5 ± 7.1 years. Serum markers for persistent HBV infection, including HBsAg (measured with ARCHITECT HBsAg QT, Abbott Japan, Tokyo, Japan) and HBV DNA (measured with the Amplicor HBV test, Roche Diagnostics, Branchburg, NJ), were negative in all 132 patients. HCV infection was confirmed in 95 of the 132 patients by detection of both HCV antibody and HCV RNA with the Amplicor HCV test, version 2.0 (Roche Diagnostics) in serum (HCV-related HCC). HCV antibody and HCV RNA were negative in the remaining 37 patients (non-viral HCC). Patient characteristics of both subgroups are shown in table 1. HBV surface antibody (anti-HBs), measured with ARCHITECT anti-HBs (Abbott Japan), was positive in 27 (28.4%) patients with HCV-related HCC and in 5 (13.5%) patients with non-viral HCC. HBV core antibody (anti-HBc), measured with ARCHITECT anti-HBc (Abbott Japan), was positive in 49 (51.6%) patients with HCV-related HCC and in 19 (51.4%) patients with non-viral HCC.

The entire protocol was approved by the hospital ethics committee and carried out in compliance with the Helsinki Declaration. Written informed consent was obtained prior to sampling of stored serum from all patients and prior to the measurement of serum HBV DNA from patients who were alive at the time of the study.

Detection and Quantification of Low-Level HBV DNA in Serum

Detection and quantification of HBV DNA was carried out with the COBAS TaqMan HBV test (Roche Diagnostics) according to Weiss et al. [6]. HBV DNA was manually isolated from a 500-μl of serum sample using a generic preparation sample kit (High Pure 16 System Viral Nucleic Acid Kit, Roche Diagnostics). A known number of quantitation standard (QS) molecules was introduced into each specimen during sample lysis and carried throughout the specimen preparation, amplification, and detection steps, serving as a QS and inhibition control. The DNA was eluted in a volume of approximately 80 μl, of which 50 μl was used for PCR in a reaction mixture of 100 μl, amplifying a 105-bp segment of the precore-core region. The cycles in which the fluorescence becomes detectable for target HBV and QS are used to calculate the target HBV concentration.

Table 2. Patients in whom low-level HBV DNA was detected from serum

Sex	Age years	Child-Pugh class	HCV RNA	Anti-HBs	Anti-HBc	Tumor size, cm	Observation period, days	Outcome	Serum HBV DNA concentration copies/ml
M	75	A	p	p	p	2.9	1,232	alive	60
M	61	A	p	n	n	1.1	2,878	alive	126
F	78	B	n	n	n	2.0	339	dead	98

HCV RNA = Hepatitis C virus RNA; Anti-HBs = hepatitis B virus surface antibody; Anti-HBc = hepatitis B virus core antibody; p = positive; n = negative.

Results and Discussion

HBV DNA was detected in 2 of the 95 (2.1%) HCC patients with chronic HCV infection and in 1 of the 37 (2.7%) HCC patients without hepatitis virus infection. HCC patients in whom low-level HBV DNA was detected by the COBAS TaqMan HBV test are shown in table 2. Anti-HBs and anti-HBc were positive in 1 patient, whereas both were negative in the other 2 patients. All 3 patients in whom HBV DNA was detected had cirrhosis at the time of diagnosis of HCC. The serum HBV DNA level was 60–126 copies/ml.

The association of overt HBV infection with HCC has been established; however, whether the pathogenesis of HCC can be attributed to occult HBV infection remains controversial. The reported frequency of the detection of low-level HBV DNA in serum has varied significantly among HBsAg-negative patients with HCC [7–15].

The calculated prevalence of occult HBV infection depends strongly on the sensitivity of the detection method for HBV DNA. Although HBV DNA PCR is the most sensitive test currently available for detection of HBV DNA molecules, its sensitivity varies. Low sensitivity can result in underestimation of the rate of occult HBV infection and, conversely, a false-positive HBV DNA detection can result in overestimation. A highly sensitive but specific HBV PCR assay, therefore, is essential for determining precisely the prevalence of occult HBV infection.

This is the first study to evaluate the prevalence of low-level HBV DNA in serum by the commercially available COBAS TaqMan HBV test in HBsAg-negative patients with HCC in Japan where the prevalence of HBsAg-positive individuals is reportedly under 1% and where around 15% of HCC develops in HBsAg-positive patients [16]. The COBAS TaqMan real-time HBV test is reported to have high reproducibility and a high range of detection and quantification, in comparison to the COBAS Amplicor HBV test; it can reportedly detect HBV of less than 50 copies/ml; the 95% hit rate is reportedly 35 copies/ml (6.7 IU/ml) [5, 17]. Although the presence of the nick in minus strand of HBV could impair the sensitivity, it still has high sensitivity for the detection of HBV DNA, in comparison to other methods that are commercially available. The HBV virus level in serum is usually less than 10^4 copies/ml in patients with occult HBV infection. The COBAS TaqMan real-time HBV test, therefore, can detect serum HBV DNA in many patients with occult HBV infection. Indeed, in the present study, the HBV DNA level was 60, 126, and 98 copies/ml in the 3 patients in whom HBV DNA was detected.

In the present study that used a sensitive real-time PCR assay, the prevalence of occult HBV in serum was very low in HBsAg-negative patients in Japan, including those with and without HCV infection. This indicated a low association of circulating low-level HBV (one of the forms of occult HBV) with the development of HCC in patients with HBsAg-negative HCC. Thus, by evaluation with serum samples, we did not find evidence for an association between the circulating low-level HBV and the development of HCC in HBsAg-negative patients.

In a more recent report, the COBAS TaqMan HBV test has a high sensitivity for the detection of HBV DNA as well as high specificity [17]. However, further evaluation is needed to confirm the actual prevalence of circulating low-level HBV in HBsAg-negative patients with HCC using various detection methods for HBV DNA in serum. In addition, because 'occult HBV infection' contains the detection of HBV in liver tissue or HBV integration in HBsAg-negative patients, further investigations are needed to clarify the effect of entire 'occult HBV infection' as an etiological agent in hepatocarcinogenesis of HBsAg-negative patients, including the prevalence of the detection of HBV in liver tissue and the prevalence of integrated HBV using the various detection methods.