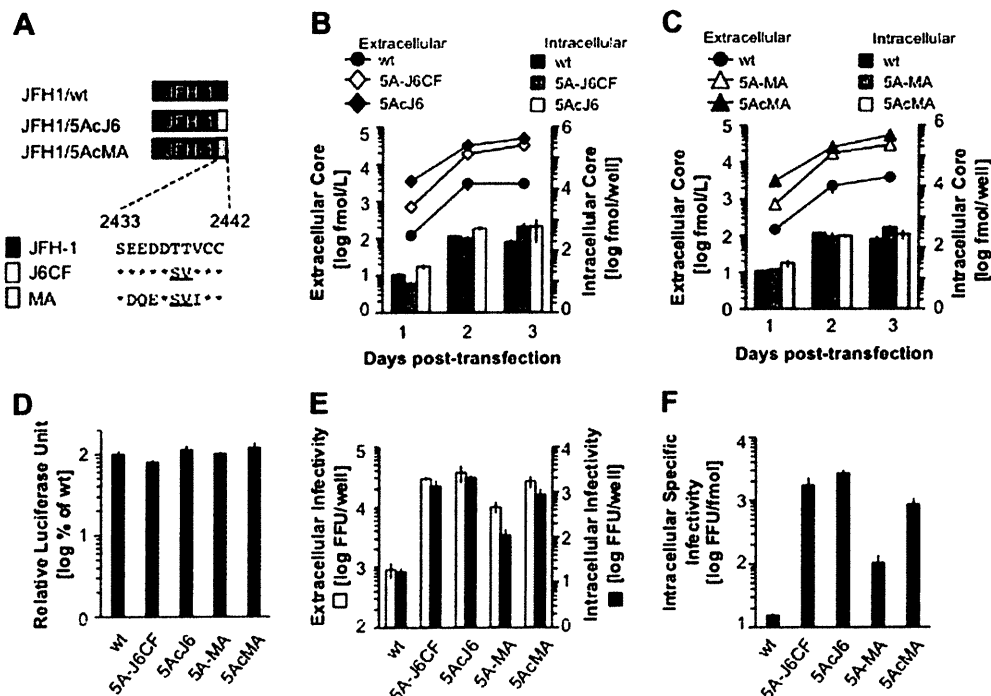


**Fig. 1.** Production and replication of recombinant viruses with NSSA of strains of genotypes 1 and 2. (A) Huh7.5.1 cells were transfected with *in vitro* synthesized RNA of JFH1/wt and indicated recombinants. The amount of extracellular (line graph) and intracellular (bar graph) HCV core protein was determined at the indicated time points. Assays were performed in triplicate, and means  $\pm$  standard deviation are plotted. (B) Huh7.5.1 cells were transfected with subgenomic replicon RNA of JFH1/wt and indicated recombinants. Luciferase activity at day 2 was measured. Replication levels of JFH1/wt and indicated recombinants were calculated as fold increases at 4 h and are expressed as percentages of JFH1/wt. (C) Huh7-25 cells were transfected with RNA of JFH1/wt and recombinants. Forty-eight hours after transfection, extra- and intracellular infectivities were determined by inoculating into naïve Huh7.5.1 cells. (D) Intracellular specific infectivity of JFH1/wt and indicated recombinants.



**Fig. 2.** C-terminal amino acids in NSSA were responsible for the enhanced virus production of recombinant viruses with NSSA of genotype 2 strains. (A) Alignment of C-terminal amino acids in NSSA of JFH-1, J6CF, and MA. Identical amino acids are indicated by asterisks. The indicated number represents the position of the amino acid in the entire polyprotein of JFH-1. (B) Huh7.5.1 cells were transfected with RNA of JFH1/wt, 5A-J6CF, and 5AcJ6. The amount of extracellular (line graph) and intracellular (bar graph) core proteins were quantified at the indicated time points. (C) Huh7.5.1 cells were transfected with RNA of JFH1/wt, 5A-MA, and 5AcMA. The amount of extracellular (line graph) and intracellular (bar graph) core proteins were quantified at the indicated time points. (D) Huh7.5.1 cells were transfected with subgenomic replicon RNA of JFH1/wt and indicated recombinants. Luciferase activity at day 2 was measured. Replication levels of JFH1/wt and indicated recombinants were calculated as the fold increase at 4 h and are expressed as percentages of JFH1/wt. (E) Huh7-25 cells were transfected with RNA of JFH1/wt and recombinant viruses. Forty-eight hours after transfection, extra- and intracellular infectivities were determined by inoculating into naïve Huh7.5.1 cells. (F) Intracellular specific infectivities of JFH1/wt and indicated recombinants.

JFH1/5AcJ6 and 5AcMA were approximately 20-fold higher than that of the JFH1/wt ( $p < 0.05$ ), and were slightly higher than those of JFH1/5A-J6CF and 5A-MA. We also determined the specific intracellular infectivity of each recombinant virus to assess virus assembly (Fig. 2F). As with extra- and intracellular infectivities, the specific intracellular infectivities of JFH1/5AcJ6 and 5AcMA were more than 10-fold higher than that of the JFH1/wt ( $p < 0.05$ ), and were slightly higher than those of JFH1/5A-J6CF and 5A-MA. These results suggest that these C-terminal amino acids of NS5A are responsible for enhanced assembly of intra-genotypic recombinant viruses JFH1/5A-J6CF and 5A-MA.

### 3.3. Amino acid substitutions at the C-terminus of NS5A accelerate cleavage kinetics between NS5A and NS5B

To investigate whether substitution of the C-terminus of NS5A affects the cleavage kinetics between NS5A and NS5B, we performed pulse-chase assays using a T7-based expression system. Immunoprecipitations were performed with an NS5B-specific antibody and immunocomplexes were analyzed on a 7.5% SDS-PAGE (Supplementary Fig. A). Fully processed NS5B and an uncleaved NS5A–NS5B precursor with a size of approximately 130 kDa could be detected for JFH1/wt and recombinant constructs JFH1/5AcJ6 and 5AcMA. In the case of JFH1/wt, the amount of uncleaved precursor was gradually decreased but still detectable at 4 h of the chase period. On the other hand, in the case of the recombinant constructs, JFH1/5AcJ6 and 5AcMA, the amounts of precursor were reduced more rapidly and were undetectable by 4 h of chase. To assess the kinetics of the cleavage, the percentages of uncleaved NS5A–NS5B precursor at the examined time points were plotted and analyzed using nonlinear regression (Supplementary Fig. B). Rapid cleavage kinetics was observed in JFH1/5AcJ6 and 5AcMA transfected cells as compared with JFH1/wt. These observations suggest that substitutions at the C-terminus of NS5A of these recombinant viruses are responsible for the accelerated cleavage kinetics between NS5A and NS5B, and might be associated with enhanced infectious viral particle assembly.

### 3.4. Susceptibility of recombinant HCV to the NS5A inhibitor BMS-790052

Using developed JFH-1 based inter- and intra-genotypic recombinant viruses, we assessed their susceptibility to the NS5A inhibitor BMS-790052 [12]. After transfection with synthesized HCV RNA, cells were treated with different concentrations of BMS-790052 for 2 days and intracellular HCV core protein levels were

determined. No cytopathic effects were observed at the concentrations used (data not shown). As shown in Fig. 3, the intracellular core protein levels of JFH1/wt and recombinant viruses were inhibited to different extents. Recombinant viruses with NS5A of genotype 1, JFH1/5A-H77 and 5A-Con1, showed higher susceptibility to BMS-790052 as compared with JFH1/wt, while JFH1/5A-J6CF and 5A-MA showed much lower susceptibility. To compare the susceptibilities, the effective concentrations required to inhibit 50% of intracellular core protein level ( $EC_{50}$ ) were determined, because the intracellular core protein levels of these recombinant viruses were at almost the same level at day 2 after transfection (Fig. 1A). The  $EC_{50}$  of JFH1/wt and recombinant viruses with NS5A of genotype 1, JFH1/5A-H77 and 5A-Con1, were 6.4, 3.1, and 1.4 pM, respectively, and do not conflict with results using replicon systems reported previously [12]. In contrast, recombinant viruses with NS5A of genotype 2, JFH1/5A-J6CF and 5A-MA, were more resistant to BMS-790052, and  $EC_{50}$  values were 1.5 and  $>5$  nM, respectively. Collectively, the anti-HCV effect of the specific NS5A inhibitor BMS-790052 showed strain and genotype dependency. In particular, the NS5A of genotype 2 strains, J6CF and MA, excepting JFH-1, showed 300- to 1000-fold lower susceptibility to BMS-790052 compared with the NS5A of genotype 1 strains, H77 and Con1.

## 4. Discussion

HCV NS5A is essential for replication and infectious virus production, similar to other nonstructural proteins possessing enzymatic activities, including NS3 (a serine protease) and NS5B (an RNA-dependent RNA polymerase). Currently, these nonstructural proteins are being targeted to establish anti-viral compounds to improve the outcome of therapy for chronic HCV infection, and several inhibitors for these proteins are entering into clinical trials. A great deal of interest has also been shown in the development of NS5A inhibitors, and one potent inhibitor, BMS-790052, has recently been described [12]. In this study, to assess strain and genotype dependent susceptibility for this inhibitor, we generated recombinant HCV with NS5A from strains other than JFH-1, because a limited number of strains are available in the HCV cell culture system. We replaced NS5A of JFH-1 with those of genotype 1 and 2 strains, and observed efficient replication and infectious virus production in cell culture.

The replication efficiencies of these NS5A recombinant viruses were almost the same, whereas virus production levels into the culture medium were very different from JFH1/wt (Fig. 1A and

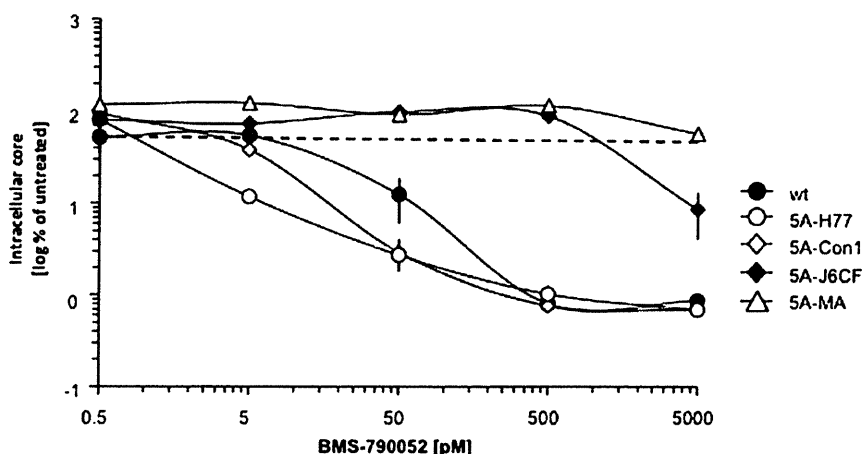


Fig. 3. Susceptibility of JFH1/wt and recombinant viruses to the NS5A inhibitor. Huh7.5.1 cells were transfected with RNA of JFH1/wt and recombinant viruses and treated with serially diluted BMS-790052 for 48 h. The amounts of intracellular HCV core protein were quantified and normalized against untreated control set to 100%.

**Table 1**  
Amino acid substitutions in NS5A of strains used, and reported resistant mutations to BMS-790052.

AA <sup>a</sup>	Strains used in this study						Reported resistant mutations <sup>b</sup>			Ref.
	Entire	NS5A	JFH1	H77	Con1	J6CF	MA	1a	1b	
2004	28	F	M	L	F	L	T (683)	T (20)	–	[20]
2006	30	K	Q	R	K	K	E (24,933) K (24,317) H (1450) R (1217) M (350) V (3350)	E (6)	–	[20]
2007	31	L	L	L	M	M	L (233) L (17)	F (5) M (3) V (23)	M (170)	[12,20]
2008	32	P	P	P	P	P	–	–	–	[20]
2068	93	A	A	C	C	C	–	–	E (150)	[12]
2069	93	Y	Y	Y	Y	Y	C (1850) H (5367) N (47,017)	H (19) N (28)	H (130–1400)	[12,20]

<sup>a</sup> AA, amino acid position which are according to entire polyprotein (Entire) and NS5A of JFH1.

<sup>b</sup> Fold resistance as compared with parental amino acid is indicated in parentheses.

B). Enhanced virus production was detected in recombinant viruses replaced with NS5A of genotype 2 strains, while reduced virus production was observed in recombinant viruses replaced with NS5A of genotype 1 strains (Fig. 1A). The single cycle virus production assay revealed that this enhanced virus production with NS5A of genotype 2 was due to efficient viral particle assembly (Fig. 1D). To analyze the mechanism of efficient virus assembly by NS5A of strains J6CF and MA, we focused on the cleavage between NS5A and NS5B. Several reports have shown that amino acids in the C-terminus of NS5A influence the cleavage [15,16]. Thus, we used recombinant JFH-1 viruses harboring amino acids of J6CF and MA in the C-terminus of JFH-1 NS5A, and assessed replication and infectious virus production. We found that both of these recombinant viruses, JFH1/5AcJ6 and 5AcMA, showed more enhanced virus assembly (Fig. 2F), and reasoned that the amino acid substitutions T2438S and T2439V were responsible for the enhanced infectious virus production. In pulse-chase assays for the cleavage of NS5A and NS5B, accelerated cleavage was observed in recombinant viruses JFH1/5AcJ6 and 5AcMA. Uncleaved NS5A–NS5B disappeared earlier in JFH1/5AcJ6- and 5AcMA-transfected cells than in JFH1/wt-transfected cells (Supplementary Fig. A). Taken together, the enhanced virus assembly observed with JFH1/5A-J6CF and 5A-MA depended on the C-terminal amino acid substitutions in NS5A, possibly through accelerated cleavage kinetics between NS5A and NS5B. The reason for the correlation between accelerated cleavage and enhanced virus assembly is still unknown. Accelerated cleavage may lead to an increased amount of mature NS5A used for virus assembly or affect the interaction with the core protein, which has been reported to be important for infectious virus assembly [17]. Another possibility is the interaction between the C-terminus of NS5A and some host factor(s) involved in virus particle assembly, such as apolipoprotein E [18]. Amino acid substitutions at this region may alter the potency of this interaction directly. A previous report has also shown that another mutation in this region, V2440L, is associated with delayed cleavage kinetics between NS5A and NS5B, but enhanced virus assembly [14]. Further investigation will be necessary to clarify this mechanism and to solve the discrepancy.

Using this cell culture system with NS5A recombinant viruses, we assessed strain and genotype dependent susceptibility to the novel NS5A inhibitor, BMS-790052. This potent inhibitor successfully inhibited replication of JFH1/wt and recombinant viruses with NS5A of genotype 1 strains (Fig. 3). However, it showed limited effectiveness on recombinant viruses with NS5A of genotype 2 strains. This high efficacy for genotype 1 strains makes sense because this inhibitor and its lead compound were identified using genotype 1a and 1b subgenomic replicons [19].

During preparation of this paper, another study was published describing an HCV cell culture system with NS5A-substituted recombinant viruses [20]. That study used a J6/JFH-1 chimeric virus that is known to have high virus production efficiency, but not natural viruses, and established nine recombinant viruses with NS5A from strains of eight different subtypes. They found that recombinant viruses with NS5A of strains of genotypes 1a, 1b, 4a, 5a, and 6a were sensitive, and strains of genotypes 2a and 3a were resistant to the NS5A inhibitor, data that are consistent with our own observations. In addition, we found that recombinant virus with NS5A of genotype 2b, which is the one of the predominant genotypes in Japan, was also resistant to the compound. Resistant mutations to BMS-790052 have been reported and are frequently observed in the N-terminus of NS5A, suggesting inhibition of membrane localization and dimerization of NS5A (Table 1) [12,20]. Among these reported mutations, one of the most potent, 2006E/K/H/R (amino acid position (AA) 30 in NS5A), is found in all strains but H77, and another potent resistant mutation, 2007F/M/V (AA 31 in NS5A), is also found in J6CF and MA. Thus, the lower susceptibilities of recombinant viruses JFH1/5A-J6CF and 5A-MA, as compared with JFH1/wt, might be due to the latter mutation. Based on an analysis of the database of submitted strains (Hepatitis Virus Database; <http://s2as02.genes.nig.ac.jp/index.html>), this resistant mutation, 2007M, is detected in 84.2% and 79.0% of genotype 2a and 2b strains, respectively, whereas it is observed in only 0.2% of genotype 1a and 3.8% of genotype 1b strains [20,21]. From these observations, most of genotype 2a and 2b strains may be resistant to BMS-790052, although these are known to be sensitive to interferon [22].

In conclusion, we established JFH-1 based recombinant viruses by replacement of NS5A with those from strains of genotypes 1 and 2. All the generated recombinant viruses could replicate and produce infectious viruses in cell culture, and were useful to assess the genotype and strain dependency to a novel NS5A inhibitor. The strategy of using recombinant virus will facilitate not only a better understanding of the strain-specific roles of NS5A in the HCV lifecycle, but also aid in developing and testing specific inhibitors against NS5A from different genotypes and strains.

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#### Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2011.05.144.

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Short  
CommunicationStructural requirements of virion-associated  
cholesterol for infectivity, buoyant density and  
apolipoprotein association of hepatitis C virusMami Yamamoto,<sup>1,2</sup> Hideki Aizaki,<sup>1</sup> Masayoshi Fukasawa,<sup>3</sup>  
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Our earlier study has demonstrated that hepatitis C virus (HCV)-associated cholesterol plays a key role in virus infectivity. In this study, the structural requirement of sterols for infectivity, buoyant density and apolipoprotein association of HCV was investigated further. We removed cholesterol from virions with methyl  $\beta$ -cyclodextrin, followed by replenishment with 10 exogenous cholesterol analogues. Among the sterols tested, dihydrocholesterol and coprostanol maintained the buoyant density of HCV and its infectivity, and 7-dehydrocholesterol restored the physical appearance of HCV, but suppressed its infectivity. Other sterol variants with a  $3\beta$ -hydroxyl group or with an aliphatic side chain did not restore density or infectivity. We also provide evidence that virion-associated cholesterol contributes to the interaction between HCV particles and apolipoprotein E. The molecular basis for the effects of different sterols on HCV infectivity is discussed.

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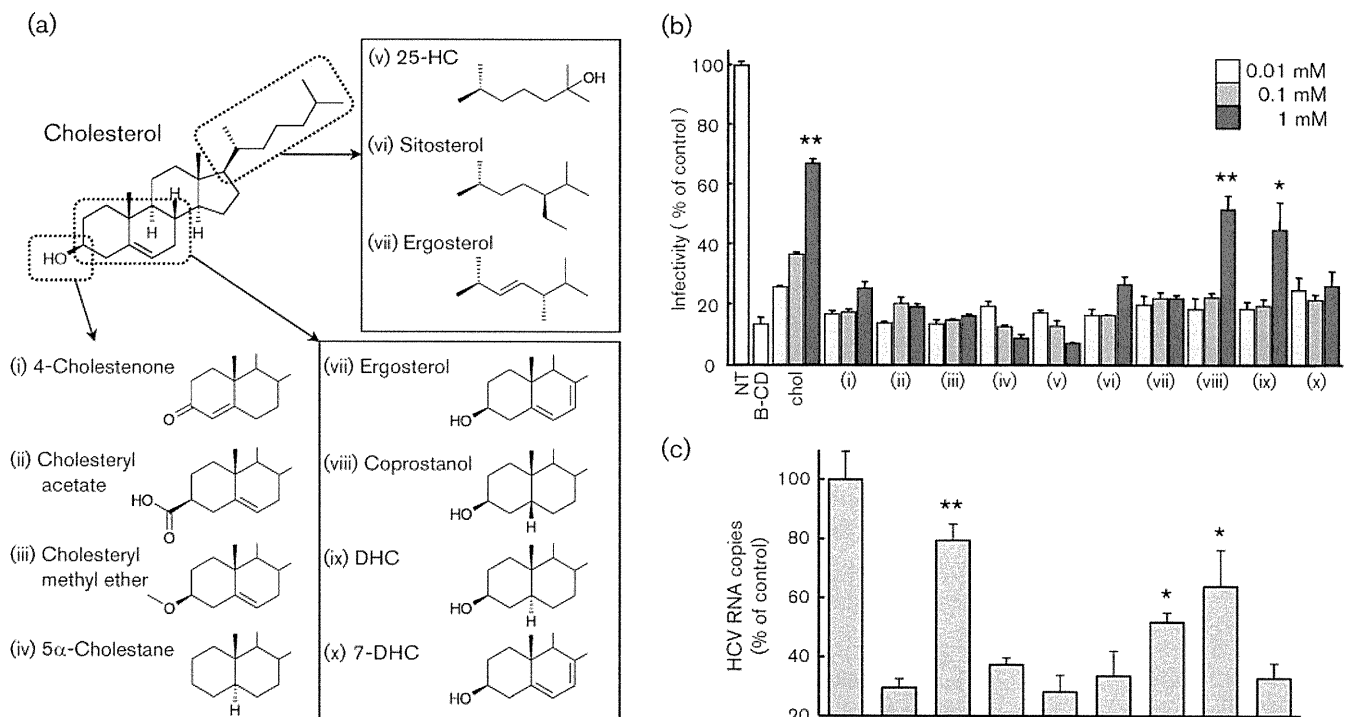
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Hepatitis C virus (HCV) is a major cause of liver diseases, and is an enveloped, plus-strand RNA virus of the genus *Hepacivirus* of the family *Flaviviridae*. The mature HCV virion is considered to consist of a nucleocapsid, an outer envelope composed of the viral E1 and E2 proteins and a lipid membrane. Production and infection of several enveloped viruses, such as human immunodeficiency virus type 1 (HIV-1), hepatitis B virus and varicella-zoster virus (Bremer *et al.*, 2009; Campbell *et al.*, 2001; Graham *et al.*, 2003; Hambleton *et al.*, 2007), are dependent on cholesterol associated with virions. However, except for HIV-1 (Campbell *et al.*, 2002, 2004), there is limited information about the effects of replacing cholesterol with sterol analogues on the virus life cycle. We demonstrated the higher cholesterol content of HCV particles compared with host-cell membranes, and that HCV-associated cholesterol plays a key role in virion maturation and infectivity (Aizaki *et al.*, 2008). Recently, by using mass spectrometry, Merz *et al.* (2011) identified cholesteryl esters, cholesterol,

phosphatidylcholine and sphingomyelin as major lipids of purified HCV particles.

To investigate further the effect of the structural requirement for cholesterol on the infectivity, buoyant density and apolipoprotein association of HCV, depletion of virion-associated cholesterol and substitution of endogenous cholesterol with structural analogues (Fig. 1a) was used in this study. HCVcc (HCV grown in cell culture) of the JFH-1 isolate (Wakita *et al.*, 2005), prepared as described previously (Aizaki *et al.*, 2008), was treated with 1 mM methyl  $\beta$ -cyclodextrin (B-CD), which extracts cholesterol from biological membranes, for 1 h at 37 °C. The cholesterol-depleted virus was then incubated with exogenous cholesterol or cholesterol analogues at various concentrations for 1 h. After removal of B-CD and free sterols by centrifugation at 38 000 r.p.m. (178 000 g) for 2.5 h, the treated particles were used to infect Huh7 cells, kindly provided by Dr Francis V. Chisari (The Scripps Research Institute, La Jolla, CA, USA), and their infectivity was determined by quantifying the viral core protein in cells using an enzyme immunoassay (Ortho-Clinical Diagnostics) at 3 days post-infection (p.i.). Virus infectivity, which fell to <20% after B-CD treatment, was

A supplementary table and figure are available with the online version of this paper.



**Fig. 1.** Role of virion-associated cholesterol analogues in virus infection. (a) Structures of sterols used in this study. Variations in the 3 $\beta$ -hydroxyl group (lower left), aliphatic side chain (upper right) or ring structure (lower right) of cholesterol are shown. (i–x) Compounds studied in (b) and (c). (b) Effect of replenishment with sterols on HCV infectivity. Intracellular HCV core levels were determined at 72 h p.i. as the indicator of infectivity, which is represented as a percentage of the untreated HCVcc level (NT). (c) Effects of virion-associated sterols on virus internalization. HCV RNA copies in cells after virus internalization were quantified and are shown as percentages of the untreated HCVcc level (NT). (b, c) Means  $\pm$  SD of four samples are shown. \* $P$  < 0.05; \*\* $P$  < 0.01, compared with B-CD-treated virus (unpaired Student's  $t$ -test). Data are representative of at least two experiments.

recovered by addition of cholesterol at 0.01–1 mM in a dose-dependent manner (Fig. 1b). Among the cholesterol analogues tested, variants with a 3 $\beta$ -hydroxyl group (4-cholestenone, cholesteryl acetate, cholesteryl methyl ether and 5 $\alpha$ -cholestane) or variants with an aliphatic side chain [25-hydroxycholesterol (25-HC), sitosterol and ergosterol] exhibited no or little effect on the recovery of infectivity of B-CD-treated HCV (Fig. 1b, lanes i–vii). In contrast, addition of variants in the structure of the sterol rings [coprostanol or dihydrocholesterol (DHC)] at 1 mM restored infectivity to around 50% compared with non-treated virus control (Fig. 1b, lanes viii and ix). Other variants in the ring structure [7-dehydrocholesterol (7-DHC) and ergosterol, which is also a variant with an aliphatic side chain as indicated above] did not show any increase in the infectivity of B-CD-treated virus (Fig. 1b, lanes x and vii).

We demonstrated previously that HCV-associated cholesterol plays an important role in the internalization step of the virus, but not in cell attachment during virus entry (Aizaki *et al.*, 2008). The effect of virion-associated cholesterol analogues on virus attachment to cells and

following internalization was determined. HCVcc, treated with B-CD with or without subsequent replenishment with sterols, was incubated with Huh7-25-CD81 cells, which stably express CD81 (Akazawa *et al.*, 2007), for 1 h at 4  $^{\circ}$ C. As an internalization assay, the incubation temperature was shifted to 37  $^{\circ}$ C post-binding procedure and maintained for 2 h. The cells were then treated with 0.25% trypsin for 10 min at 37  $^{\circ}$ C, by which >90% of HCV bound to the cell surface was removed (data not shown; Aizaki *et al.*, 2008). Internalized HCV was quantified by measuring the viral RNA in cell lysates by real-time RT-PCR (Takeuchi *et al.*, 1999). B-CD treatment or supplementation with sterols of B-CD-treated HCV had little or no effect on virus attachment to the cell surface (data not shown). Regarding virus internalization (Fig. 1c), treatment of HCVcc with 1 mM B-CD resulted in approximately 70% reduction of viral RNA. The reduced level of the internalized HCV recovered markedly to approximately 80% of the untreated HCVcc level by replenishment with 1 mM cholesterol. In agreement with the results shown in Fig. 1(b), addition of coprostanol or DHC to the B-CD-treated virus caused a significant recovery of virus internalization, suggesting that coprostanol and DHC associated with the

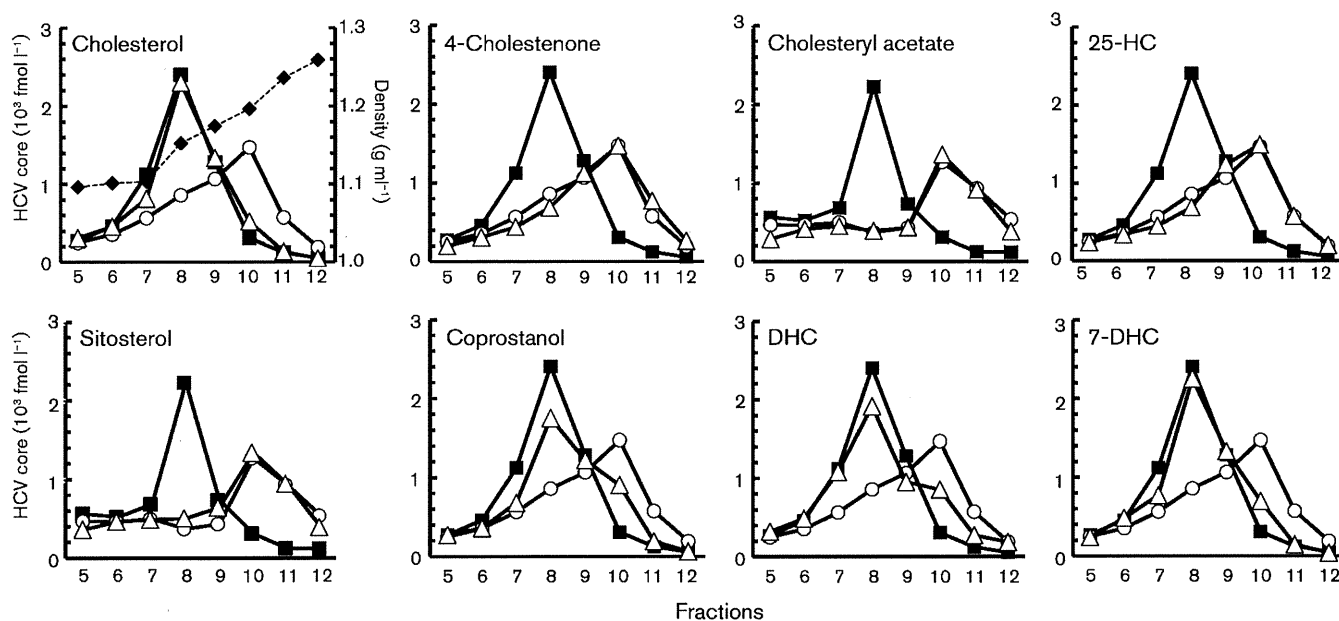
virion have the ability to play a role in HCV internalization into cells, in a manner comparable to cholesterol (Fig. 1c, lanes viii and ix). No or only a little recovery of virus internalization was observed by loading with other cholesterol analogues, such as 4-cholestenone, 5 $\alpha$ -cholestane, 25-HC or 7-DHC (Fig. 1c, lanes i, iv, v and x).

To monitor the effect of cholesterol analogues on the physical characteristics of HCV, we next investigated buoyant-density profiles by using sucrose density-gradient centrifugation, in which untreated, B-CD-treated and sterol-replenished HCVcc were concentrated and layered onto continuous 10–60% (w/v) sucrose density gradients, followed by centrifugation at 35 000 r.p.m. (151 000 g) for 14 h. Fractions were collected and analysed for the core protein. Fig. 2 shows that the virus density became higher after treatment with B-CD and that cholesterol-replenished virus shifted the density of B-CD-treated HCV to the non-treated level. Consistent with the result shown in Fig. 1(b), no effect on restoration of the buoyant densities of HCV was observed using variants with modifications in either the 3 $\beta$ -hydroxyl group (4-cholestenone, cholesteryl acetate and 5 $\alpha$ -cholestane) or the aliphatic side chain (25-HC and sitosterol). In contrast, variants in the sterol ring structure (coprostanol, DHC and 7-DHC) had an ability to recover the density of B-CD-treated virus to that of non-treated virus.

Incorporation efficiency of the sterols into the cholesterol-depleted HCVcc was further determined by gas chromatography with flame ionization detection (see Supplementary Table S1, available in JGV Online). Under the experimental

conditions used, exogenously supplied cholesterol after B-CD treatment was able to restore cholesterol content in HCVcc almost to initial levels. When 4-cholestenone, cholesteryl acetate, 25-HC, DHC or 7-DHC was added to B-CD-treated HCVcc, virion-associated sterol levels were 146, 157, 68, 96 or 73%, respectively, of that of the non-treated control. The proportion of cholesterol analogues to the total sterols incorporated was  $\geq 30\%$  when 4-cholestenone, cholesteryl acetate, DHC or 7-DHC was used; however, the proportion in the case of 25-HC was only 3%. It may be that the hydrophilic modification of the aliphatic side chain leads to poor association with HCVcc.

Collectively, exogenous variants with the 3 $\beta$ -hydroxyl group, such as 4-cholestenone and cholesteryl acetate, can be incorporated into B-CD-treated HCVcc, but resulted in no recovery of virus infectivity, indicating the importance of the 3 $\beta$ -hydroxyl group of cholesterol associated with the virus envelope in HCV infectivity. In contrast, two variants with modification in their sterol ring structures, coprostanol and DHC, have the ability to substitute for cholesterol. However, 7-DHC, another variant within the sterol ring, is incorporated readily into the depleted virion and restores the virus density, HCV replenished with 7-DHC is not infectious. These facts suggest that reduced forms of the sterol ring (coprostanol and DHC) in virion-associated cholesterol can be permitted for maintaining virus infectivity. However, a molecule with an additional double bond in the ring structure (7-DHC) seems to fail to exhibit infectivity, presumably because the change reduces structural flexibility in the

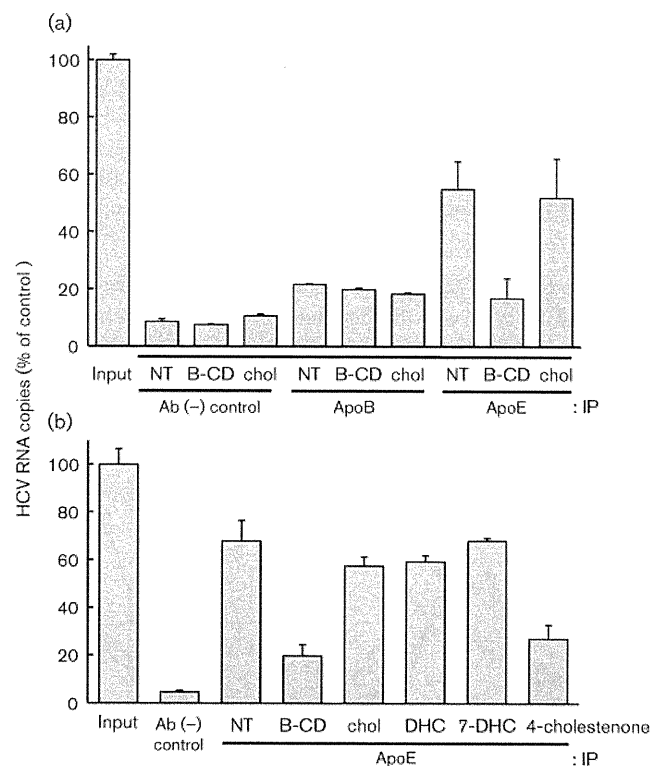


**Fig. 2.** Sucrose density-gradient profiles of lipid-modified HCV. Core protein concentration in each fraction of untreated HCVcc (■), B-CD-treated HCVcc (○) or HCVcc replenished with sterols (△) was determined. Corresponding densities of fractions are shown as a dashed line (◆).

sterol molecule and consequently in the virion structure. Coprostanol and DHC are *cis* and *trans* isomers, which are often known to have different physical properties. However, based on their molecular models, these two sterols, as well as cholesterol, possibly have similar spatial arrangements of the aliphatic side chain, the hydroxyl group and four-ring region because of their structural flexibility. In contrast, the spatial arrangement of 7-DHC does not seem comparable to that of cholesterol. Campbell *et al.* (2004) reported that replacement of HIV-1-associated cholesterol with raft-inhibiting sterols, including coprostanol, suppresses HIV-1 infectivity, whereas replacement with raft-promoting analogues such as DHC and 7-DHC (Megha *et al.*, 2006; Wang *et al.*, 2004; Xu & London, 2000; Xu *et al.*, 2001) maintains infectivity, demonstrating the importance of the raft-promoting properties of virion-associated cholesterol in HIV-1 infectivity (Campbell *et al.*, 2004). It is therefore likely that HCV-associated cholesterol is involved, at least in part, in virus infectivity via a molecular basis independent of lipid-raft formation.

The density of blood-circulating HCV is heterogeneous, ranging approximately from  $<1.06$  to  $1.25 \text{ g ml}^{-1}$ , and it is proposed that low-density virus is associated with very-low-density lipoprotein (VLDL) and/or low-density lipoprotein (LDL) (André *et al.*, 2002; Thomssen *et al.*, 1993). It has recently been demonstrated that the pathway for VLDL assembly plays a role in assembly and maturation of infectious HCVcc (Icard *et al.*, 2009). HCVcc with low density, which is presumably associated with VLDL or VLDL-like lipoproteins, was found to possess higher infectivity than that with high density (Lindenbach *et al.*, 2006). This study, as well as our earlier work, indicated that removal of cholesterol from HCVcc by B-CD increased the buoyant density of the virus and reduced its infectivity. Thus, one may hypothesize that the virion-associated cholesterol plays a role in the formation of a complex with lipoproteins or apolipoproteins. To address this, the interaction between apolipoproteins and HCVcc with or without B-CD treatment was investigated by co-immunoprecipitation (Co-IP kit; Thermo Scientific). Virus samples were subjected separately to AminoLink Plus coupling resin, which was conjugated with a monoclonal antibody (mAb) against apolipoprotein E (ApoE) or apolipoprotein B (ApoB), and incubated at  $4^\circ\text{C}$  for 4 h. After washing, total RNAs were extracted from the resulting resin beads by using TRIzol reagent (Invitrogen), followed by quantification of HCV RNA as described above (Takeuchi *et al.*, 1999). As indicated in Fig. 3(a), only a fraction of HCVcc was precipitated with an anti-ApoB mAb. In contrast, an anti-ApoE mAb was able to coprecipitate a considerable amount of the virus. It is of interest that B-CD-treated HCVcc hardly reacted with the mAb; however, the cholesterol-replenished virus was found to recover its reactivity, suggesting a role for virion-associated cholesterol in the formation of the HCV-lipoprotein/apolipoprotein complex. The results obtained are consistent with findings indicating that HCVcc can be

captured with anti-ApoE antibodies, but capture with anti-ApoB antibodies is inefficient (Chang *et al.*, 2007; Hishiki *et al.*, 2010; Huang *et al.*, 2007; Jiang & Luo, 2009; Merz *et al.*, 2011; Nielsen *et al.*, 2006; Owen *et al.*, 2009), as well as with a recent model of structures of infectious HCV, in which HCVcc looks like ApoE-positive and primarily ApoB-negative lipoproteins (Bartenschlager *et al.*, 2011). We further tested the ApoE distribution in the density-gradient fractions of HCVcc samples (see Supplementary Fig. S1, available in JGV Online). With or without cholesterol depletion, ApoE was detected at a wide range of concentrations:  $1.04 \text{ g ml}^{-1}$  (fraction 1) to  $1.17 \text{ g ml}^{-1}$  (fraction 9). However, its level in the fractions at  $1.10 \text{ g ml}^{-1}$  (fraction 5) to approximately  $1.17 \text{ g ml}^{-1}$  was moderately decreased in the case of B-CD-treated virus.



**Fig. 3.** Effect of virion-associated sterols on HCV-apolipoprotein interaction. (a) HCVcc samples with no treatment (NT), B-CD-treated (B-CD) or replenished with cholesterol (chol) were incubated with an amine-reactive resin coupling either an anti-ApoB mAb (ApoB) or an anti-ApoE mAb (ApoE). Control resin that is composed of the same material as above, but is not activated, was used as a negative control [Ab (-) control]. (b) B-CD-treated HCVcc was incubated with cholesterol (chol), DHC, 7-DHC or 4-cholestenone, followed by immunoprecipitation with the resin coupling with anti-ApoE mAb. (a, b) HCV RNAs in the immunoprecipitates were quantified and are indicated as percentages of the amount of input HCVcc RNA. Means  $\pm$  SD of three samples are shown. Data are representative of three experiments.



Whether cholesterol analogues could have a comparable role in HCV association with lipoprotein was examined further (Fig. 3b). Addition of DHC or 7-DHC, but not 4-cholestenone, to B-CD-treated HCVcc resulted in the recovery of coprecipitation of the virus with anti-ApoE. The results are correlated with the effect of sterols on the restoration of the buoyant densities of lipid-modified HCVcc (Fig. 2), suggesting that virion-associated cholesterol variants with modification in the sterol rings, but not in either the  $3\beta$ -hydroxyl group or the aliphatic side chain, may tolerate the interaction between HCV and ApoE-positive lipoprotein.

Given that 7-DHC restored the association of HCV with ApoE and virion buoyant density, but did not restore infectivity, cholesterol and/or its analogues might affect the ability of virion membranes to fuse with the cell, independent of ApoE association. As cholesterol is an important mediator of membrane fluidity, one may hypothesize that HCV-associated cholesterol is involved in infectivity through modulation of the membrane fluidity. It has been reported that, in patients with Smith–Lemli–Opitz syndrome, a disorder of the cholesterol-synthesis pathway, cholesterol content decreases and 7-DHC increases in the cell membranes, leading to alteration of phospholipid packing in the membrane and abnormal membrane fluidity (Tulenko *et al.*, 2006).

It is now accepted that maturation and release of infectious HCV coincide with the pathway for producing VLDLs, which export cholesterol and triglyceride from hepatocytes. This study revealed roles for the structural basis of virion-associated cholesterol in the infectivity, buoyant density and apolipoprotein association of HCV. Although it was shown that HCV virions in infected patients, so-called lipo-viro particles, exhibited certain biochemical properties such as containing ApoB, ApoC and ApoE (Diaz *et al.*, 2006; Bartenschlager *et al.*, 2011), our studies provide useful information and the basis for future investigations toward a deeper understanding of the biogenesis pathway of infectious HCV particles.

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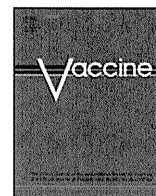
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## Production and characterization of HCV particles from serum-free culture

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### ABSTRACT

Hepatitis C virus (HCV) is a major cause of liver cancer, and it is therefore important to develop a prophylactic strategy for HCV infection. In recent years, a system for cell culture of the infectious HCV particle has been established, and the inactivated particle has potential as an antigen for vaccine development. In this study, we aimed to establish highly efficient HCV particle purification procedures using the following serum-free culture of HCV particles. First, naïve human hepatoma Huh7 cells were grown in serum-free medium that was supplemented with human-derived insulin, transferrin and sodium selenite. Then, *in vitro* transcribed JFH-1 or J6/JFH-1 chimeric HCV-RNA was transfected into the serum-free conditioned Huh7 cells. Infectious HCV was secreted into the culture supernatant with the same efficiency as that from cells cultured in FBS-containing medium. The HCV-core protein and RNA continued to be detected in the culture supernatant when the infected cells were subcultured in serum-free medium. Sucrose gradient centrifugation analyses indicated that the profiles of HCV-core, HCV-RNA and the infectivity of HCV particles were almost identical between HCV from FBS-supplemented and serum-free cultures. We further determined that anti-CD81, anti-SR-BI and anti-E2 antibodies inhibited infection by serum-free cultured HCV to a greater extent than infection by HCV from FBS-supplemented cultures. These HCV particles also differed in the level of associated apolipoproteins: the ApoE level was lower in serum-free cultured HCV. ApoB and ApoE antibody-depletion assays suggested that infection of serum-free cultured HCV was independent of ApoB and ApoE proteins. These data suggest that lipids conjugated with HCV affect infection and neutralization.

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### 1. Introduction

Hepatitis C virus (HCV) is an enveloped virus that belongs to the *Hepacivirus* genus of the *Flaviviridae* family. HCV is a human pathogen that is a major cause of chronic hepatitis, liver cirrhosis and hepatic carcinoma. HCV therapy mainly involves treatment with pegylated-interferon and ribavirin; however, these agents are not very effective for patients with high titer HCV-RNA and geno-

type 1. Thus, it is necessary to develop new, more effective therapies and preventive care treatments for HCV. It was discovered that a genotype 2a strain, JFH-1, efficiently replicated in Huh7 cells [1]. Moreover, an *in vitro* culture system that generates infectious HCV has also been successfully developed using the JFH-1 genome [2–4]. Recently, it has been shown to be possible to produce various chimeric HCVs by replacement of the JFH-1 structural protein region with the same region from other strains [5]. These chimeric HCV particles are expected to lead to a HCV vaccine as well as to new pharmaceuticals.

Huh7 is a human hepatoma cell line that was established in 1982 [6]. This cell line can be cultured in serum-free medium supplemented with selenium. Serum-free culture has advantages for the simple purification and preparation of animal-origin-free virus particles. In this study, we successfully produced HCV particles in serum-free culture and compared the properties of these particles to those from FBS-supplemented cultures. Interestingly, serum-free cultured HCV was susceptible to CD81-, SR-BI- and HCV-E2-neutralizing antibodies. It was recently suggested that HCV particles associate with lipids to form viro-lipo particles [7–9], and it has also been shown that HCV particles can associate with

**Abbreviations:** HCV, hepatitis C virus; ITS, insulin-transferrin-selenium; MOI, multiplicity of infection; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium; VLDL, very-low-density lipoprotein.

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lipids to form exosomes [10,11]. We examined apolipoprotein association of serum-free cultured HCV. We found that this virus had a lower ApoE level than HCV from serum-supplemented cultures and that infection by this virus was apolipoprotein-independent.

## 2. Materials and methods

### 2.1. Cell culture

Huh7, Huh7.5.1 ([4], a generous gift from Dr. Francis V. Chisari), Huh7-25 and Huh7-25-CD81 [12] cell lines were cultured in 5% CO<sub>2</sub> at 37 °C in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (DMEM-10). Our previous FACS analysis indicated that Huh7-25 cells do not express CD81, and that Huh7-25-CD81 constitutively express CD81, on the cell surface [12]. For serum-free culture, the cells were conditioned and cultured in DMEM/F-12 supplemented with Insulin-Transferrin-Selenium-X (ITS) (Invitrogen, Carlsbad, CA).

### 2.2. Establishment of serum-free cultured cells

Sub-confluent Huh7 cells that were cultured in DMEM-10 were passaged in 10-cm dishes (Nunc, Rochester, NY) in DMEM containing 5% FBS. The cells were then sequentially passaged in DMEM containing 2, 1 and 0.5% FBS and were ultimately passaged in serum-free medium. The cells were detached for passage in serum-free culture using TrypLE Select (Invitrogen).

### 2.3. Cell growth assay

Cell growth was assayed by MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay using the CellTiter 96<sup>®</sup> AQueous One Solution Cell Proliferation Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. In brief,  $1 \times 10^4$  cells were seeded into a 96-well culture plate (IWAKI, Tokyo, Japan) in 100  $\mu$ L of media, and 20  $\mu$ L of the assay solution was added into each well at the appropriate time. After incubation for 1 h at 37 °C, the absorbance of the solution at 490 nm was measured.

### 2.4. Plasmids

pJFH1 and pJ6/JFH1 were generated as previously reported [2,13].

### 2.5. RNA synthesis

RNA synthesis was performed as described previously [14]. Briefly, the pJFH1 and pJ6/JFH1 plasmids were digested with *Xba* I and were treated with Mung Bean nuclease (New England Biolabs, Beverly, MA). The digested plasmid DNA fragment was then purified and was used as a template for RNA synthesis. HCV-RNA was synthesized *in vitro* using a MEGAscript<sup>™</sup> T7 kit (Ambion, Austin, TX). The synthesized RNA was treated with DNaseI, followed by acid phenol extraction to remove any remaining template DNA.

### 2.6. RNA transfection

RNA transfection was performed as described previously [15]. Briefly, trypsinized cells were washed with Opti-MEM I<sup>™</sup> reduced-serum medium (Invitrogen) and were resuspended at a density of  $7.5 \times 10^6$  cells/mL in Cytomix buffer [1]. RNA (10  $\mu$ g) that was synthesized from pJFH1 or pJ6/JFH1 was mixed with 400  $\mu$ L of the cell suspension and was transferred into an electroporation cuvette (Precision Universal Cuvettes, Thermo Hybrid, Middlesex, UK). The cells were then pulsed at 260 V and 950  $\mu$ F with the Gene Pulser II<sup>™</sup>

apparatus (Bio-Rad, Hercules, CA). Transfected cells were immediately transferred to a 6-well plate, in which each well contained 3 mL of culture medium.

### 2.7. Infectivity titration

Huh7.5.1 cells were employed to determine the infectivity titer using end point dilution and immunofluorescence as described below. Briefly, each sample was serially diluted 5-fold in DMEM-10 and a 100- $\mu$ L aliquot was used to inoculate Huh7.5.1 cells. Infection was examined 72 h post-inoculation by immunofluorescence using a mouse monoclonal anti-Core antibody 2H9 and Alexa 488-conjugated secondary anti-mouse IgG antibody. The infectious foci were counted. The titer was then calculated and is indicated as focus forming units per mL (FFU/mL).

### 2.8. HCV inhibition assay

To analyze the inhibitory effects of anti-CD81 and anti-SR-BI against virus infection, naïve Huh7.5.1 cells ( $2 \times 10^4$ ) were seeded into a 48-well plate and were incubated for 1 h at 37 °C with JS-81 or rat anti-SR-BI serum ([16], a generous gift from Dr. H. Barth) that was serially diluted with DMEM. Mouse IgG1 (Sigma, St. Louis, MO) and rat pre-immune serum were used as controls for JS-81 and anti-SR-BI, respectively. Antibodies were removed and the cells were washed once with PBS. The cells were then inoculated with viruses (MOI 0.1) from FBS-supplemented or serum-free culture for 3 h, and were then washed with PBS and cultured with DMEM-10 for 72 h. The cells were washed three times with PBS and 100  $\mu$ L of Passive Lysis Buffer (Promega) was added into each well. Cell lysates were collected and HCV-core concentrations were measured as described below.

To analyze the inhibitory effects of anti-E2 against HCV particles, viruses that were purified from FBS-supplemented or serum-free culture ( $2 \times 10^3$  FFU) were mixed with mouse anti-E2 (AP33, a kind gift from Genentech, Inc.) antibody, and were then incubated for 30 min at room temperature. Naïve Huh7.5.1 cells ( $1 \times 10^4$ ) were seeded into a poly-D-lysine coated 96-well plate, and cells were inoculated with the virus-antibody mixtures, which were serially diluted with DMEM-10, and, after 3 h, the mixtures were removed and the cells were washed once with PBS. DMEM-10 was added into each well, and the cells were cultured for 72 h. The cells were fixed with methanol for 15 min at –20 °C, and the infected cells were stained with rabbit anti-N55A antibody using immunofluorescence as described above [17]. Percentage infection was calculated from the infectious titer of each diluted virus.

### 2.9. Sucrose density gradient analysis and HCV purification

Supernatants (4 mL) of J6/JFH-1 HCV cells were layered on top of a preformed continuous 10–60% sucrose gradient in 10 mM Tris, 150 mM NaCl, and 0.1 mM EDTA (TNE buffer). HCV-core levels, HCV-RNA titer and infectious titers of the media are shown in the supplementary table. The gradients were centrifuged using an SW41 rotor (Beckman Coulter, Fullerton, CA) at 35,000 rpm for 16 h at 4 °C, and fractions (500  $\mu$ L each) were collected from the bottom of the tube. The density of each fraction was estimated by weighing a 100- $\mu$ L drop from fractions of a gradient run.

Partially purified HCV was prepared by collecting the peaks of HCV-core and HCV-RNA and was used for the infection assay and for characterization.

### 2.10. Quantification of HCV-core protein and RNA

To estimate the levels of HCV-core proteins, the concentration of HCV-core proteins was measured. Aliquots of samples were

assayed using the HCV Core ELISA kit (Ortho Clinical Diagnostics, Tokyo, Japan). Viral RNA was isolated from harvested culture media or from sucrose density gradient fractions using the QiaAmp Viral RNA Extraction kit (Qiagen, Tokyo, Japan). Copy numbers of HCV-RNA were determined by the real-time detection reverse transcription-polymerase chain reaction (RTD-PCR) using an ABI Prism 7500 fast sequence detector system (Applied Biosystems, Tokyo, Japan) [18].

### 2.11. Immunoprecipitation of HCV particles

Protein G-Sepharose (GE Healthcare, Little Chalfont, UK) was mixed with DMEM-10 for 1 h at 4°C, and was spun down by centrifugation for 1 min at 5000 rpm (TOMY, Tokyo, Japan). HCV particles ( $1 \times 10^7$  copies HCV-RNA) were mixed with the resin and were incubated overnight at 4°C with rotation. The sample was centrifuged for 1 min at 5000 rpm, and the supernatant was then collected. A 7.5 µL aliquot of anti-human ApoB (AB742, Millipore, Billerica, MA) or anti-human ApoE polyclonal antibody (AB947, Millipore) was added into the pre-cleared virus fluid (100 µL), and the mixture was incubated overnight at 4°C. Mouse IgG (5 µg, Sigma) was used as a control. The mixture was mixed with the resin and incubated for 1 h at 4°C, with rotation. The supernatants were collected following centrifugation and the pellets were then washed twice with PBS and suspended in DMEM-10. Viral RNA was eluted from both the supernatants and the suspended pellets using the QIAamp Viral RNA mini kit (Qiagen). The HCV-RNA titer present in each total RNA from the supernatant and the pellet was evaluated, and the infectivity of the supernatant was measured by inoculation of naïve Huh7.5.1 cells.

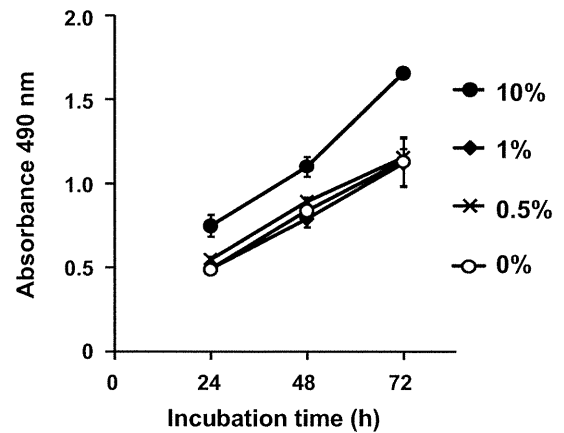
## 3. Results

### 3.1. Establishment of serum-free cultured Huh7 cells

Huh7 cells are routinely maintained in our laboratory by culture in 10% FCS-supplemented medium. To examine HCV particles produced from infected cells cultured under serum-free conditions, we first established a serum-free culture system which allowed the proliferation of Huh7 cells. It was previously reported that Huh7 cells could be cultured in serum-free media that contains selenium [6]. We therefore examined the growth of Huh7 cells following gradual reduction of the level of FBS and ultimately culture in completely serum-free, selenium-supplemented (ITS-containing) media. The cells could be passaged and cultured over a long period in this medium, although the observed growth, as assayed using an MTS assay, was slightly lower than that of FBS-supplemented cultures for all the cell lines used in this study (Fig. 1 and Supplementary Fig. S1). Based on this result, we used ITS-supplemented media for the evaluation of serum-free cultured HCV.

### 3.2. Production of HCV particles from serum-free cultures

We next tested the efficiency of HCV particle production in serum-free culture. *In vitro* transcribed HCV-RNAs were transfected into the CD81-negative Huh7-25, and the CD81 positive Huh7-25-CD81 cell lines. The re-infection rate is known to be negligible when Huh7-25 is used [19]. When synthetic RNAs of JFH-1 or J6/JFH-1 strains were transfected, the HCV-core protein and HCV-RNA were detected in the culture media, and each medium was infectious for naïve Huh7 cells (Fig. 2, Supplementary Table). The specific infectivity of each medium (the values of the infectivity titer divided by the values of the HCV-core protein or of HCV-RNA) of J6/JFH-1 HCV was higher than that of JFH-1 (Fig. 2C, Supplementary Table). These results showed that infectious HCV was secreted into the



**Fig. 1.** Cell proliferation assay of serum-free cultured Huh7 cells. Huh7 cells that were seeded into a 96-well plate ( $1 \times 10^4$ /well) were sequentially grown in DMEM/F-12 media containing 10%, 1%, 0.5% and 0% fetal bovine serum. At indicated times, an MTS cell proliferation assay was performed using a commercial kit.

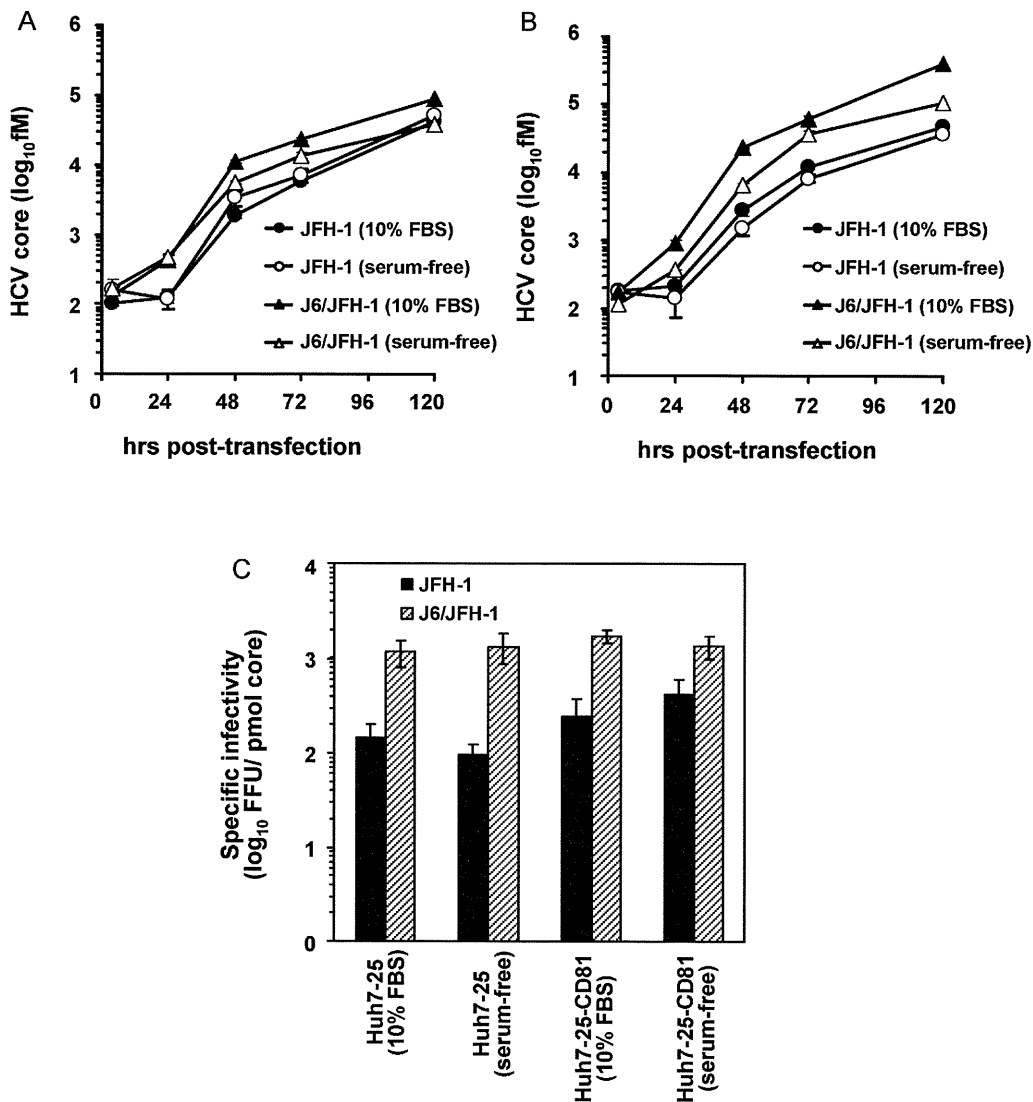
culture medium of both cell lines. The slightly higher HCV production of Huh7-25-CD81 cells may be due to re-infection of secreted virus particles. To determine if HCV-infected cells could be cultured for several passages in serum-free medium, serum-free cultured Huh7 cells were inoculated with infectious J6/JFH-1 chimeric HCV at multiplicity of infection (MOI) of 0.2 and were then cultured for a long period, following which the HCV-core protein and HCV-RNA in the culture medium was analyzed. The HCV-core protein and HCV-RNA were continuously detected in serum-free media, and their level was almost equal to that of infected FBS-supplemented Huh7 culture (Fig. 3).

### 3.3. Characterization of serum-free cultured HCV by sucrose density gradient analysis

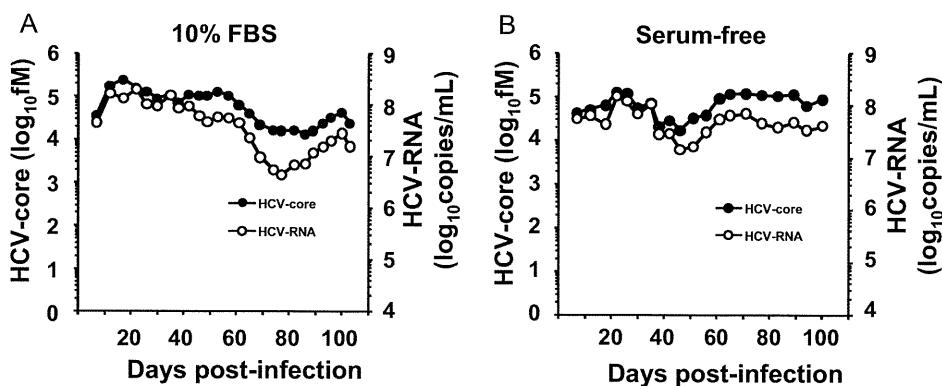
We next compared the characteristics of HCV viruses produced under serum-free and serum-supplemented conditions by density gradient analysis. Each infectious supernatant was layered on top of a preformed continuous 10–60% sucrose gradient and centrifuged. Eighteen fractions were obtained and HCV-core and RNA titers of each fraction were determined. The detected virus titers in each density fraction were different mainly due to differences in the amount of input virus, as shown in the supplementary table. As previously reported, infectivity of all viruses was observed in fractions of lower density (approximately 1.10 g/mL sucrose) than those in which the peaks of HCV-core and HCV-RNA were detected (Fig. 4), although the specific infectivity of serum-free cultured HCV was slightly lower than that of FBS-supplemented HCV. These results suggested that the infectious HCV produced in the media by serum-free cultures had similar characteristics to those of HCV produced by serum-supplemented cultures. In addition, the virus particles produced from CD81-positive and -negative cells exhibited similar density profiles (Compare Fig. 4A, B vs. C, D).

### 3.4. Antibodies differentially inhibit HCV from serum-free and serum supplemented cultures

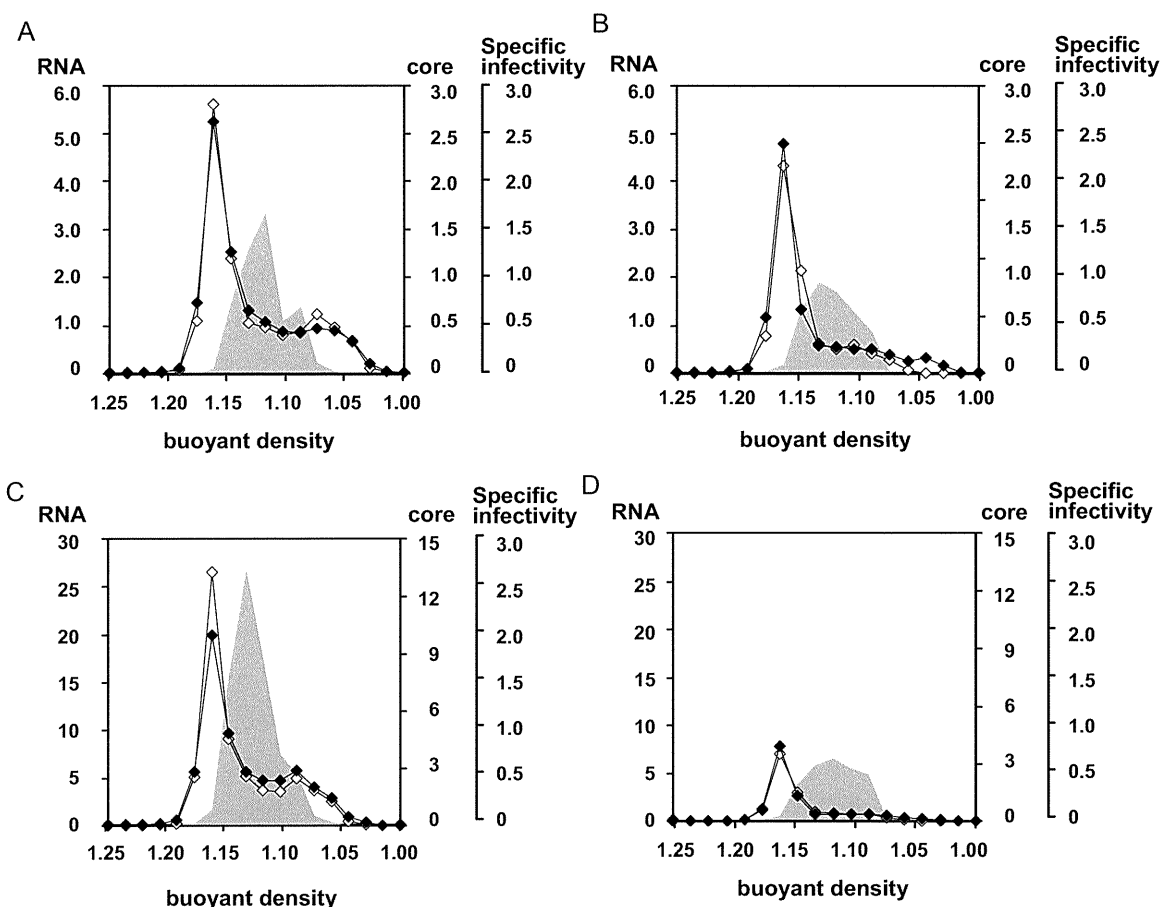
We next examined antibody inhibition of cell infection by HCV derived from serum-free or serum-supplemented cultures. CD81 and SR-BI are candidate cellular receptors for HCV infection. We first determined the inhibitory effect of anti-CD81 and anti-SR-BI antibodies on infection of serum-free cultured HCV. Interestingly, HCV infection by HCV derived from serum-free and serum-supplemented cultures was differently inhibited by these



**Fig. 2.** HCV production from serum-free cultured Huh7 cells transfected with *in vitro* transcribed JFH-1 and J6/JFH-1 RNA. *In vitro* transcribed JFH-1 and J6/JFH-1 RNA was transfected into Huh7-25 (A) and Huh7-25-CD81 (B) cells that were grown under the indicated serum conditions. The culture supernatant was collected 4, 24, 48, 72 and 120 h post-transfection, and the HCV-core protein levels were analyzed using ELISA. All data were measured in triplicate, and are shown as means  $\pm$  SD. Infectivity of each supernatant that was collected 120 h post-transfection was analyzed by infectivity titration, and specific infectivity was calculated by dividing the mean value of the infectivity titer by that of the HCV-core protein (C). All data were measured in triplicate, and are shown as means  $\pm$  SD. Profiles of HCV-core, HCV-RNA and infectivity are indicated in the Supplementary Table.



**Fig. 3.** HCV production from serum-free cultured Huh7 cells infected with J6/JFH-1 HCV. Huh7 cells that were grown in 10% FBS-supplemented (A) or serum-free (B) DMEM/F-12 were inoculated with the J6/JFH-1 virus (MOI, 0.2), and media of sub-cultures were collected. The HCV-core (closed circles) and RNA (open circles) were analyzed using ELISA and RTD-PCR, respectively.



**Fig. 4.** Density gradient analysis of the supernatants derived from infected serum-free cultured Huh7 cells. *In vitro* transcribed J6/JFH-1 RNA was transfected into Huh7-25 (A and B) and Huh7-25-CD81 (C and D) cells that were cultured under 10% FBS-supplemented (A and C) or serum-free (B and D) conditions. Culture supernatants (4 mL) were collected 5 days post-transfection, and were then layered on top of a preformed continuous 10–60% sucrose gradient in TNE buffer. The gradients were centrifuged using an SW41 rotor at 35,000 rpm for 16 h at 4 °C, and fractions (500  $\mu$ L each) were collected from the bottom of the tube. The buoyant density (g/mL, x-axis), the levels of HCV-core ( $\times 10^2$  pM, closed diamonds) and HCV-RNA ( $\times 10^8$  copies/mL, open diamonds), and the specific infectivity for naïve Huh7.5.1 cells (FFU/pmol core, shown in gray) of each fraction were analyzed as described in Section 2.

antibodies (Fig. 5A and B). Next, to confirm that the anti-E2 antibody, which has been shown to bind HCV particles, inhibits HCV infection, HCV was pre-incubated with the anti-E2 antibody AP33 and inoculated into Huh7 cells. As shown in previous reports [20,21], AP33 inhibited HCV infection. However, its inhibitory effect was different for serum-free and serum-supplemented cultured HCV. Thus, infection of serum-free cultured HCV displayed the highest susceptibility to this antibody (Fig. 5C).

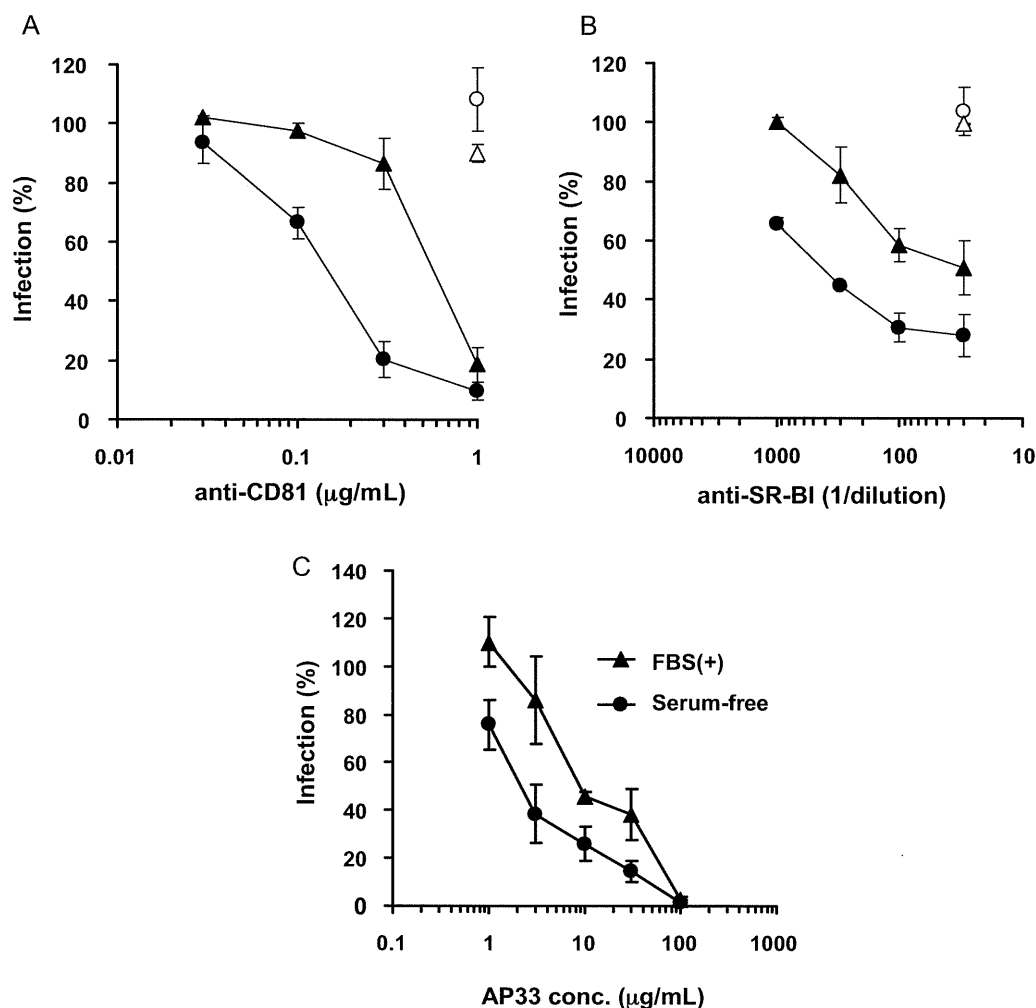
It has also been recently reported that VLDL associates with HCV and affects infectious particle formation and infection [7–9,22,23]. We therefore determined whether apolipoproteins associate with serum-free cultured HCV by immunoprecipitation of apolipoproteins from the culture media with anti-human apolipoprotein antibodies, followed by analysis of the viral titer in the pellet and the supernatant. HCV particles from both serum-free and serum-supplemented cultures were associated with both ApoB and ApoE (Fig. 6A). The percent of HCV from FBS-supplemented and serum-free cultures respectively that was associated with ApoB was  $13.22 \pm 0.09\%$  and  $16.84 \pm 0.08\%$  ( $p < 0.05$ , *t*-test) and the percent associated with ApoE was  $20.77 \pm 0.33\%$  and  $10.04 \pm 0.04\%$  ( $p < 0.005$ , *t*-test). Thus, serum-free HCV particles had a larger amount of associated ApoB, and a smaller amount of ApoE, than HCV from serum-supplemented cultures. We next determined whether depletion of ApoE affects viral infectivity by measurement of the infectivity titers of the virus in the supernatant following ApoE precipitation. This experiment showed that the infectivity of

HCV from FBS-supplemented cultures, but not of HCV from serum-free cultures, was down-regulated by depletion of ApoB and ApoE (Fig. 6C). These results indicated that apolipoprotein associates differently with viral particles derived from FBS-supplemented and serum-free cultures, and, further, that the infectivity of HCV derived from serum-free culture is only weakly affected by the associated apolipoprotein. These data therefore suggest that, unlike HCV from serum supplemented culture, and in contrast to previous reports regarding HCV infection, infection of HCV derived from serum-free culture may be apolipoprotein-independent. However, further studies are required to confirm this possibility.

#### 4. Discussion

In this study, we established a serum free cell culture system for the production of HCV particles, and compared the characteristics of these particles to those of HCV particles derived from serum-supplemented cultures. The particles derived from serum-free culture were infectious, suggesting that these particles would provide an appropriate antigen for the development of antibodies and vaccines. The serum-free cultured HCV could infect naïve Huh7 cells. Furthermore, sucrose density gradient analysis indicated that the profiles of HCV-core protein and HCV-RNA of serum-free cultured HCV were almost the same as those of HCV from FBS-supplemented cultures. Under serum-free conditions, HCV components (core protein and RNA) tended to be





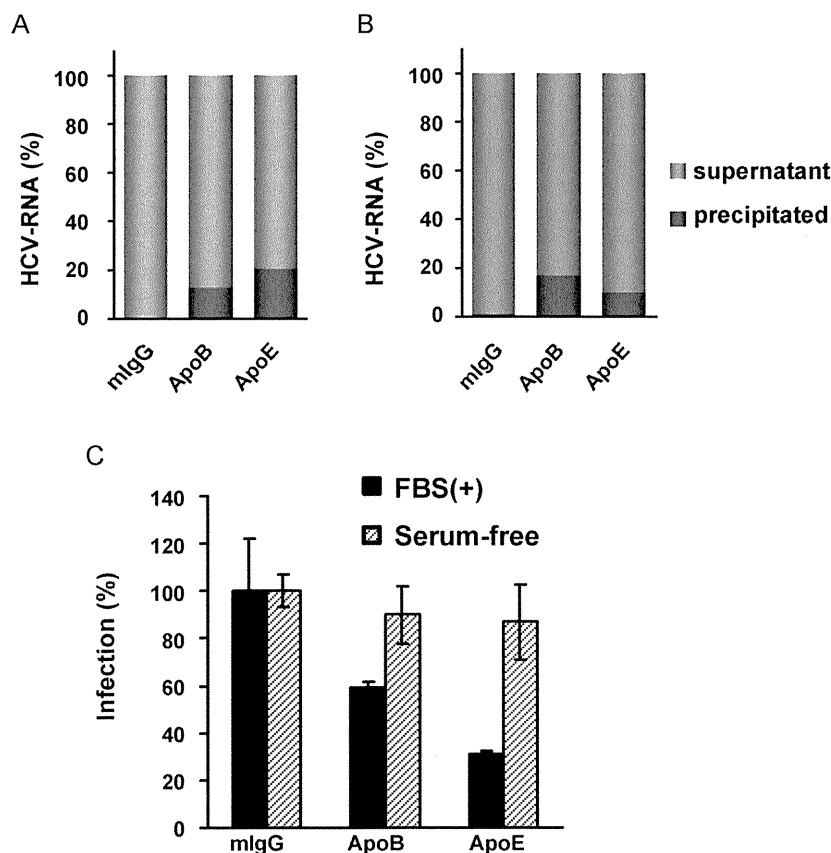
**Fig. 5.** Inhibition of serum-free cultured HCV infection by anti-CD81, anti-SR-BI and anti-E2 antibodies. Huh7.5.1 cells were pre-incubated with serially diluted anti-CD81 (JS-81, panel A) or anti-SR-BI (panel B) antibody for 1 h, and infectious J6/JFH-1 virus (MOI 0.1) obtained from FBS-supplemented (triangles) or serum-free (circles) culture was inoculated into each well for 72 h. Cells were lysed and HCV-core protein was measured. As controls, 1 μg/mL of mouse IgG (open triangles) and 30-fold diluted rat serum (open circles) were used. For incubation of virus particles with antibody, infectious J6/JFH-1 virus obtained from FBS-supplemented (closed triangle) or serum-free (closed circle) cultures was mixed with the indicated concentration of anti-E2 (AP33, panel C) antibody, and was then inoculated into naïve Huh7.5.1 cells. Infectivity was calculated by titration.

lost from fractions of lower density. Since exosome-like multivesicular particles have been reported to be present in lower density fractions [10], production of multivesicular particles may be different between serum-free and serum-supplemented cultures. When culture supernatants were used, the specific infectivity of serum-free cultured HCV was almost identical to that of FBS-supplemented HCV. However, infectivity of fractions separated on sucrose density gradients was lower for serum-free cultured HCV than for FBS-supplemented HCV cultures. These data may indicate that host factors that contribute to HCV infection differed under different culture conditions. Following infection and subsequent long-term culture under either serum-free or serum-supplemented conditions, HCV-core and RNA levels were identical for the first 40 days, but after this time period the ratio of HCV-core protein:RNA increased in both cultures. This result suggested that long-term culture may induce HCV-core protein which did not have HCV-RNA. As shown in Fig. 3, virus production under serum-free conditions seems to be more stable over 100 days than production under 10% FBS conditions. Fluctuation of viral titer during continuous cultivation of HCV-infected cells has been previously reported [24]. This fluctuation is dependent on the appearance of non-permissive cells with low or no CD81 expression. It is thus

likely that these non-permissive cells appear more rapidly under 10% FBS culture conditions than under serum-free conditions. This result may be due to the higher cellular growth rate of these cells in serum-supplemented culture as shown in Fig. 1 and Supplementary Fig. S1.

Our study highlighted some differences between HCV that was produced under serum-free and serum-supplemented conditions. Thus, interestingly, serum-free cultured HCV showed a higher susceptibility to antibody inhibition of viral infection than HCV from serum-supplemented cultures. The multiplicity of infection (MOI) of partially purified HCV was calculated and an MOI of 0.02 was used for HCV infection of naïve Huh7 cells. Although infection by HCV produced under both culture conditions was inhibited by anti-CD81, anti-SR-BI and anti-E2 antibodies in a dose-dependent manner, the degree of antibody inhibition at each dose was stronger for serum-free cultured HCV than for HCV from serum-supplemented cultures (Fig. 5). The anti-CD81 and anti-SR-BI antibody targets the host cells, since CD81 and SR-BI are candidate cellular receptors for HCV. In contrast, the anti-E2 antibody AP33 is a neutralizing antibody for HCV [20,21], and targets virus particles. The antibody inhibition result obtained therefore suggested that HCV infection was affected by contaminating pro-





**Fig. 6.** Immunoprecipitation of cell-cultured HCV with anti-ApoB and ApoE antibodies.  $1 \times 10^7$  HCV-RNA copies of the J6/JFH-1 virus were incubated with mouse IgG, anti-ApoB or anti-ApoE antibodies, and were then precipitated using protein G-Sepharose. Virus RNA was extracted from the supernatant and the precipitated resin, and HCV-RNA was analyzed using RTD-PCR. All experiments were performed in triplicate, and data are shown as percentages of the mean (A: serum-supplemented, B: serum-free cultured, HCV). Infectivity of the antibody-depleted virus (supernatant) was determined by inoculation into naïve Huh7.5.1 cells. All experiments were performed in triplicate, and data are shown as means  $\pm$  SD. All data are indicated as percentage of infection, in which the infectivity (FFU/mL) of the supernatant from the mouse IgG (mlgG) precipitation was designated as 100% (C).

teins or by characteristics of HCV that arose due to the different culture conditions used for HCV production. Since CD81-negative Huh7 cells were also able to produce infectious HCV, and since anti-CD81 and anti-E2 antibodies similarly inhibited infection by HCV produced in serum-free culture (data not shown), it is highly unlikely that viral incorporation of CD81 that is produced by CD81-expressing virus-producing cells modulates anti-CD81-inhibition of viral infection. An alternative possibility was that the different antibody inhibition results may have been due to differences in the level, or type of apolipoprotein associated with HCV. HCV associates with lipoproteins in human sera [25], and it has been reported that the envelope proteins of HCV interact with lipoproteins [26]. Moreover, in a recent study, VLDL was reported to associate with HCV particles and affect viral infection and virus secretion [22,23]. Mature VLDL is composed of the apolipoproteins B, C-II and E, as well as lipid, and ApoB and ApoE have been reported to be important for the infectivity of HCV particles [22,23,27–29]. We found that the content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different (Fig. 6A). Thus, serum-free HCV particles had a greater amount of associated ApoB, and a lesser amount of ApoE, than HCV from serum-supplemented cultures. Since ApoE is usually only found in mature VLDL, the serum-free cultured HCV may associate with immature VLDL. A second difference related to associated apolipoprotein of HCV grown under different serum conditions is that the infectivity of HCV from serum-supplied culture, but not that of serum-free cultured HCV, was down-regulated by antibody depletion of ApoB and ApoE. This result suggested that the infec-

tion of HCV from serum-supplemented culture, but not infection of serum-free cultured HCV, depended on apolipoproteins. It is possible that production of HCV in serum-free culture results in the formation of HCV particles that differ in lipid and lipoprotein composition from particles produced under serum-supplemented conditions. Consequently, the affinity of serum-free cultured HCV for its cellular receptor might have been altered, and its susceptibility to antibody inhibition of infectivity may have been increased. However, further analysis of the lipid content of HCV grown under different serum conditions is necessary to confirm this point.

Production of HCV using a serum-free culture system has advantage for vaccine development because there is low protein contamination, it facilitates simple viral purification procedures, and it does not involve the use of animals. Furthermore, lipoproteins associated with virus particles may shield conserved epitopes. Purified virus particles produced under serum-free conditions may have these shielded epitopes exposed and thus potentially become stronger immunogens for the induction of neutralizing antibodies. In this study, serum-free cultured HCV was infectious *in vitro*, and the viral particles had a form similar to that of native enveloped viruses. However, we have not yet confirmed the antigenic or immunogenetic properties of serum-free cultured HCV, which would be important for its potential use as a vaccine. Nevertheless, we have recently designed a system for the purification of HCV particles (Morikawa, unpublished data) which will allow testing of these parameters. If these purified particles have high antigenicity, then a HCV vaccine that is derived from HCV produced in cell culture may be available in the future.

## 5. Conclusions

Infectious HCV could be efficiently produced in serum-free culture. However, the serum-free cultured HCV was highly susceptible to anti-CD81, anti-SR-BI and anti-E2 inhibition of infectivity. The content of ApoB and ApoE in HCV from serum-supplemented and serum-free cultures was different and the infectivity of serum-free cultured HCV appeared to be independent of apolipoproteins. Therefore, lipids conjugated with HCV may affect virus infection and neutralization.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.vaccine.2011.04.069.

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# In Vivo Adaptation of Hepatitis C Virus in Chimpanzees for Efficient Virus Production and Evasion of Apoptosis

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Hepatitis C virus (HCV) employs various strategies to establish persistent infection that can cause chronic liver disease. Our previous study showed that both the original patient serum from which the HCV JFH-1 strain was isolated and the cell culture-generated JFH-1 virus (JFH-1cc) established infection in chimpanzees, and that infected JFH-1 strains accumulated mutations after passage through chimpanzees. The aim of this study was to compare the *in vitro* characteristics of JFH-1 strains emerged in each chimpanzee at early and late stages of infection, as it could provide an insight into the phenomenon of viral persistence. We generated full-genome JFH-1 constructs with the mutations detected in patient serum-infected (JFH-1/S1 and S2) and JFH-1cc-infected (JFH-1/C) chimpanzees, and assessed their effect on replication, infectious virus production, and regulation of apoptosis in cell culture. The extracellular HCV core antigen secreted from JFH-1/S1-, S2-, and C-transfected HuH-7 cells was 2.5, 8.9, and 2.1 times higher than that from JFH-1 wild-type (JFH-1/wt) transfected cells, respectively. Single cycle virus production assay with a CD81-negative cell line revealed that the strain JFH-1/S2, isolated from the patient serum-infected chimpanzee at a later time point of infection, showed lower replication and higher capacity to assemble infectious virus particles. This strain also showed productive infection in human hepatocyte-transplanted mice. Furthermore, the cells harboring this strain displayed lower susceptibility to the apoptosis induced by tumor necrosis factor  $\alpha$  or Fas ligand compared with the cells replicating JFH-1/wt. **Conclusion:** The ability of lower replication, higher virus production, and less susceptibility to cytokine-induced apoptosis may be important for prolonged infection *in vivo*. Such control of viral functions by specific mutations may be a key strategy for establishing persistent infection. (HEPATOLOGY 2011;00:000–000)

Currently, approximately 200 million people are infected with hepatitis C virus (HCV) and are at continuous risk of developing chronic liver diseases such as chronic hepatitis, liver cirrhosis, and

hepatocellular carcinoma.<sup>1,2</sup> Although acute HCV infection elicits innate and adaptive immune responses, the virus successfully evades clearance in approximately 75% of infected individuals.<sup>3,4</sup> The mechanisms by

*Abbreviations:* Ag, antigen; CTL, cytotoxic T lymphocytes; FasL, Fas ligand; HCV, hepatitis C virus; JFH-1cc, cell culture-generated JFH-1 virus; JFH-1/wt, JFH-1 wild-type; MFI, mean fluorescence intensity; NK, natural killer, NS, nonstructural; PARP, poly(adenosine diphosphate ribose) polymerase; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; TUNEL, terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling.

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which HCV leads to persistent infection at a high frequency are not yet fully understood. Lack of appropriate animal models, except chimpanzees, has rendered such studies difficult. Human hepatocyte-transplanted mice,<sup>5,6</sup> a useful small animal model to study HCV infection, are unsuitable to study the mechanisms of virus persistence because of a lack of B and T cell-mediated immunity.

HCV is a noncytopathic positive-stranded RNA virus of the *Flaviviridae* family. It primarily infects hepatocytes of humans and chimpanzees, where, thanks to error-prone RNA-dependent RNA polymerase, the infected virus accumulates a high number of mutations rapidly, thus providing opportunity for selection of viruses that have the ability to escape the immune system and establish persistent infection. Deciphering the strategies employed by HCV to establish persistence can be helpful in the development of new strategies to eradicate the virus and to stop disease progression. Until recently, the lack of an HCV strain having the ability to establish infection *in vivo* and *in vitro* was a substantial hindrance in studying the molecular mechanisms of virus persistence. This problem was solved by the identification of an HCV strain, JFH-1, that was isolated from a fulminant hepatitis patient and found to be capable of replicating and assembling infectious virus particles in chimpanzees as well as in cell culture.<sup>7-10</sup> This clone can be used to study the molecular mechanisms by which HCV evades the host immune system and causes chronic infection.

In a previous report, we inoculated patient serum from which the JFH-1 strain was originally isolated and cell culture-generated JFH-1 virus (JFH-1cc) into two different chimpanzees.<sup>11</sup> HCV established infection in both animals within 3 days of inoculation. In the JFH-1cc-infected chimpanzee, genome sequence of predominant infecting virus at week 2 was identical to JFH-1 wild-type (JFH-1/wt [in this study, this abbreviation was used instead of JFH-1 to distinguish it from other variant strains]), and the infecting virus has four synonymous and seven nonsynonymous mutations at week 7. In the JFH-1 patient serum-infected chimpanzee, 19 synonymous and six nonsynonymous mutations were observed in predominantly circulating virus at week 2, and this number increased to 35 synonymous and 17 nonsynonymous mutations at the later stage of infection course (week 23).<sup>11</sup> From these observations, we presumed that the isolates evolved in each chimpanzee at later stages of infection might have some advantage over the viruses isolated at earlier time points for survival in infected animals. Thus, in this study, we generated JFH-1 variants con-

taining the mutations observed in these animals and assessed their effect on replication and infectious virus production in cell culture. Furthermore, we examined the effects of infection of these strains to tumor necrosis factor  $\alpha$  (TNF- $\alpha$ )- or Fas ligand (FasL)-mediated apoptosis.

## Materials and Methods

The complete Materials and Methods are provided in the Supporting Information.

## Results

**Effects of Mutations Identified in Chimpanzees.** To investigate the effect of mutations on virus phenotype, we generated constructs containing the mutations observed in JFH-1 patient serum-infected chimpanzee and JFH-1cc-infected chimpanzee at various time points. The JFH-1 variants JFH-1/S1 and JFH-1/S2 contain the mutations observed in the patient serum-infected chimpanzee at week 2 and week 23, respectively, and JFH-1/C contains the mutations observed in the JFH-1cc-infected chimpanzee at week 7 (Supporting Table 1). The replication and virus production capacity of these variants in HuH-7 cells was compared with that of JFH-1/wt. After electroporation of *in vitro*-synthesized full-genome RNA of JFH-1/wt and variant strains, extracellular and intracellular HCV RNA and core antigen (Ag) were measured (Fig. 1). At day 5 posttransfection, all constructs displayed similar intracellular HCV RNA levels. However, extracellular HCV RNA level of JFH-1/C was 1.6 times higher than that of JFH-1/wt. Likewise, extracellular HCV RNA level of JFH-1/S2 was 3.4 times higher than that of JFH-1/S1 (Fig. 1A). Intracellular HCV core Ag levels of JFH-1/S2 and C were  $240.9 \pm 58.2$  and  $189.8 \pm 42.1$  fmol/mg protein, respectively, and were significantly lower ( $P < 0.005$ ) than that of JFH-1/S1 ( $526.1 \pm 58.2$  fmol/mg protein) and JFH-1/wt ( $511.7 \pm 32.9$  fmol/mg protein) at day 1, but reached comparable levels at day 5 posttransfection. On the other hand, extracellular HCV core Ag level of JFH-1/C was 2.2 times higher than that of JFH-1/wt, and that of JFH-1/S2 was 3.6 times higher than that of JFH-1/S1 at day 5 posttransfection (Fig. 1B). Transfection efficiency of these strains, indicated by intracellular HCV core Ag levels at 4 hours posttransfection, was almost identical (data not shown).

**Single Cycle Virus Production Assay.** For detailed analysis of the effects of these mutations on different stages of the virus lifecycle, we used a Huh7-25 cell