新世界ザルであるタマリンおよびマーモセット感染実験は当センター感染症実験施設にて実施した。感染性分子クローン pGBB は Dr. Bukh (NIAID, NIH, USA) より分与を受けた。pGBB から in vitro transcription により得られたウイルスゲノム RNA をサルに接種後４週で全採血した plasma を以後のウイルス接種用ストックとした。ウイルス感染サルよりケタミン麻酔下で定期的に採血し、得られた血液について血清生化学検査、plasma 中ウイルス量及び抗体価測定を行った。血液および組織中のウイルスRNA量測定はリアルタイムPCR法を用いた。pGBB よりサブクローニングしたCore 発現ベクターを導入した大腸菌からリコンビナントCore蛋白を得て、これによるELISA系を構築して抗体価測定を行った。なおすべての動物実験は、倫理面を含めて医薬基盤研究所動物実験委員会の審査・承認を得て実施した。

C. 研究結果

（１）ウイルス接種ルートによる病態への影響に関する解析

感染性分子クローン pGBB から in vitro transcription により得られたウイルス RNA を２頭のタマリン肝臓に接種したところ、二頭ともに接種後２～８週にわたり顕著なウイルス血症（10^9～10^{10} copies/ml）および ALT, AST 値の上昇を主徴とする急性Ｃ型肝炎様症状を発症した。次に、上記タマリン由来 plasma をタマリン２頭に経肝臓接種したところ、どちらの個体においても先の実験同様に顕著なウイルス血症が認められた。この際、肝炎マーカーである ALT 値は接種後２週～３週でピークとなり、８～１０週後の血中ウイルス消失と共にバックグラウンドレベルに復した（図１）。一方、同様のタマリン由来 plasma を経静脈接種すると、前述の感染個体と同程度のウイルス血症が認められるにも関わらず、ALT 値の上昇ははるかに緩やかとなりピークに達するのに接種後７～９週を要した（図１）。このことは、経肝臓による接種と比べ、経静脈による接種では肝炎惹起が遅れることを意味するものである。昨年度の本研究にて報告したように、GBV-B は HCV 同様に肝臓のみならず血液リンパ系組織を中心に多様な指向性を示すことから、経静脈による接種では肝外組織を主としたウイルス複製がおこったことを示唆するものと考えられた。

（２）ウイルス感染後の長期フォローアップによるウイルス・免疫応答の推移に関する解析

近年、新世界ザルであるマーモセットはタマリンと同様に GBV-B に感受性であることが報告されている（Bright et al.: J Virol 78, 2062, 2004）。マーモセットは実験用霊長類として汎用されておりタマリンと比較して入手し易いことから、モデル動物の選択肢のひとつとして有用と考えられた。昨年度は、Bright らのデータを追試する目的でマーモセットとタマリンに実験感染を行いウイルス動態の比較を行なったところ、マーモセットではタマリンの場合と比較して１～２桁程度低い血中ウイルス量を示していた。今年度は、タマリン、マーモセット両方における長期フォローアップを行ない、ウイルス・免疫応答の変化を調査した。

今回調査したタマリン４頭では、感染後約１年半の観察期間において亜急性期以後ウイルスゲノムは検出されなかった。ところが、マーモセットでは感染後約１年半を経過した時点でも、なお４頭中２頭でウイルスゲノムが検出されていることを見出した。特に、１

頭は間欠的にウイルスが検出されることから、GBV-B は生体内で潜伏感染しうることが示唆される（図２）。

そこで、この２例における慢性感染の原因を探る目的で、抗ウイルス抗体価の推移を比較検討した（図３、４）。タマリン４例および亜急性期以降ウイルスが検出されなかったマーモセット２例では、感染８～１２週に抗コア・NS3 抗体価どちらも急激な上昇を示し、抗体価がピークに達する時期と相反して血中ウイルスが検出限界以下となる。その後抗体価は徐々に低下していく。一方マーモセットにおける慢性感染２例では、抗体誘導が顕著に遅延していることが明らかとなった。このことは、何らかの理由でウイルス感染により誘導される宿主免疫応答が充分機能しなかったことを示唆していた。

D. 考察

（１）ウイルス接種ルートによる病態への影響に関する解析

本研究結果より、同じウイルスを異なる経路により感染させた場合、ほぼ同程度の血中ウイルス量の推移を示すにも関わらず、その病態に違いが見られることが明らかとなった。ヒトにおける HCV 感染は主に輸血や血液製剤などであることから、今回の経静脈感染は本サロゲートモデルを HCV 感染へ外挿するにあたってはより適当な感染方法であると考えられる。

昨年度の研究成果で、GBV-B を経肝臓で感染させても肝臓以外の諸組織で感染すること、さらに個体によってはむしろ肝臓以外の組織（主に脾臓・リンパ組織）での感染が優勢となりうることを報告した（Microbes Infection, 印刷中）。今回の解析で経静脈接種した個体において、急性期（～４週）に血中ウイルス量が高値を示すにもかかわらず、肝炎マーカー上昇が殆ど見られなかったが、この際肝外組織を主としたウイルス複製がおこったと考えるのが妥当であろう。もしくは、肝臓で効率よく感染・複製が生じたが肝臓への障害が起こらなかったとする仮説も考えられるが、同じ接種ウイルスを用いていることからそのような異なる細胞障害活性を示すとは考えにくい。経静脈接種した個体において急性期に何ら有意な臨床症状が認められなかった事から、HCV 感染においても同様な経過をたどることが推定される。しかしヒトの場合、臨床症状を呈さない限り見過ごしがちとなることから、今後そのような視点での解析が必要と考えられる。

（２）ウイルス感染後の長期フォローアップによるウイルス・免疫応答の推移に関する解析

今回の結果において、GBV-B はマーモセットにおいて長期間に渡り持続感染しうることが明らかとなった。これまでの知見では、タマリンにおいて長期持続感染例が３例報告されている。しかしいずれも異なる研究グループで例外的に見出されたケースであり、再現性は認められていない。またどのケースでも一定レベルの高いウイルス血症を伴っている。一方本研究で見られたマーモセットでの長期持続感染例は始めてであり、しかも４例中２例が慢性化したことは特筆すべき点である。さらに、１例ではヒトにおける HCV 感染例で見られる間欠的（回帰的）ウイルス血症が認められた。このことは、少なくともマーモセットでは、非常に低レベルの（検出限界以下の）ウイルス複製状態でも完全には排除されず生体内で潜伏感染し、何らかの刺激もしくはウイルス側の変異といった原因により再活

性化しうることを表わしている。このような感染例を詳細に解析することにより、HCV 慢性感染機序の解明に有用な情報が得られるものと期待される。

本研究結果より、マーモセットにおけるGBV-B 感染の慢性化原因として、抗体誘導が顕著に遅延していたことが一因として挙げられる。特にこの２例では、急性期における血中ウイルス量が低レベルであった、もしくはウイルス血症が顕著に遅延した事から、感染初期においてある程度以下のウイルスレベルでは有効な免疫応答を惹起し得ないものと推測される。一方、HCV では高い頻度でウイルスゲノムに変異が生じ、これが免疫応答の回避に有効に働くことが知られている。我々の予備的実験結果でも、慢性感染マーモセット由来ウイルスゲノムには多くのアミノ酸置換を伴う変異が認められている。これらの変異が GBV-B 慢性感染に果たす役割を明らかにする目的で、慢性感染例由来ウイルスゲノムにおける経時的な適合変異の有無について解析を進めているところである。

E. 結論

今年度は、本サロゲートモデルの HCV 感染への外挿を念頭において、ウイルス接種ルートによる病態への影響、及びウイルス感染後の長期フォローアップによるウイルス・免疫応答の推移に関する解析を行なった。その結果、(i) ウイルス感染経路によりその病態に大きな影響が見られること、(ii) マーモセットにおいて初めて長期に渡り持続感染を呈する２例を見出したこと、(iii) 感染初期における血中ウイルスレベルおよび免疫応答レベルがマーモセットにおける慢性化を左右しうること、を明らかにした。これらの知見は本サロゲート霊長類モデルが抗 HCV 薬・ワクチンの有効性評価系としてのみでなく、C 型肝炎における急性期の意義や慢性化メカニズムを解明する上でも有用であることを示唆するものと考えられた。

F. 研究発表

1. 論文発表

Hara M , Kikuchi T, Sata T, Nakajima N, Ami Y, Sato Y, Tanaka K, Narita T, Ono F, Akari H, Terao K, Mukai R. Detection of SRV/D shedding in body fluids of cynomolgus macaques and comparison of partial gp70 sequences in SRV/D-T isolates. Virus Genes, in press.

Ishii K, Iijima S, Kimura N, Lee Y-J, Ageyama N, Yagi S, Yamaguchi K, Maki N, Yoshizaki S, Machida S, Suzuki T, Iwata N, Sata T, Terao K, Miyamura T, Akari H: GBV-B as a pleiotropic virus: Distribution of GBV-B in extrahepatic tissues *in vivo*. *Microbes and Infection, in press.*

2. 学会発表

Iijima S, Lee Y-J, Arold S, Strebel K, Akari H. Tripartite hydrophobic residues as a potential myristoyl pocket are a determinant for MHC-I down-regulation by HIV-1 Nef. Cold Spring Harbor meeting on Retroviruses, New York, May 2006.

Lee Y-J, Iijima S, Arold S, Strebel K, Akari H. Identification of a new functional domain on Vif to modulate Gag processing by its N-terminus. Cold Spring Harbor meeting on Retroviruses, New York, May 2006.

明里宏文：医薬基盤研究所におけるサル類を用いた感染症動物モデル開発．第１回人獣共通感染症セミナー、宮崎、平成１８年６月

Yokota T, Iijima S, Kubodera T, Ishii K, Ageyama N, Yingwei C, Lee Y-J, Maki N,

Mizusawa H, Akari H: Efficient regulation of viral replication by systemically administered siRNA in a non-human primate surrogate model for hepatitis C. 第１２回日本遺伝子治療学会、東京、平成１８年８月

川合　覚、明里宏文、揚山直英、寺尾恵治、小野文子、片貝祐子、松田　肇. サル・重症マラリア疾患モデルに対する MRI 解析システムの構築、第 5 回分子寄生虫・マラリアフォーラム、東京、2006 年 10 月

李永仲、飯島沙幸、明里宏文. Identification of a new functional domain in the N-terminus of HIV-1 Vif for modulating Gag processing. 第５４回日本ウイルス学会学術集会、平成１８年１１月

明里宏文、石井孝司、飯島沙幸、槇昇、森健一、吉崎佐矢香、横田隆徳、木村展之、片貝祐子、揚山直英、岩崎優紀、鈴木哲朗、宮村達男. C 型肝炎のサロゲート霊長類モデル：GBV-B 長期持続感染マーモセットの解析. 第５４回日本ウイルス学会学術集会、平成１８年１１月

李永仲、飯島沙幸、明里宏文：HIV-1 Vif 蛋白における Gag p2/NC プロセシング制御に関わる新しい機能ドメイン. 第２０回日本エイズ学会学術集会、平成１８年１２月

明里宏文、石井孝司、飯島沙幸、槇昇、森健一、吉崎佐矢香、木村展之、片貝祐子、揚山直英、岩崎優紀、鈴木哲朗、宮村達男. C 型肝炎の霊長類サロゲートモデルの開発：GBV-B 長期持続感染マーモセットの解析とその意義. 第１３回サル類疾病国際ワークショップ、つくば、平成１８年１２月

G. 知的財産権の出願・登録状況

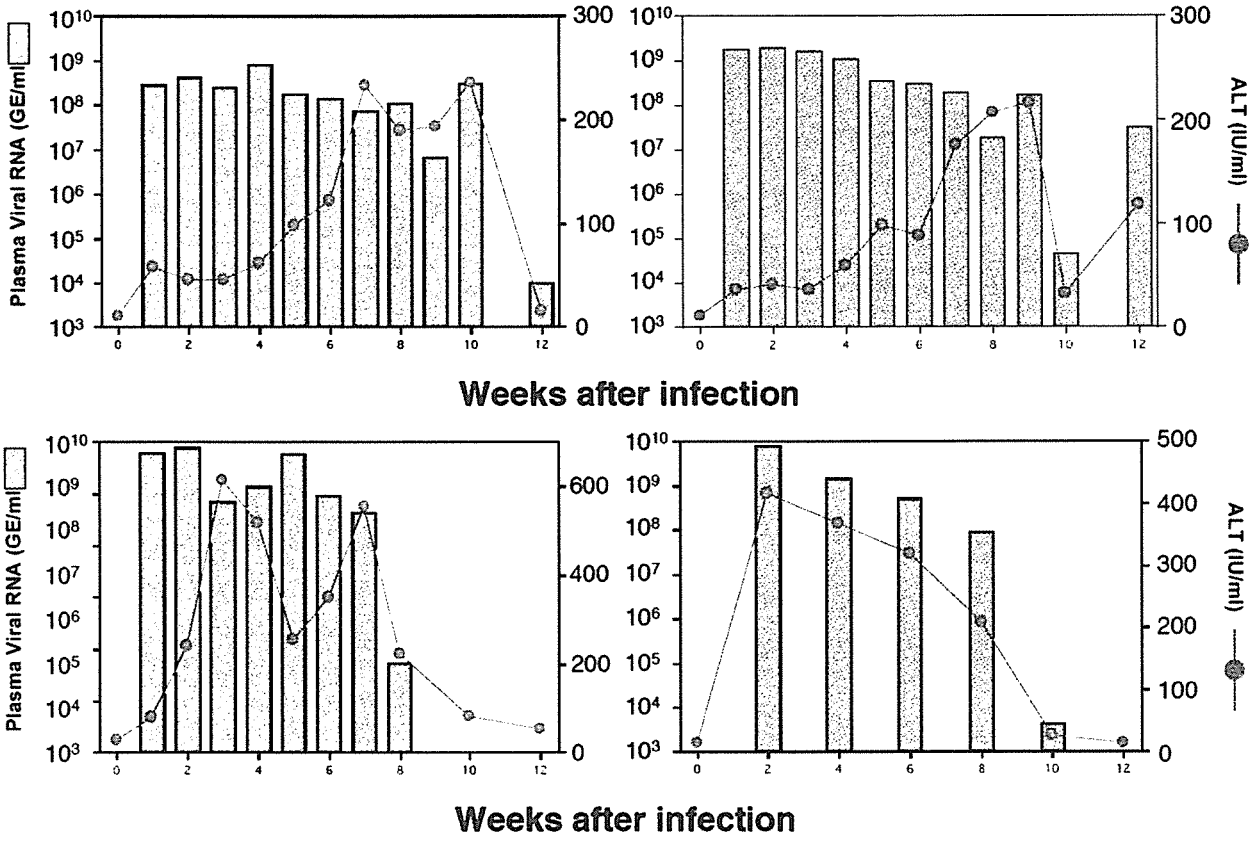

図１： GBV-B/タマリン接種実験結果：ウイルス接種ルートによる影響

上段：経静脈接種２例、下段：経肝臓接種２例における感染１２週までのウイルスロードおよびALT値

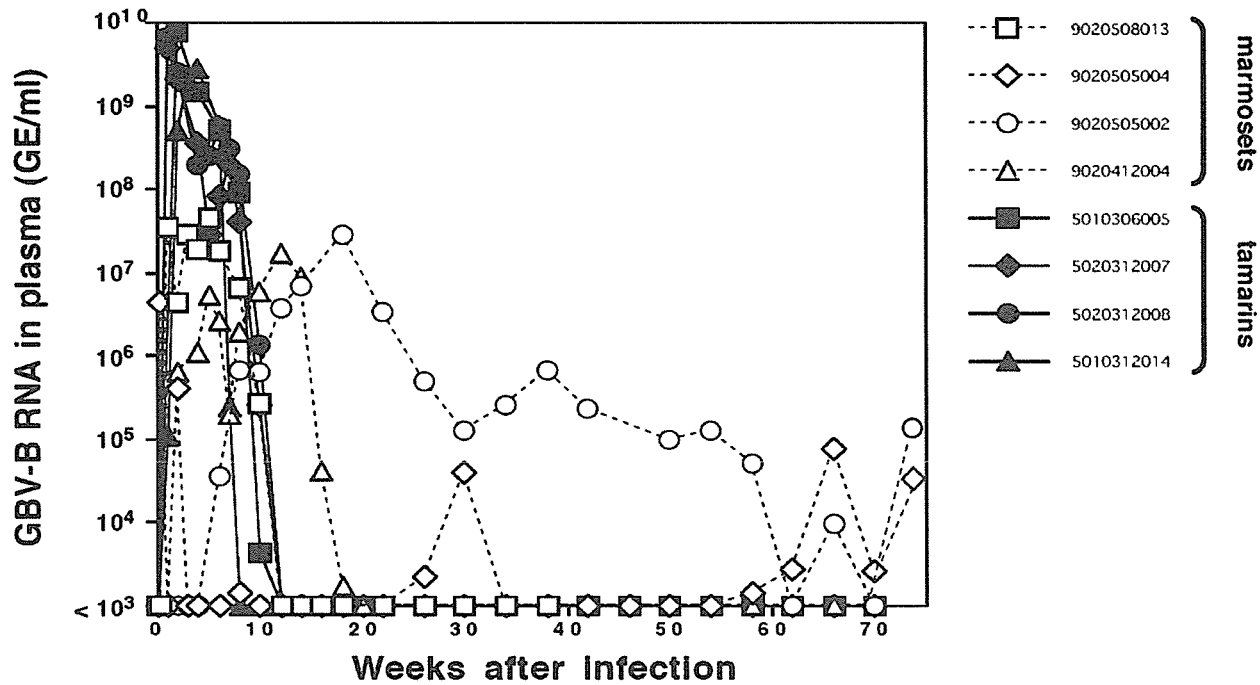

図２： GBV-B感染マーモセット、タマリンにおけるウイルス動態：長期フォローアップ

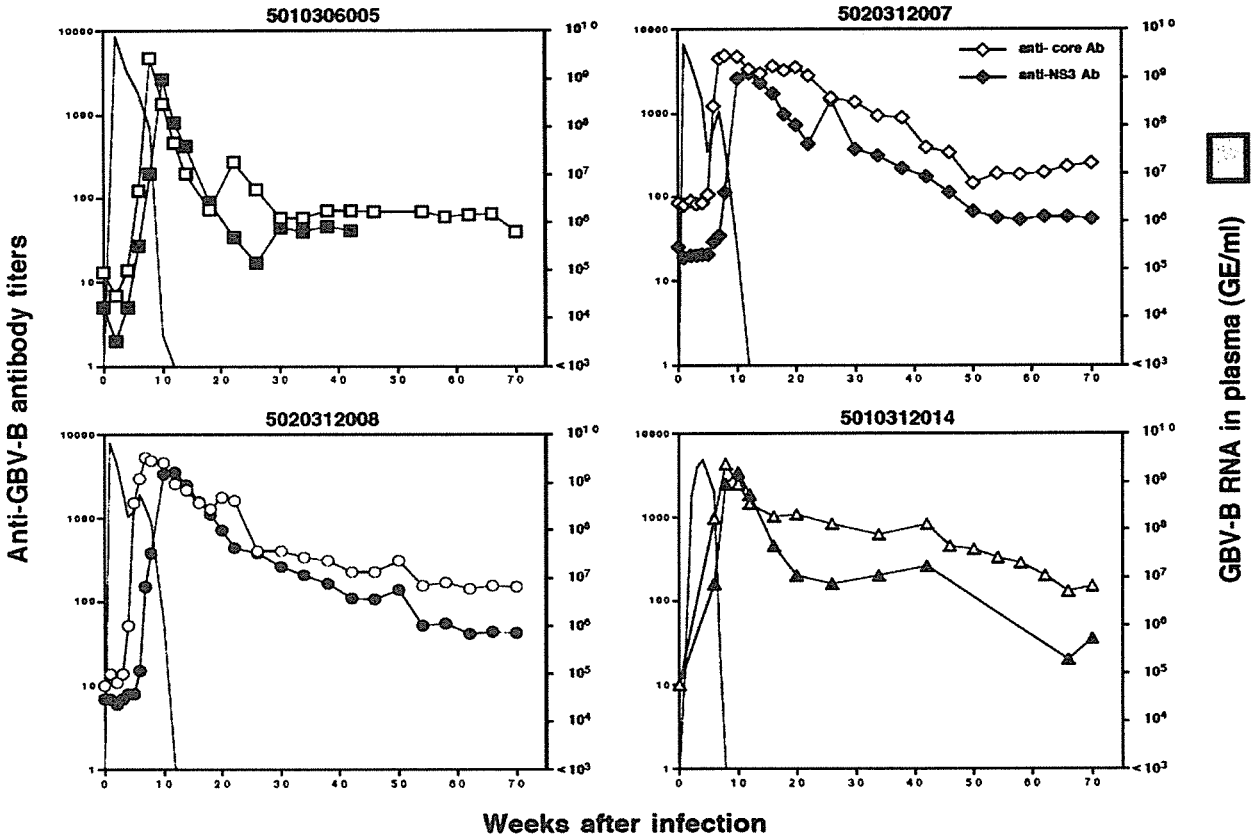

図３： GBV-B 感染タマリンにおける抗 GBV-B 抗体価とウイルス動態の相関

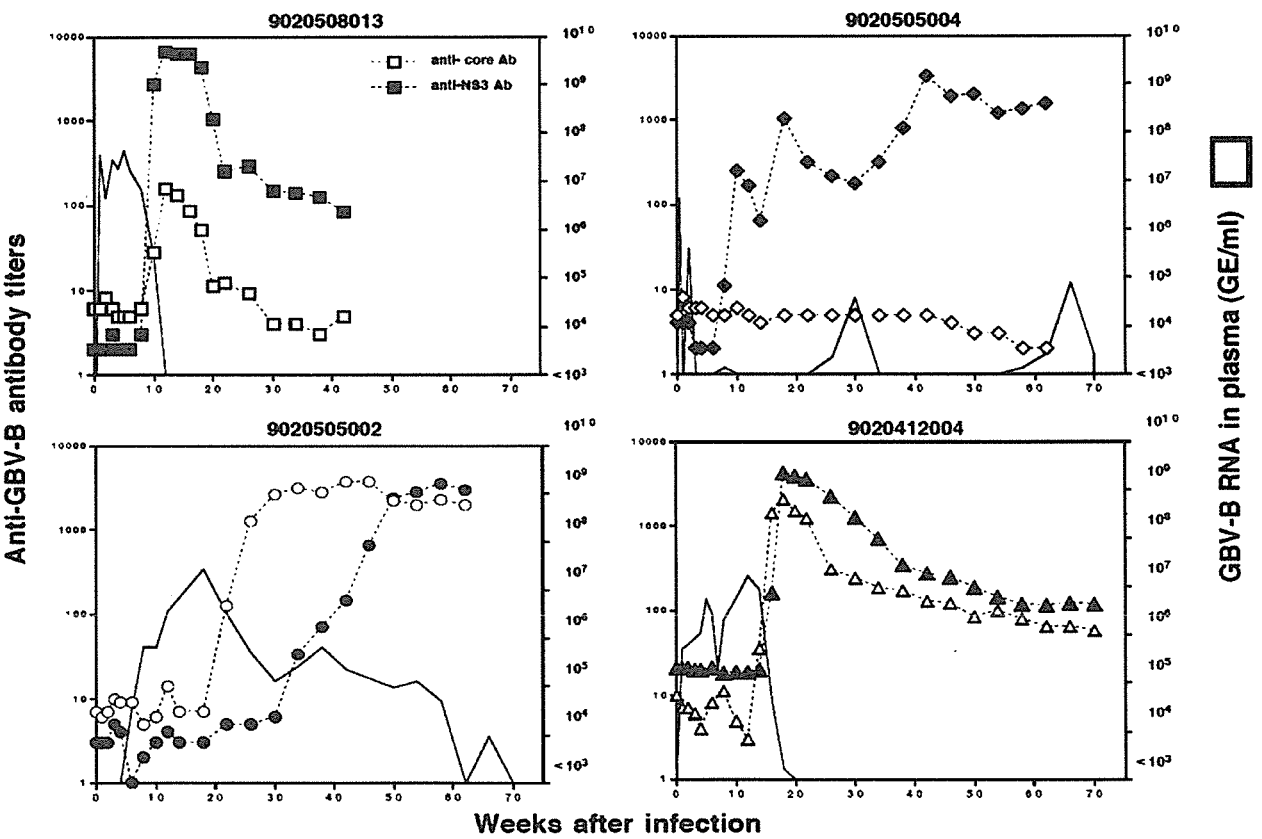

図４： GBV-B 感染マーモセットにおける抗 GBV-B 抗体価とウイルス動態の相関

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
Suzuki T, Aizaki H, Murakami K, Shoji I, and Wakita T.	Molecular biology of hepatitis C virus.	J. Gastroenterol	in press		2007
Shirakura M, Murakami K, Ichimura T, Suzuki R, Shimoji T, Fukuda K, Abe K, Sato S, Fukasawa M, Yamakawa Y, Nishijima M, Moriishi K, Matsuura Y, Wakita T, Suzuki T, Howley PM, Miyamura T, and Shoji I.	E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein.	J. Virol.	81	1174-1185	2007
Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, Murata S, Tanaka K, Suzuki T, Miyamura T, Koike K, Matsuura Y.	Critical role of PA28 γ in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis	Proc. Natl. Acad. Sci. USA	104	1661-1666	2007
Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T, Miyamura T, Koike K, and Matsuura Y.	Involvement of PA28gamma-dependent pathway in insulin resistance induced by hepatitis C virus core protein.	J. Virol.	81	1727-1735	2007
Nakai K, Okamoto T, Kimura-Someya T, Ishii K, Lim C-K,Tani H, Matsuo E, Abe T, Mori Y, Suzuki T, Miyamura T, Nunberg JH, Moriishi K, and Matsuura Y.	Oligomerization of hepatitis C virus core protein is crucial for interaction with cytoplasmic domain of E1 envelope protein.	J. Virol.	80	11265-11273	2006
Okamoto T, Nishimura Y, Ichimura T, Suzuki K, Miyamura T, Suzuki T, Moriishi K, and Matsuura Y.	Hepatitis C virus RNA replication is regulated by FKBP8 and Hsp90.	EMBO J.	25	5015-5025	2006
Baek K-H, Park H-Y, Kang C-M, Kim S-J, Jeong S-J, Hong E-K, Park J-W, Sung Y-C, Suzuki T, Kim C-M, and Lee C-W.	Overexpression of hepatitis C virus NS5A protein induces chromosome instability via mitotic cell cycle dysregulation.	J. Mol. Biol.	359	22-34	2006
Murakami K, Ishii K, Ishihara Y, Yoshizaki S, Tanaka K, Gotoh Y, Aizaki H, Kohara M, Yoshioka H, Mori Y, Manabe N, Shoji I, Sata T, Bartenschlager R, Matsuura Y, Miyamura T,	Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b.	Virology	351	381-392	2006

and Suzuki T.					
Sato S, Fukasawa M, Yamakawa Y, Natsume T, Suzuki T, Shoji I, Aizaki H, Miyamura T, and Nishijima M.	Proteomic Profiling of Lipid Droplet Proteins in Hepatoma Cell Lines Expressing Hepatitis C Virus Core Protein.	J. Biochem.	139	921-930	2006
Fukasawa M, Tanaka Y, Sato S, Ono Y, Nitahara-Kasahara Y, Suzuki T, Miyamura T, Hanada K, and Nishijima M.	Enhancement of de novo fatty acid biosynthesis in hepatic cell line Huh7 expressing hepatitis C virus core protein.	Biol. Pharm. Bull.	29	1958-1961	2006
Suzuki T, and Suzuki R.	Maturation and assembly of hepatitis C virus core protein.	In: Molecular Biology of the Flavivirus.		295-312	2006
Masaki T, Matsuura T, Ohkawa K, Miyamura T, Okazaki I, Watanabe T, and Suzuki T.	All-trans retinoic acid down-regulates human albumin gene expression through the induction of C/EBPbeta-LIP.	Biochem J.	397	345-353	2006
Shimoike T, Koyama C, Murakami K, Suzuki R, Matsuura Y, Miyamura T, and Suzuki T.	Down-regulation of the internal ribosome entry site (IRES)-mediated translation of the hepatitis C virus: critical role of binding of the stem-loop IIId domain of IRES and the viral core protein.	Virology	345	434-445	2006
Saito M, Matsuura T, Masaki T, Maehashi H, Shimuzu K, Hataba Y, Iwahori T, Suzuki T, and Braet F.	Reconstruction of liver organoid using a bioreactor.	World J. Gastroenterol.	12	1881-1888	2006
Hamazaki H, Ujino S, Miyano-Kurosaki N, Shimotohno K, Takaku H.	Inhibition of hepatitis C virus RNA replication by short hairpin RNA synthesized by T7 RNA polymerase in hepatitis C virus subgenomic replicons.	Biochem Biophys Res Commun.	343	988-994	2006
Ishii N, Watashi K, Hishiki T, Goto K, Inoue D, Hijikata M, Wakita T, Kato N, Shimotohno K.	Diverse effects of cyclosporine on hepatitis C virus strain replication.	J Virol.	80	4510-4520	2006
Naka K, Abe K, Takemoto K, Dansako H, Ikeda M, Shimotohno K, Kato N.	Epigenetic silencing of interferon-inducible genes is implicated in interferon resistance of hepatitis C virus replicon-harboring cells.	J Hepatol.	44	869-878	2006
Shimakami T, Honda M, Kusakawa T, Murata T, Shimotohno K, Kaneko S, Murakami S.	Effect of hepatitis C virus (HCV) NS5B-nucleolin interaction on HCV replication with HCV subgenomic replicon.	J Virol.	80	3332-3340	2006

Tsumura A, Hayakawa T, Kumaki Y, Takebayashi S, Sakaue M, Matsuoka C, Shimotohno K, Ishikawa F, Li E, Ueda HR, Nakayama J, Okano M.	Maintenance of self-renewal ability of mouse embryonic stem cells in the absence of DNA methyltransferases Dnmt1, Dnmt3a and Dnmt3b.	Genes Cells	11	805-814	2006
Murata T, Shimotohno K.	Ubiquitination and proteasome-dependent degradation of human eukaryotic translation initiation factor 4E.	J Biol Chem.	281	20788-20800	2006
Hamazaki H, Ujino S, Miyano-Kurosaki N, Shimotohno K, Takaku H.	Inhibition of hepatitis C virus RNA replication by short hairpin RNA synthesized by T7 RNA polymerase in hepatitis C virus subgenomic replicons.	Biochem Biophys Res Commun.	343	988-994	2006
Goto K, Watashi K, Murata T, Hishiki T, Hijikata M, Shimotohno K.	Evaluation of the anti-hepatitis C virus effects of cyclophilin inhibitors, cyclosporin A, and NIM811.	Biochem Biophys Res Commun.	343	879-884	2006
Suzuki H, Kaneko H, Tamai N, Miyano-Kurosaki N, Hashimoto K, Shimotohno K, Takaku H.	Suppression of HCV RNA replication by baculovirus-mediated shRNA expression.	Nucleic Acids Symp Ser	49	49:339-340	2005
Hamazaki H, Ujino S, Abe E, Miyano-Kurosaki N, Shimotohno K, Takaku H.	RNAi expression mediated inhibition of HCV replication.	Nucleic Acids Symp Ser	48	307-308	2006
Komohara Y, Yano H, Shichijo S, Shimotohno K, Itoh K, Yamada A.	High expression of APOBEC3G in patients infected with hepatitis C virus.	J Mol Histol.	37	327-332	2006
Hamazaki H, Takahashi H, Shimotohno K, Miyano-Kurosaki N, Takaku H. I	Inhibition of hcv replication in HCV replicon by shRNAs.	Nucleosides Nucleotides Nucleic Acids.	25	801-805	2006
Aly HH, Watashi K, Hijikata M, Kaneko H, Takada Y, Egawa H, Uemoto S, Shimotohno K.	Serum-derived hepatitis C virus infectivity in interferon regulatory factor-7-suppressed human primary hepatocytes.	J Hepatol.	46	26-36	2007
Zhang J, Yamada O, Yoshida H, Sakamoto T, Araki H, Shimotohno K	Helper virus-independent trans-replication of hepatitis C virus-derived minigenome.	Biochem Biophys Res Commun.	352	170-176	2007
Nomura- Takigawa Y, Nagano-Fujii M, Deng L, Kitazawa S, Ishido S, Sada K, Hotta H	Non-structural protein 4A of Hepatitis C virus accumulates on mitochondria and renders the cells prone to undergoing mitochondria-mediated apoptosis.	J Gen Virol	87	1935-1945	2006

Deng L, Nagano-Fujii M, Tanaka M, Nomura-Takigawa Y, Ikeda M, Kato N, Sada K, Hotta H	NS3 protein of Hepatitis C virus associates with the tumour suppressor p53 and inhibits its function in an NS3 sequence-dependent manner.	J Gen Virol	87	1703-1713,	2006
Yonashiro R, Ishido S, Kyo S, Fukuda T, Goto E, Matsuki Y, Ohmura-Hoshino M, Sada K, Hotta H, Yamamura H, Inatome R, Yanagi S	A novel mitochondrial ubiquitin ligase plays a critical role in mitochondrial dynamics.	EMBO J	25	3618-3626	2006
Kim KI, Kim SR, Sasase N, Taniguchi M, Harada S, Kinoshita K, Kim SH, Akimoto Y, Shikata M, Kimura N, Izawa S, Ohtani A, Nakao K, Motojima M, Kinoshita M, Hirai M, Ohzu M, Hirooka T, Nabeshima S, Ishii F, Tanaka K, Hotta H	2'-,5'-Oligoadenylate synthetase response ratio predicting virological response to PEG-interferon-alpha2b plus ribavirin therapy in patients with chronic hepatitis C.	J Clin Pharm Ther	31	441-446	2006
Vallet S, Gouriou S, Nkontchou G, Hotta H, Vilerio M, Legrand-Quillien MC, Beaugrand M, Trinchet JC, Nousbaum JB, Dény P, Gaudy C, Goudeau A, Picard B, Payan C	Is hepatitis C virus NS3 protease quasispecies heterogeneity predictive of progression from cirrhosis to hepatocellular carcinoma?	J Viral Hepat	14	96-106	2007
El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim S R, and Hotta H	Prediction of efficient virological response to pegylated interferon/ribavirin combination therapy by NS5A sequences of hepatitis C virus and anti-NS5A antibodies in pretreatment sera.	Microbiol Immunol	in press		2007
Lu L, Li C, Fu Y, Thaikruea L, Thongswat S, Maneekarn N, Apichartpiyakul C, Hotta H, Okamoto H, Netski D, Pybus O G, Murphy D, Hagedorn C H, Nelson K E	Complete genomes for hepatitis C virus subtypes 6f, 6i, 6j and 6m:viral genetic diversity among Thai blood donors and infected spouses.	J Gen Virol	in press		2007
Ii M. Seya T.	A novel cyclohexene derivative, TAK-242, selectively inhibits Toll-like receptor 4-mediated cytokine production through suppression of intracellular signaling	Molec. Pharmacol.	69	1288-1295	2006

Sakurai F. Seya T.	Crucial role of the short consensus repeats 1 and 2, not the cytoplasmic domain, of human CD46 on infection of subgroup B adenovirus serotype 35	J. Control Release	113	271-278	2006
Wrackmeyer U. Seya T.	Intelectin: a novel lipid reft-associated protein in the enterocyte brush border	Biochemistry	45	9188-9197	2006
Sasai M. Seya T.	NAP1 (NAK-associated protein 1) participates in both the TLR3 and the cytoplasmic pathways in type I interferon induction	J. Immunol.	177	8676-8683	2006
Ishii A. Seya T.	Lamprey Toll-like receptors with properties distinct from those of variable lymphocyte receptors	J Immunol.	178	397-486	2007
Niimi K. Seya T.	Toll-like receptor 3-mediated eotaxin-1 release from human bronchial smooth muscle cells	J. Immunol.	178	489-495	2007
Akazawa T. Seya T.	Antitumor NK activation induced by the Toll-like receptor3-TICAM-1 (TRIF) pathway in myeloid dendritic cells	Proc. Natl. Acad. Sci. USA.	104	252-257	2007
Sukenobu N. Seya T.	Protein kinase Cd associates TIRAP/Mal and regulates NF-kB and mitogen-activated protein kinases in Toll-like receptor signaling.	Molec. Immunol.	44	2257-2264	2007
Azuma M. Seya T.	Inhibition of lipid A-mediated type I interferon induction by Bactericidal/peameability-increasing protein (BPI).	Biochem. Biophys. Res. Commun	354	574-578	2007
Ebihara T. Seya T.	NKG2D ligands are induced on human dendritic cells by TLR ligand stimulation and RNA virus infection	Int. Immunol.	in press		2007
Shingai M. Seya T.	Inhibition of the TLR pathway for interferon-beta induction by soluble G protein of respiratory syncytial virus	J. Virol.	in press		2007

Ishii A. Seya T.	Phylogenetic and expression analysis of amphibian Xenopus Toll-like receptors	Immunogenetics	in press		2007
Hirofumi Uto, Katsuhiro Hayashi, Kazunori Kusumoto, Satoru Hasuike, Kenji Nagata, Mayumi Kodama, Akio Ido, Michinori Kohara, Sherri O. Stuver, Hirohito Tsubouchi	Spontaneous elimination of hepatitis C virus RNA in individuals with persistent infection in a hyperendemic area of Japan	Hepatology Research	34	28-34	2006
Tsunamasa Watanabe, Masayuki Sudoh, Makoto Miyagishi, Masaaki Arai, Kazuaki Inoue, Kazunari Taira, Makoto Yoshiba, Michinori Kohara.	Targeting the Hepatitis C Virus genome with RNA interference using highly effective and non-toxic long double-stranded RNA	Gene Theraphy	13	883-892	2006
Takuya Umehara, Masayuki Sudoh, Fumihiko Yasui, Chiho Matsuda, Yukiko Hayashi, and Michinori Kohara	Serine palmitoyltransferase inhibitor suppresses HCV replication in a mouse model	Biochem. Biophys. Res. Commun.	346	67-73	2006
Yoshiyuki Tanaka, Junko Kato, Michinori Kohara, Mark S. Galinski	Antiviral effects of glycosylation and glucose trimming inhibitors on human parainfluenza virus type 3	Antiviral Res.	72	1-9	2006
Kaito M, Ishida S, Tanaka H, Horiike S, Fujita N, Adachi Y, Kohara M, Konishi M, Watanabe S	Morphology of hepatitis C and hepatitis B virus particles as detected by immunogold electron microscopy	Med Mol Morphol.	39	63-71	2006
Hironori Nishitsuji, Michinori Kohara, Mari Kannagi, and Takao Masuda	Effective Suppression of Human Immunodeficiency Virus Type 1 through Combination of Short- or Long-hairpin RNAs Targeting Essential Sequences for Retroviral Integration	J. Virology	80	7658-66	2006
Masahiro Kitabatake, Shingo Inoue, Fumihiko Yasui, Shoji Yokochi, Masaaki Arai, Kouichi Morita, Hisatoshi Shida, Minoru Kidokoro, Fukashi Murai, Mai Quynh Le, Kouji Matsushima and Michinori Kohara	SARS-CoV spike protein recombinant vaccinia virus efficiently induces neutralizing antibodies in spite of pre-immunization with vaccinia virus	Vaccine	25	630-637	2006

Kazuaki Inoue, Takuya Umehara, Urs T. Ruegg, Fumihiko Yasui, Tsunamasa Watanabe, Hiroshi Yasuda, Makoto Yoshiba, Jean-Maurice Dumont, Pietro Scalfaro, Michinori Kohara	Evaluation of the cyclophilin inhibitor DEBIO-025 in hepatitis C virus-infected chimeric mice in vivo	Hepatology	in press		2007
楳原琢哉、坂本洋、須藤正幸、小原道法	スフィンゴ脂質生成とC型肝炎ウイルス	BIO Clinica	22	90-95	2007
Abe K, Nozaki A, Tamura K, Ikeda M, Naka K, Dansako H, Hoshino H, Tanaka K, Kato N.	Tandem repeats of lactoferrin-derived anti-hepatitis C virus peptide enhance antiviral activity in cultured human hepatocytes.	Microbiol. Immunol.	51	117-125	2007
Yuasa K, Naganuma A, Sato K, Ikeda M, Kato N, Takagi H, Mori M.	Zinc is a negative regulator of hepatitis C virus RNA replication.	Liver Int.	26	1111-1118	2006
Ikeda M, Abe K, Yamada M, Dansako H, Naka K, Kato N.	Different Anti-HCV profiles of statins and their potential for combination therapy with interferon.	Hepatology	44	117-125	2006
Naka K, Dansako H, Kobayashi N, Ikeda M, Kato N.	Hepatitis C virus NS5B delays cell cycle progression by inducing interferon-b via Toll-like receptor 3 signaling pathway without replicating viral genomes.	Virology	346	348-362	2006
Koike K	Hepatitis C virus infection presenting with metabolic disease by inducing insulin resistance	Intervirology	49	51-57	2006
Koike K, Miyoshi H	Oxidative stress and hepatitis C viral infection	Hepatol Res	34	65-76	2006

Koike K	Oxidative stress and apoptosis in hepatitis C: the core issue	J Gastroenterology	41	292-294	2006
Okuse C, Yotsuyanagi H, Nagase Y, Kobayashi Y, Yasuda Y, Koike K, Iino S, Suzuki M, Itoh F	Risk Factors for Retinopathy Associated with Interferon Alpha-2b and Ribavirin Combination Therapy in Patients with Chronic Hepatitis C	World J Gastroenterol	12	3759-3759	2006
Koike K	Antiviral treatment of hepatitis C: present status and future prospects	J Infect Chemother	12	227-232	2006
Takahashi H, Yotsuyanagi H, Yasuda K, Koibuchi T, Suzuki M, Kato T, Nakamura T, Iwamoto A, Nishioka K, Iino S, Koike K, Itoh F	Molecular epidemiology of hepatitis A virus in metropolitan areas in Japan	J Gastroenterol	41	981-986	2006
Koike K, Tsukada K, Yotsuyanagi H, Moriya K, Kikuchi Y, Oka S, Kimura S	Prevalence of Coinfection with Human Immunodeficiency Virus and Hepatitis C Virus in Japan	Hepatol Res	37	2-5	2007
Koike K	Pathogenesis of HCV-associated HCC: dual-pass carcinogenesis through the activation of oxidative stress and intracellular signaling	Hepatol Res	In press		2007
Koike K	Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways	J Gastroenterol Hepatol	In press		2007
Yotsuyanagi H, Koike K	Mechanisms underlying drug resistance in antiviral treatment for infections with hepatitis B and C viruses	J Gastroenterol	In press		2007
Suzuki Y, Yotsuyanagi H, Okuse C, Nagase Y, Takahashi H, Moriya K, Suzuki M, Koike K, Iino S, Itoh F	Fatal liver failure caused by reactivation of lamivudine-resistant hepatitis B virus: A case report	World J Gastroenterol	In press		2007
Kato H., Takeuchi O., Sato S., Yoneyama M., Yamamoto M., Matsui K., Uematsu S., Jung A., Kawai T., Ishii K. J.,	Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses.	Nature	441	101-105	2006

Yamaguchi O., Otsu K., Tsujimura T., Koh C.-S., Sousa C. R., Matsuura Y., Fujita T., and Akira S.					
Shimazu T, Komatsu Y, Nakayama K-I, Fukazawa H, Horinouchi S, Yoshida M.	Regulation of SV40 large T-antigen stability by reversible acetylation.	Oncogene	25	7391-7400	2006
Murakami Y, Yamagoe S, Noguchi K, Takebe Y, Takahashi N, Uehara Y, Fukazawa H.	Ets-1-dependent expression of vascular endothelial growth factor receptors is activated by latency-associated nuclear antigen of Kaposi's sarcoma-associated herpesvirus through interaction with daxx.	J. Biol. Chem.	281	28113-28121	2006
Masumi A, Fukazawa H, Shimazu T, Yoshida M, Ozato K, Komuro K, Yamaguchi K.	Nucleolin is involved in interferon regulatory factor-2-dependent transcriptional activation.	Oncogene	25	5113-5124	2006
Ishii K, Iijima S, Kimura N, Lee Y-J, Ageyama N, Yagi S, Yamaguchi K, Maki N, Yoshizaki S, Machida S, Suzuki T, Iwata N, Sata T, Terao K, Miyamura T, Akari H	GBV-B as a pleiotropic virus: Distribution of GBV-B in extrahepatic tissues in vivo	Microbes and Infection	in press		2007
Hara M , Kikuchi T, Sata T, Nakajima N, Ami Y, Sato Y, Tanaka K, Narita T, Ono F, Akari H, Terao K, Mukai R	Detection of SRV/D shedding in body fluids of cynomolgus macaques and comparison of partial gp70 sequences in SRV/D-T isolates	Virus Genes	in press		2007

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Production of infectious hepatitis C virus particles in three-dimensional cultures of the cell line carrying the genome-length dicistronic viral RNA of genotype 1b

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Abstract

We show that a dicistronic hepatitis C virus (HCV) genome of genotype 1b supports the production and secretion of infectious HCV particles in two independent three-dimensional (3D) culture systems, the radial-flow bioreactor and the thermoreversible gelation polymer (TGP), but not in monolayer cultures. Immunoreactive enveloped particles, which are 50–60 nm in diameter and are surrounded by membrane-like structures, are observed in the culture medium as well as at the endoplasmic reticulum membranes and in dilated cytoplasmic cisternae in spheroids of Huh-7 cells. Infection of HCV particles is neutralized by anti-E2 antibody or patient sera that interfere with E2 binding to human cells. Finally, the utility of the 3D-TGP culture system for the evaluation of antiviral drugs is shown. We conclude that the replicon-based 3D culture system allows the production of infectious HCV particles. This system is a valuable tool in studies of HCV morphogenesis in a natural host cell environment.

Keywords: Hepatitis C virus; Replication; Three-dimensional culture; Virus particle

Introduction

Infection with hepatitis C virus (HCV) currently represents a major medical and socioeconomic problem. HCV is a main causative agent of chronic hepatitis, cirrhosis, and hepatocellular carcinoma, and there are an estimated 170 million HCV carriers worldwide (Choo et al., 1989). The standard treatments for HCV infection are interferon alpha (IFN-α) in combination with ribavirin (RBV) or, more recently, a polyethylene glycol-modified form of IFN-α; however, sustained response is seen in only ~50% of treated patients (Davis et al., 2003; Manns et al., 2001). Further development of new anti-HCV drugs and vaccines has been obstructed by the lack of either a small animal model or a robust cell culture system capable of supporting viral replication and the production of infectious progeny.

HCV is a small enveloped RNA virus belonging to the family Flaviviridae and harboring a single-stranded RNA genome with

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positive polarity. A precursor polyprotein of ~3000 amino acids (aa) is encoded by a large open reading frame. This polyprotein is cleaved by cellular and viral proteases to give rise to a series of structural and nonstructural proteins (Choo et al., 1991; Grakoui et al., 1993; Hijikata et al., 1991). The establishment of selectable dicistronic HCV RNAs that are capable of autonomous replication in human hepatoma Huh-7 cells was a significant breakthrough in HCV research (Blight et al., 2000; Lohmann et al., 1999) and has provided an important tool for the study of HCV replication mechanisms and for screening antiviral drugs (Frese et al., 2001; Guo et al., 2001). This replicon system was first developed to replicate only viral subgenomic RNAs but has been further expanded to enable the replication of genome-length dicistronic RNAs (Ikeda et al., 2002; Pietschmann et al., 2002). Although the viral genome replicates and all HCV proteins are properly processed in this system, virus particle production has not yet been achieved. A number of researchers (Date et al., 2004; Kato et al., 2001, 2003) have developed an HCV genotype 2a replicon (JFH-1) that efficiently replicates in a variety of human cells. Recently, it has been demonstrated that the full-length JFH-1 genome or a chimeric genome using JFH-1 and J6, a related genotype 2a strain, produces infectious particles in cell cultures (Lindenbach et al., 2005; Wakita et al., 2005; Zhong et al., 2005). More recently, production of infectious genotype 1a virus (Hutchinson strain) using similar experimental systems has been described (Yi et al., 2006). These complete HCV culture systems produce robust levels of infectious virus and provides a powerful tool for HCV research. However, to date their applications have not been extended to constructs based on strains of genotype 1b, which is highly prevalent worldwide.

We previously demonstrated that differentiated human hepatoma FLC4 cells transfected with in vitro transcribed HCV genomic RNA can produce and secrete infectious particles in three-dimensional (3D) radial-flow bioreactor (RFB) culture (Aizaki et al., 2003). This RFB system was initially aimed to develop artificial liver tissue, and the bioreactor column consists of a vertically extended cylindrical matrix through which liquid medium flows continuously from the periphery toward the center of the reactor (Kawada et al., 1998). In RFB culture, human hepatocellular carcinoma-derived cells can grow spherically or cubically, and they retain liver functions such as albumin synthesis (Kawada et al., 1998; Matsuura et al., 1998) and drug-metabolizing activity mediated by cytochrome P450 3A4 (Iwahori et al., 2003).

In the present study, two kinds of 3D culture techniques, the RFB and the thermoreversible gelation polymer (TGP), were used for the production and secretion of infectious HCV particles by using a dicistronic HCV genome derived from genotype 1b. We also demonstrate that these 3D culture systems are useful for evaluating anti-HCV drugs.

Results

Secretion of HCV-LPs from RCYM1 carrying genome-length dicistronic HCV RNA cultured in RFB culture

We first assessed the replicative capacity of selectable genome-length HCV RNAs in FLC4 cells. However, no G418-resistant colonies were observed, indicating that FLC4 cells do not support replication of these HCV RNAs (data not shown). Therefore, subsequent experiments were carried out with a stable Huh-7 cell line, RCYM1, which supports full-length HCV RNA replication and which was developed by transfection of the cells with genome-length dicistronic RNA derived from the Con1 clone I389neo/core-3′/NK 5.1 (genotype 1b)

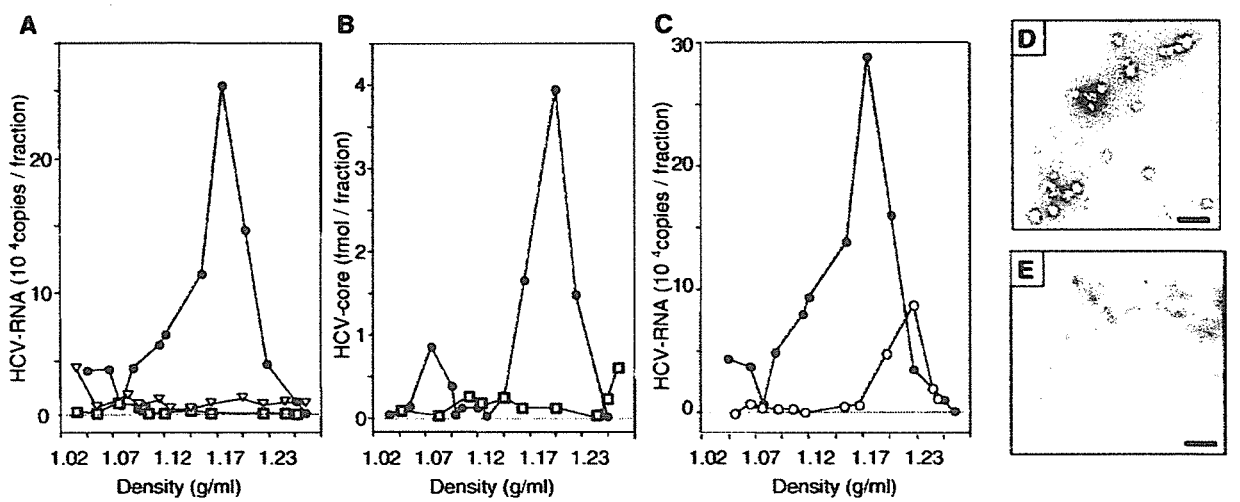

Fig. 1. Sucrose density gradient analysis of culture supernatants of RCYM1 cells. Culture media collected from radial-flow bioreactor (RFB)-cultured RCYM1 (closed circles), monolayer-cultured RCYM1 (open squares), and RFB-cultured 5–15 cells (open triangles) were fractionated as described in Materials and methods. (A) HCV RNA in each fraction was measured by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). Mean values of duplicates were plotted against the density of the corresponding fraction. (B) HCV core protein in each fraction was determined by enzyme-linked immunosorbent assay (ELISA). Mean values of duplicates were plotted against the density. (C) Culture medium of RFB-cultured RCM1 cells were treated with 0.2% NP40 (open circles), followed by centrifugation in a sucrose gradient. Each fraction was tested for HCV RNA by real-time RT-PCR. (D, E) Electron microscopy analysis. Samples were prepared from the 1.18 g/ml fraction of culture media collected from RFB-cultured (D) or monolayer-cultured (E) RCYM1 cells.

(Pietschmann et al., 2002). The HCV RNA level in RCYM1 cells was approximately 5×10^6 copies/μg total RNA as determined by real-time reverse transcriptase-polymerase chain reaction (RT-PCR). The expression and subcellular localization of HCV protein were confirmed by Western blotting and immunofluorescence analysis (data not shown). To develop 3D RFB cultures, first we loaded RCYM1 cells onto an RFB column by flowing cell suspension, after which the cells were attached to carrier beads. Cells proliferated within the 3D matrix, and culture medium was circulated radially through the column.

In order to investigate whether HCV-like particles (HCV-LPs) were secreted from RCYM1 cells in the RFB culture system, we fractionated culture fluid collected after 5–10 days of culture by continuous 10–60% (wt/vol) sucrose density gradient centrifugation. HCV RNA and core protein were predominantly detected in the 1.15–1.20 g/ml fractions, with maximal detection in the 1.18 g/ml fraction (Figs. 1A and B). In the same experiment using 5–15 cells, in which a subgenomic HCV replicon replicates, no peak similar to that observed in RCYM1 cells corresponding to HCV RNA was detected. In both RCYM1 cells and 5–15 cells in the RFB culture system, a substantial amount of HCV RNA was detected in the 1.03–1.07 g/ml fractions (Fig. 1A). Consistent with a previous report by Pietschmann et al. (2002), these RNAs released from cells with a subgenomic replicon did not correspond to virus particles. When an equivalent number of RCYM1 cells were cultured in a monolayer culture system, limited amounts of HCV RNA and core protein were detected in the culture supernatant (Figs. 1A and B).

The mature HCV virion is thought to have a nucleocapsid and an outer envelope composed of a lipid membrane with viral envelope glycoproteins. Culture fluids were treated with NP40 in order to solubilize lipids and were then subjected to sucrose density gradient centrifugation. HCV RNA sedimented to a density of 1.22 g/ml rather than 1.18 g/ml (Fig. 1C), indicating that the density of HCV particles became higher due to de-envelopment. Transmission electron microscopy (TEM) of the 1.18 g/ml fraction, which was subjected to negative staining after concentration, revealed particle structures with diameters of 30–60 nm and a major particle size of 50 nm (Fig. 1D). No similar particle-like structures were observed in the same density fraction of the RCYM1 monolayer culture (Fig. 1E) or in the 1.23 g/ml fraction of the RCYM1-RFB culture (data not shown). These results indicate that, in the RFB system, the production and secretion of HCV-LPs is possible with a selectable dicistronic HCV genome.

Production and secretion of HCV-LPs from spheroid culture of RCYM1 cells using TGP

In the 3D RFB culture system for RCYM1 cells, extracellular secretion of HCV-LPs was observed. Based on this observation, we hypothesized that morphological changes occurring in 3D culture, such as polarity formation, promote advantageous in the assembly of viral proteins, particle formation, and extracellular secretion. To examine whether similar phenomena could be observed in other 3D culture systems, we investigated HCV-LP expression using a 3D culture system with TGP as a carrier.

TGP is a biocompatible polymer made from conjugates of polyethyleneglycol and poly-*N*-isopropylacrylamide, which is a thermoresponsive polymer composed of *N*-isopropylacrylamide and *n*-butylmethacrylate. The TGP solution possesses sol-gel transition properties; it is water soluble (sol phase) at temperatures below the transition temperature, and it is insoluble (gel phase) above it. It is possible to manipulate the transition temperatures through molecular engineering. The transition temperature for TGP in the present experiments was approximately 20 °C.

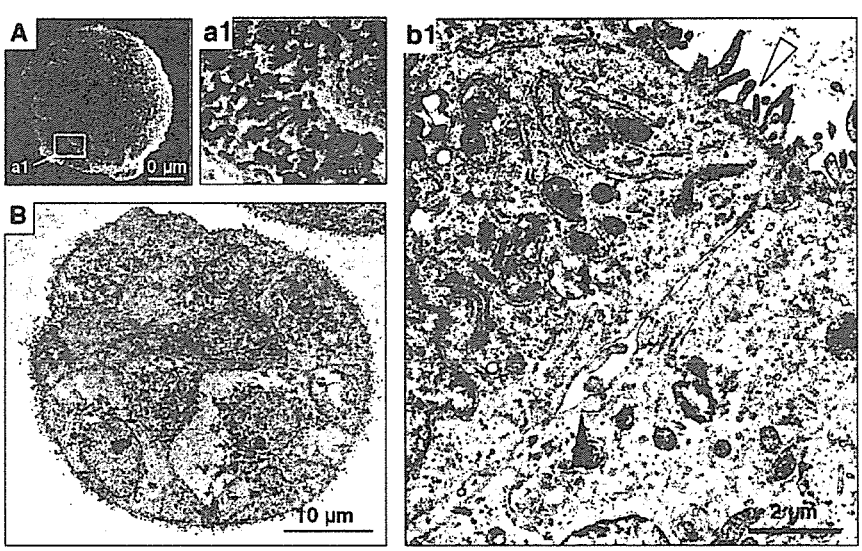

Fig. 2. Huh-7 and RCYM1 cells form spheroids in thermoreversible gelation polymer (TGP). Scanning electron microscopy (A and a1) and transmission electron microscopy (B and b1) of RCYM1 cells cultured in TGP for 8 days. Open arrowhead, microvilli; closed arrowheads, bile canaliculi-like structures.

RCYM1 cells, which were seeded into the TGP, formed three-dimensional compacted aggregates called spheroids after 3 days of culture, and numerous spheroids with diameters of approximately 1 mm were observed after 7–10 days of culture. After 8 days of culture, the spheroids were fixed and examined by scanning electron microscopy (Figs. 2A and a1) and ultrathin sections were examined by TEM (Figs. 2B and b1). Well-developed microvilli, a feature of polarized epithelium, were observed on the cell surface (Figs. 2A and a1). Bile canaliculi-like structures were also observed within intercellular spaces, and they appeared to be connected via tight junctions (Figs. 2B and b1). This cytomorphology, similar to that observed in the RFB culture (Kawada et al., 1998; Matsuura et al., 1998), correlated well with the features of mature liver tissue.

It is known that the replication of HCV replicons in Huh-7 cells depends on host cell growth. We found that the growth of RCYM1 cells in the TGP culture system was significantly slower than that of cells in monolayer culture (Fig. 3A). Accordingly, the expression of HCV proteins (Fig. 3B) in the RCYM1 spheroids was apparently lower compared to those observed in the monolayer cells. The viral RNA copy number in the spheroids was approximately one tenth of that in the monolayer culture (data not shown). The results of sucrose density gradient analysis of culture supernatant demonstrated co-sedimentation of HCV RNAs and core proteins at a density of 1.15–1.20 g/ml, with a peak at 1.18 g/ml (Figs. 3C and D). This distribution was consistent with the pattern obtained in RFB culture (Figs. 1A and B). It should be noted that in these experiments, lower cell numbers were used in the 3D cultures than in the monolayer cultures because of the slower growth of cells. As estimated from the quantitative data of the 1.15–1.20 g/ml fractions of the culture supernatants, 0.1–1 copies of HCV RNA/cell/day are produced and assembled into viral particles in the TGP-cultured RCYM1 cells.

TEM analysis of the 1.18 g/ml fraction after negative staining showed particle structures with a diameter of 50–60 nm and spike-like projections (Fig. 3E). Observation of ultrathin sections indicated a lipid bilayer-like membrane structure with a

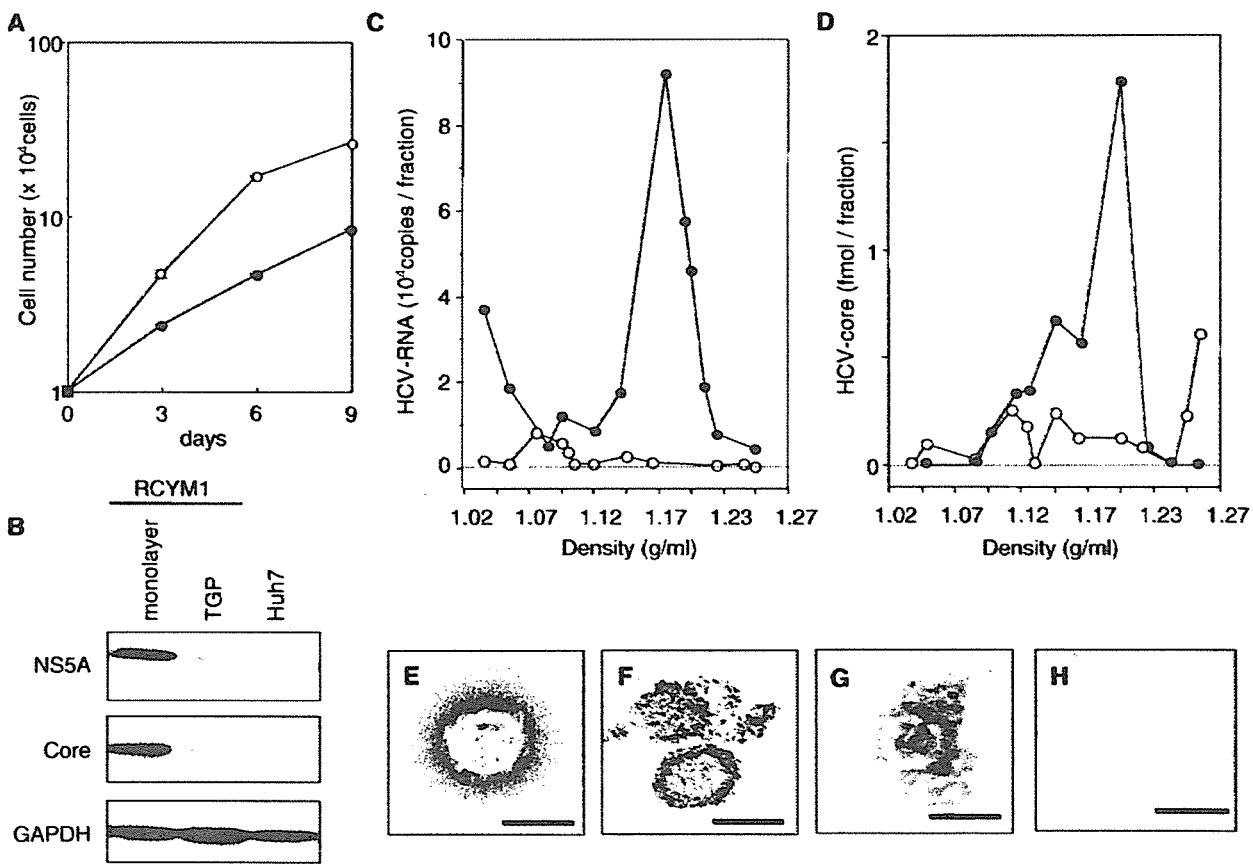

Fig. 3. Expression of HCV proteins in RCYM1 cells and secretion of viral particles in TGP culture. (A) Cell growth curves of the TGP (closed circles) and monolayer (open circles) culture of RCYM1 cells. Cells were harvested at days 0, 3, 6, and 9 postinoculation and cell numbers were determined. (B) Western blotting of HCV core and NS5A proteins in RCYM1 cells and control Huh-7 cells. (C, D) Sucrose density gradient analysis of culture supernatants of RCYM1 cells. The culture supernatants were fractionated as described in Materials and methods. HCV RNA (C) and core protein (D) in each fraction were determined by ELISA and real-time RT-PCR, respectively. Representative data from three independent experiments are shown. Closed circles, TGP culture; open circles, monolayer culture. (E–H) Electron microscopy of HCV-like particles (HCV-LPs) in the supernatants of TGP-cultured RCYM1 cells. (E) Negative staining of HCV-LPs in the 1.18 g/ml density fraction. There was no spherical structure in 1.05 g/ml density fraction, as shown in panel H. (F) Ultrathin section of HCV-LPs. Precipitated HCV-LP samples were prepared from the 1.18 g/ml fraction as described in Materials and methods. (G) Immunogold labeling of HCV-LPs with an anti-E2 antibody in the 1.18 g/ml density fraction. Gold particles, 5 nm; scale bars, 50 nm.

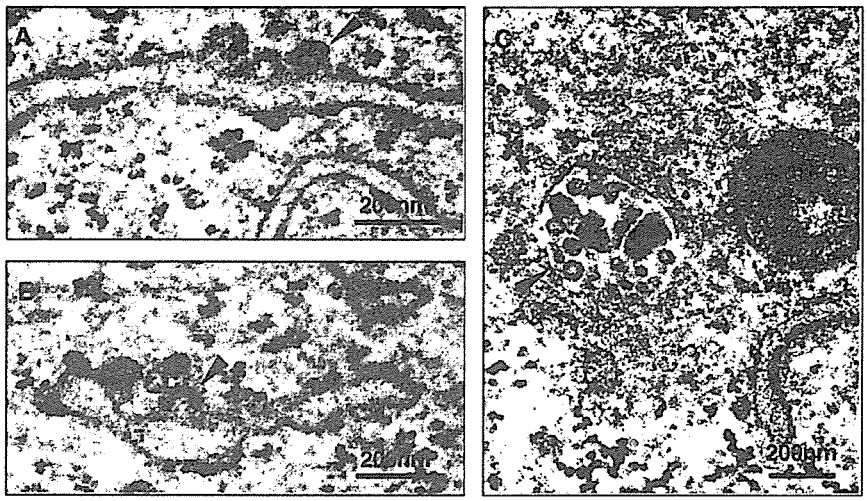

Fig. 4. Electron microscopy of ultrathin sections of RCYM1 cells grown in TGP. HCV-LPs in TGP-cultured RCYM1 cells. Spherical virus-like particles 50–60 nm in diameter (arrowheads) were observed at the ER membranes (A, B) and in the cytoplasmic vesicles (C).

width of approximately 5 nm (Fig. 3F). Immunoelectron microscopic study using anti-E2 antibody revealed HCV envelope protein(s) on the particle surface (Fig. 3G). Substantial amounts of HCV RNA were detected in the 1.03–1.05 g/ml fractions of the supernatant (Fig. 3C); however, HCV-LP structures were not observed in these fractions (Fig. 3H). These results were consistent with those from the RFB system, as shown above. The efficacy of 3D cell culture systems in virion formation was thus demonstrated in both the RFB and TGP culture systems using human liver-derived cells.

Ultrastructural localization of HCV-LPs in TGP-cultured spheroids of RCYM1 cells

We next determined the intracellular localization of HCV-LPs produced in RCYM1-TGP culture at the ultrastructural level by electron microscopic (EM) analysis of ultrathin sections. Spherical particles having membrane-like structures with short surface projections (diameter, 50–60 nm) were observed primarily at the endoplasmic reticulum (ER) membrane (Fig. 4A) as well as in the dilated cisternae of the ER (Fig. 4B). In vesicles, these virus-like particles were frequently associated with amorphous materials (Fig. 4C). In a previous study, Shimizu et al. (1996) report that virus-like particles with similar morphology and size were observed in human B cells infected with HCV. No similar particle-like structures were observed in RCYM1 cells in monolayer culture or in subgenomic replicon 5–15 in cells in TGP culture (data not shown).

In order to determine whether the virus-like particles observed by conventional TEM in the present experiment were HCV-LPs, we conducted immunoelectron microscopic analysis with anti-core antibody and anti-E1 antibody. Double-labeling experiments showed that the virus-like particles associated with the ER membrane exhibited immunoreactivity for both HCV proteins, and that the E1 protein surrounded the core proteins (Fig. 5A). To the best of our knowledge, this is the first report to clearly demonstrate that the viral envelope protein surrounds the core protein in HCV particle formation. As a negative control, thin sections prepared from subgenomic RNA containing 5–15 cells were stained with these antibodies and were found to exhibit negligible levels of background immunostaining (data not shown).

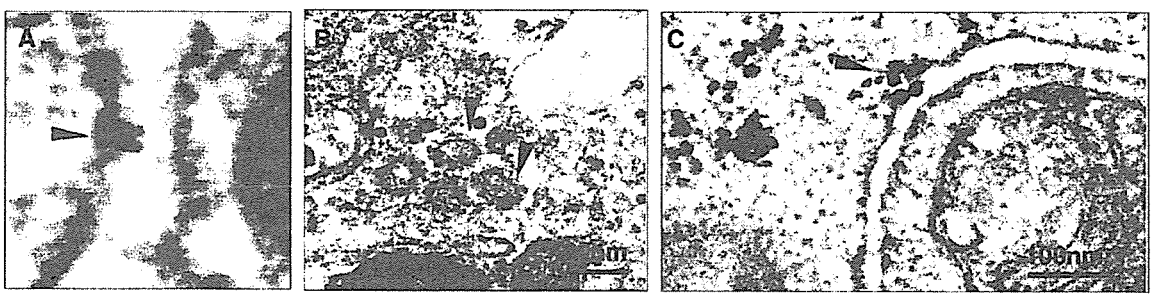

Fig. 5. Immunoelectron microscopy of ultrathin sections of TGP-cultured RCYM1 cells. (A) Double immunostaining with anti-E1 and anti-core monoclonal antibodies. Core protein-specific gold particles (10 nm in diameter) and E1 protein-specific gold particles (5 nm in diameter) formed rosettes on the surface of the ER membrane. (B and C) Silver-intensified immunogold staining with anti-core (B) and anti-E1 (C) antibodies. The second antibody conjugated with gold particles 1.4 nm in diameter was applied, followed by enlargement of the particles by the silver enhancement reagent. Arrowheads indicate virus-like particles reacting with anti-core and/or anti-E1 antibodies.