E. 結論

本結果は、マーモセットGBV-B感染モデルが慢性C型肝炎のモデル動物確立に向けたブレークスルーとなる重要な知見であり、今後の抗HCV薬・ワクチンの有効性評価系としてのみでなく、C型肝炎慢性化メカニズムを解明する上でも貴重な情報をもたらすものと期待される。

F. 研究発表

1. 論文発表

1. 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 9, 515-521, 2007.
2. Yokota T, Iijima S, Kubodera T, Ishii K, Katakai Y, Ageyama N, Chen Y, Lee Y-J, Unno N, Nishina K, Iwasaki Y, Maki N, Mizusawa H, Akari H: Efficient regulation of viral replication by systemically administered siRNA with cationic liposome in a non-human primate surrogate model for hepatitis C. Biochemical and Biophysical Research Communications 361, 294-300, 2007.

2．学会発表

1. 明里宏文：サルを用いた病原体感染実験実施におけるコンプライアンスとバイオセーフティ．第１４３回日本獣医学会学術集会シンポジウム、平成１９年４月
2. 岩崎優紀、明里宏文：C型肝炎霊長類サロゲートモデルの開発．日本レトロウイルス研究会学術集会、平成１９年７月
3. 岩崎優紀、飯島沙幸、木村展之、片貝祐子、揚山直英、明里宏文：霊長類サロゲートC型肝炎モデル：マーモセットにおけるくすぶり型慢性GBV-B感染．第１４３回日本獣医学会学術集会、平成１９年９月
4. Akari H, Ishii K, Iwasaki Y, Iijima S, Maki N, Mori K-i, Katakai Y, Kimura N, Yoshizaki S, Ageyama N, Yokota T, Suzuki T, Miyamura T: Development of chronic GBV-B infection in marmosets with smoldering plasma viremia. 14th International Symposium on Hepatitis C virus and related viruses. September, 2007.
5. 岩崎優紀、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、鈴木哲朗、神奈木真理、宮村達男、明里宏文：C型肝炎サロゲート霊長類モデル：GBV-Bは新世界ザルに潜伏感染する．第５５回日本ウイルス学会学術集会、平成１９年１１月

G. 知的財産権の出願・登録状況

特になし

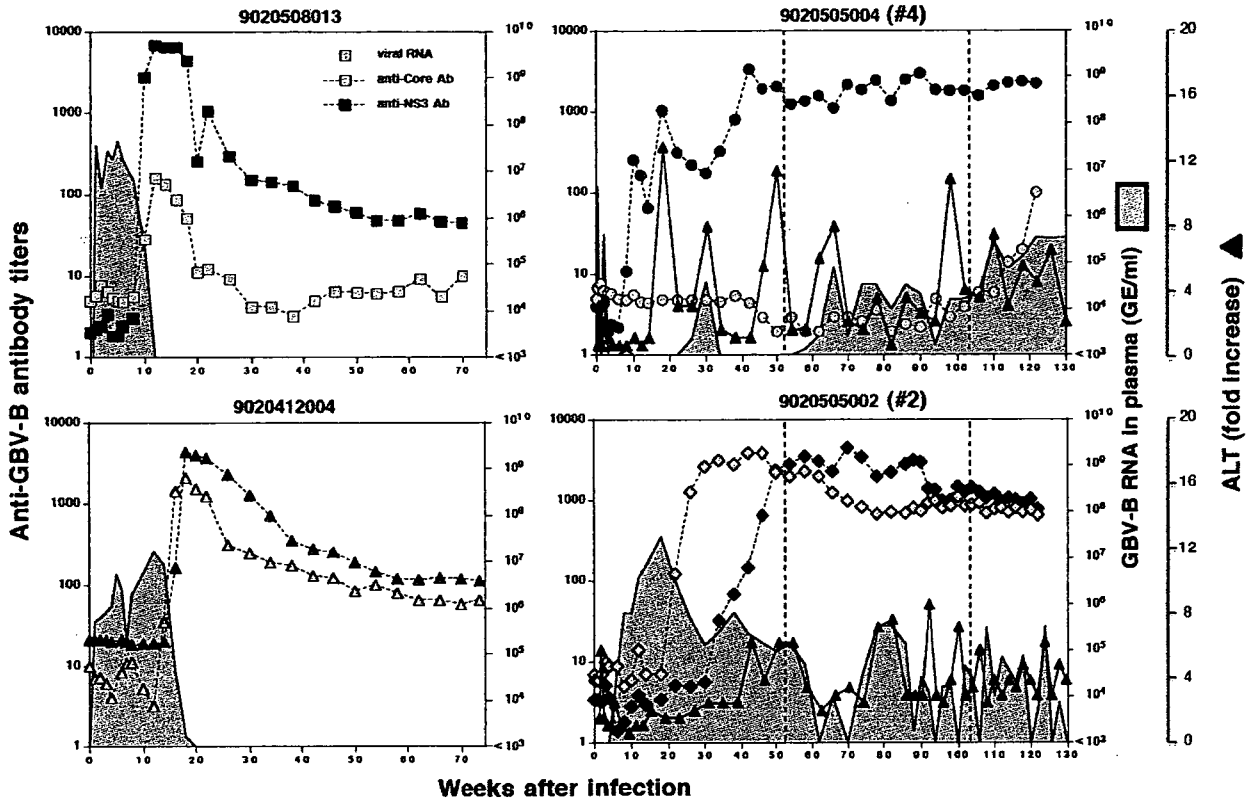

図１： マーモセットGBV-B 接種後の各種マーカー長期フォローアップ

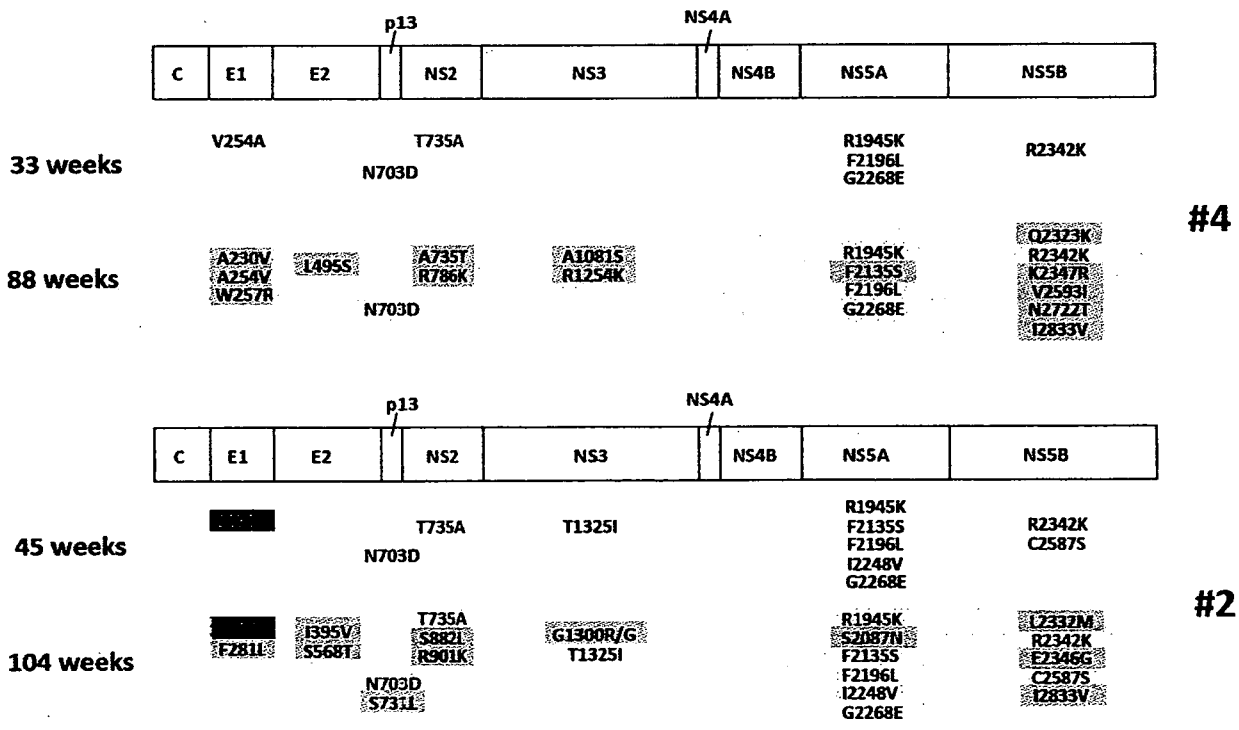

図２：長期持続感染マーモセットにおけるGBV-B 各蛋白の経時的アミノ酸変異

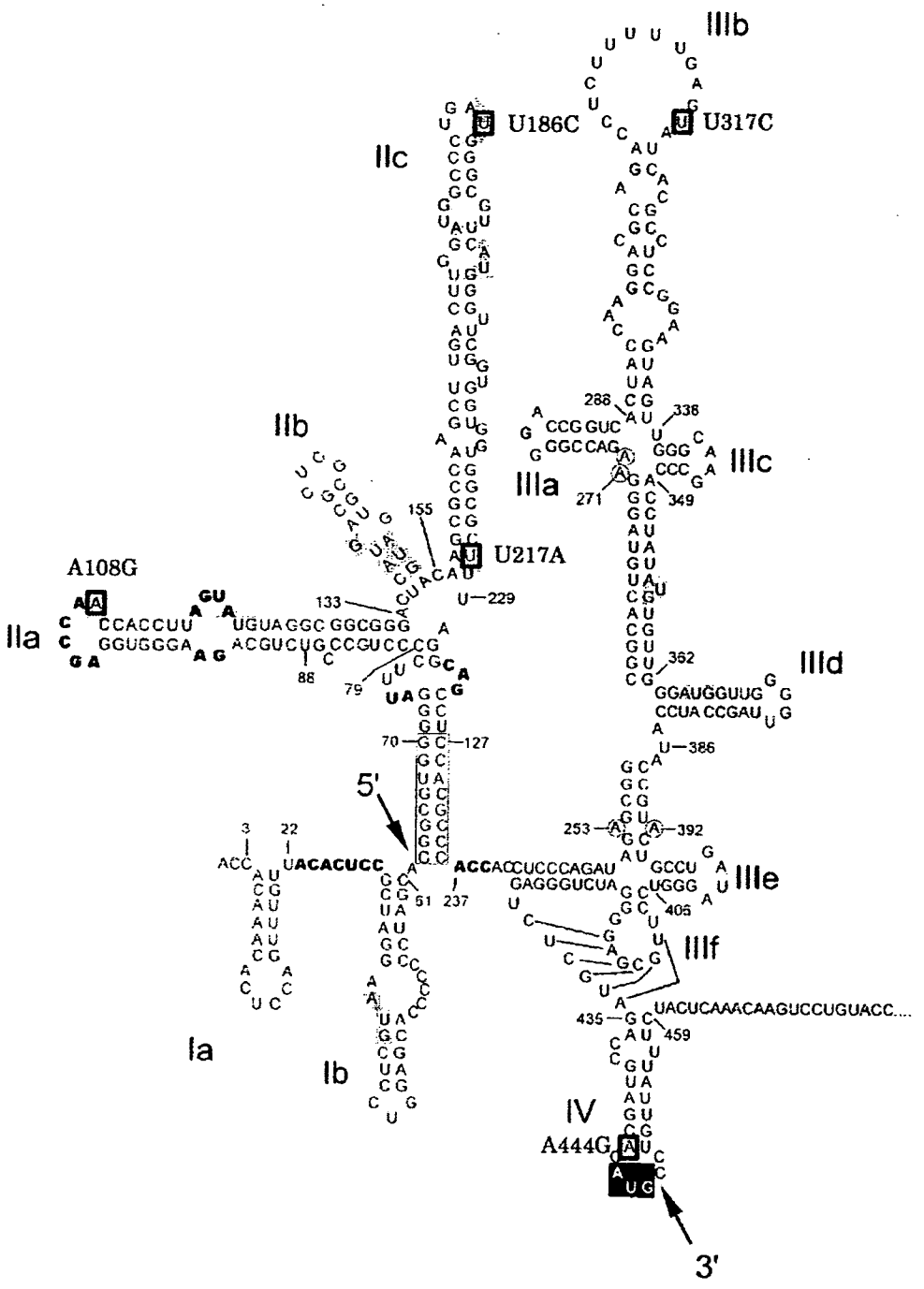

図３： 長期持続感染マーモセット由来GBV-B 5'UTRにおける遺伝子変異

Ⅲ．研究成果の刊行に関する一覧表

研究成果の刊行に関する一覧表

書籍

無し

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Aizaki H, Morikawa K, Fukasawa M, Hara H, Inoue Y, Tani H, Saito K, Nishijima M, Hanada K, Matsuura Y, Lai MMC, Miyamura T, Wakita T, Suzuki T.	Critical role of virion-associated cholesterol and sphingolipid in hepatitis C virus infection	J. Virol.			In press
Okamoto T, Omori H, Kaname Y, Abe T, Nishimura Y, Suzuki T, Miyamura T, Yoshimori T, Moriishi K, Matsuura Y	A single amino acid mmutation in hepatitis C virus NS5A disrupting FKBP8 interaction impairs viral replication.	J. Virol.	82	3480-3489	2008
Taguwa S, Okamoto T, Abe T, Mori Y, Suzuki T, Moriishi K, Matsuura Y.	Human butyrate-induced transcript 1 interacts with hepatitis C virus NS5A and regulates viral replication.	J. Virol.	82	2631-2641	2008
Murakami K, Inoue Y, Hmwe SS, Omata K, Hongo T, Ishii K, Yoshizaki S, Aizaki H, Matsuura T, Shoji I, Miyamura T, Suzuki T.	Dynamic behavior of hepatitis C virus quasispecies in a long-term culture of the three-dimensional radial-flow bioreactor system.	J. Virol. Methods.	148	174-181	2008
Suzuki T, Ishii K, Aizaki H, Wakita T.	Hepatitis C viral life cycle.	Adv. Drug Deliv. Rev.	59	1200-1212	2007
Suzuki T, Aizaki H, Murakami K, Shoji I, Wakita T.	Molecular biology of hepatitis C virus.	J. Gastroenterol.	42	411-423	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, Shoji I.	E6AP ubiquitin ligase mediates ubiquitylation and degradation of hepatitis C virus core protein.	J. Virol.	81	1174-1185	2007
Miyamoto H, Moriishi K, Moriya K, Murata S, Tanaka K, Suzuki T,	Involvement of the PA28gamma-dependent pathway in insulin resistance	J. Virol.	81	1727-1735	2007

Miyamura T, Koike K, Matsuura Y.	induced by hepatitis C virus core protein.				
Moriishi K, Mochizuki R, Moriya K, Miyamoto H, Mori Y, Abe T, Murata S, Tanaka K, Miyamura T, Suzuki T, Koike K, Matsuura Y.	Critical role of PA28gamma in hepatitis C virus-associated steatogenesis and hepatocarcinogenesis.	Proc. Natl. Acad. Sci. USA.	104	1661-1666	2007
Mizutani T, Endoh D, Shirato K, Shimizu H, Fukushi S, Saijo M, Sakai K, Kwang L, Ito M, Nerome R, Takasaki T, Ishii K, Suzuki T, Kurane I, Morikawa S, Nishimura H.	Rapid genome sequencing of RNA viruses.	Emerg. Infect. Dis.	13	322-324	2007
Murayama A, Date T, Morikawa K, Akazawa D, Miyamoto M, Kaga M, Ishii K, Suzuki T, Kato T, Mizokami M, Wakita T.	The NS3 helicase and NS5B-to-3'X regions are important for efficient hepatitis C virus strain JFH-1 replication in Huh7 cells.	J. Virol.	81	8030-8040	2007
Inoue Y, Murakami K, Hmwe SS, Aizaki H, Suzuki T.	Transcriptomic comparison of human hepatoma Huh-7 cell clones with different hepatitis C virus replication efficiencies.	Jpn. J. Infect. Dis.	60	173-178	2007
Watashi K, Shimotohno K.	Chemical genetics approach to hepatitis C virus replication: cyclophilin as a target for anti-hepatitis C virus strategy	Rev Med Virol.	17	245-250	2007
Arimoto K, Takahashi H, Hishiki T, Konishi H, Fujita T and Shimotohno K	Negative regulation of the RIG-I signaling by the novel ubiquitin ligase RNF125	Proc. Natl. Acd. Sci. USA	104	7500-7505	2007
Watashi K, Inoue D, Hijikata M, Goto K, Aly HH, Shimotohno K.	Anti-hepatitis C virus activity of tamoxifen reveals the functional association of estrogen receptor with viral RNA polymerase NS5B	J.Biol. Chem.	282	32765-32769	2007
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	2008
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

El-Farrash MA, Aly HH, Watashi K, Hijikata M, Egawa H, Shimotohno K.	In vitro infection of immortalized primary hepatocytes by HCV genotype 4a and inhibition of virus replication by cyclosporin	Microbiol Immunol	51	127-133	2007
Miyanari Y, Atsuzawa K, Usuda N, Watashi K, Hishiki T, Zayas M, Bartenschlager R, Wakita T, Hijikata M, Shimotohno K	The lipid droplet is an important organelle for hepatitis C virus production	Nat Cell Biol	9	1089-9710	2007
Arimoto KI, Konishi H, Shimotohno K	UbcH8 regulates ubiquitin and ISG15 conjugation to RIG-I	Mol Immunol	45	1078-1091	2007
Inubushi S, Nagano-Fujii M, Kitayama K, Tanaka M, An C, Yokozaki H, Yamamura H, Nuriya H, Kohara M, Sada K, Hotta H.	Hepatitis C virus NS5A protein interacts with and negatively regulates the non-receptor protein-tyrosine kinase Syk.	J Gen Virol (in press)			2008
Goto E, Mito-Yoshida M, Uematsu M, Aoki M, Matsuki Y, Ohmura-Hoshino M, Hotta H, Miyagishi M, Ishido S.	An excellent monitoring system for surface ubiquitination-induced internalization in mammals	PLoS ONE	3(1):	e1490	2008
Nishise Y, Saito T, Sugahara K, Ito JI, Saito K, Togashi H, Nagano-Fujii M, Hotta H, Kawata S.	Risk of hepatocellular carcinoma and secondary structure of hepatitis C virus (HCV) NS3 protein amino-terminus, in patients infected with HCV subtype 1b.	J Infect Dis.	196(7):	1006-1009,	2007.
El-Shamy A, Sasayama M, Nagano-Fujii M, Sasase N, Imoto S, Kim SR, 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 pre-treatment sera.	Microbiol Immunol	51(4)	471-482	2007
Lu L, Li C, Fu Y, Thaikruea L, Thongswat S, Maneekarn N, Apichartpiyakul C, Hotta H, Okamoto H, Netski D, Pybus OG, Murphy D, Hagedorn CH, Nelson KE.	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.	88(5)	1505-1518,	2007.
Matsuki Y, Ohmura-Hoshino M, Goto E,	Novel regulation of MHC class II function in B cells.	EMBO J.	26(3):	846-854,	2007.

Aoki M, Mito-Yoshida M, Uematsu M, Hasegawa T, Koseki H, Ohara O, Nakayama M, Toyooka K, Matsuoka K, Hotta H, Yamamoto A, Ishido S.					
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(2):	96-106,	2007.
Akazawa T., Seya T.	Tumor regression induced by adoptive transfer of MyD88-transfected dendritic cells in a mouse model.	FEBS Lett..	581	3334-3340	2007
Shingai M., Seya, T.	Differential type I interferon (IFN) inducing abilities of wild-type vs. vaccine strains of measles virus.	J. Immunol.	179	6123-6133	2007
Funami K., Seya, T.	Spatiotemporal mobilization of TICAM-1 in response to dsRNA.	J. Immunol.	179	6867-6872	2007
Shime H., Seya, T.	Tumor-secreted lactic acid promotes IL-23-IL-17 proinflammatory pathway.	J. Immunol.	180	In press	2008
Ebihara T., Seya, T.	Hepatitis C virus (HCV)-infected apoptotic cells extrinsically modulate dendritic cell function to activate T cells and NK cells.	Hepatology	?	In press	2008
Tsunamasa Watanabe, Kohara M, et al.	Liver target delivery of small interfering RNA to the HCV gene by lactosylated cationic liposome	*J. Hepatology*	47	744-750	2007
Tsunamasa Watanabe, Kohara M, et al.	Therapeutic application of RNA interference for Hepatitis C Virus	Advanced Drug Delivery Reviews	59	1263-1276	2007
Naoya Sakamoto, Kohara M, et al.	Inhibition of hepatitis C virus infection and expression in vitro and in vivo by recombinant adenovirus expressing short hairpin RNA	*J. Gastroenterology. Hepatology*	In press		2008
Totsugawa T, Kohara	Survival of liver failure pigs by	*J. Hepatology*	47	74-82	2007

M,.et al.	transplantation of reversibly immortalized human hepatocytes with Tamoxifen-mediated self-recombination				
Toshie Mashiba, Kohara M, et al.	Identification of CTL epitopes in hepatitis C virus by a genome-wide computational scanning and a rational design of peptide vaccine	*Immunogenetics*	59	197-209	2007
Shin-ichiro Nakagawa, Kohara M, et al.	Inhibition of Hsp90 suppresses HCV replication in replicon cell lines and in chimeric mice with humanized liver	*Biochem. Biophys. Res. Commun.*	353	882-888	2007
Kazuaki Inoue, Koahra M, et al.	Non-immunosuppressive cyclosporin DEBIO-025 with interferon shows synergistic anti-HCV effect in chimeric mouse	*Hepatology*	45	921-928	2007
Kitabatake M, Kohara M,. et al.	SARS-CoV spike protein recombinant Vaccinia virus efficiently induces neutralizing antibodies in spite of pre-immunization with vaccinia virus	*Vaccine*	25	630-637	2007
Abe K, Kato N, et al.	Cell culture-adaptive NS3 mutations required for the robust replication of genome-length hepatitis C virus RNA.	Virus Research	125	88-97	2007
Ariumi Y, Kato N, et al.	DDX3 DEAD box RNA helicase is required for hepatitis C virus (HCV) RNA replication.	Journal of Virology	81	13922-13926	2007
Ikeda M, Kato N, et al.	Modulation of host metabolism as a target of new antivirals.	Advanced Drug Delivery Reviews	59	1277-1289	2007
Abe K, Kato N, et al.	Serum-free cell culture system supplemented with lipid-rich albumin for hepatitis C virus (strain O of genotype 1b) replication.	Virus Research	125	162-168	2007
Dansako H, Kato N, et al.	Limited suppression of the interferon-beta production by hepatitis C virus serine protease in cultured human hepatocytes.	FEBS Journal	274	4161-4176	2007
Ikeda M, Kato N, et al.	Life style-related diseases of the digestive system: cell culture system for the screening of anti-HCV	Journal of Pharmacologi	105	145-150	2007

	reagents: suppression of HCV replication by statins and synergistic action with interferon.	cal Sciences			
Yano M, Kato N, et al.	Comprehensive analysis of the effects of ordinary nutrients on hepatitis C virus RNA replication in cell culture.	Antimicrobial Agents and Chemotherapy	51	2016-2027	2007
Peng LF, Kato N, et al.	Identification of novel epoxide inhibitors of hepatitis C virus replication using a high-throughput screen	Antimicrobial Agents and Chemotherapy	51	3756-3759	2007
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	37	S38-43	2007
Koike K	Hepatitis C virus contributes to hepatocarcinogenesis by modulating metabolic and intracellular signaling pathways	J Gastroenterol Hepatol	22	S108-111	2007
Yotsuyanagi H, Koike K	Mechanisms underlying drug resistance in antiviral treatment for infections with hepatitis B and C viruses	J Gastroenterol	42	329-335	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	13	964-969	2007
Aono J, Yotsuyanagi H, Miyoshi H, Tsutsumi T, Fujie H, Shintani Y, Moriya K, Okuse C, Suzuki M, Yasuda K, Iino S, Koike K.	Amino acid substitutions in S region of hepatitis B virus in the sera from patients with acute hepatitis.	Hepatol Res	37	731-739	2007
Ichibangase T, Moriya K, Koike K, Imai K	A novel proteomics method revealed disease-related proteins in the liver of hepatitis C mouse model.	J Proteome Res	6	2841-2849	2007
Okuse C, Yotsuyanagi H, Koike K.	Hepatitis C as a Systemic Disease: Virus and Host Immunologic Responses Underlie Hepatic and	J Gastroenterol	42	857-865	2007

	Extrahepatic Manifestations.				
Hashimoto M, Sugawara Y, Tamura S, Kaneko J, Matsui Y, Moriya K, Koike K, Makuuchi M.	Impact of new methicillin-resistant Staphylococcus aureus carriage postoperatively after living donor liver transplantation.	Transplant Proc	39	3271-3275	2007
Tanaka N, Moriya K, Kiyosawa K, Koike K, Aoyama T.	Hepatitis C virus core protein induces spontaneous and persistent activation of peroxisome proliferator-activated receptor α in transgenic mice: Implications for HCV-associated hepatocarcinogenesis.	Int J Cancer	122	124-31	2007
Koike K, Kikuchi Y, Kato M, Takamatsu J, Shintani Y, Tsutsumi T, Fujie H, Miyoshi H, Moriya K, Yotsuyanagi H.	Prevalence of Hepatitis B Virus Infection in Patients with Human Immunodeficiency Virus in Japan.	Hep Res	38	310-314	2007
Tanaka N, Moriya K, Kiyosawa K, Koike K, Gonzalez FJ, Aoyama T.	PPAR-α is essential for severe hepatic steatosis and hepatocellular carcinoma induced by HCV core protein.	J Clin Invest	118	683-694	2008
Hashimoto M, Sugawara Y, Tamura S, Kaneko J, Matsui Y, Moriya K, Koike K, Makuuchi M.	Methicillin-resistant Staphylococcus aureus infection after living-donor liver transplantation in adults	Transpl Infect Dis		In press	2008
Koike K, Tsutsumi T, Miyoshi H, Shinzawa S, Shintani Y, Fujie H, Yotsuyanagi H, Moriya K.	Molecular Basis for the Synergy between Alcohol and Hepatitis C Virus in Hepatocarcinogenesis	J Gastroenterol Hepatol		In press	2008
Ishizaka N, Ishizaka Y, Seki G, Nagai R, Yamakado M, Koike K.	Association between hepatitis B/C viral infection, chronic kidney disease and insulin resistance in individuals undergoing general health screening.	Hepatol Res		In press	2008
Newell P, Villanueva A, Friedman SL, Koike K, Llovet JM.	Experimental models of hepatocellular carcinoma	J Hepatol		In press	2008
Abe T., Kaname Y., Hamamoto I., Tsuda Y., Wen X., Taguwa S., Moriishi K., Takeuchi O., Kawai T., Kanto T., Hayashi N., Akira S.,	Hepatitis C Virus Nonstructural Protein 5A Modulates TLR-MyD88-Dependent Signaling Pathway in the Macrophage Cell Lines.	J. Virol.	81	8953-8966	2007

Matsuura Y.					
Mori Y., Yamashita T., Tanaka Y., Tsuda Y., Abe T., Moriishi K., Matsuura Y.	Processing of Capsid Protein by Cathepsin L Plays a Crucial Role in Replication of the Japanese Encephalitis Virus in Neural and Macrophage Cells.	J. Virol.	81	8477-8487	2007
Tani H., Komoda Y., Matsuo E., Suzuki K., Hamamoto I., Yamashita T., Moriishi K., Fujiyama K., Kanto T., Hayashi N., Owsianka A., Patel A.H., Whitt M.A., Matsuura Y.	Replication-competent recombinant vesicular stomatitis virus encoding hepatitis C virus envelope proteins.	J. Virol.	81	8601-8612	2007
Yamamoto M., Uematsu S., Okamoto T., Matsuura Y., Sato S., Kumar H., Satoh T., Saitoh T., Takeda K., Ishii K.J., Takeuchi O., Kawai T., Akira S.	Enhanced TLR-mediated NF-IL6 dependent gene expression by Trib1 deficiency.	J. Exp. Med.	204	2233-2239	2007
Moriishi K., Matsuura Y.	Host factors involved in the replication of hepatitis C virus.	Rev. Med. Virol.	17	343-354	2007
Noguchi, K., Fukazawa, H., Murakami, Y., Takahashi, N., Yamagoe, S., Uehara, Y.	Gamma-herpesviruses and cellular signaling in AIDS-associated malignancies..	Cancer Sci	98	1288-1296	2007
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	9	515-521	2007
Yokota T, Iijima S, Kubodera T, Ishii K, Katakai Y, Ageyama N, Chen Y, Lee Y-J, Unno N, Nishina K, Iwasaki Y, Maki N, Mizusawa H, Akari H	Efficient regulation of viral replication by systemically administered siRNA with cationic liposome in a non-human primate surrogate model for hepatitis C	Biochemical and Biophysical Research Communications	361	294-300	2007

Ⅳ. 研究成果の刊行物・別冊

JVI Accepts, published online ahead of print on 26 March 2008
J. Virol. doi:10.1128/JVI.02530-07

A Critical Role of Virion-Associated Cholesterol and Sphingolipid in Hepatitis C Virus Infection

Hideki Aizaki[1], Kenichi Morikawa[1], Masayoshi Fukasawa[2], Hiromichi Hara[1], Yasushi Inoue[1], Hideki Tani[3], Kyoko Saito[2], Masahiro Nishijima[2], Kentaro Hanada[2], Yoshiharu Matsuura[3], Michael M. C. Lai[4], Tatsuo Miyamura[1], Takaji Wakita[1], and Tetsuro Suzuki[1,*]

[1] Department of Virology II and [2] Department of Biochemistry and Cell Biology, National Institute of Infectious Diseases, Tokyo 162-8640, Japan, [3] Department of Molecular Virology, Research Institute for Microbial Diseases, Osaka University, Osaka 565-0871, Japan, and [4] Department of Molecular Microbiology and Immunology, University of Southern California, Los Angels, CA 90033-1054, USA.

*Corresponding author. Mailing address:
Tetsuro Suzuki, PhD
Department of Virology II
National Institute of Infectious Diseases
1-23-1 Toyama, Shinjuku-ku, Tokyo 162-8640, Japan.
Tel: 81 3 5285 1111
Fax: 81 3 5285 1161
E-mail: tesuzuki@nih.go.jp

Running title: Role of virion-associated lipids in HCV infection

Word count for the Abstract: 153 words

ABSTRACT

In this study, we establish that cholesterol and sphingolipid associated with hepatitis C virus (HCV) particles are important for virion maturation and infectivity. In a recently-developed culture system enabling study of the complete life cycle of HCV, mature virions were enriched with cholesterol as assessed by the cholesterol/phospholipids molar ratio of virion and cell membranes. Depletion of cholesterol from the virus or hydrolysis of virion-associated sphingomyelin almost completely abolished HCV infectivity. Supplementation of cholesterol-depleted virus with exogenous cholesterol enhanced infectivity to untreated control values. Cholesterol-depleted or sphingomyelin-hydrolyzed virus had markedly defective internalization but no influence on cell attachment. Significant portions of HCV structural proteins partitioned into cellular detergent-resistant, lipid raft-like, membranes. Combined with the observation that inhibitors of the sphingolipid biosynthetic pathway block virion production, but not RNA accumulation, of a JFH-1 isolate, our findings suggest that altering the lipid composition of HCV particles might be a useful approach when designing anti-HCV therapy.

INTRODUCTION

Hepatitis C virus (HCV) is recognized as a major cause of chronic liver disease, including chronic hepatitis, hepatic steatosis, cirrhosis, and hepatocellular carcinoma. It presently affects approximately 200 million people worldwide (26). HCV is an enveloped positive-strand RNA virus belonging to the *Hepacivirus* genus of the *Flaviviridae* family. Its genome of ~9.6 kb encodes a polyprotein precursor of ~3000 residues and the structural proteins (core, E1, and E2) reside in its N-terminal region.

Little is known about the assembly of HCV and its virion structure since efficient production of authentic HCV particles has only recently been achieved. Nucleocapsid assembly generally involves oligomerization of the capsid protein and encapsidation of genomic RNA. This process is thought to occur upon interaction of the core protein with viral RNA, and this core-RNA interaction may induce a change from RNA replication to packaging. As with related viruses, the mature HCV virion likely consists of a nucleocapsid and outer envelope composed of a lipid membrane and envelope proteins. Expression of the structural proteins in mammalian cells has been observed to generate virus-like particles with similar ultrastructural properties to HCV virions (4, 29). Packaging of these HCV-like particles into intracellular vesicles as a result of budding from the endoplasmic reticulum (ER) has also been observed (8, 34). However, HCV structural proteins are observed both in the ER and Golgi apparatus (45). Moreover, complex N-linked glycans have been detected on the surface of HCV particles isolated from patient sera, suggesting that the glycans transit through the Golgi (44). Interactions between the core and E1/E2 proteins are thought to determine viral morphology, and are mediated through a cytoplasmic loop present in the polytopic form of E1 (35). Recently,

ourselves and others have identified a unique HCV genotype 2a isolate, JFH-1, that is able to replicate and produce high levels of infectious virus in culture (HCVcc)(54, 56), enabling us to investigate new aspects of the HCV life cycle.

In this study, we examine the importance of cholesterol and sphingolipid in association with the HCV membrane in virion maturation and virus infectivity. Mature HCV particles are rich in cholesterol. Cholesterol depletion or hydrolysis of sphingolipid from HCV particles results in a loss of infectivity. We further demonstrate a requirement for virion-associated cholesterol and sphingolipid for viral entry.

MATERIALS AND METHODS

Cell Culture. The human hepatoma cell line Huh-7, which is permissive to HCV infection, was obtained from Francis V. Chisari (The Scripps Research Institute). Human embryonic kidney 293T cells were cultured in DMEM-10% FBS. Huh-7 cell lines, which carry subgenomic replicon RNA of either the JFH-1 (20) or N strain (11, 17), were cultured as previously described (21, 46).

Reagents. The primary antibodies used in this study were mouse monoclonal antibodies against vesicular stomatitis virus-glycoprotein (VSV-G)(Sigma, St. Louis, MO), HCV E1 (54) and E2 (Biodesign International, Saco, ME), caveolin-2 (New England Biolads, Beverly, MA), CD81 (BD Pharmingen, Franklin Lake, NJ), as well as rabbit polyclonal antibodies against calnexin (Stressgen, Ann Arbor, MI) and HCV core (48). ISP-1/myriocin, cholesterol, and heparinase I were purchased from Sigma, and recombinant *Bacillus cereus* SMase was obtained from Higeta Shoyu (Tokyo, Japan). (1R, 3R)-N-(3-hydroxy-1-hydroxymethyl-3-phenylpropyl) dodecanamide (HPA-12), which was synthesized as described (24), is a gift from Dr. Shu Kobayashi (University of Tokyo).

Plasmids. pCAE1 and pCAE2 contain HCV cDNAs spanning E1 region (aa 192-383) with a FLAG tag at the N-terminus and E2 (aa 384-809) region with a Myc tag at the N-terminus of NIHJ1 strain (1), respectively, under the control of the CAG promoter (38). pCAV340V and pCAV711V consist of the ectodomains of E1 and E2, respectively, with the N-terminal signal sequences, transmembrane, and cytoplasmic domains derived from VSV-G, as described (50) (Fig. 4D).

Virus production. The plasmid pJFH1, containing full-length cDNA of the JFH-1 isolate, was used to generate HCVcc as described (23, 33, 34, 54). In vitro transcribed RNA from the linearized pJFH1 was delivered to Huh-7 cells by electroporation. Culture supernatants

were collected at 72 h post-transfection, clarified by low-speed centrifugation, passed through a 0.45-mm filter, and concentrated using Amicon Ultra-15 (Millipore, Bedford, MA) or by ultracentrifugation (23). Infectious titers, HCV RNA, and core protein concentrations of the viral stocks were ~ 5 x 10^3 focus forming units per ml, ~1 x 10^7 copies/ml, and ~1 x 10^4 fmol/L, respectively. HCVcc was isolated by a combination of ultrafiltration, ion exchange chromatography, heparin affinity chromatography, and sucrose density ultracentrifugation (33, K. Morikawa and T. Wakita, unpublished data). Pseudotyped VSV containing E1 and E2 proteins of HCV genotype 1a, H77c, isolate (HCVpv) was generated as previously described (51). Briefly, 293T cells transiently expressing E1 and E2 proteins (H77 strain) were infected with VSVdelG-GFP/G, in which the G envelope gene was replaced with a green fluorescent protein and pseudotyped with VSV-G.

Determination of cholesterol and phospholipid content of HCVcc and infected cells. Cellular and viral lipids were extracted from isolated HCVcc and from uninfected and infected Huh-7 cells. Cholesterol content was determined using the cholesterol oxidase method as previously described (15). Total phospholipid content was determined using the method of Rouser et al. (42).

Cholesterol depletion and replacement. To remove cholesterol from the HCV envelope, stock samples of HCVcc were treated with methyl-β-cyclodextrin (B-CD) in Dulbecco's modified Eagle medium (DMEM) (Sigma) supplemented with 10% fetal bovine serum (FBS) (Sigma) and nonessential amino acids (Invitrogen, Carlsbad, CA) for 1 h at 37°C, followed by centrifugation at 100,000 g for 3 h to form a pellet, which was resuspended in 0.5 ml of the medium. In order to replenish cholesterol, the medium of HCVcc treated with 5 mg/ml B-CD was replaced with DMEM containing various concentrations of exogenous

cholesterol (Sigma) and incubated for 1 h, followed by centrifugation to form a pellet. In order to perform HCVcc infection assays, Huh-7 cells were infected with HCVcc with or without the above treatment for 1 h at 37°C and then washed as described above. Viral core protein in the cells and in the supernatant was quantified 72 h later using HCV core ELISA (Ortho-Clinical Diagnostics, Tokyo, Japan).

SMase treatment. HCVcc was treated with sphingomyelinase (SMase) at various concentrations in DMEM for 1 h at 37°C and then centrifuged at 100,000 g for 3 h to form a pellet, which was resuspended in 0.5 ml of medium for the infection assays.

HCVcc binding and internalization assays. To monitor binding, cells grown in a 6-well plate were preincubated for 1 h at 4°C, after which B-CD- or SMase-treated HCVcc were bound to the cells for 1 h at 4°C. As a measure of virus internalization, following the virus binding procedure, the cells were warmed to 37°C and maintained for 2 h, after which they were treated with 0.25% trypsin for 10 min at 37°C. Huh7-25, which is a CD81-negative Huh-7 subclone (3), was used to ensure removal of surface-bound virus by trypsin treatment. For both the binding and internalization assays, the resulting cells as described above were washed with ice-cold PBS, followed by lysis with TRIzol reagent (Invitrogen). Cell-associated virus was quantified by measuring the amount of HCV RNA in the cell lysate by the real-time RT-PCR method (2, 34). The heparinase treatment of cells was performed as previously described (33).

HCV replication assay in HCVcc infected- or replicon cells. HCV subgenomic replicon cells or cells infected with HCVcc were treated with various concentrations of inhibitors for 72 h. Total RNA was isolated from replicon cells using TRIzol Reagent (Invitrogen), followed by quantification of HCV RNA by real-time RT-PCR as previously described (2, 34). Levels of core protein in the culture supernatant of HCVcc-infected cells were tested