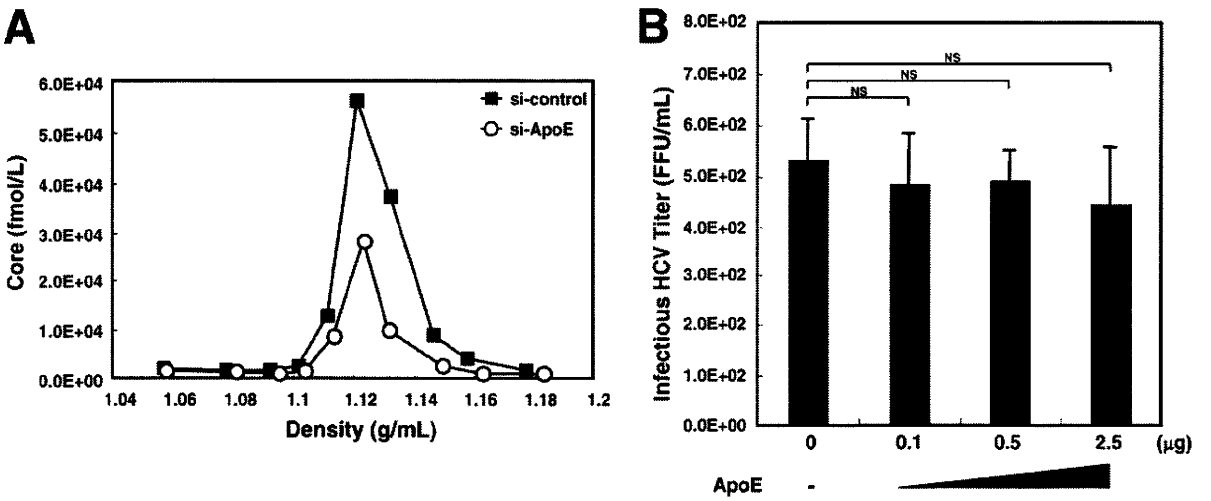

FIG. 1. Nature of HCV produced from ApoE knockdown HuH7.5 cells. (A) Buoyant density analysis. HuH7.5 cells were seeded onto 60-mm-diameter dishes. Cells were transfected with siRNA (si-control or si-ApoE). Four hours after transfection, cells were infected with HCVcc. Forty-eight hours after inoculation, the concentrated culture medium was fractionated using 14 to 54% iodixanol density gradient centrifugation at 36,000 rpm for 16 h at 4°C. The buoyant density profile is represented by the amount of core protein (in femtomoles per liter) in each fraction. Data from a representative of three experiments are shown. (B) Analysis of the infectivity of HCVcc produced from ApoE knockdown HuH7.5 cells after incubation with recombinant ApoE. HCVcc from cells in which ApoE expression was silenced was incubated alone or with different doses of human recombinant ApoE3 at 37°C for 2 h. Then the reaction mixtures were inoculated into naïve HuH7.5 cells. Forty-eight hours after infection, titers of infectious HCV were quantified by a focus-forming unit assay. The average values for three independent experiments are shown; error bars, standard deviations of the means. *P* values were determined by comparison (by Student's *t* test) with HCVcc that was not treated with recombinant ApoE3. NS, not significant ($P > 0.05$).

ApoE for infectivity (4, 6, 15, 27). However, it is uncertain when ApoE associates with HCV during the processes of morphogenesis and the secretion of infectious HCV particles. ApoE by itself can be released from ApoE-producing cells; thus, it is possible that HCV associates with ApoE after being secreted into the culture medium. However, this possibility is less likely, because the infectivity of HCV secreted from cells lacking ApoE expression was not rescued by incubation with different doses of recombinant ApoE (Fig. 1B).

To study the association of ApoE with HCV during the process of infectious virus production, we analyzed the production and infectivity of HCV produced by cells expressing mutant ApoE that is not secreted, due to the addition of Lys-Asp-Glu-Leu (KDEL), an endoplasmic reticulum (ER) retention signal, to its C terminus (Fig. 2A). sh-#3 cells were transfected with an ApoE3 or ApoE3-KDEL expression plasmid followed by HCV infection. Western blot analysis revealed that ApoE3 and ApoE3-KDEL were produced abundantly in transfected cells (Fig. 2B). As expected, ApoE3-KDEL accumulated inside cells, whereas ApoE3 localized both inside and outside cells (Fig. 2B). The expression of actin, NS5A, ApoB, and α1-antitrypsin in the transfected cells was unchanged (Fig. 2B). Additionally, the levels of extracellular and intracellular HCV core protein in the two types of transfected cells were not significantly different (Fig. 2C).

Next, we analyzed HCV infectivity in the extracellular and intracellular fractions of these cells. Infectious HCV was recovered from cells ectopically expressing ApoE3 (Fig. 2D, left). However, cells producing ApoE3-KDEL released very few infectious virus particles into the culture medium; instead, the infectious virus accumulated in the intracellular fraction (Fig. 2D, right). These results suggest that infectious virus particles constituted with ApoE are produced inside the cells and that the release of these infectious particles depends on the secretion of ApoE.

The ApoE isoform affects the infectivity of HCV. ApoE is a multifunctional protein that plays central roles in lipid metabolism and neurobiology. It has three major isoforms (ApoE2, ApoE3, and ApoE4) that have different effects on lipid and neuronal homeostasis. These isoforms differ by amino acid substitutions at one or two sites (residues 130 and 176). ApoE3 is the most common isoform, and there have been no reports of diseases associated with ApoE3. On the other hand, ApoE2 is the major risk factor for type III hyperlipoproteinemia, and ApoE4 is the major risk factor for Alzheimer's disease (20). ApoE2 has lower affinity for the LDLR than ApoE3 and ApoE4. Since lipoprotein receptors, including the LDLR and scavenger receptor class B, member I (SR-BI), are suspected of acting as receptors for HCV infection (3, 24, 27, 34), we hypothesized that the ApoE isoform may affect HCV infectivity. To clarify this hypothesis, we analyzed the production of infectious HCV from cells expressing different isoforms of ApoE.

Plasmids expressing ApoE isoforms were transfected into HuH7.5 cells in which endogenous ApoE3 had been knocked down (sh-#3). Although the level of ectopically expressed ApoE was higher than that of endogenous ApoE, the levels of ectopically expressed ApoE and the levels of ApoE secreted into the culture medium were not different for cell groups expressing different ApoE isoforms (Fig. 3A). The replication of the HCV genome, as determined by the amounts of NS5A and core, was unaffected by the expression of different isoforms of ApoE (Fig. 3A). The amount of extracellular core in ApoE isoform-expressing cells was one-half of that in control

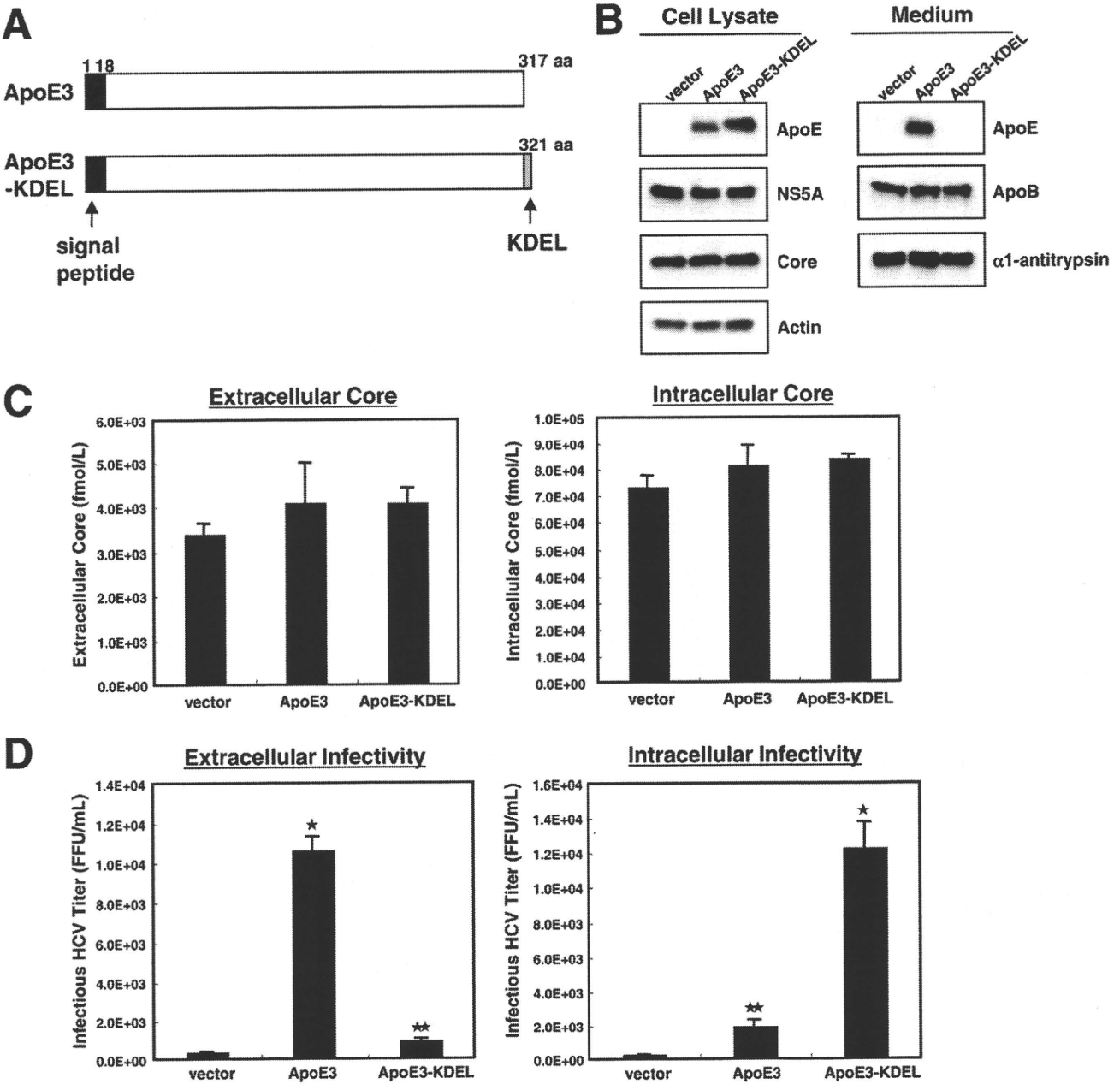

FIG. 2. The release of infectious HCV into the culture medium depends on the secretion of ApoE. (A) Schematic representation of ApoE3 and the ApoE3-KDEL mutant. (B) Verification of the expression of ectopic ApoE3 and ApoE3-KDEL. The sh-#3 cells were seeded onto 100-mm-diameter dishes. Cells were transfected with either pCAG (vector), pCAG-ApoE3 (ApoE3), or pCAG-ApoE3-KDEL (ApoE3-KDEL). Four hours after transfection, cells were inoculated with JFH1. Forty-eight hours after inoculation, cell lysates and supernatants were analyzed for the production of ApoE and its mutant by Western blotting with anti-ApoE antibodies. The expression of actin, α1-antitrypsin, ApoB, core, and NS5A was also examined. (C) The amounts of core in the culture supernatant and intracellular fractions were determined by a core-specific ELISA. The cells and transfections were the same as for panel B. (D) Analysis of HCV infectivity. The culture medium or cell lysate was inoculated into naïve HuH7.5 cells. The titers of infectious HCV were quantified by focus-forming unit assays. The average values for three independent experiments are shown; error bars, standard deviations of the means. The cells and transfections were the same as for panel B. *P* values were determined by comparison (by Student's *t* test) with cells expressing the control vector. *, $P < 0.0005$; **, $P < 0.005$.

cells and was not significantly different among cell groups expressing different ApoE isoforms (Fig. 3B, vector versus ApoE2 to ApoE4). We also noticed that the amounts of HCV RNA in extracellular fractions from cells expressing different ApoE isoforms were not significantly different (data not shown). To examine the production of infectious virus, the culture media of these cells were inoculated into naïve HuH7.5 cells, and infectivity was assayed 48 h after infection. Interestingly, ApoE2-expressing cells released substantially less infectious HCV into the culture medium than did ApoE3- or

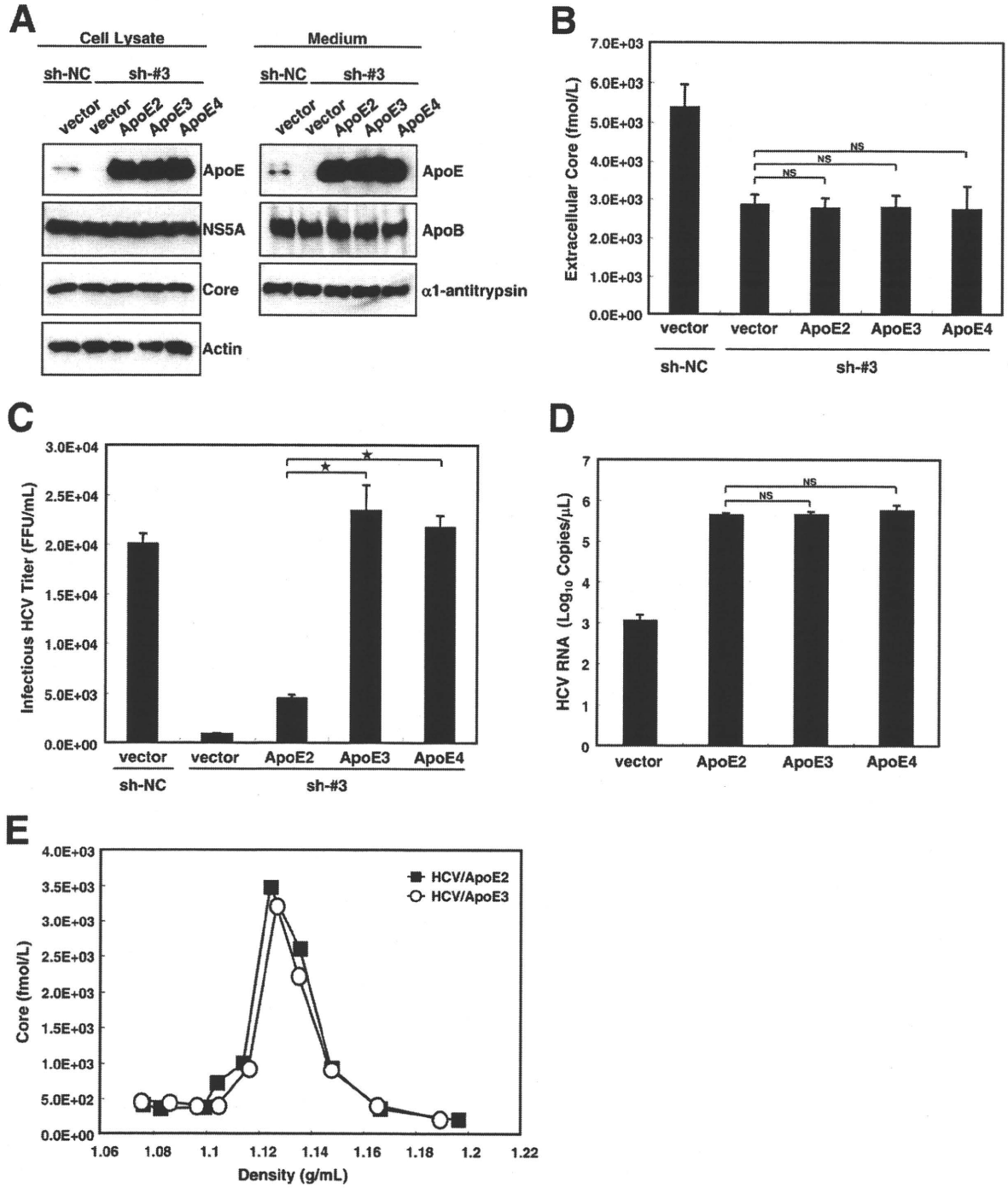

FIG. 3. ApoE isoforms affect HCV infectivity. (A) Verification of expression of ectopically introduced ApoE and the effect of ApoE isoforms on HCV genome replication. sh-NC cells and sh-#3 cells were seeded onto 60-mm-diameter dishes. Cells were transfected with either pCAG (vector), pCAG-ApoE2 (ApoE2), pCAG-ApoE3 (ApoE3), or pCAG-ApoE4 (ApoE4). Four hours after transfection, cells were inoculated with JFH1. Forty-eight hours after inoculation, the expression of ApoE, ApoB, NS5A, core, actin, and α1-antitrypsin in cell lysates and/or supernatants was analyzed by Western blotting using relevant antibodies. (B) The release of HCV core into the culture supernatant by cells expressing different ApoE isoforms was measured by a core-specific ELISA. The cells and transfections in panels B to D were the same as those in panel A. *P* values were determined by comparison (by Student's *t* test) with HCVcc from sh-#3 cells expressing the control vector. NS, not significant ($P > 0.05$). (C) Amount of infectious HCV that egressed from HCV-infected cells. Culture media of the indicated cells were inoculated into naïve HuH7.5 cells. Forty-eight hours after infection, naïve HuH7.5 cells were infected with the supernatant. Forty-eight hours after infection, the titers of infectious HCV were quantified by a focus-forming unit assay. *P* values were determined by comparison (by Student's *t* test) with HCVcc from sh-#3 cells expressing ApoE2. ⋆, $P < 0.0005$. (D) Association of HCV with ApoE isoforms. HCV released into the culture medium from cells bearing each ApoE isoform was incubated with an anti-HA antibody. RNA was extracted from the immunoprecipitant and subjected to quantification by quantitative RT-PCR. *P* values were determined by comparison (by Student's *t* test) with HCVcc from sh-#3 cells expressing ApoE2. NS, not significant ($P > 0.05$). (E) Density gradient analysis of HCVcc containing ApoE2 or ApoE3. Concentrated HCVcc from cells expressing ApoE2 or ApoE3 was fractionated using 14 to 54% iodixanol density gradient centrifugation at 36,000 rpm for 16 h at 4°C. The buoyant density profile is represented by measuring the amount of core protein (in femtomoles per liter). HCV/ApoE3, HCV bearing ApoE3; HCV/ApoE2, HCV bearing ApoE2. Data from a representative of three experiments are shown.

ApoE4-expressing cells (Fig. 3C). We examined the possibility that the affinity of ApoE for HCV differs by isoform. HA-tagged ApoE isoform expression plasmids were transfected into sh-#3 cells, which were then infected with HCV. The culture media from the infected cells were incubated with an anti-HA antibody, and the immunocomplexes were recovered. RNA was extracted from the complexes and analyzed by quantitative RT-PCR. The amounts of HCV RNA in the immune complexes obtained from the culture media of cells expressing different ApoE isoforms were almost the same (Fig. 3D). Furthermore, we examined whether a difference between the densities of ApoE2-containing HCV and ApoE3-containing HCV can be correlated with the difference in infectivity (Fig. 3E). There is no significant density shift between these two viruses to explain the difference in infectivity, suggesting the importance of factors other than particle density for HCV infectivity. These results suggest that the ability of HCV-associated ApoE isoforms to bind to the LDLR seems to be responsible for isoform-based differences in the infectivity of HCV.

HCV requires the LDLR and SR-BI expression for full infectivity. Previously, the LDLR and SR-BI were shown to be involved in HCV infection, possibly through HCV-associating ApoE that functions as a ligand to those molecules. However, there is controversy as to which receptor, together with other receptors, such as CLDN1 and CD81, is involved in more importantly in HCV entry (3, 16, 24, 27, 34). To investigate this point, we conducted an infectivity analysis using ApoE2- and ApoE3-complemented HCV with HuH7.5 cells that were depleted of the LDLR and/or SR-BI by treatment with specific siRNAs. Western blot analysis showed that the levels of the LDLR and SR-BI were substantially reduced by treatment with their specific siRNAs (Fig. 4A). These cells were infected with HCV produced by HuH7.5 cells expressing ectopic ApoE3 (HCV bearing ApoE3 [HCV/ApoE3]) or ApoE2 (HCV bearing ApoE2 [HCV/ApoE2]), and infectivity was analyzed 48 h after infection (Fig. 4B). We observed significant reductions in HCV/ApoE3 infection of cells in which the LDLR or SR-BI was silenced, as expected on the basis of reports from other groups (3, 24, 27, 34). Assuming that LDLR and SR-B1 function independently in the step of HCV entry, a further reduction in infectivity would be expected for cells in which both the LDLR and SR-BI are silenced. However, this was not the case. The reduction in infectivity was almost the same as those for cells in which either the LDLR or SR-BI was silenced (Fig. 4B). The levels of HCV/ApoE2 entry into cells with both or either LDLR or SR-BI knocked down were also nearly the same (Fig. 4B).

Next, we conducted infectivity assays of HCV/ApoE2 and HCV/ApoE3 after treating them with antibodies against various candidate molecules for the HCV receptor (Fig. 4C). Anti-CLDN1 and anti-CD81 strongly inhibited HCV infection, as shown in Fig. 4C. Anti-LDLR and anti-SR-BI antibodies showed only moderate inhibition of HCV/ApoE3 infection (Fig. 4C, compare with inhibition by anti-CLDN1 and anti CD81 antibodies). Taken together, our results suggest the importance of both the LDLR and SR-BI for HCV infection, possibly through the function of HCV-associating ApoE.

DISCUSSION

Accumulating evidence suggests that HCV is complexed with lipoproteins and that it exhibits both viral and lipoprotein characteristics, leading to the recognition of HCV as an LVP. However, it is not known how the nature of lipoproteins associated with HCV is involved in the cycle of virus proliferation. HCV was found to be secreted in a manner that parallels the formation of VLDL by experiments that used an MTP inhibitor or ApoB knockdown (10, 14). In these studies, the suppression of ApoB significantly impaired the production of both VLDL and HCV. In contrast, another study reported a lesser contribution of ApoB to HCV production (15). On the other hand, ApoE knockdown severely interfered with the production of infectious virus (see below).

We showed here that ApoE is required for HCVcc infectivity for HuH7.5 cells, which is consistent with reports showing that the inhibition of ApoE production leads to reduced HCVcc infectivity (4, 6, 15, 27). Depletion of ApoE resulted in a significant reduction in the infectivity not only of JFH1 but also of TNS2J1, the chimeric HCVcc composed of the structural region of HCV-1b and a nonstructural region derived from JFH1, although the replication efficiencies of the genomes were unchanged (see Fig. S1B in the supplemental material). Thus, the requirement of ApoE for infectious HCV production may be unrelated to the HCV genotype.

ApoE seems to have an additional role in regulating virus assembly/release besides its role in virus entry. Chang et al. report a severe reduction in HCV particle assembly/release following ApoE knockdown (6). We analyzed the amount of HCV by measuring the level of core as well as virus RNA in the culture medium from ApoE-silenced HuH7.5 cells. Under this condition, the production and secretion of ApoE were severely suppressed (see Fig. S1A in the supplemental material). However, we observed only a 50% reduction of both core and HCV RNA levels in the culture medium (see Fig. S1B for core; data for HCV RNA not shown), in strong contrast to the data of Chang et al. Since ApoE knockdown does not affect replicon activity, we established several HuH7.5 clones that stably silenced the production of ApoE. Using some of those clones, sh-#3 and sh-#12, we observed the same result: only a ~50% reduction of HCV particle release upon HCV infection (see Fig. S1E). At present we cannot explain the difference between our results and those of Chang et al. However, it could be due to a difference in RNA transfection reagents that may affect cell variability or in HuH7.5 cells that might have been genetically modified during a prolonged period of cultivation after distribution from the original supplier.

ApoE associates with NS5A (4, 9, 15). Since NS5A is suggested to be involved in virus particle assembly (2, 21, 31), it is possible that ApoE participates, at least in part, in virus particle assembly by interacting with NS5A, as suggested by others (4, 15). However, the facts that the release of virus particles into the culture medium from cells in which ApoE is silenced is not completely suppressed and that HCV retained inside cells expressing the ApoE3-KDEL mutant and not released into culture medium indicate that ApoE is not an essential factor for assembly and release.

As for the effect of ApoE on HCV infectivity, we observed a dramatic reduction following ApoE knockdown (see Fig. S1B

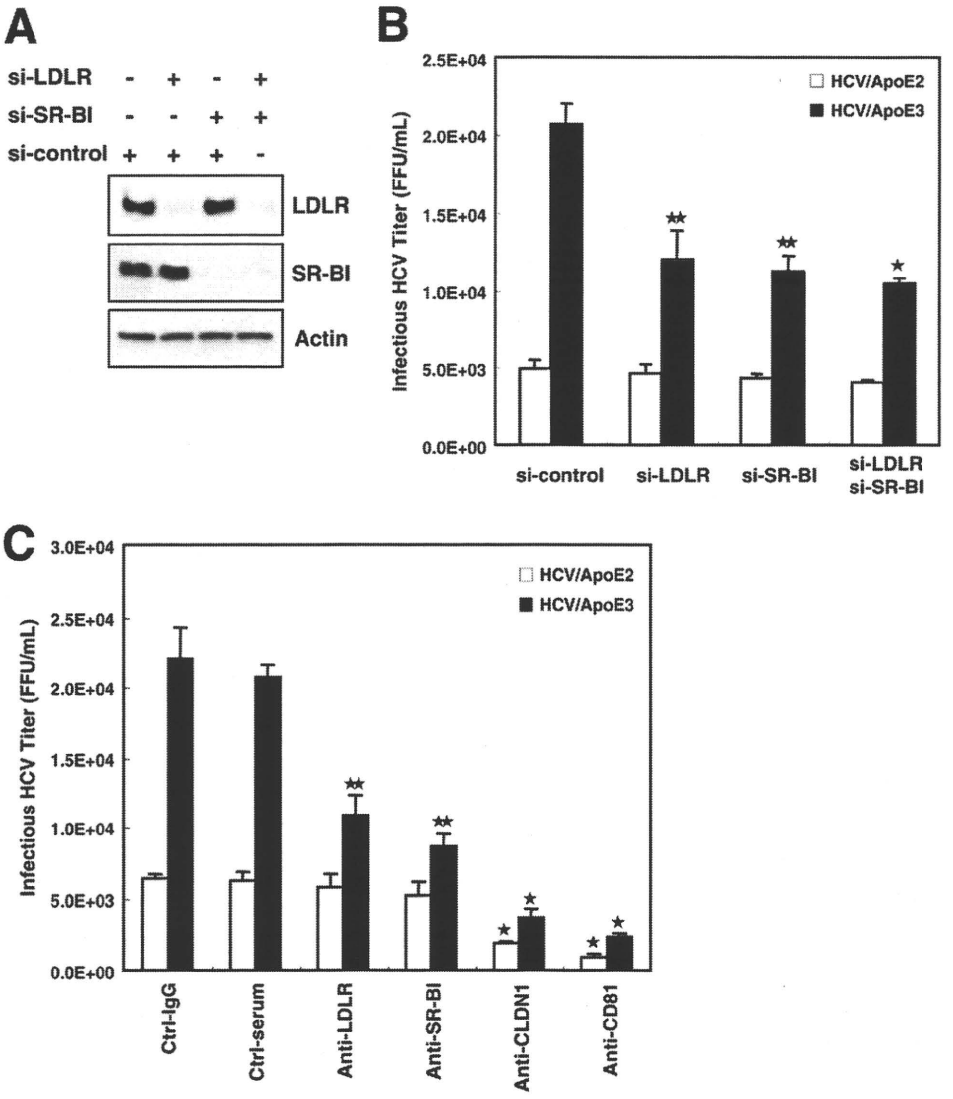

FIG. 4. Suppression of LDLR and SR-BI results in reduced HCV infection. (A) Knockdown of the LDLR and SR-BI by siRNA. HuH7.5 cells were seeded onto 24-well plates and transfected with siRNA (si-control, si-LDLR, and/or si-SR-BI). Twenty-four hours after transfection, cell lysates were analyzed for the expression of the LDLR, SR-BI, and actin by Western blotting. (B) Analysis of HCV infectivity for HuH7.5 cells in which either the LDLR, SR-BI, or both were silenced. Cells were transfected with siRNA (si-control, si-LDLR, and/or si-SR-BI). Twenty-four hours after transfection, HCVcc produced from cells bearing ApoE2 (open bars) or ApoE3 (filled bars) was serially diluted and inoculated. Forty-eight hours after infection, infected cells were counted by fluorescence microscopy after staining with an anti-NS5A antibody. HCV/ApoE3, HCV bearing ApoE3; HCV/ApoE2, HCV bearing ApoE2. *P* values were determined by comparison (by Student's *t* test) with si-control-treated cells. *, $P < 0.0005$; **, $P < 0.005$. (C) HuH7.5 cells were preincubated with control goat IgG (Ctrl-IgG), control rat preimmune serum (Ctrl-serum), or a goat anti-LDLR, rat anti-SR-BI, rat anti-claudin 1 (anti-CLDN1), or mouse anti-CD81 antibody for 1 h at 37°C before infection with serially diluted HCVcc from HuH7.5 cells expressing ApoE2 (open bars) or ApoE3 (filled bars). Forty-eight hours after infection, infected cells were stained with anti-NS5A antibodies and counted by fluorescence microscopy. The average values for three independent experiments are shown; error bars, standard deviations of the means. *P* values were determined by comparison (by Student's *t* test) against cells treated with preimmune serum. *, $P < 0.0005$; **, $P < 0.005$.

and S1E in the supplemental material). Thus, we think that ApoE affects HCV infectivity severely but affects virus assembly and/or release only slightly. We tried to find a difference between HCV derived from HuH7.5 cells and HCV from ApoE-depleted HuH7.5 cells by density gradient centrifugation, because a correlation between low HCV particle density and infectivity has been known. However, we could not see any difference in buoyant density to explain the difference in infectivity (Fig. 1A). We think that association with ApoE does not drastically change the physical nature of HCV. It will be important, however, to look for differences in the physical and biochemical nature of HCV in detail, including lipid contents, the status of glycoproteins such as E1 and E2, and so on, in the future.

An association between ApoE and secreted HCV was observed (see Fig. S3A in the supplemental material) (6, 15, 27). To obtain insight into the function of ApoE in the virus life cycle, and particularly in the step of infectious virus secretion,

we analyzed the infectious virus released into the culture medium from cells expressing the ApoE mutant ApoE3-KDEL, which is not secreted, because it contains the ER retention signal peptide KDEL. We confirmed that ApoE3-KDEL was not secreted into the culture medium (Fig. 2B). Indirect immunofluorescence showed that the majority of ApoE3-KDEL was retained on the ER, whereas ApoE3 was localized on the ER and the Golgi apparatus (data not shown). The amount of virus released into the culture medium from cells expressing ApoE3-KDEL was almost the same as that from cells expressing ApoE3 (Fig. 2C). However, the production of infectious virus in the culture medium was severely suppressed, since the infectious HCV accumulated in the cell lysate (Fig. 2D). The accelerated level of intracellular accumulation of infectious virus, which was higher than that in cells expressing ApoE3, suggests that the secretion of "infectious" virus depends on ApoE production and secretion. The amounts of HCV released into the culture medium from cells expressing ApoE3-KDEL and ApoE3 were almost the same as that released by cells lacking endogenous ApoE expression (Fig. 2C and D), indicating that ApoE affects the assembly and release of noninfectious virus into the culture medium only slightly but mainly affects the release of infectious virus. Our result also suggests that the association of ApoE and HCV occurs prior to the secretion of the virus from cells, which is required for the virus to gain infectivity. Incubation of recombinant ApoE with HCV produced from ApoE knockdown cells did not show a significant increase in HCV infectivity (Fig. 1B). Moreover, no interaction of ApoE with HCV *in vitro* was observed (data not shown); this observation confirms the establishment of the association before virus secretion.

The physical structure of HCV as an LVP is uncertain. It is not known if the association of ApoE with infectious virus depends on a coassociation with or integration of lipoprotein. We observed the importance of a substrate of lipoprotein lipase (LPL), which associates with HCV, for infectivity (29). LPL hydrolyzes triglycerides in VLDL and converts them to intermediate-density lipoproteins (IDL). When HCV produced from HuH7.5 cells was treated with LPL followed by hepatic lipase, the density of the virus was shifted higher than the density prior to treatment, and infectivity was simultaneously lost. Importantly, the amount of ApoE associated with HCV was reduced (29). This observation suggests that the interaction of ApoE with HCV depends on the presence of a virus-associated triglycerol ester, most likely a lipid component of lipoprotein. This observation also suggests the importance of an association of lipoprotein with HCV in order to maintain the function of ApoE for HCV infection. However, more study is needed to clarify how ApoE interacts with HCV to increase infectivity.

The ApoE gene is polymorphic, with three common alleles, *apoE2*, *apoE3*, and *apoE4*, which produce 3 isoforms of ApoE. Because the ApoE isoforms have different affinities for the LDLR (ApoE2 has low affinity, while ApoE3 and ApoE4 have high affinity), we analyzed the effect of ApoE isoforms on HCV infectivity. HCV/ApoE3 and HCV/ApoE4 showed almost the same infectivity as the control virus produced from HCV-infected HuH7.5 cells expressing endogenous ApoE3 (Fig. 3C). However, the infectivity of HCV/ApoE2 was about one-fifth that of HCV/ApoE3, even though the level of virus particles was almost the same as that of HCV/ApoE3 (Fig. 3B and C). Assuming that the LDLR plays a role as a receptor of HCV (24, 27, 33), this observation is in agreement with the fact that the binding affinity of ApoE for the LDLR is well correlated with the difference in HCV infectivity by ApoE isoforms.

HCV/ApoE2 showed reduced infectivity for LDLR-silenced HuH7.5 cells (Fig. 4B). Since the level of infectivity was almost the same as that for control cells, entry through SR-BI on HuH7.5 cells was not utilized by HCV/ApoE2. This is also suggested by analysis of the infectivity of HCV/ApoE2 for doubly silenced HuH7.5 cells (Fig. 4B). Because the possibility remains that ApoE2 interacts weakly with both the LDLR and SR-BI, although the binding regions for the LDLR and SR-BI on the ApoE molecule do not overlap (17), we analyzed the infectivity of HCV/ApoE3 for cells in which either the LDLR, SR-BI, or both were silenced (Fig. 4B). As expected on the basis of reports from other groups, suppression of infectivity for LDLR- or SR-BI-silenced HuH7.5 cells was observed. However, to our surprise, infectivity was not further reduced for doubly silenced cells (Fig. 4B). This result suggests that both the LDLR and SR-BI are required for virus entry. The absence of either of these proteins would result in a reduction in infectivity. It is important to determine whether these receptor molecules function independently or cooperatively for HCV entry.

When infectivity was analyzed by treating HCV with an anti-SR-BI or anti-LDLR antibody, the infectivity of HCV remained at a level higher than that of HCV treated with an anti-CLDN1 or anti-CD81 antibody (Fig. 4C). Further, a significant level of HCV/ApoE3 infectivity for LDLR- and SR-BI-silenced HuH7.5 cells was observed (Fig. 4B). These data suggest the presence of another receptor molecule(s) that is relevant to the LDLR and SR-BI regarding the ability to interact with ApoE.

The functional importance of ApoE for HCV infectivity is not limited to the HCVcc used in the present study. An epidemiological study of Caucasians with persistent chronic hepatitis indicates a notable absence of the ApoE2/ApoE2 genotype in HCV antibody-positive individuals (28), which is in agreement with the lower infectivity of ApoE2-bearing HCVcc. The authors did not describe any role for ApoE2 on HCV infectivity. However, our results strongly suggest that HCV produced from ApoE2-bearing individuals is eliminated quickly because it is less infectious. It will be important to conduct a virological study of HCV obtained from individuals carrying different isoforms of ApoE in the future.

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Lipoprotein lipase and hepatic triglyceride lipase reduce the infectivity of hepatitis C virus (HCV) through their catalytic activities on HCV-associated lipoproteins

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ABSTRACT

The effect of lipolysis by lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) on hepatitis C virus (HCV) infection was evaluated. First, medium from HuH7.5 cells bearing HCV genome replication was treated with LPL. LPL treatment led to reduced HCV infectivity, shifted HCV to higher densities, and lowered the amount of apolipoprotein E-associated HCV. The effect of endogenous HTGL secreted from HuH7.5 on HCV infectivity was next examined. Neutralization of HTGL by an anti-HTGL antibody resulted in suppression of LPL-induced reduction in infectivity of HCV-bearing medium, while knockdown of HTGL by siRNA led to increased HCV infectivity irrespective of LPL. HCV in medium from HTGL knockdown cells was found in fractions with a lower density. These results indicate that changes in the nature of HCV-associated lipoproteins by LPL and/or HTGL affect HCV infectivity, suggesting that association of HCV with specific lipoproteins is important for HCV infectivity.

Introduction

It is estimated that about 3% of the world's population are infected with hepatitis C virus (HCV). The virus often establishes a chronic infection with a high risk of developing cirrhosis and hepatocellular carcinoma. Because an overall 50% of patients do not experience significant benefits from the current pegylated interferon and ribavirin-based combination therapy, establishing an accurate and detailed understanding of the mechanisms of the HCV life cycle is an important step in resolving this world health problem.

HCV is a positive strand RNA virus, belonging to the *Flaviviridae* family. It has a single open reading frame encoding virus proteins flanked by 5′- and 3′-untranslated regions. Virus proteins are translated as a precursor polyprotein and processed into structural (Core, E1 and E2) and non-structural (NS) proteins by cellular and viral proteases. The viral genome is replicated in the virus replication complex composed of the NS proteins.

Very-low-density lipoprotein (VLDL), which transports endogenously synthesized triglyceride (TG)/cholesterol (CH) from liver to peripheral tissues, is synthesized in hepatocytes by the two-step processes (Olofsson and Borèn, 2005); first, a small quantity of TG becomes associated with apolipoprotein B (ApoB), resulting in ApoB-containing VLDL precursor, catalyzed by microsomal triglyceride transfer protein (MTP). Second, the precursor fuses with a large droplet of TG/CH associating other apolipoproteins, such as apolipoprotein E (ApoE) to form mature VLDL. In human sera, some HCV associates with VLDL-like or low-density lipoprotein (LDL)-like lipoproteins (Thomssen et al., 1992, 1993). Because of these associations, HCV is called a lipo-viro-particle (LVP) (André et al., 2002). In cultured cells, HCV assembly and release is dependent on the production of ApoB-containing VLDL (Huang et al., 2007; Gastaminza et al., 2008; Icard et al., 2009), while the presence of ApoE is more crucial for production of infectious HCV (Chang et al., 2007; Jiang and Luo, 2009). These findings strongly suggest importance of the association of HCV with lipoproteins in HCV infectivity, although the precise mechanism by which these lipoproteins endow HCV with infectivity remains elusive.

Lipoprotein lipase (LPL) and hepatic triglyceride lipase (HTGL) are secreted proteins functioning as key enzymes in lipid metabolism, both of which hydrolyze TG in lipoproteins. LPL mainly converts VLDL to intermediate-density lipoprotein (IDL) (Braun and Severson, 1992; Mead et al., 2002), whereas HTGL is predominant in the conversion of IDL to LDL (Connelly, 1999; S.-Fojo et al., 2004). In addition, both lipases can mediate cellular binding and uptake of lipoproteins.

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Until now the effect of LPL on HCV was reported by two groups (Thomssen and Bonk, 2002; Andréo et al., 2007). Thomssen and Bonk (2002) treated HCV-positive human sera with LPL derived from *Pseudomonas* spp. (LPL-Ps) and measured HCV RNA titer afterward. They observed destruction of HCV RNA by this treatment, which was blocked by the presence of RNase inhibitors. Because this destruction was dependent on catalytic activity of LPL-Ps, the authors suggested that direct disruption of HCV by this enzyme activity, most likely lysis of virus membranous components, made viral RNA sensitive for RNase present in the reaction mixture. On the other hand, Andréo et al. (2007) reported the effect of bovine LPL on HCV infectivity. They observed that bovine LPL reduced HCV infectivity through its bridging effect between HCV-associated lipoprotein and cells. They described that HCV infectivity was suppressed after association with cellular components followed by subsequent cell entry of HCV, suggesting that LPL-mediated inactivation of HCV required cell interaction.

The former study (Thomssen and Bonk, 2002) focused on the physical change of HCV structure by LPL from *Pseudomonas* spp. that is phylogenetically different from mammals and, thus, it is not clear if the LPL-Ps is physiologically relevant to mammalian LPL. In the latter (Andréo et al., 2007), bovine LPL was shown to reduce HCV infectivity through its bridging effect between HCV-associated lipoprotein and cells. However, the mechanism to reduce HCV infectivity through the interaction with cells in the presence of bovine LPL remains elusive. Thus, so far, the effects of lipolysis of HCV-associated lipoprotein by LPL on HCV infectivity are unclear. Taking account of the studies demonstrating that HCV is associated with lipoproteins, lypolysis activity of LPL and/or HTGL may have direct effects on the property and the infectivity of HCV. Here, we examined the effects of bovine LPL and endogenous human HTGL on the physiological characteristics of HCV-associated lipoproteins and further evaluated the role of lipoproteins on HCV infectivity. We found that LPL and HTGL directly altered the physiological characteristics as well as the infectivity of HCV through their catalytic activities.

Results

HCV infectivity was reduced by bovine LPL treatment

In order to evaluate the association of HCV with lipoproteins and its role in HCV infectivity, we analyzed HCV for sensitivity to LPL. Culture medium of HuH7.5 cells bearing HCV JFH1 genome replication was used as the virus source for this study. The HCV-bearing medium was incubated with bovine LPL (Sigma) and the infectivity was evaluated by adding the LPL-treated medium to naïve HuH7.5 cells. The percentage of HCV-positive cells was dramatically reduced in an LPL dose-dependent manner when HuH7.5 cells were exposed to LPL-treated HCV-bearing medium (Fig. 1a), while treatment with heated LPL, which lost enzymatic activity (Fig. 1b), did not show significant reduction in infectivity (Fig. 1a). Anti-LPL antibody inhibited the reduction in HCV infectivity (Suppl. Fig. 1), indicating that this reduction mainly resulted from LPL itself.

Since enzymatic activity of LPL is shown to inhibit the establishment of HCV infection in cultured cells after virus entry (Andréo et al., 2007), it is possible that our observation results from the same effect of LPL through interaction with cells after virus entry as previously reported. To address the question whether LPL acts on the HCV infectivity through enzymatic effect on the HCV-bearing medium or not, LPL activity was confined to the HCV-bearing medium by using orlistat (Sigma), which inhibits enzymatic activity of LPL (Fig. 1c). When the HCV-bearing medium was treated with orlistat-inactivated LPL, a reduction in infectivity was not observed (Fig. 1d). After treatment of HCV-bearing medium with LPL, we added orlistat to the medium to suppress LPL activity and subsequently inoculated the medium containing inactivated LPL into HuH7.5 cells. Under this condition, LPL-induced reduction in HCV infectivity through interaction with cells, if any, should be suppressed. Therefore, we are able to evaluate the effect of LPL activity on the HCV-bearing medium itself. Significant reduction of HCV infectivity was observed even suppressing LPL activity through interaction with cells (Fig. 1e). These results indicated that LPL could reduce infectivity of HCV which was independent of cellular interaction.

LPL, at the concentration used in this study to reduce HCV infectivity, did not alter the infectivity of Sendai virus (SeV) (Fig. 1f). Therefore, the effects of LPL were likely limited to certain viruses such as HCV due to the nature of LVP. The LPL-induced reduction in HCV infectivity likely results from the lipolytic alteration of a lipoprotein-like structure associating or integrating with HCV. From these results, it is strongly suggested that LPL reduce HCV infectivity through its lipolytic effect on lipoproteins associated with HCV.

Experiments using an MTP inhibitor indicate dependence of HCV release from culture cells on the production of lipoprotein such as VLDL (Huang et al., 2007; Gastaminza et al., 2008). We demonstrated that hydrolysis of HCV-associated lipoprotein by LPL led to a reduction of HCV infectivity. These results imply that HCV could be released from cells as a complex with lipoprotein and that their association could be required for not only their release but also HCV infectivity.

LPL treatment shifted HCV to higher densities

Since the size and buoyant density of VLDL is altered by lipolysis, we speculated that LPL treatment might shift HCV to a higher buoyant density. To examine this possibility, the HCV-bearing medium treated with LPL was ultracentrifuged through iodixanol gradients. Thirty fractions were collected. Core, HCV RNA, and infectivity in each fraction were quantified (Figs. 2a to c). The Core peak shifted from 1.118 toward 1.128 g/ml due to LPL treatment with a dose-dependent manner (Fig. 2a). Fractions with densities from 1.107 to 1.115 g/ml of the undigested HCV sample showed significantly higher infectivity than all other fractions of higher density from the same sample (Fig. 2b). All the LPL-treated fractions showed no infectivity or infectivity lower than that of the untreated fractions (Fig. 2b).

To exclude the possibility that bovine LPL disrupted HCV RNA structure, we quantified HCV RNA in each fraction from untreated and LPL-treated (500 μg/ml) HCV samples. The amount of Core and HCV RNA from all the fractions was not changed by LPL treatment (Figs. 2a and c), indicating that the amount of HCV nucleocapsid estimated by the amount of Core and HCV RNA was not affected by treatment with LPL used in this study. The peak of HCV RNA was observed at a density of the 1.115 g/ml in the untreated control sample, whereas the peak shifted to a density of 1.128 g/ml in the LPL treatment (Fig. 2c). This peak shift in HCV RNA distribution coincided to the shift in peak of Core. Therefore, we suggest that LPL hydrolyzes lipid components from HCV-associated lipoproteins, which results in shifting HCV to higher buoyant densities without substantial changes to the HCV nucleocapsid.

LPL treatment reduced association of HCV with ApoE

To further clarify the effect of LPL on the structure of HCV-associated lipoprotein, we analyzed the association of HCV with ApoB and ApoE. Each fraction of the density gradient centrifugation of LPL-untreated and -treated HCV was subjected to immunoprecipitation using polyclonal antibodies specific for ApoB or ApoE. Subsequently, the immunocomplexes were subjected to RNA extraction followed by quantitative real-time RT-PCR (qRT-PCR). Complexes containing HCV RNAs co-immunoprecipitated with anti-ApoB and anti-ApoE antibodies from LPL-untreated HCV distributed from densities of 1.105 to 1.13, having a peak at 1.115 (Figs. 2d and e), while those from LPL-treated HCV were found in fraction with higher densities (Figs. 2d and e).

LPL affected the association between HCV and ApoB-positive lipoprotein to some extent (Fig. 2d), while it is noticed that the amount

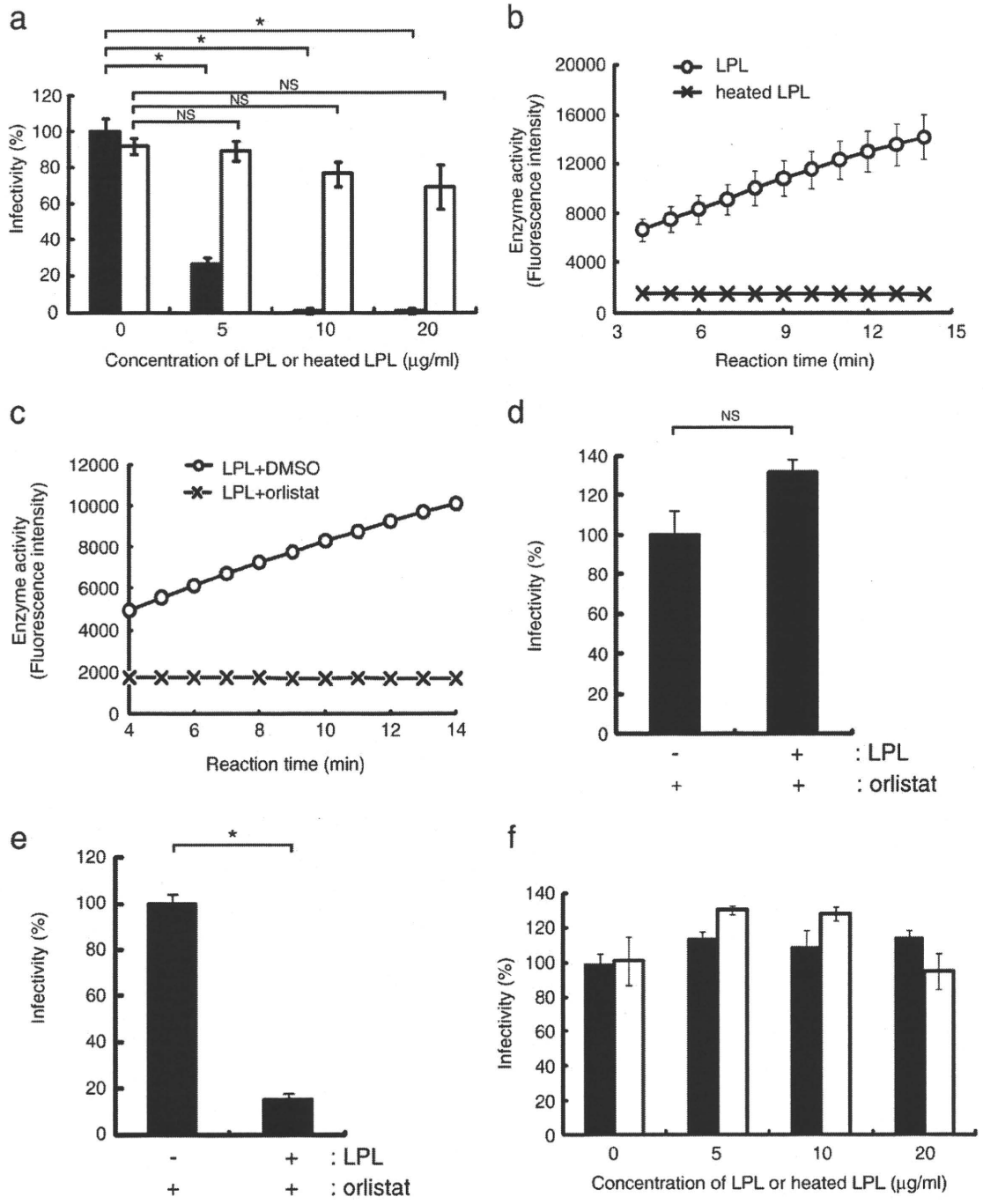
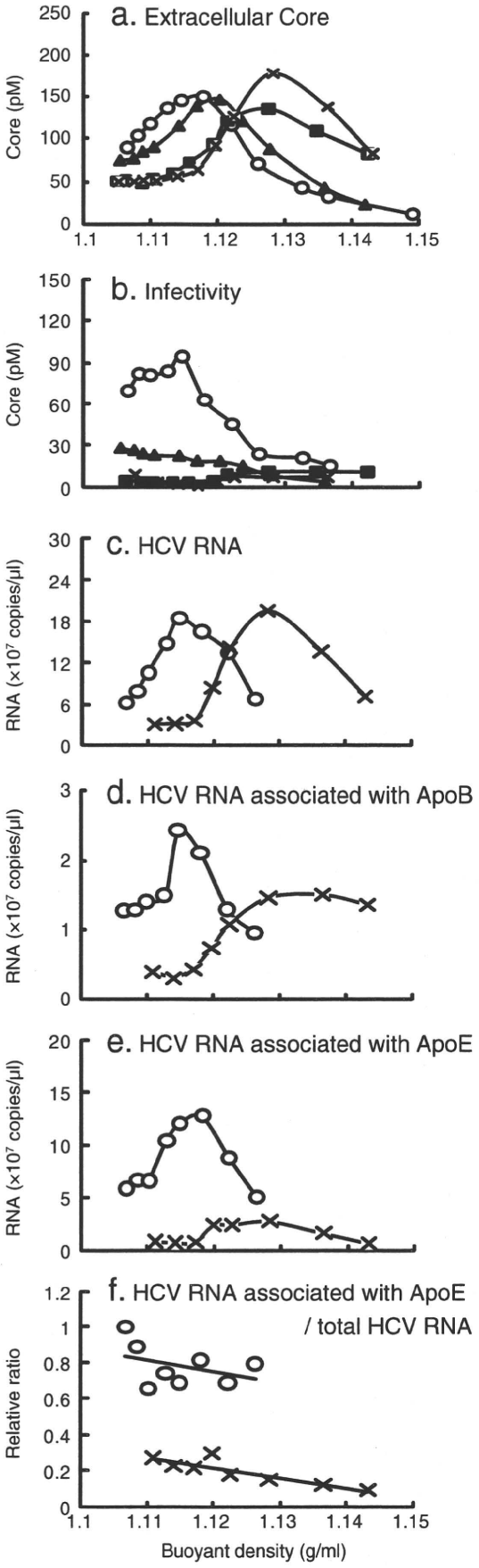


Fig. 1. LPL treatment reduced infectivity of the HCV-bearing medium in a dose-dependent manner. a. The HCV-bearing medium was pretreated with bovine LPL (▪) or heat-inactivated LPL (□) (Fig. 2b) at 37 °C for 1 h before inoculation with HuH7.5 cells. Cells were fixed at 24 h post-inoculation and subjected to immunofluorescence staining. The mean percentage of HCV-positive cells relative to LPL-untreated medium is shown with the standard deviation (n = 3). Statistically significant differences ($p<0.001$) are indicated by asterisks (Student's *t*-test): NS (not significant, $p>0.01$). b. Activities of LPL and inactivated LPL by heating at 100 °C for 5 min were determined. The mean value and standard deviation are shown (n = 3). c. Activities of LPL on incubation with orlistat were determined. LPL (10 μg/ml) and orlistat (25 μg/ml) were mixed and subjected to determine lipase activity. Orlistat completely inhibited LPL activity. The mean value and standard deviation are shown (n = 3). d. The HCV-bearing medium was pretreated with LPL (10 μg/ml) and orlistat (25 μg/ml) at 37 °C for 1 h before inoculation. Cells were subjected to immunofluorescence staining at 24 h post-inoculation. The mean percentage of HCV-positive cells relative to LPL-untreated medium is shown with the standard deviation (n = 3). There was no statistically significant difference observed (Student's *t*-test): NS $p>0.01$. e. Orlistat (25 μg/ml) was added just before HuH7.5 cells were inoculated with the HCV-bearing medium pretreated with LPL (10 μg/ml). Cells were subjected to immunofluorescence staining at 24 h post-inoculation. The mean percentage of HCV-positive cells relative to LPL-untreated medium is shown with the standard deviation (n = 3). Statistically significant differences are indicated by asterisks (Student's *t*-test): *$p<0.001$. f. SeV, whose genome contains the gene coding Green Fluorescent Protein (GFP), was pretreated with bovine LPL (▪) or heat-inactivated LPL (□) (Fig. 2b) at 37 °C for 1 h before inoculation with HeLa cells. Expression of GFP in infected cells was observed under microscope at 24 h post-inoculation. The mean percentage of SeV-positive cells relative to LPL-untreated medium is shown with the standard deviation (n = 3). LPL did not significantly affect the infectivity of SeV ($p>0.01$).



of HCV RNA in the complex associating with ApoE from LPL-treated sample was remarkably reduced (Fig. 2e). Thus, it is indicated that HCV is associated with both ApoB and ApoE but that the association with ApoE is more closely related to HCV infectivity than that with ApoB.

The ratio of HCV RNA in the complex associating with ApoE to the total HCV RNA was dramatically lower in LPL-treated samples than in PBS-treated samples (Fig. 2f). HCV with the same buoyant density showed a different ratio of the association with ApoE between LPL-treated and -untreated samples (Fig. 2f). It is indicated that HCV with the same buoyant density might have heterogeneous characteristics, especially in the association with ApoE. Though LPL affects the buoyant density of HCV, buoyant density may not become a direct indicator of HCV infectivity.

LPL and HTGL reduced HCV infectivity

Generally, two successive lipolytic steps, sequentially catalyzed by LPL and HTGL, convert VLDL through IDL to LDL (Braun and Severson, 1992; Connelly, 1999; Mead et al., 2002; S.-Fojo et al., 2004). Hepatocytes produce HTGL but not LPL (Braun and Severson, 1992; Connelly, 1999). If HuH7.5 cells produce HTGL into culture medium, addition of LPL to the HCV-bearing medium from HuH7.5 cells would lead to two successive lipolytic actions by exogenous LPL and endogeneous HTGL. To analyze this possibility, we examined HTGL expression in HuH7.5 cells. Expression of LPL mRNA in hepatocyte-derived cell lines, HuH7.5 and HepG2, was lower compared to that in the 293T cell line derived from kidney (Fig. 3a). We observed higher expression of HTGL mRNA in HuH7.5 and HepG than in 293T (Fig. 3a). HTGL activity was detected in medium from HuH7.5 cells, which was higher than that in medium from 293T cells (Fig. 3b) and showed good agreement to the level of mRNA (Fig. 3a), though HTGL activity was not detected in medium from HepG2 cells.

Then, to evaluate the role of HTGL on LPL-induced reduction of HCV infectivity, the HCV-bearing medium was incubated with LPL in the presence of a neutralizing antibody against HTGL and infectivity was measured. Since LPL activity itself was not affected by the anti-HTGL antibody treatment (data not shown), we expected that HCV-associated lipoprotein should be protected against HTGL in the presence of the anti-HTGL antibody. In fact, LPL-induced reduction in HCV infectivity was partly suppressed by the presence of the anti-HTGL antibody (Fig. 3c). The infectivity was not fully restored after treatment with the anti-HTGL antibody from the LPL-induced suppressive status, which indicates that HTGL plays a partial role in the LPL effect but that LPL also plays a role. This result implied that two lipases could reduce HCV infectivity through changing the lipoprotein-like structure and that, conversely, infectivity of HCV was regulated by the lipoprotein-like structure that associated with HCV.

HTGL reduced HCV infectivity through its catalytic activity, irrespective of LPL activity

To confirm the reductive effect of HTGL on HCV infectivity, the endogenous expression of HTGL was suppressed by siRNA specific to

Fig. 2. Shift of buoyant density of HCV as well as detachment of ApoE by LPL treatment. The HCV-bearing medium treated with PBS or LPL was subjected to centrifugation in an iodixanol gradient. After ultracentrifugation, aliquots of 30 consecutive fractions were collected and analyzed for (a) Core, (b) infectivity, (c) HCV RNA, and HCV RNA in (d) ApoB-associated and (e) ApoE-associated complexes. For a and b, PBS (○), 5 (▲), 50 (■), and 500 (×) μg/ml of LPL-treated samples, and for c–f, PBS (○) and 500 (×) μg/ml of LPL-treated samples were subjected. a. Core in each fraction was measured by ELISA (Ortho Clinical Diagnostics). b. HuH7.5 cells were inoculated with aliquots of each fraction for 2 h. Core ELISA of the culture medium was performed at 24 h post-inoculation. c. RNA was extracted from each fraction and subjected to cDNA synthesis followed by quantitative PCR. d. Measurement of HCV RNA in the complexes associated with ApoB. e. Measurement of HCV RNA in the complexes associated with ApoE. f. Ratio of HCV RNA in the complexes associated with ApoE versus the total HCV RNA in each fraction. The values were obtained by dividing the amounts of RNA of Fig. 3e by those of Fig. 3c.

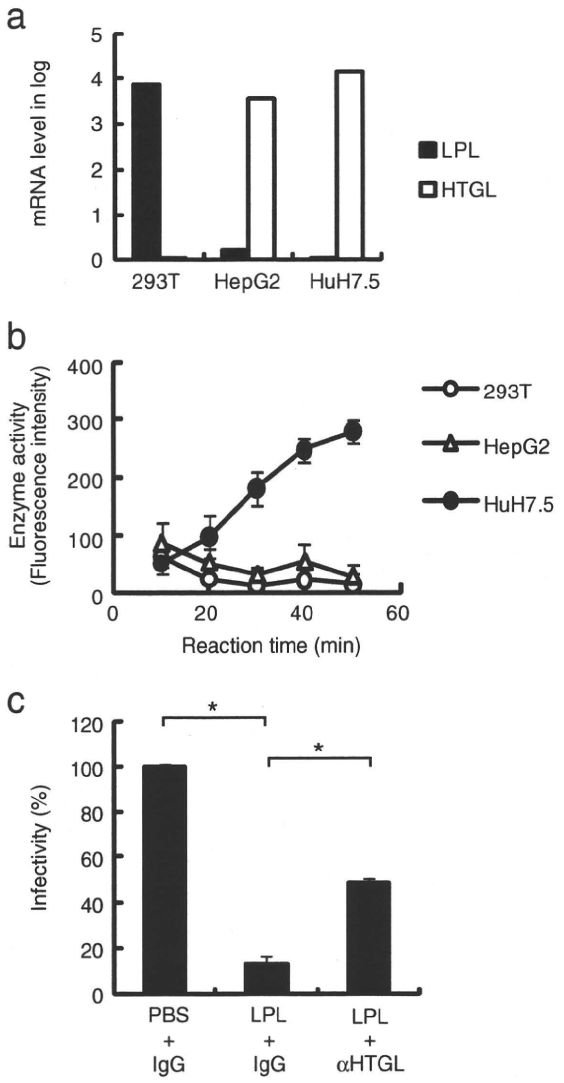


Fig. 3. Treatment of the HCV-bearing medium with exogenous LPL led to subsequent digestion by endogenously produced HTGL, resulting in loss of HCV infectivity. a. Expression of LPL and HTGL in HuH7.5, HepG2 and 293T cells. The mean of LPL mRNA expression level in 293T cells relative to the level in HuH7.5 cells is shown with standard deviation (n = 3) and vice versa for HTGL (n = 3). b. Validation of HTGL activity. The culture medium from HuH7.5, HepG2 and 293T cells was concentrated to 20 times using centricon YM-30 (Millipore), and was analyzed for HTGL activity. The mean and standard deviation are shown (n = 3). c. The HCV-bearing medium was incubated with anti-HTGL antibody before LPL treatment. HuH7.5 cells were inoculated with the medium and then subjected to immunofluorescence staining at 24 h post-inoculation. The mean percentage of HCV-positive cells relative to medium incubated with PBS and IgG is shown with the standard deviation (n = 3). Statistically significant differences are indicated by asterisks (Student's *t*-test): $^{*}p<0.001$.

HTGL (Fig. 4a). The level of HTGL mRNA in cells transfected with siRNA targeting HTGL was lower than one 6th of the control cells transfected with non-target siRNA (Fig. 4a). The activity of secreted HTGL was slightly lower in HTGL knockdown cells than the control (Fir. 4b). It may be because HTGL is so stable at protein level that its activity remains 48 h after transfection of siRNA. The presence of infectious HCV in the cells as well as the amounts of Core secreted into the culture medium was not significantly different between HTGL knockdown cells and the control (Fig. 4a). ApoB and ApoE were secreted at the same level from HTGL knockdown cells and the control cells (Fig. 4c). These results indicate that HTGL knockdown did not affect the production of either HCV or lipoproteins. Interestingly, the infectivity of the culture medium was about 2 folds higher than that in culture medium from control cells (Fig. 4a). The HCV-bearing medium from HTGL knockdown cells was ultracentrifuged through iodixanol gradients. We collected 80 fractions from the gradients and analyzed for Core. The Core peak was shifted to a lower buoyant density in the medium from HTGL knockdown cells compared to the control (Fig. 4d). Thus, HTGL could hydrolyze HCV-associated lipoproteins in the medium derived from HuH7.5 cells irrespective of LPL, leading to a reduction of infectivity.

Discussion

Our results indicate that bovine LPL reduced HCV infectivity through its catalytic activity. Since the same doses of LPL did not impair the infectivity of SeV (Fig. 1f), it is likely that HCV-associated lipoprotein is targeted by the LPL and that the lipoprotein associating with HCV plays a pivotal role in HCV infectivity. Previously, it was shown that LPL-Ps disrupted HCV (Thomssen and Bonk, 2002) and that bovine LPL suppressed HCV infectivity through uncertain mechanisms within cells after bridging HCV with cells by bovine LPL (Andréo et al., 2007). We detected Core and HCV RNA from the LPL-treated HCV-bearing medium almost at the same level as the untreated medium (Figs. 2a and c). Thus, bovine LPL at the concentration used in this study did not destroy the HCV structure as reported by LPL-Ps (Thomssen and Bonk, 2002). LPL seems to have at least two distinct functions to reduce HCV infectivity; one is through its de-lipidation activity observed here and another is its suppressive activity observed after being associated with cells (Andréo et al., 2007). We used an LPL inhibitor, orlistat, to delineate these two suppressive functions of LPL on HCV. After incubation of the HCV-bearing medium with LPL at a concentration of 10 μg/ml, orlistat was added so that catalytic activity of LPL could be suppressed upon contact of HCV with cells. HCV infectivity was significantly reduced even under this condition (Fig. 1e). This result indicates that de-lipidation by LPL is a major cause of HCV inactivation. However, it is worth mentioning that the residual infectivity was higher than that observed in the conditions of infection assay conducted without orlistat (Fig. 1e, compare the lane with the same dose (10 μg/ml) of LPL in Fig. 1a), which indicates the presence of another inactivating mechanism suggested by Andréo et al. (2007) though the contribution is not as high.

The experiments using neutralizing antibody against HTGL (Fig. 3c) indicated that both LPL and HTGL have some roles in LPL-induced reduction in HCV infectivity. Knockdown of HTGL resulted in HCV with higher infectivity and a lower density in the medium (Figs. 4a and d). HTGL is the predominant enzyme in the lipolysis of IDL, but also hydrolyzes TG in all lipoproteins to some extent (Connelly, 1999). Thus, it is suggested that endogenous HTGL has lipolytic activity on HCV-associated lipoprotein irrespective to LPL functions. Taking account of the effect of LPL and HTGL on HCV infectivity, their expression is disadvantageous for HCV. There were not much differences in expressions of LPL and HTGL between HuH7.5 cells and HepG2 cells (Fig. 3a), suggesting that expression of lipases does not explain HuH7.5 as an excellent cell line for HCV.

Our study shows that LPL and/or HTGL change(s) the nature of HCV-associated lipoproteins, leading to reduced HCV infectivity. This indicates that the association of HCV with certain lipoprotein-like VLDL is important for HCV infectivity. Recently, ApoE was shown to be important for HCV infectivity as a ligand of the HCV receptor (Owen et al., 2009). It is conceivable that the reduction of ApoE in accordance to lipolysis of HCV associating lipoproteins is a direct cause of the reduction of HCV infectivity.

We used bovine LPL at 2–20 μg/ml to reduce HCV infectivity (Fig. 1a). It is expected that the same doses of human LPL could reduce HCV infectivity. However, heparin-treated human plasma contains around 100 ng/ml of LPL; most of LPL is bound to heparan sulfate proteoglycan (HSPG) at the cell surface and

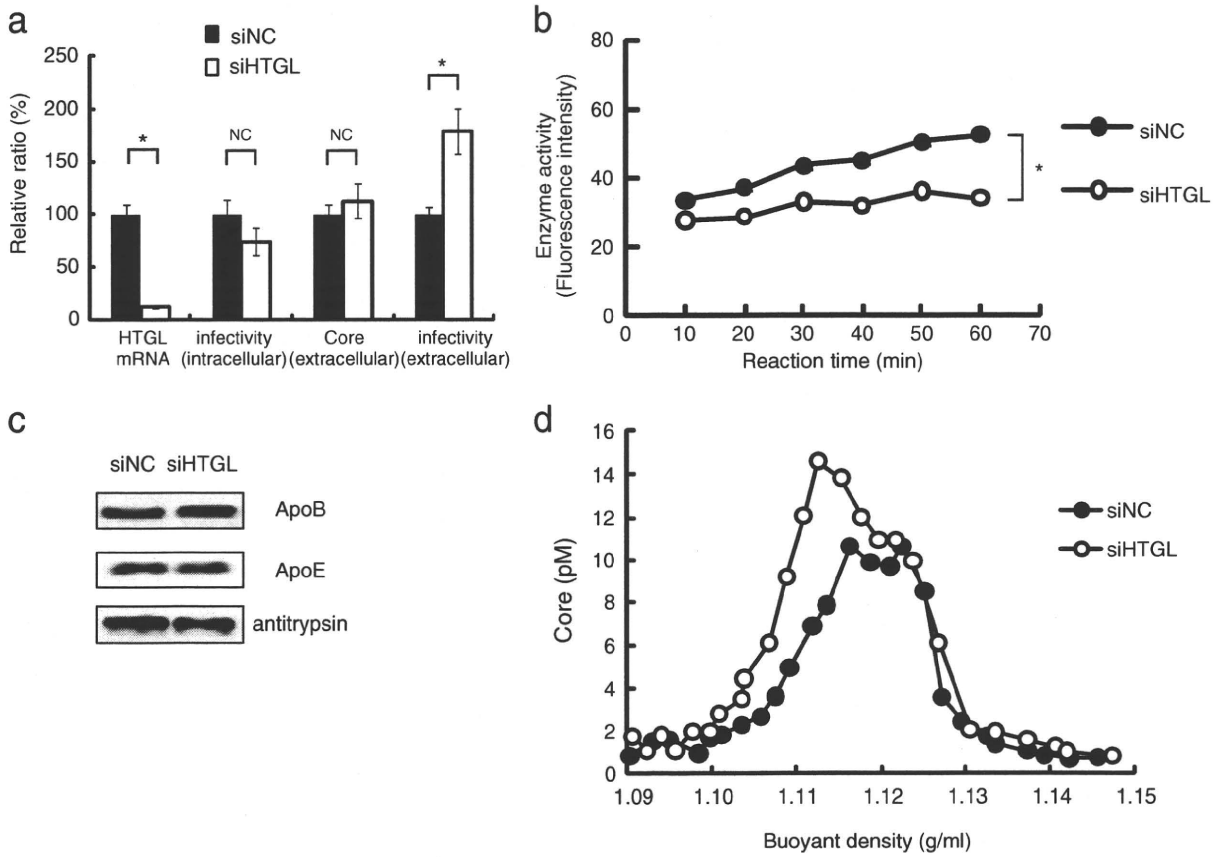


Fig. 4. HCV with higher infectivity and a lower buoyant density in the medium from HTGL knockdown cells. a. HTGL mRNA, infectivity of HCV within cells, extracellular Core and infectivity of HCV in the medium are shown. The mean percentages relative to control cells are shown with the standard deviation (mRNA, Core; n = 3, infectivity; n = 5). Statistically significant differences in HTGL mRNA level and extracellular infectivity are indicated by asterisks (Student's *t*-test): $^*p<0.001$. There was no statistically significant difference in intracellular infectivity and extracellular core (Student's *t*-test): NS $p>0.01$. b. Activities of HTGL were determined. After secretion, most of the HTGL is bound to heparan sulfate proteoglycans (HSPG) at the cell surface (Connelly, 1999; S.-Fojo et al., 2004). Both HTGLs bound and unbound to HSPG are expected to act on lipoproteins in the medium. Thus, in order to evaluate activities of HTGL acting on lipoproteins in the medium, heparin (10 U/ml) was added to the medium to release HTGL bound to HSPG at 24 h post-transfection of siRNA. The medium was harvested at 48 h post-transfection, passed through 0.45 μm filter (Iwaki) to eliminate cell debris and used for the assay. Statistically significant difference in HTGL activity at 60 min is indicated by asterisks (Student's *t*-test): $^*p<0.001$. c. Detection of apolipoproteins (ApoB and ApoE) and α-1 antitrypsin as standard secreted in culture medium from HTGL knockdown cells and control cells. d. Buoyant density of HCV produced from HTGL knockdown cells. The HCV-bearing medium from HTGL knockdown cells was subjected to centrifugation in an iodixanol gradient. After ultracentrifugation, aliquots of 80 consecutive fractions were collected and analyzed for Core by ELISA. The data are presented from 25 fractions around Core peak from a single representative experiment of three experiments. There was a significant difference in the buoyant density of the Core peak between HTGL knockdown cells and the control (Student's *t*-test, $p<0.001$).

heparin leads to a release of LPL bound to HSPG (Kern et al., 1990). Therefore, it is conceivable that HCV infectivity could be reduced through activities of LPL in the circulation at a lesser efficiency than observed in this work. Here, we demonstrated that the hydrolyzing activity of HTGL as well as LPL affects HCV infectivity. Considering this lipase-induced reduction in HCV infectivity in the circulation, it is important for virus to infect the proximal hepatocytes before entering circulation. In other words, the activities of LPL and/or HTGL may be one host mechanism to resist invasion and spread of HCV.

Materials and methods

Cell culture

HuH7.5, HeLa, 293T and HepG2 cells were maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin.

Virus source

HuH7.5 cells were transfected with HCV 2a strain, JFH1 RNA genome and maintained in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Three days before harvest, medium was replaced with Opti-Pro (Invitrogen) supplemented with 0.1% BSA. Culture medium was filtered with 0.45 μm filter (Iwaki) and used as virus source (the HCV-bearing medium).

HCV infectivity

To determine HCV infectivity in the medium, HuH7.5 cells were inoculated with the HCV-bearing medium (MOI of 0.5) at 37 °C for 2 h. Cells were washed with PBS and incubated in DMEM supplemented with 10% FBS, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were fixed at 24 h post-inoculation and subjected to immunofluorescence staining using serum from a HCV-positive individual to detect HCV-infected cells. Three to five fields under microscope were randomly selected. Cells in a field were counted and the percentage of HCV-positive cells to total cells (around 500–1000) was calculated.

To determine HCV infectivity within cells, cells were collected by trypsinization. After washing with PBS, cells were incubated with water and passed through a 27-gauge needle (Terumo) at ten times. After centrifugation, supernatant was passed through a 0.45 μm filter (Iwaki) and inoculated to HuH7.5 cells. Immunofluorescence staining was performed as mentioned above.

Lipase activity

Total lipase test (Progen) and hepatic lipase select test (Progen) were used to examine LPL activity and HTGL activity, respectively, according to the manufacturer's protocol.

Iodixanol density gradients

The HCV-bearing medium was concentrated around 50 times using amicon ultra-15 100k (Millipore) and treated with PBS or 5, 50, or 500 μg/ml of LPL at 37 °C for 1 h. The samples were applied to the top of a linear gradient formed from 17–37% iodixanol-containing PBS and spun at 36,000 rpm for 18 h at 4 °C using a SRP 41 Hitachi Ultracentrifuge rotor. Aliquots of 30 consecutive fractions were collected and used for analyses of Core, infectivity, HCV RNA, and immunoprecipitation with anti-ApoB or anti-ApoE antibodies.

For analysis of the HCV-bearing medium from HTGL knockdown cells, the concentrated samples were applied to the top of a gradient from 14–54% iodixanol-containing PBS and spun at 34,000 rpm for 20 h at 4 °C. A total of 80 consecutive fractions were collected and used for analyses of Core.

Quantification of HCV RNA

RNA was extracted from fractions of the iodixanol gradient using an RNeasy mini kit (Qiagen). Complementary DNA was prepared by incubating RNA with SuperScript III (Invitrogen) and 737R primer, a reverse RNA primer of HCV genome (Sugiyama et al., 2009). Quantitative PCR analysis was performed by 7500 Fast Real Time PCR System (Applied Biosystems). Taqman probe and primers were as follows: probe 733FB (Sugiyama et al., 2009), forward 5′-CCCTCCCGGGAGAGCCATAGTG-3′, reverse 5′-GTCTCGCGGGGGCACGCCCAAAT-3′. The copy number of HCV was determined by the standard-curve method with serial dilutions of the synthesized full-length HCV RNA.

Immunoprecipitation

Fractions from iodixanol gradient were incubated with anti-ApoB antibody (Biodesign International) or anti-ApoE antibody (Chemicon International) for 2 h at room temperature. Protein G sepharose (GE) was added and the immunocomplex was collected by centrifugation. The pellets were used for RNA extraction.

Expression of LPL and HTGL

RNA was extracted from cells using RNeasy mini kit (Qiagen). Complementary DNA was prepared by incubating RNA with Super-Script III (Invitrogen) and oligo(dT) as an universal primer. Quantitative PCR analysis was performed with primers specific for LPL (Lindegaard et al., 2005), for HTGL (Sirvent et al., 2004), and for GAPDH (Suzuki et al., 2008). The LPL and HTGL mRNA expression level was calibrated with the level of GAPDH mRNA expression.

Neutralization of HTGL

The HCV-bearing medium was incubated with IgG rabbit (Santa Cruz) or anti-HTGL antibody (sc-21007, Santa Cruz) at 4 °C overnight, followed by PBS or LPL treatment at 37 °C for 1 h. HuH7.5 cells were inoculated with the medium at 37 °C for 2 h and subjected to immunofluorescence staining as explained above.

Knockdown of HTGL

siRNA (siGENOME SMART pool M-008743-00-0005, Thermo) at a final concentration of 40 nM was transfected with HuH7.5 cells using siLentFect reagent (Bio-Rad). The HCV-bearing medium (MOI = 0.5) was inoculated with cells at 4 h post-transfection. Then, fresh medium was replaced 2 h later. At 24 h post-transfection, medium was replaced with Opti-pro (Invitrogen) supplemented with 0.1% BSA. At 48 h post-transfection, culture medium and cells were used for analyses of RNA, infectivity, Western blotting and buoyant density.

Western blotting

Western blotting was performed using anti-ApoB antibody (Biodesign International), anti-ApoE antibody (Innogenetics) and anti-α-1 antitrypsin antibody (Biodesign). Detection was carried out using ECL plus reagent (GE).

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Sphingomyelin Activates Hepatitis C Virus RNA Polymerase in a Genotype-Specific Manner▽†

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Hepatitis C virus (HCV) replication and infection depend on the lipid components of the cell, and replication is inhibited by inhibitors of sphingomyelin biosynthesis. We found that sphingomyelin bound to and activated genotype 1b RNA-dependent RNA polymerase (RdRp) by enhancing its template binding activity. Sphingomyelin also bound to 1a and JFH1 (genotype 2a) RdRps but did not activate them. Sphingomyelin did not bind to or activate J6CF (2a) RdRp. The sphingomyelin binding domain (SBD) of HCV RdRp was mapped to the helix-turn-helix structure (residues 231 to 260), which was essential for sphingomyelin binding and activation. Helix structures (residues 231 to 241 and 247 to 260) are important for RdRp activation, and 238S and 248E are important for maintaining the helix structures for template binding and RdRp activation by sphingomyelin. 241Q in helix 1 and the negatively charged 244D at the apex of the turn are important for sphingomyelin binding. Both amino acids are on the surface of the RdRp molecule. The polarity of the phosphocholine of sphingomyelin is important for HCV RdRp activation. However, phosphocholine did not activate RdRp. Twenty sphingomyelin molecules activated one RdRp molecule. The biochemical effect of sphingomyelin on HCV RdRp activity was virologically confirmed by the HCV replicon system. We also found that the SBD was the lipid raft membrane localization domain of HCV NS5B because JFH1 (2a) replicon cells harboring NS5B with the mutation A242C/S244D moved to the lipid raft while the wild type did not localize there. This agreed with the myriocin sensitivity of the mutant replicon. This sphingomyelin interaction is a target for HCV infection because most HCV RdRps have 241Q.

Hepatitis C virus (HCV) has a positive-stranded RNA genome and belongs to the family *Flaviviridae* (21). HCV chronically infects more than 130 million people worldwide (34), and HCV infection often induces liver cirrhosis and hepatocellular carcinoma (19, 28). To date, pegylated interferon (PEG-IFN) and ribavirin are the standard treatments for HCV infection. However, many patients cannot tolerate their serious side effects. Therefore, the development of new and safer therapeutic methods with better efficacy is urgently needed.

Lipids play important roles in HCV infection and replication. For example, the HCV core associates with lipid droplets and recruits nonstructural proteins and replication complexes to lipid droplet-associated membranes which are involved in the production of infectious virus particles (24). HCV RNA replication depends on viral protein association with raft membranes (2, 30). The association of cholesterol and sphingolipid with HCV particles is also important for virion maturation and infectivity (3). The inhibitors of the sphingolipid biosynthetic pathway, ISP-1 and HPA-12, which specifically inhibit serine palmitoyltransferase (SPT) (23) and ceramide trafficking from the endoplasmic reticulum (ER) to the Golgi apparatus (37), suppress HCV virus production in cell culture but not viral RNA replication by the JFH1 replicon (3). Other serine SPT inhibitors (myriocin and NA255) inhibit genotype 1b replication (4, 29, 33). Very-low-density lipoprotein (VLDL) also interacts with the HCV virion (15).

Sakamoto et al. reported that sphingomyelin bound to HCV RNA-dependent polymerase (RdRp) at the sphingomyelin binding domain (SBD; amino acids 230 to 263 of RdRp) to recruit HCV RdRp on the lipid rafts, where the HCV complex assembles, and that NA255 suppressed HCV replication by releasing HCV RdRp from the lipid rafts (29). In the present study, we analyzed the effect of sphingomyelin on HCV RdRp activity *in vitro* and found that sphingomyelin activated HCV RdRp activity in a genotype-specific manner. We also determined the sphingomyelin activation domain and the activation mechanism. Finally, we confirmed our biochemical data by a HCV replicon system.

MATERIALS AND METHODS

HCV RNA polymerase. A C-terminal 21-amino-acid deletion was made to the HCV RdRps of strains HCR6 (genotype 1b) (36), NN (1b) (35), Con1 (1b) (5), JFH1 (2a) (36), J6CF (2a) (25), H77 (1a) (7), and RMT (1a), and the mutants

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were purified from bacteria as described previously (36). HCR6 (1b) RdRp with the mutation L245A [RdRp(L245A)] or I253A [RdRp(I253A)] or the double mutation L245A and I253A [RdRp(L245A/I253A)]; JFH1 (2a) RdRp with the mutation(s) A242C/S244D, A242, S244D, or T251Q; J6CF (2a) RdRp with the mutation(s) R241Q, S244D, or R241Q/S244D; and H77 (1a) RdRp(A238S/Q248E) were introduced using an *in vitro* mutagenesis kit (Stratagene) and the oligonucleotides listed in Table S1 in the supplemental material. HCR6 (1b) His_6-tagged RdRp(L245A/I253A) was removed from pET21b/KM (36) and cloned into the BamHI/XhoI site of pGEX-6P-3 (GE), resulting in pGEXHCVHCR6RdRp(L245A/I253A).

***In vitro* HCV transcription.** *In vitro* HCV transcription was performed as described previously (36). Briefly, following 30 min of preincubation without ATP, CTP, or UTP, 100 nM HCV RdRp was incubated in 50 mM Tris-HCl (pH 8.0), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, 1 mM dithiothreitol (DTT), 0.5 mM GTP, 50 μM ATP, 50 μM CTP, 5 μM [α-^{32}P]UTP, 200 nM RNA template (SL12-1S), 100 U/ml human placental RNase inhibitor, and the lipid (amount indicated below) at 29°C for 90 min. ^{32}P-labeled RNA products were subjected to 6% polyacrylamide gel electrophoresis (PAGE) containing 8 M urea. The resulting autoradiograph was analyzed with a Typhoon Trio plus image analyzer (GE).

RNA filter binding assay. An RNA filter binding assay was performed as described previously (36). Briefly, 100 nM HCV RdRp and 100 nM ^{32}P-labeled RNA template (SL12-1S) were incubated with or without 0.01 mg/ml egg yolk sphingomyelin in 25 μl of 50 mM Tris-HCl (pH 7.5), 200 mM monopotassium glutamate, 3.5 mM $MnCl_2$, and 1 mM DTT at 29°C for 30 min. After incubation, the solutions were diluted with 0.5 ml of TE (50 mM Tris-HCl [pH 7.5], 1 mM EDTA) buffer and filtered through nitrocellulose membranes (0.45-μm pore size; Millipore). The filter was washed five times with TE buffer, and the bound radioisotope was analyzed by Typhoon Trio plus after being dried.

Enzyme-linked immunosorbent assay (ELISA). Ninety-six-well microtiter plates (Corning) were coated with 250 ng of egg yolk sphingomyelin in ethanol by evaporation at room temperature. After the wells were blocked with phosphate-buffered saline (PBS) and 3% bovine serum albumin (BSA), they were incubated with 1 pmol of the HCV RdRp of HCR6 (1b) wild type (wt) or L245A, I253A, or L245A/I253A mutant; NN (1b); H77 (1a); RMT (1a); J6CF (2a); or JFH1 (2a) wt or A242C/S244D, A242, S244D, or T251Q mutant in Tris-buffered saline (50 mM Tris-HCl [pH 7.5] and 150 mM NaCl) for 1.5 h at room temperature. After being blocked with 3% BSA, the bound HCV RdRp was detected by adding rabbit anti-HCV RdRp serum (1:5,000) (see Fig. S1 in the supplemental material) (17) before incubation with a horseradish peroxidase (HRP)-conjugated anti-rabbit IgG antibody (1:5,000; Southern Biotech). The optical density at 450 nm (OD_{450}) was measured with a Spectra Max 190 spectrophotometer (Molecular Devices) using a TMB (3,3′,5,5′-tetramethylbenzidine) Liquid Substrate System (Sigma).

HCV subgenomic replicon. A D244S mutation was introduced into the HCV strain NN (1b) subgenomic replicon pLMH14 (35), resulting in pLMH(NN)5B(D244S) [where 5B(D244S) is the NS5B protein with the mutation D244S]. The A242C/S244D mutation was introduced into the HCV JFH1 (2a) replicon, pSGR-JFH1/luc (25), resulting in pSGR-JFH1/luc5B(A242C/S244D). The HpaI and XbaI fragment of pSGR-JFH1 (18) was replaced with that of pSGR-JFH1/luc5B(A242C/S244D), resulting in pSGR-JFH15B(A242C/S244D). The A238S/Q248E mutation was introduced into HCV H77 (1a) replicon pHCVrep13(S2204I)/Neo (7) after the neomycin gene was replaced by the firefly luciferase gene [pH77(I)/luc] by insertion of AflII and AscI sites (see Table S1 in the supplemental material), resulting in pH77(I)/luc5B(A238S/Q248E). Subgenomic replicon RNA was transcribed *in vitro* by T7 RNA polymerase using MegaScript (Ambion) after the replicon plasmids were linearized by XbaI (strain NN and JFH1 replicons) or HpaI (strain H77 replicon). Subgenomic replicon RNA was stored at −80°C after being purified by phenol-chloroform extraction and ethanol precipitation.

Replicon assay with myriocin. Huh7.5.1 cells were kindly provided by F. Chisari and were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco) (38). HCV replicon RNA (10 μg) was transfected into 4×10^6 Huh7.5.1 cells (1×10^7/ml) in OptiMEM I (Gibco) by electroporation (GenePulser Xcell; Bio-Rad) at 270 V, 100 Ω, and 950 μF. After transfection, the cells were plated in 12-well plates incubated in DMEM–10% FBS. At 6 h after transfection, cells were treated with 0, 5, and 50 nM myriocin. At 4, 54, and 78 h after transfection (48 and 72 h after myriocin treatment), the cells were harvested, and luciferase activity was measured using a Dual-Glo luciferase assay kit and a GloMax 96 Microplate Luminometer (Promega). Luciferase activity was normalized against the activity at 4 h after transfection (26).

HCV JFH1 wt and NS5B(A242C/S244D) replicon cells. Huh7/scr cells were kindly provided by F. Chisari of the Scripps Research Institute and were maintained in Dulbecco's modified Eagle's medium (Gibco) with 10% fetal bovine serum (Gibco). RNA (10 μg each) from SGR-JFH1 and SGR-JFH1 with the mutations A242C/S244D in NS5B [NS5B(A242C/S244D) was transfected into 4×10^6 Huh7/scr cells (1×10^7/ml) in OptiMEM I (GIBCO) by electroporation (GenePulser Xcell; Bio-Rad) at 270 V, 100 Ω, and 950 μF. After transfection, the cells were plated in 10-cm dishes and incubated in DMEM–10% FBS with 1.0 and 0.5 mg/ml G418 (Gibco). JFH1 wt and NS5B(A242C/S244D) replicon cells were maintained in DMEM–10% FBS and 0.5 mg/ml G418.

Membrane floating assay. JFH1 wt and NS5B(A242C/S244D) replicon cells were suspended in two packed cell volumes of hypotonic buffer (10 mM HEPES-NaOH [pH 7.6], 10 mM KCl, 1.5 mM $MgCl_2$, 2 mM DTT, and 1 tablet/25 ml of EDTA-free protease inhibitor cocktail tablets [Roche]) and disrupted by 30 strokes of homogenization in a Dounce homogenizer using a tight-fitting pestle at 4°C. After nuclei were removed by centrifugation at 2,000 rpm for 10 min at 4°C, the supernatant (postnuclear supernatant [PNS]) was treated with 1% Triton X-100 in TNE buffer (25 mM Tris-HCl [pH 7.6] 150 mM NaCl, 1 mM EDTA) for 30 min on ice. The lysates were supplemented with 40% sucrose and centrifuged at 38,000 rpm in a Beckman SW41 Ti rotor (Beckman Coulter) overlaid with 30% and 10% sucrose in TNE buffer at 4°C for 14 h.

Western blotting. Western blotting using anti-HCV RdRp (17), rabbit anti-NS3 (32), anti-NS5A (16) and anti-caveolin-2 was performed as previously published (17).

Reagent. Egg yolk sphingomyelin, cholesterol phosphocholine, myriocin, and rabbit anti-caveolin-2 antibodies were purchased from Sigma. Hexanoyl sphingomyelin, C_6-ceramide, C_8-β-D-glucosyl ceramide, and C_8-β-D-lactosyl ceramide were purchased from Avanti Polar Lipids. [α-^{32}P]UTP was purchased from New England Nuclear.

Statistical analysis. Significant differences were evaluated using *P* values calculated from a Student's *t* test.

Nucleotide sequence accession number. The sequence of HCV RMT has been deposited in the GenBank under accession number AB520610.

RESULTS

Sphingomyelin activation of HCV RNA polymerases of various genotypes. There are several sequence variations in the sphingomyelin binding domain (SBD; amino acids 231 to 260 of HCV RdRp) among HCV genotypes (see Fig. 7A). In order to compare the RdRps of different genotypes of HCV, we purified RdRp from genotypes 1b (strains HCR6, NN, and Con1), 1a (H77 and MRT), and 2a (JFH1 and J6CF) (see Fig. S2 in the supplemental material). First, the effect of ethanol on HCV HCR6 (1b) RdRp transcription was examined because lipids were suspended in ethanol before they were added to the HCV transcription reaction mixture. We found that 2% ethanol did not inhibit HCV transcription (see Fig. S3 in the supplemental material); therefore, all subsequent experiments were performed using less than 2% ethanol.

The kinetics of sphingomyelin activation were analyzed using egg yolk sphingomyelin for HCR6 (1b) RdRp wt (Fig. 1A) and subtype 2a (JFH1 and J6CF) RdRps (Fig. 1B), and *N*-hexanoyl-D-*erythro*-sphingosylphosphorylcholine (hexanoyl sphingomyelin) was used for HCR6 (1b) RdRp wt (Fig. 1C) and subtype 1a (H77 and RMT) RdRps (Fig. 1D). The egg yolk sphingomyelin activation curve of HCR6 (1b) RdRp wt at low concentrations (<0.01 mg/ml) was sigmoid. The transcription activity of HCR6 (1b) RdRp wt increased in a dose-dependent manner. It was activated 11-fold at 0.01 mg/ml and then plateaued (14-fold activation) at 0.1 mg/ml. However, JFH1 (2a) and J6CF (2a) RdRps were activated 2.5-fold and 2.2-fold, respectively, at 0.01 mg/ml sphingomyelin, at which point they plateaued.

Egg yolk sphingomyelin is a mixture. In order to obtain the optimal molar ratio for sphingomyelin activation of HCR6 (1b)

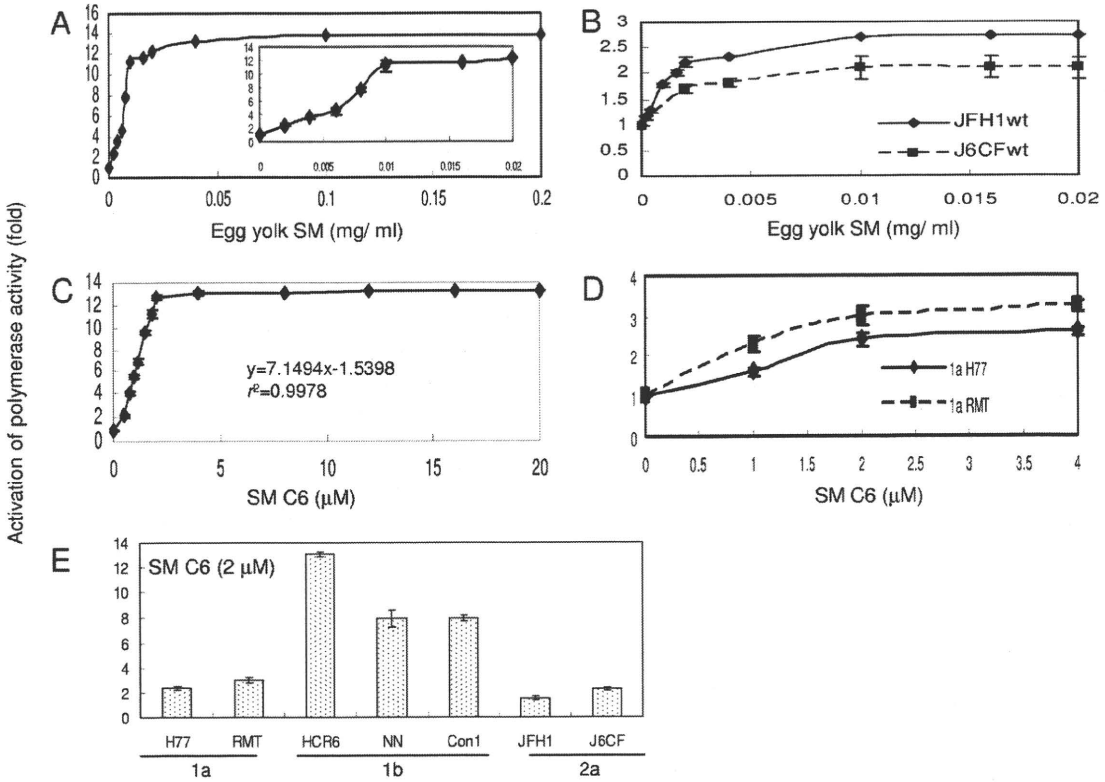

FIG. 1. Sphingomyelin activation of HCV RNA polymerases. (A) Activation kinetics of HCV HCR6 (1b) RdRp wt by egg yolk sphingomyelin (SM). The inset shows activation produced by 0 to 0.02 mg/ml egg yolk sphingomyelin. Activation kinetics of HCV 2a (JFH1 and J6CF) RdRps by egg yolk sphingomyelin (B) and of HCV HCR6 (1b) RdRp wt by hexanoyl sphingomyelin (SM C6) (C). In panel C, the first order of the graph was fitted by linear regression; the calculated equation is indicated in the graph. (D) Activation kinetics of HCV 1a (H77 and RMT) RdRps by hexanoyl sphingomyelin. (E) Activation effect of hexanoyl sphingomyelin on HCV RdRp of various genotypes. HCV RdRp (100 nM) was incubated with or without 2 μM SM C6. The names of the RdRps are indicated below the graph. Mean ± standard deviation of the activation ratio was calculated from three independent experiments.

RdRp wt, its activation kinetics were calculated using hexanoyl sphingomyelin (Fig. 1C, SM C6). The equation for the first-order ratio of hexanoyl sphingomyelin activation according to linear regression fitting was as follows: $y = 7.1494x - 1.5398$, where y is the activation ratio and x is the sphingomyelin concentration ($r^2 = 0.9978$). RdRp activation had almost plateaued at 2 μM hexanoyl sphingomyelin. The activation kinetics of JFH1 (2a) and J6CF (2a) RdRps in egg yolk sphingomyelin were biphasic and plateaued at 0.01 mg/ml. Those of RMT (1a) and H77 (1a) RdRps in hexanoyl sphingomyelin were also biphasic and plateaued at 2 μM. The curve of the first order was fitted by linear regression. The molar ratio of RdRp to hexanoyl sphingomyelin at its plateau was calculated as 1:20.

Because RdRp activation had almost plateaued at 2 μM hexanoyl sphingomyelin, we compared the effect of sphingomyelin on 100 M concentrations of RNA polymerases of the HCV 1a, 1b, and 2a genotypes using 2 μM hexanoyl sphingomyelin (Fig. 1E and Table 1).

Helix-turn-helix structure for sphingomyelin binding and activation. Sphingomyelin binds to the SBD peptide (see HCV SBD in Fig. 7) (29). Initially, we tested whether SBD was the sphingomyelin binding site in HCV RdRp by ELISA (Fig. 2A and Table 1). When the L245 and I253 residues of the SBD peptide were mutated to A, sphingomyelin binding activity was lost (29). We introduced the same mutations in HCV HCR6 (1b) RdRp and purified HCR6 (1b) RdRp with mutations L245A, I253A, and L245A/I253A. Because the C-terminal His-tagged HCR6 RdRp(L245A/I253A) was not soluble, it was solubilized by tagging of glutathione *S*-transferase (GST) sequence at the N terminus but lost polymerase activity. As the L245A/I253A mutant had lost its polymerase activity, polymerase activation was tested only for L245A and I253A (Fig. 2B and Table 1). These results confirmed that SBD located in the finger domain (residues 230E to 263G) successfully achieved sphingomyelin binding in HCV RdRp and that sphingomyelin did not bind to the SBD when the helix-turn-helix structure had been destroyed by the L245A or I253A mutation (29).

The sphingomyelin binding activities of genotype 1a and 2a RdRps were also tested (Fig. 2 and Table 1). Both JFH1 and J6CF were tested for genotype 2a because J6CF (2a) RdRp had an additional amino acid difference at position 241 in the SBD, and its sphingomyelin binding activity was very low (Fig. 2A and 7A; Table 1). J6CF (2a) RdRp(R241Q) showed the same sphingomyelin binding activity as HCR6 (1b) RdRp wt, indicating that 241Q was the critical amino acid for sphingomyelin binding. J6CF (2a) RdRp(S244D) and RdRp(R241Q/S244D) also showed higher sphingomyelin binding activity

TABLE 1. Summary of sphingomyelin activation of HCV RNA polymerase activities

Parameter	Value for the parameter by RdRp genotype, strain, and variant[a]																					
	1b							1a						2a								
	HCR6					NN	Con1	RMT	H77		J6CF				JFH1							
	wt	L245A	I253A	L245A/I253A	D244S	wt	wt	wt	wt	A238S/Q248E	wt	R241Q	S244D	R241Q/S244D	wt	A242C	S244D	A242C/S244D	T251Q
SM binding (%)[b]	100	24.3	30.8	15.5	78.7	93.4	117	144	86.7	82.5	19.3	118	53.1	80.2	70.4	75.5	93.1	92.4	80.7
Activation of polymerase (*n*-fold)[c]	13.0 (11.2)[d]	(2.8)[d]	(2.5)[d]	ND	3.6	7.9	7.9	3.0	2.0	8.1	2.3	4.3	5.6	3.4	1.6	1.0	3.1	4.4	1.8
Activation of RNA binding (*n*-fold)[c]	4.5	2.6	1.7	ND	1.9	ND	ND	ND	1.4	3.3	1.5	3.6	3.2	1.7	1.3	ND	ND	1.4	ND

[a] Numbers were averaged from three independent experiments. ND, not done.
[b] Egg yolk sphingomyelin (SM; 250 ng) was used.
[c] Hexanoyl sphingomyelin (2 μM) was used.
[d] Egg yolk sphingomyelin (0.01 mg/ml) was used.

than the wt ($P < 0.001$) but lower binding than the R241Q mutant. However, S244D showed higher RdRp activation than R241Q ($P < 0.005$), while the RdRp activation ratio of the double mutant (R241Q/S244D) was lower than that of S244D or R241Q, although all of them activated RdRp with sphingomyelin ($P < 0.005$) (Fig. 2A and C and Table 1). For JFH1, when the JFH1 RdRp SBD was modified (A242C/S244D) to allow it to bind with more sphingomyelin than the wt ($P < 0.005$), the mutant JFH1 RdRp(A242C/S244D) was activated more than the wt by sphingomyelin ($P < 0.005$) (Fig. 2A and C; Table 1). The sphingomyelin binding activity of JFH1 RdRp(T251Q) was 80.7% of that of HCR6 (1b), and its activation ratio was 1.8-fold. These results agree that SBD is both the sphingomyelin activation and binding domain and that the domains for these two activities are somehow different.

We determined which amino acid, 242C or 244D, enhanced sphingomyelin binding by comparing HCR6 (1b) and JFH1 (2a) RdRps. Sphingomyelin binding of HCR6 (1b) RdRp(D244S) was 79% of that of the wt ($P < 0.005$) (Fig. 2A and Table 1), and its activation by sphingomyelin was only 3.6-fold (Fig. 2C and Table 1). The sphingomyelin binding of JFH1 (2a) RdRp(A242C) and RdRp(S244D) increased to 75.5% and 93.1%, respectively, of HCR6 (1b) RdRp wt (Fig. 2A and Table 1). This was significantly higher than that of JFH1 (2a) RdRp wt ($P < 0.005$), and the sphingomyelin activation of JFH1 (2a) RdRp(A242C) and RdRp(S244D) was increased 1.0-fold and 3.1-fold, respectively ($P < 0.005$) (Fig. 2C and Table 1). From these mutation analyses of the J6CF and JFH1 RdRps, we concluded that 244D enhanced sphingomyelin binding and RdRp activation.

HCV 1a RdRps were not activated even though sphingomyelin bound to them (Fig. 1E and 2A and Table 1). We then tried to elucidate the domains responsible for sphingomyelin activation. There are 14 amino acids (residues 19, 25, 81, 111, 120, 131, 184, 270, 272, 329, 436, 464, 487, and 540) unique to genotype 1a RdRp in the region of residues 1 to 570 and two amino acid differences unique to 1a RdRp in SBD, i.e., 238A and 248Q (see Fig. 6A). Initially, we focused on the SBD and introduced the A238S and Q248E mutations into the H77 (2a) RdRp SBD (Fig. 2A and D and Table 1). The sphingomyelin binding activity of H77 (2a) RdRp(A238S/Q248E) was similar to that of H77 (2a) RdRp wt. The sphingomyelin activation ratio of H77 (2a) RdRp(A238S/Q248E) was increased 8.1-fold, leading us to conclude that these mutations are essential to sphingomyelin activation.

Effect of lipids on HCV RNA polymerase activity. In order to elucidate the structure of the lipids involved in activation of HCV RdRp, D-lactosyl-β-1,1′-*N*-octanoyl-D-*erythro*-sphingosine [C_8-lactosyl(β) ceramide], D-glucosyl-β1-17-N-octanoyl-D-*erythro*-sphingosine (C_8-β-D-glucosyl ceramide), *N*-hexanol-D-*erythro*-sphingosine (C_6-ceramide), and cholesterol were tested for their abilities to activate RdRp. The relative polymerase activities of 100 nM HCV HCR6 (1b) RdRp activated with 0.01 mg/ml egg yolk sphingomyelin, 2 μM hexanoyl sphingomyelin, 8 μM C_8-lactosyl(β) ceramide, 12 μM C_8-β-D-glucosyl ceramide, 12 μM C_6-ceramide, and 0.02 mg/ml cholesterol were 11.2, 13.0, 5.66, 4.19, 1.12, and 2.25 of that without lipids, respectively (Fig. 3A). The amount of lipids that gave the maximum activation was calculated from the kinetics of the lipids bound to HCR6 (1b) and JFH1 (2a) RdRps (Fig. 3B and

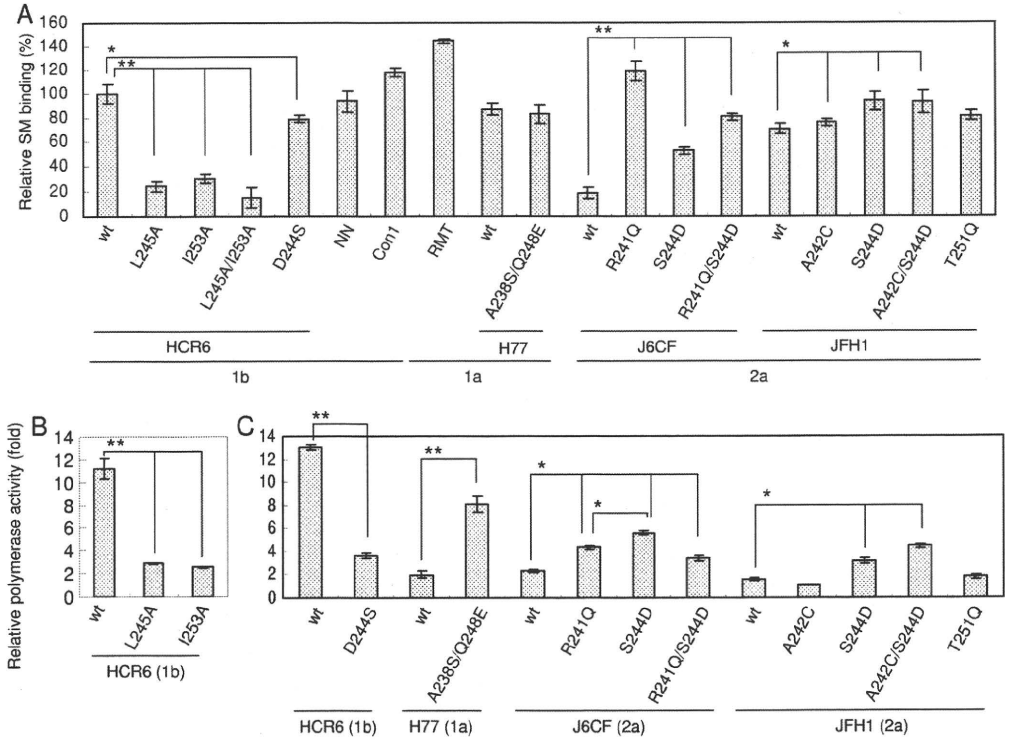

FIG. 2. Sphingomyelin binding and activation of HCV RNA polymerase sphingomyelin binding domain mutants. Names of RdRps are indicated below the graphs. (A) Egg yolk sphingomyelin (SM) binding activity relative to that of HCR6 (1b) RdRp wt. Mean ± standard deviation of the binding was calculated from three independent experiments. (B) Egg yolk sphingomyelin activation of HCR6 (1b) RdRps. RdRps (100 nM) were incubated with or without 0.01 mg/ml egg yolk sphingomyelin. (C) Hexanoyl sphingomyelin activation of the RdRps (RdRp names are indicated below the graphs). HCV RdRps (100 nM) were incubated with or without 2 μM hexanoyl sphingomyelin. The mean ± standard deviation of the activation ratio was calculated from three independent experiments. *, $P < 0.005$; ** $P < 0.001$.

C). C_8-lactosyl(β) ceramide and C_8-β-D-glucosyl ceramide activated HCR6 (1b) RdRp compared with the linear regression kinetics of the reaction with hexanoyl sphingomyelin as it plateaued (Fig. 1C and 3B). Cholesterol activated HCR6 (1b) RdRp slightly but did not activate JFH1 (2a) RdRp (Fig. 3C). We therefore concluded that the phosphocholine of sphingomyelin bound to the SBD of HCV RdRp because the order of HCV RdRp activation was hexanoyl sphingomyelin > C_8-lactosyl(β) ceramide > C_8-β-D-glucosyl ceramide, and C_6-ceramide did not activate HCV HCR6 (1b) RdRp. The polarity of the phosphocholine of sphingomyelin is important for HCV RdRp activation (see Fig. S5 in the supplemental material).

In order to test whether phosphocholine activated HCV RdRp (Fig. 3D), HCR6 (1b) RdRp was incubated with 0.4, 2, 20, 100, and 400 μg and 2, 4, 11, 54, and 100 mg of phosphocholine. Up to 400 μg of phosphocholine did not affect RdRp activity, but more than 2 mg of phosphocholine inhibited RdRp activity.

Effect of sphingomyelin on the template RNA binding of HCV RNA polymerase. The mechanism of HCV RdRp activation was analyzed. RNA polymerase changes its conformation throughout the different transcription steps, and template binding is the first step of transcription (9). Therefore, the effect of sphingomyelin on template RNA binding activity was tested (Fig. 4A and Table 1). Sphingomyelin enhanced the template RNA binding of HCR6 (1b) RdRp wt but not that of JFH1 (2a), H6CF (2a), or H77 (1a) wt RdRp. When the A238S/Q248E mutation was introduced into H77 (1a) RdRp, the RNA binding was enhanced. J6CF (2a) RdRp R241Q and S244D mutants showed similar enhancement of RNA binding, but the R241Q/S244D double mutant did not. The activation effect of RNA binding of HCR6 (1b) RdRp mutants L245A, I253A, and D244S was lower than that of RdRp wt. JFH1 (2a) RdRp wt and RdRp(A242C/S244D) showed similar RNA binding activation levels. Based on a comparison of the sphingomyelin activation of HCR6 (1b) RdRp wt and its mutants which lost sphingomyelin binding with J6CF (2a) RdRp wt and the R241Q and S244D mutants and H77 (1a) RdRp wt and the A238S/Q248E mutant, we concluded that polymerase activation by sphingomyelin was induced mainly via activation of the template RNA binding of RdRp. RNA binding activity of JFH1 (2a) RdRp wt and RdRp(A242C/S244D) was almost saturated because RNA binding of these RdRps was not activated by sphingomyelin (see Fig. S4 in the supplemental material).

HCV RdRp has to be bound with sphingomyelin before or at the same time as it binds to template RNA. After RdRp had bound to the template RNA, sphingomyelin did not enhance template RNA binding strongly (Fig. 4B).

Effect of the sphingomyelin binding domain mutations for HCV replicon activity with myriocin. In order to confirm sphingomyelin activation of HCV polymerase activity in a viral replication system, HCV replicon activity of the loss-of-function mutant HCV NN (1b) NS5B(D244S) and the gain-of-