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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

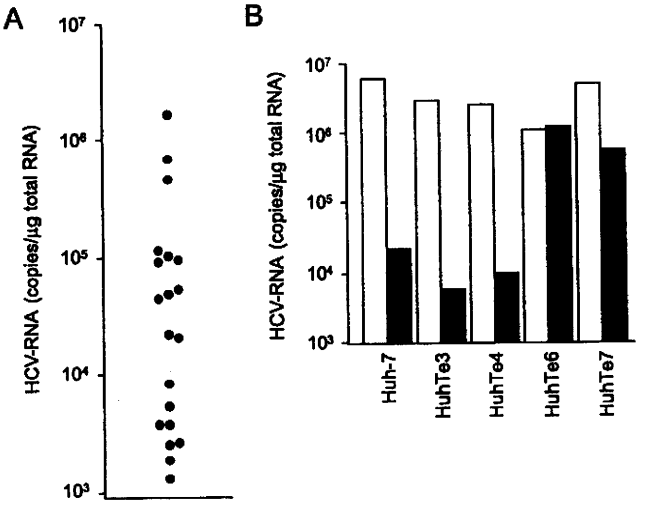

Fig. 1. Copy numbers of HCV RNA after transfection of Huh-7 cells and Huh-7 subclones with subgenomic HCV replicon RNA derived from pFKI389neo/NS3-3'/NK5.1. (A) Transfected Huh-7 cells were subjected to G418 selection. Eighteen colonies were isolated after transfection and cultured in a 96-well plate 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 different replication assays were used to demonstrate that the efficiency of HCV RNA replication in HuhTe6 cells is greater than that in HuhTe4 cells.

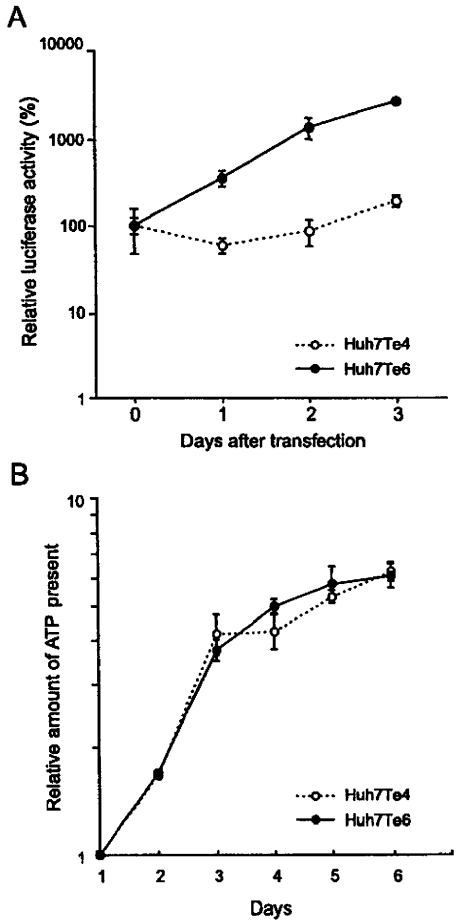

Fig. 2. Cell growth of HuhTe4 and HuhTe6 cells, and transient replication of the HCV replicon. (A) Transient replication of the HCV replicon carrying a luciferase gene in HuhTe4 (open circles) and HuhTe6 (closed circles) cells. Replicon RNA was transfected into the cells by electroporation. Luciferase 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.

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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C型肝炎に関する最近の情報

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はじめに

1988年，米国カイロン社が輸血後非A非B型肝炎の原因ウイルスとして，C型肝炎ウイルス（HCV）の遺伝子のクローニングに成功した。その後，このウイルスに対する各種診断技術が開発され，血液スクリーニングに導入されたため，輸血によるC型肝炎の発生は激減した。しかしながら，現在我が国には150万人以上，全世界にも約1.7億人もの感染者が存在すると推定されており，肝硬変，肝癌と進行する可能性もあり，公衆衛生上最も重要な病原ウイルスの1つである。本稿では，C型肝炎に関する最近の話題とそれの理解に必要な病気の特徴について述べたい。

C型肝炎の最新の話題

最近，（1）薬害C型肝炎訴訟と（2）肝炎患者の個人情報が厚労省の地下倉庫に放置されていた，という2つの血液製剤によるC型肝炎問題が連日ニュースで大きく取り上げられている。（1）の薬害C型肝炎訴訟は，HCVに汚染された血液製剤フィブリノーゲンを止血剤として投与されたことで，HCVに感染したとして患者171人が国と製薬会社を相手取って総額100億円を超える損害賠償を求めた訴訟であり，2002年10月に東京・大阪から始まり，全国の5ヵ所の集団訴訟に広がった。5つの地裁とも判決は製薬会社の責任を認め，このうち4つの地裁では国の責任も指摘されている。（2）は，2002年8月に血液製剤フィブリノーゲンを投与されC型肝炎に感染した可能性がある418人のリストを旧三菱ウエルファーマ（現田辺三菱製薬）が厚労省に報告したものの，厚労省と製薬会社のいずれも患者本人に検査や治療を呼びかけてこなかったことから，「治療機会を奪われた」ということで問題となっている。最近，こうした状況下で大阪高裁が患者と国・製薬会社の双方に和解を勧告したことから，与野党がそれぞれウイルス性肝炎治療の患者支援策を打ち出し，一気に長年の懸案解決に向けた動きが始まっている。

疫　学

我が国のC型肝炎，肝硬変，肝癌患者の数は約50万人，一方症状が現れていない「無症候性キャリア」は110～140万人と推定されている。全国の日赤血液センターにおける初回献血者のデータに基づく2000年時点のHCV抗体陽性率は，年齢が上がるとともに増え，60～69歳で3.38%である。

HCVの感染経路としては，感染血液の輸血，経静脈的薬物濫用，入れ墨，針治療，観血的医療行為などが考えられる。母子感染は妊婦がHCV-RNA陽性の場合，出生児が感染する確率は10%程度と言われている。また，血液透析に伴うHCV新規感染の発生は平均年率2%程度の頻度あるといわれ，歯科診療における潜在的な感染の可能性も示唆されている。ニュースで取り上げられている血液製剤フィブリノーゲンを投与された約28万人のうち感染者は約1万人と推計されている。我が国のC型肝炎患者のうち，輸血歴を有するものは3～5割程度にすぎず，多くの患者で感染経路は不明である。

HCV感染に伴って急性肝炎を発症した後，30～40%ではウイルスが検出されなくなり，肝機能が正常化するが，残りの60～70%はHCVキャリアになり，多くの場合，急性肝炎からそのまま慢性肝炎へ移行する。慢性肝炎から自然寛解（治療なしでウイルスが消える）する確率は0.2%と非常に稀で，10～16%の症例は初感染から平均20年の経過で肝硬変に移行する。肝硬変の症例は，年率5%以上と高率に肝細胞癌を発症する。肝癌死亡総数は年間3万人を越え，いまだに増加傾向にあるが，その約8割がC型肝炎を伴っている。

現行のスクリーニングシステム実施下では，輸血その他の血液製剤による新たなC型肝炎の発生は限りなくゼロに近づいている。現在，米国では薬物濫用者を中心に年間25,000人の新たなHCV感染者が発生しているが，日本ではHCVによる新たな急性肝炎の発症は2001年以降年間40～70人程度と大変少なく抑えられている。以上から，国のC型肝炎対策の基本は，多くの国民に対してC型肝

12/27 再校

炎ウイルス検査を行い，早期に感染の有無を確認し，感染者に対して適切な治療を行うことと考えられている。さらに，上記のような病気について正しい知識を普及させることは，感染者の就業・入所・入学等に伴う偏見・差別等を防ぐためにも重要である。

臨床症状

C型肝炎では全身倦怠感に引き続き，比較的徐々に食欲不振，悪心・嘔吐，右季肋部痛，上腹部膨満感，濃色尿などが見られるようになる。一般的に，C型肝炎ではA型やB型肝炎とは異なり，劇症化することは少なく，黄疸などの症状も軽い。慢性肝炎ではほとんどが無症状で，倦怠感などの自覚症状を訴えるのは2～3割にすぎない。肝硬変で非代償期まで進行すると黄疸，腹水，浮腫，肝性脳症による症状である羽ばたき振戦（手指が震える），意識障害などが出現するようになる。肝細胞癌を合併すると，初期は無症状であるが末期になると肝不全に陥り，他の癌と同様に悪液質の状態となる。以上のように，C型肝炎の問題点は症状が全くない潜伏期間が20～30年に及ぶこともあるため，治療の機会がなく悪化させるケースが少なくないことである。

診断

C型肝炎のもうひとつの問題点は，HCVに感染していても肝機能検査では正常を示すことが多いことである。そこで，HCV感染の有無を判定する方法としては，HCV血清抗体の検出と核酸・抗原の検出の2種類が用いられている。一般的には，初めにHCV抗体検査が行われる。この抗体検査で陽性となった場合，（1）HCVに感染しているキャリア状態，（2）過去に感染し，現在ウイルスは排除された状態，の2つの可能性が考えられる。このようなHCVキャリア と感染既往者とを適切に区別するため，HCV-RNAの検出を行う。また，急性C型肝炎においてもHCV抗体の陽性化には感染後通常1～3ヵ月を要するため，この時期の確定診断には HCV-RNA定性検査が行われる。

治療

2002年に発足したC型肝炎等緊急総合対策では，保健所，老人保健，政府管掌健康保険等による肝炎ウイルス検査を導入し，ハイリスクグループ（1992年以前に輸血を受けた者，輸入およびそれと同等のリスクを有する非加熱血液凝固因子製剤を投与された者，1994年以前にフィブリノーゲン製剤（フィブリン糊を含む）を投与された者，大きな手術・臓器移植を受けた者，薬物濫用者，入れ墨・ボディピアスをしている者，その他過去に健康診断等で肝機能異常を指摘されているにも関わらず，その後肝炎検査を実施していない者等）を重点対象としつつ，一定年齢以上の全ての国民を対象にC型肝炎検査を行う体制が構築された。しかしながら，健診の受診率がそれほど高くなく，実際老人保健事業のC型肝炎ウイルスの節目検診で25～30%の受診率であった。また，その検診で要精密となった者のうち実際に二次医療機関を受診したのは8割程度，さらに，二次医療機関でも専門の医療機関を受診された方はその約半分という状況で，健診と治療連携における課題がある。

C型肝炎の治療の中心はインターフェロン（IFN）である。従来の単独投与に加え，2001年からリバビリンとの併用療法に医療保険が適用されるようになり，2002年からIFNの保険適用上の投与期間の制限が撤廃，2003年からペグインターフェロン，2005年からIFN自己注射承認，2006年から代償性肝硬変もIFNの適応，と治療法は年々進歩している。一般に，IFNによってHCVが排除されるのは30%程度，リバビリンとの併用療法の場合で約40%と言われているが，IFN療法でウイルスを排除できなかった場合でも，肝炎の進行を遅らせ，肝癌の発生を抑制，遅延させる効果が期待できる。

以上のように治療法は進歩しているが，実際には適切な治療法が選択されない場合や中断してしまうという問題点がある。そこで，二次医療圏に1ヵ所程度の専門医療機関，都道府県に1ヵ所の肝疾患診療連携拠点病院というものを置き，かかりつけ医と専門医療機関の連携を進め，治療水準の均てん化（全国どこでも一定以上の同じ水準の治療を受けられること）を計りつつ，高度専門的集学的な治療を適応できる医療機関の確保を目指している。また，IFN治療は，治療費が月に約7万円と高額で患者にとって大きな負担になっているが，IFN治療で癌患者が減り医療費抑制にもつ

ながることが知られているので，検査費だけでなく治療費にも公的助成することが検討されている。

まとめ

ここ十数年の間にHIV，HCV，SARS等が新たに発見され，さらに現在鳥インフルエンザの脅威が叫ばれている。HIV，HCVでは血液製剤による感染を防ぐ有効な対策が講じられるのが遅れ，被害が広がったと問題になっている。また，分子生物学の発達によって新たな治療法の開発が試みられている。最近では，インフルエンザ治療薬タミフルもその副作用から「薬害」ではないかと問題視されている。このように生命科学や医学研究の発達によって，より医療の現場は一層複雑になってきている。一方，今後も未知の感染症や治療法の想定外の副作用などが発生する恐れもあり，そのような状況で我々は如何に準備していけば良いか，大変難しい問題である。筆者は医学生時代に「病気を診ずして病人を診よ」と教わってきたことを覚えている。これは，医療者は疾病そのものの診断や治療だけにとらわれることなく，病をもっている人の心の痛みをよく理解し，患者さんを全人的に診て治療することの重要性を訴えているものである。国，製薬会社，医療関係者，マスコミなどが患者の視点に立ってこのような精神を実践することで，新しい事態により早く的確に対応できると思われる。

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特集2　C型肝炎ウイルスによる発癌機構とその治療

3. C型肝炎ウイルスの感染粒子形成機構

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効率のよいウイルス産生細胞系が確立されていなかったため，C型肝炎ウイルス（HCV）の生活環研究の中で感染粒子の形成機構に関する解析は最も遅れていた．JFH-1株の出現により，感染から分泌までウイルス生活環全体に亘る解析が可能となり，粒子形成の分子機構研究が大きな展開を見せている．細胞内の脂肪滴及びその周辺の膜構造が粒子形成の場として働くことが示された．我々は，ゲノム複製調節に関与することが知られていたHCV非構造蛋白NS5Aが粒子形成にも関与することを示し，粒子形成の初期過程において，新たに作られたウイルスRNAがNS5A蛋白によって捕捉され，更にこのNS5A-HCV RNA複合体がCore蛋白と会合することがRNAパッケージングの引き金になるというモデルを提唱した．また，感染性粒子表面のコレステロール，スフィンゴ脂質が粒子構造の維持，感染性に重要であることを示す知見を得た．ウイルス非構造蛋白，脂質，脂質結合因子がHCV粒子のアセンブリー，輸送などにどのように関与しているかを明らかにすることが粒子形成機構研究の鍵になるものと思われる．

はじめに

「レプリコンシステム」「シュードタイプウイルス」「JFH-1株」これらはこの10年間のHCV研究の進展に大きく寄与した実験手法，研究材料である．HCVの生活環に関する基礎研究は，効率のよい培養細胞系が確立されていなかったため必ずしも順調には進んでいなかった．1999年にはじめてHCV RNA複製実験系としてレプリコンシステムが導入され，ゲノム複製機構に関する研究が大きな進展をみせた．また，レトロウイルスまたは水疱性口内炎ウイルスのエンベロープ蛋白質を欠損させ，代わりにHCVのエンベロープ蛋白質を持ったシュードタイプウイルスは感染モデルとして有用であることがわかった．そして2005年，HCV JFH-1株を用いた効率のよい感染増殖細胞系が確立されるに至り，長らく困難を極めた感染から分泌までの全ステップの分子機構の研究が可能となった．

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HCV培養細胞系の試みと感染増殖細胞系の樹立

C型肝炎患者血清中のHCVを培養細胞に感染させウイルス増殖細胞系を作製する方法はHCVゲノム発見当初から試みられてきた．生体内での標的細胞である肝細胞を由来とする細胞株，またリンパ球系細胞で数多く感染，複製が調べられたが，観察できるウイルス量は低いレベルであり，詳細なウイルス研究への応用は難しい状況であった．我々は，三次元化細胞培養システムであるラジアルフロー型バイオリアクター（RFB）及び温度感受性ハイドルゲル（TGP）を利用してHCV培養細胞系の構築を行った．単層培養系に比べより本来の肝組織に近い立体的な培養環境化の方が肝炎ウイルスの感染増殖に適しているのではないかという発想であった．実際に，これらの三次元培養系では細胞あたりのアルブミン産生，分泌能や肝特異的薬物代謝酵素の発現が亢進していること，極性細胞の特徴である細胞間ジャンクション構造が形成されることが示されている[12, 17, 20, 24]．RFB培養肝細胞株に患者血清感染，またHCVゲノムcDNAをトランスフェクションし高密度培養を行うことでウイルスの複製増殖を確認した[1]．さらに，遺伝子型1bのダイシストロニックゲノム（全長レプリコン）を保持した細胞株をTGP培養することで単層培養系では認め

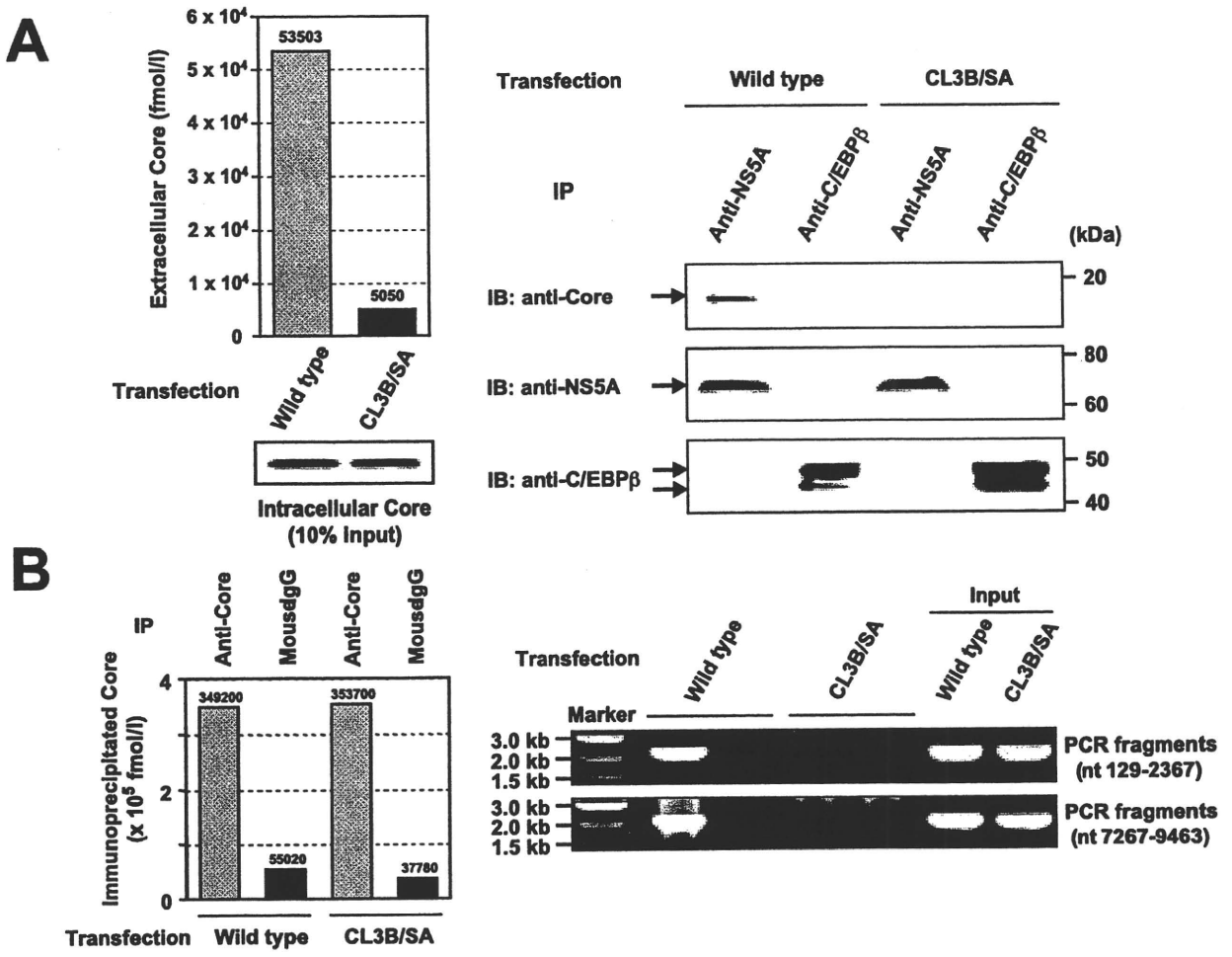

図1 NS5A domain III 変異が HCV 産生，Core-NS5A 相互作用，Core-associated viral RNA へ及ぼす影響
(A) NS5A domain III cluster 3-B のセリン/アラニン置換変異を持つ JFH-1 ゲノム（CL3B/SA）または野生型ゲノムを導入した Huh-7 細胞の細胞内ウイルス蛋白レベル（Intracellular Core）と細胞外分泌ウイルスレベル（Extracellular Core）を調べた（左図）．これらの HCV 発現細胞ライセートを抗 NS5A 抗体で免疫沈降し抗 Core または抗 NS5A 抗体でウエスタンブロッティングを行った（右図）．(B) 免疫沈降-RT-PCR 法による Core-associated HCV RNA の検出．

られなかったウイルス粒子の産生を観察した[24]．しかしながら，依然としてその HCV 増殖レベルは必ずしも高いものではなく，よりウイルス産生効率にすぐれ，汎用性の高い培養系の登場が待望されていた．

JFH-1 株は，遺伝子型 2a の劇症患者の急性期血清から単離された HCV クローンで，レプリコンシステムによる解析から，他の多くのクローンで見られるような適応変異を伴わずに高いゲノム複製効率を有することが示された[15,16]．次に，JFH-1 株の全長 cDNA から合成された RNA をヒト肝癌細胞株 Huh-7（通常の単層培養）へ導入することで，感染性粒子の産生，分泌が観察され，この HCV 粒子はチンパンジーにも感染性を有することも示された[32]．さらに，感染実験に HCV 複製感受性の高い Huh-7 由来細胞株（Huh7.5, Huh-7.5.1）を用いることで，感染力価 10^4-10^5 の培養上清が得られること，感染後 2-3 週間でほぼ 100% の細胞が HCV 陽性となること，が明らかとなった[18,33]．

HCV 粒子形成における非構造蛋白 NS5A の役割

現在，このようにして確立された JFH-1 株による HCV 感染増殖細胞系を用いて，HCV 生活環の研究が活発に行われている．粒子形成の分子機構に関するこれまでの研究成果の中で最もインパクトを与えたものは，HCV の粒子形成には細胞内脂肪滴が重要な役割を果たすという発見である[22]．かねてから，構造蛋白 Core の一部が細胞の脂肪滴に存在することが知られていたが，非構造蛋白の細胞内局在を詳細に調べた結果，NS5A 蛋白などが小胞体の他に脂肪滴近傍にも存在すること，脂肪滴周辺で Core 蛋白を取り巻くようにして非構造蛋白が存在することが見出された．さらに，脂肪滴と会合できないような変異ゲノムを発現させたところ，感染性 HCV 粒子は産生されなくなることが示さ

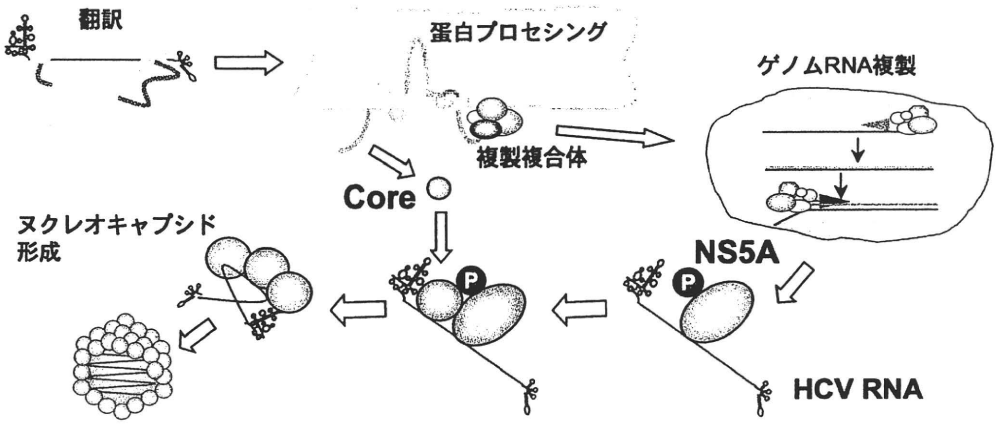

図 2　HCV 粒子形成初期過程における NS5A 蛋白の役割のモデル
前駆体蛋白からプロセシングされた HCV 蛋白のうち，非構造蛋白（NS3, 4A, 4B, 5A, 5B）は宿主因子とともに複製複合体を形成しゲノム RNA 複製を行う．新生された HCV RNA と NS5A 蛋白との複合体が Core 蛋白と会合することがヌクレオキャプシド形成の引き金になる．

れた．

一方，HCV ゲノム複製を可視化する試みから，NS5A 蛋白の C 末端領域（domain III ；後述）に In-frame で GFP を挿入することにより，ゲノム複製細胞が簡便にモニターできることがレプリコンシステムで示された[4, 23]．同様の解析は全長ゲノム発現系でも行われ，確かにこのような変異体からウイルスの産生は観察されるものの，その産生効率は野生型に比べ明らかに低下しているようであり[26]，我々もそれを確認した．そこでこれらの知見から，NS5A 蛋白，特にその C 末端領域は HCV 粒子形成になんらかの役割を担っているのではないかと考えた．

NS5A はリン酸化蛋白で，低リン酸化型（56 kDa）と高リン酸化型（58 kDa）が存在し，HCV ゲノム複製に必須であることが示されている．3 種類のドメイン構造を有し，N 末端側の domain I は立体構造が解かれ RNA 結合能を有する．Domain II にはインターフェロン感受性に関係する ISDR（Interferon sensitivity determining region）が含まれているが，domain II，III とも構造，機能について十分に解析されていない．NS5A domain III には，リン酸化に関与するセリン残基のクラスターが二ヶ所（cluster 3-A, 3-B）存在し，これらは HCV クローン間でよく保存されている．我々は domain III のリン酸化がウイルス産生に及ぼす影響を調べるため，種々の部分欠損または置換変異体を構築し，ゲノム複製，粒子産生能を解析した．その結果，cluster 3-B の 3 セリン残基のうち，任意の 2 残基または 3 残基をアラニンへ置換することにより，ゲノム複製は野生型と同等であるものの，産生されるウイルス量が顕著に低下することを見出した．また，このような変異に伴って NS5A 蛋白のリン酸化レベルが低下することも確認した[19]．

NS5A domain III の変異が HCV 生活環のどのステップに影響を及ぼすのかを調べることによって，粒子形成機構における NS5A 蛋白の機能解明につながるものと思われる．前述のように，NS5A 蛋白と Core 蛋白は脂肪滴周辺領域での近接して存在することが観察されていることから，NS5A 蛋白は Core 蛋白と結合しうるのではないかを考えた．そして実際に NS5A は Core 蛋白と相互作用すること，ウイルス産生が低下する cluster 3-B 変異体では Core 蛋白と結合できなくなること（**図 1A**），またこの変異によって NS5A 蛋白は脂肪滴周辺膜に局在できなくなること，を見出した[19]．

NS5A 蛋白はゲノム複製複合体を構成し，RNA 結合能を有している[9, 29]．そこで，NS5A 蛋白が粒子形成に関与する分子機構として，複製複合体で新生されたウイルスゲノムが NS5A 蛋白に捕捉され，さらに NS5A-Core 蛋白相互作用によってゲノム RNA がヌクレオキャプシドの場へリクルートされる，という作業仮説を考えた．これを検証するため，HCV ゲノム発現細胞のライセートを抗 Core 抗体で免疫沈降しさらにこの沈降物中の HCV RNA を long RT-PCR 法で検出した．その結果，野生型ゲノムの場合 Core 蛋白アソシエート HCV RNA が検出されたのに対し，cluster 3-B 変異ゲノムでは検出されなかった（**図 1B**）[19]．この結果は，NS5A 蛋白の cluster 3-B の変異によって Core-HCV RNA の会合が影響を受けることを示しており，NS5A-HCV RNA 複合体が Core 蛋白と会合することが Core 蛋白によるゲノムパッケージングの引き金になることを示唆している（**図 2**）．

HCV NS5A 蛋白の domain III が粒子形成にとって重要であるという知見は，最近，米国とドイツのグループからも報告された[5, 30]．粒子形成を左右するセリン残基（の一つ）が casein kinase II でリン酸化される可能性が示されているが，今後，NS5A 蛋白のリン酸化制御とウイルス粒

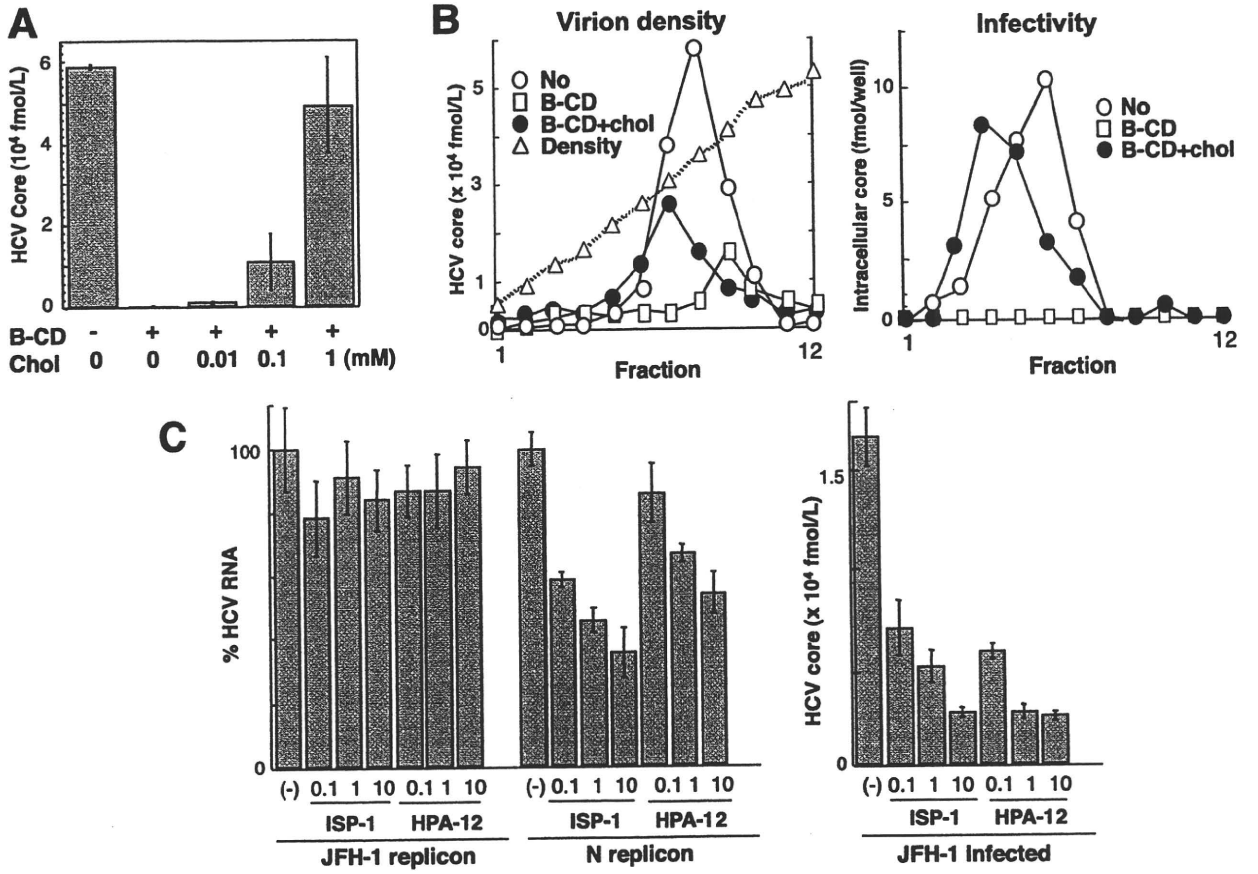

図3 HCV粒子表面コレステロールが粒子構造，感染性へ及ぼす影響（A, B）及びスフィンゴ脂質合成阻害剤によるHCV産生阻害（C）
(A) 培養上清HCVを5 mg/ml B-CD処理した後，コレステロール（Chol）を添加した．超遠心操作により薬剤を除去しHuh-7細胞に感染させ，3日後の細胞内Core量を測定した．(B) 培養上清HCVをB-CD未処理，処理，または処理後Cholを添加し，ショ糖密度勾配遠心分画した．各分画中のCore量を測定し（左図），また，各分画サンプルをHuh7細胞に感染させ3日後の細胞内Core量を測定した（右側）．(C) HCVレプリコン細胞（JFH-1 replicon, N replicon），JFH-1感染細胞にmyriocin/ISP-1または(1R,3R)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl dodecanamide（HPA-12）を加え3日間培養した．細胞内のHCV RNAまたはCore量を測定した．

子形成との関連を明らかにしていくことが重要になると思われる．また，NS5A-Core相互作用様式の詳細を解明することによって，HCV粒子形成を選択的に阻害する新たな治療薬の開発へ道が拓かれるものと期待される．

HCV粒子構造，感染性における粒子脂質成分の役割

エンベロープウイルスは小胞体，ゴルジ体，形質膜などの細胞の生体膜を被って出芽するため，細胞の膜脂質はウイルス粒子形成に重要な役割を果たしているものと考えられる．さらに，ウイルス粒子の膜脂質が宿主細胞への感染過程に関与する例も報告されている[7]．しかし，HCV粒子に含まれる脂質成分については解析が進んでおらず，その生理学的役割も不明であった．そこで我々は，培養細胞で産生させたHCV JFH-1粒子を，培養上清から，限外濾過，ショ糖密度勾配超遠心，ヘパリンアフィニティクロマトグラフィを組み合わせて，濃縮，粗精製し，このHCV粒子に含まれる脂質を生化学的に解析した．その結果，コレステロール／リン脂質モル比が細胞の膜分画に比べて有意に高値を示したことから，コレステロールに富んだ生体膜からの出芽，または粒子形成，分泌過程でのコレステロールとの会合の可能性が考えられた[3]．

次にこのHCV粒子上の膜脂質がどのような役割を果たしているかを調べるため，HCV粒子表面をmethyl-β-cyclodextrin（B-CD）で処理してコレステロールを除去した後感染させたところ，B-CDの容量依存的に感染性が低下し，B-CD処理した粒子にコレステロールを添加したところその感染性は回復した(**図3A**)[3]．また，コレステロールと親和性が高いスフィンゴ脂質の主要分子スフィンゴミエリンを加水分解するsphingomyelinase（SMase）でHCV粒子を処理することにより感染性の低下を観察した[3]．こ

れらのことはHCV genotype 1bのエンベロープを持つシュードタイプウイルスやキメラウイルスでも確認できた．以上から，ウイルス粒子表面のコレステロールとスフィンゴ脂質はウイルスの遺伝子型によらず感染に重要な役割を果たしていることが示された．

次に，HCV粒子上のコレステロールが粒子の物性に与える影響を調べた（図3B, C）．HCV産生細胞の培養上清をショ糖密度勾配遠心分画するとCore蛋白及びHCV RNAのピークは1.17 g/ml分画，感染性のピークは1.13 g/ml分画となる．このように，感染性のピークがウイルス遺伝子のそれに比べ低密度側に存在することは培養細胞系で作製したHCVの特徴の一つであるが，濃縮したこの培養上清をB-CD処理しコレステロール除去後に同様に遠心分画を行うと，Core蛋白のピークは1.20 g/ml分画に移行し，感染性はいずれの分画も検出限界以下であった．さらに，B-CD処理後の培養上清にコレステロールを添加するとCore蛋白のピークは低密度側へシフトし感染性も回復した[3]．このようなコレステロールの除去&添加によるloss- and gain-of-functionは5 mg/ml B-CD処理で観察されるが，B-CD濃度を10 mg/mlへ上げた場合はコレステロール添加によって感染性の回復は見られない．これらのことから，HCV粒子表面のコレステロールは粒子構造の維持に役立っており，コレステロールを完全に除去してしまうと粒子構造は致命的なダメージを受ける，これに対し，部分的に除去した場合の構造変化は感染性を低下させるものの，その変化は再生可能なレベルである，と考えられた．

次に，HCV粒子上のコレステロールまたスフィンゴ脂質が感染過程のどのステップに関与するのかを解析した．あらかじめコレステロール除去またはSMase処理を行ったHCV粒子の宿主細胞への吸着性は未処理ウイルスと同等であったのに対し，吸着後の細胞内への取り込みは，これらの前処理を施したHCVで顕著な低下が認められた[3]．レセプター蛋白分子とともに標的細胞内へウイルスが侵入する過程に粒子コレステロール，スフィンゴ脂質が関与する可能性が示された．

HCVゲノムは，脂質ラフトの特徴である界面活性剤不溶性の膜分画で複製することが示され[2, 27]，HCV genotype 1のゲノム複製細胞またHCVが増殖するヒト肝細胞キメラマウスに脂質ラフト構成成分であるスフィンゴミエリンの合成阻害剤myriocin/ISP-1を添加，投与することによって，HCV複製効率は顕著に低下することが報告されている[25, 31]．このmyriocin/ISP-1またはセラミド輸送阻害剤HPA-12をHCV N株（genotype 1b）またJFH-1株のサブゲノムレプリコン細胞に加えることによって，N株ではHCVゲノム複製は阻害されるものの，JFH-1株では予想に反して複製の低下はほとんど認められなかった．しかしながら，興味深いことに，JFH-1のウイルス産生系では両薬剤の容量依存的にHCV産生は抑制された（図3C）[3]．スフィンゴ脂質合成阻害剤の抗HCV効果の作用機序としてHCVゲノム複製阻害だけでなく粒子形成あるいは感染過程へも介入しうることが示唆された．

おわりに

「ウイルス非構造蛋白」「脂質」はHCVの粒子形成制御に関する代表的なキーワードとなった．本稿ではNS5A蛋白が粒子形成過程にどのように働くかを紹介したが，最近，別の非構造蛋白で前駆体蛋白のプロセシングを担っているNS2がやはり粒子形成にも関与することが報告された[11, 13, 14, 28]．しかしながらその分子機構は現在まったくと言ってよいほど不明である．一方，脂質成分，脂質代謝とHCV生活環の関連についての興味深い知見として，ウイルス産生におけるアポリポ蛋白，VLDL/LDLの重要性が示されている[6, 8, 10, 21]．脂肪滴周辺膜構造を起点とするHCVの粒子形成過程に介在する宿主蛋白の輸送・分泌経路 —おそらく脂質関連分子が含まれる— を明らかにすることは，HCVのアセンブリー，出芽から細胞外への放出までの過程を制御する分子機構の解明に直結するものと思われる．

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Involvement of nonstructural protein 5A and lipids on production of hepatitis C virus particles

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A robust system for production of recombinant infectious hepatitis C virus (HCV) has been established in 2005 and classical virological techniques are now able to be applied to the HCV research, especially regarding molecular mechanisms on virion assembly and maturation. We recently demonstrated that the C-terminal serine cluster of NS5A is a determinant of NS5A interaction with Core and the subcellular localization of NS5A. Mutation of this cluster blocks the NS5A-Core interaction, resulting in perturbation of association between Core and HCV RNA. It is thus tempting to consider that NS5A plays a key role in transporting the viral genome RNA synthesized by the replication complex to the surface of lipid droplets (LDs) or LD-associated membranes, where Core localizes, leading to facilitation of nucleocapsid formation. We also demonstrated an important role of cholesterol and sphingolipid in HCV infection and virion maturation. Specifically, mature HCV particles are rich in cholesterol. Depletion of cholesterol from HCV or hydrolysis of virion-associated sphingomyelin results in a loss of infectivity, and the addition of exogenous cholesterol restores infectivity. In addition, cholesterol and sphingolipid on the HCV membrane play a key role in virus internalization. Finally, inhibitors of the sphingolipid biosynthetic pathway efficiently block virion production.

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Interaction of Hepatitis C Virus Nonstructural Protein 5A with Core Protein Is Critical for the Production of Infectious Virus Particles

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Nonstructural protein 5A (NS5A) of the hepatitis C virus (HCV) possesses multiple and diverse functions in RNA replication, interferon resistance, and viral pathogenesis. Recent studies suggest that NS5A is involved in the assembly and maturation of infectious viral particles; however, precisely how NS5A participates in virus production has not been fully elucidated. In the present study, we demonstrate that NS5A is a prerequisite for HCV particle production as a result of its interaction with the viral capsid protein (core protein). The efficiency of virus production correlated well with the levels of interaction between NS5A and the core protein. Alanine substitutions for the C-terminal serine cluster in domain III of NS5A (amino acids 2428, 2430, and 2433) impaired NS5A basal phosphorylation, leading to a marked decrease in NS5A-core interaction, disturbance of the subcellular localization of NS5A, and disruption of virion production. Replacing the same serine cluster with glutamic acid, which mimics the presence of phosphoserines, partially preserved the NS5A-core interaction and virion production, suggesting that phosphorylation of these serine residues is important for virion production. In addition, we found that the alanine substitutions in the serine cluster suppressed the association of the core protein with viral genome RNA, possibly resulting in the inhibition of nucleocapsid assembly. These results suggest that NS5A plays a key role in regulating the early phase of HCV particle formation by interacting with core protein and that its C-terminal serine cluster is a determinant of the NS5A-core interaction.

Hepatitis C virus (HCV) infection is a major public health problem and is prevalent in about 200 million people worldwide (27, 40, 42). Current protocols for treating HCV infection fail to produce a sustained virological response in as many as half of treated individuals, and many cases progress to chronic liver disease, including chronic hepatitis, cirrhosis, and hepatocellular carcinoma (15, 31, 35, 43).

HCV is a positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family (55). Its approximately 9.6-kb genome is translated into a single polypeptide of about 3,000 amino acids (aa), in which the structural proteins core, E1, and E2 reside in the N-terminal region. A crucial function of core protein is assembly of the viral nucleocapsid. The amino acid sequence of this protein is well conserved among different HCV strains compared to other HCV proteins. The nonstructural (NS) proteins NS3-NS5B are considered to assemble into a membrane-associated HCV RNA replicase complex. NS3 possesses the enzymatic activities of serine protease and RNA helicase, and NS4A serves as a cofactor for NS3 protease. NS4B plays a role in the remodeling of host cell membranes, probably to generate the site for the replicase assembly. NS5B functions as the RNA-dependent RNA polymerase. NS5A is known to play an important but undefined role in viral RNA replication.

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NS5A is a phosphoprotein that can be found in basally phosphorylated (56 kDa) and hyperphosphorylated (58 kDa) forms (49). Comparative sequence analyses and limited proteolysis of recombinant NS5A have demonstrated that NS5A is composed of three domains (52). Domain I is relatively conserved among HCV genotypes compared to domains II and III. Analysis of the crystal structure of the conserved domain I that immediately follows the membrane-anchoring α-helix localized at the N terminus revealed a dimeric structure (53). The interface between protein molecules is characterized by a large, basic groove, which has been proposed as a site of RNA binding. In fact, its RNA binding property has been demonstrated biochemically (17). Domains II and III of NS5A are far less understood. Domain II contains a region referred to as the interferon sensitivity determining region, and this region and its C-terminal 26 residues have been shown to be essential for interaction with the interferon-induced, double-stranded RNA-dependent protein kinase (6–10, 38, 39, 48). Domain III includes a number of potential phosphoacceptor sites and is most likely involved in basal phosphorylation. This domain tolerates insertion of large heterologous sequences such as green fluorescent protein (GFP) and is not required for function of NS5A in HCV RNA replication (1, 34). However, a study with the recently established productive HCV cell culture system using genotype 2a isolate JFH-1 (28, 56, 58) demonstrated that while insertion of GFP within the NS5A region does not affect RNA replication, it does produce marked decreases in the production of infectious virus particles (41). This suggests that the C-terminal region of NS5A may affect virus particle production independent of RNA replication. Re-