Apolipoprotein E Mediates Evasion From Hepatitis C Virus—Neutralizing Antibodies

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BACKGROUND & AIMS: Efforts to develop an effective vaccine against hepatitis C virus (HCV) have been hindered by the propensity of the virus to evade host immune responses. HCV particles in serum and in cell culture associate with lipoproteins, which contribute to viral entry. Lipoprotein association has also been proposed to mediate viral evasion of the humoral immune response, though the mechanisms are poorly defined. METHODS: We used small interfering RNAs to reduce levels of apolipoprotein E (apoE) in cell culture—derived HCV—producing Huh7.5-derived hepatoma cells and confirmed its depletion by immunoblot analyses of purified viral particles. Before infection of naïve hepatoma cells, we exposed cell culture—derived HCV strains of different genotypes, subtypes, and variants to serum and polyclonal and monoclonal antibodies isolated from patients with chronic HCV infection. We analyzed the interaction of apoE with viral envelope glycoprotein 2 and HCV virions by immunoprecipitation. RESULTS: Through loss-of-function studies on patient-derived HCV variants of several genotypes and subtypes, we found that the HCV particle apoE allows the virus to avoid neutralization by patient-derived antibodies. Functional studies with human monoclonal antiviral antibodies showed that conformational epitopes of envelope glycoprotein 2 domains B and C were exposed after depletion of apoE. The level and conformation of virion-associated apoE affected the ability of the virus to escape neutralization by antibodies. CONCLUSIONS: In cell-infection studies, we found that HCV-associated apoE helps the virus avoid neutralization by antibodies against HCV isolated from chronically infected patients. This method of immune evasion poses a challenge for the development of HCV vaccines.

Keywords: Lipoviral Particle; Vaccination; Viral Escape; Lipid.

Hepatitis C virus (HCV) is a major health problem infecting approximately 130 million individuals worldwide. HCV infection typically results in a chronic infection that can lead to liver cirrhosis and hepatocellular carcinoma.1 Direct-acting antivirals have markedly improved the treatment efficacy, but limitations due to access to screening and therapy persist, highlighting the need for an effective vaccine for global control and eradication of HCV infection. A consistent hallmark of vaccines against pathogens is their reliance on immunogens that elicit neutralizing antibodies (nAbs).2 HCV vaccine development has been impeded by the viral adaptations to host immunity that enable chronic infection. Indeed, the host immune system lags behind the continuous evolution of HCV, allowing the virus to evade humoral immunity.3,4 However, the escape mechanisms from nAbs during chronic HCV infection are only partially understood. Clearly, the development of an effective vaccine requires a detailed understanding of viral evasion from host immune responses, including nAbs. Previous studies investigating the molecular mechanisms of HCV liver graft infection, identified a viral variant termed VL5,6 with efficient escape from patient nAbs.6 Functional genetics had identified phenylalanine at HCV polyprotein residue 447 as being important for neutralization escape.6 This amino acid is widely conserved among HCV isolates, as shown by its prevalence of 98.4% in all genotypes (including Jc1, Japanese fulminant hepatitis virus [JFH1], and H77) and 96.2% in genotype 1b strains (including VL).5,6 In addition, previous studies had shown that replacement of phenylalanine by leucine (F447L) rendered HCV highly susceptible to neutralization.6

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Abbreviations used in this paper: apo, apolipoprotein; E2, envelope glycoprotein 2; HCV, hepatitis C virus; HCVcc, cell culture—derived HCV; Jc1E2(FLAG), J6-JFH1 chimera with FLAG epitope in E2; JFH1, Japanese fulminant hepatitis virus; LDL, low-density lipoprotein; Luc-Jc1, J6-JFH1 chimera with luciferase reporter; mAb, monoclonal antibody; mRNA, messenger RNA; nAb, neutralizing antibody; PCR, polymerase chain reaction; siRNA, silencing RNA; TRL, triglyceride-rich lipoprotein.

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An interesting characteristic of HCV particles is their association with triglyceride-rich lipoproteins (TRL) and low-density lipoproteins (LDL) forming hybrid lipoviral particles, which results in a population of virions that are heterogeneous in buoyant density. Apolipoprotein (apo) B, E, AI, and CI have been described as lipoviral particles components. ApoE is important for both HCV infection of hepatocytes and hepatic uptake of TRL remnants, while the role of apoB, the structural protein of TRL and LDL, in the HCV life cycle is less clear. ApoCI may be involved in viral fusion, and apoAI can affect HCV replication and production. Although association with TRL and LDL has been hypothesized to contribute to viral evasion, the role of specific apolipoproteins in HCV persistence is unknown.

Given that apoE is a host protein incorporated into the HCV particle and is required for virion production, we sought to determine its functional role in viral evasion from host nAbs. Our findings reveal a previously undiscovered mechanism of viral escape specific to apoE, independent of TRL binding, and identify a residue in envelope glycoprotein 2 (E2) that contributes to this phenotype. These results define a novel challenge for the development of vaccines and immunopreventive approaches.

Materials and Methods

Patient Samples
Serum samples from patients with chronic HCV infection were obtained with informed consent and approval from the Strasbourg University Hospital’s Institutional Review Board (CPP 10-17). Sera termed 1, 2, and 4 came from patients...
infected by HCV genotype 1 and serum 3 from a genotype 2 patient.

Cells and Reagents

Huh7.5.1 and Huh7.5 green fluorescent protein cells were cultured as described previously.\textsuperscript{13} Silencing RNAs (siRNAs) targeting apoE 3' untranslated region (siApoE) (5' CUGCAGCGGGAGACCCUGU 3') or apoB and control siRNAs were from Dharmacon (Lafayette, CO). Mouse anti-apoE (ab8226) monoclonal antibody (mAb) and anti-β-actin (ab1906) for immunoblot were obtained from Abcam (Cambridge, MA). Mouse anti-apoE (1D7, 3H1, 6C5), rat anti-CD81 (QV-6A8-F2-C4), mouse anti-E2 (AP33),\textsuperscript{14–16} and human anti-E2 (CBH-23 and HC-1)\textsuperscript{17} mAbs have been described. Sheep anti-NS5A serum was a kind gift from M. Harris.\textsuperscript{18} Human anti-E2 antibody AR3B was a kind gift from M. Law.\textsuperscript{19} Anti-bodies and peptides for FLAG purification and detection were obtained from Sigma-Aldrich (St Louis, MO). Purification of IgG from patient serum was performed using MAbTrap kit from Amersham (Little Chalfont, UK).

Cell Culture–Derived Hepatitis C Virus Production, Infection, and Neutralization

Plasmids for cell culture–derived HCV (HCVcc) production of Jc1, J6-JFH1 chimera with luciferase reporter (Luc-Jc1), Jc1E2FLAG (all genotype 2a/2a), H77R2a (genotype 1a/2a),
VL:JFH1, and VL:JFH1FLAG (genotype 1b/2a) have been described previously.\textsuperscript{6,9,20–22} HCVcc were produced in Huh7.5.1 as described previously.\textsuperscript{20} Infectivity was quantified by luciferase activity or tissue culture infectious dose 50\% using anti-NS5A antibody.\textsuperscript{23} HCVcc neutralization using patient serum, IgG, and mAbs was analyzed as described previously.\textsuperscript{6}

**Silencing of Apolipoproteins in Hepatitis C Virus Producer Cells**

Silencing of apoE and apoB expression and immunoblotting in Huh7.5.1 cells was performed as described previously.\textsuperscript{24} Huh7.5.1 cells were either electroporated with HCV RNA co-electroporated with 200 pmol of either scrambled siRNA...
Tagged Viruses

GTCATCGTCGTCCTTGTAGTCCCCGTCAACGCCGGCAAAA3

FLAG (5

Neutralization experiments on viruses produced from these cells were performed using patient serum 1 at the indicated dilutions. Infection was determined by (A) end-point dilution assay, and (B) quantification of luciferase activity. Results are expressed as fold increase of neutralization relative to control serum. ApoB knockdown was confirmed 72 hours post electroporation by immunoblotting (lower panel); actin was detected as loading control. Means ± SEM from 3 experiments are shown.

Recombinant Adenoviruses and Rescue of Gene Silencing

The recombinant adenoviral genomes were generated as described previously.26 Recombinant adenoviruses Ad-apoE were generated by transfection of these plasmids into the 293T packaging cell line after PacI digestion.25 Huh7.5.1 cells were electroporated with HCV RNA and siApoE or a scramble siRNA (siCtrl). Twenty-four hours post electroporation, cells were transduced with adenoviruses expressing apoE. After 72 hours, viruses were harvested and tested in neutralization experiments and both silencing and rescue of protein expression was confirmed by immunoblotting.

Generation and Purification of E2-FLAG Tagged Viruses

The VL:JFH1 derivatives encoding a E2(FLAG) fusion protein were generated using overlap polymerase chain reaction (PCR) with 2 sets of primers: S_E1_AgeI_Con1 (5’ATAGTGGTCTGCGGAACCGGT3’) and A_E1_FLAG (5’CCCTTGTCATCGTCGTCCTTGTAGTCCCCGTCAACGCCGGCAAAA3’); S_E1 _FLAG (5’GGACGACGATGACAGGGATCAGGACGATCAGGACTCCATCACA CGACGCGG3’); and A_E2_AleI (5’TGTATGATGTCACCCATT3’). Amplicons were amplified by PCR from VL:JFH1derivates (VL:JFH1 or F447L or F447A), then combined by overlap PCR using S_E1_AgeI_Con1 and A_E2_AleI before insertion of the resulting fragments into the VL:JFH1 construct. E2(FLAG) encoding HCV viruses were purified using anti-FLAG M2 affinity gel, as described previously. Virion-associated apoE was detected by immunoblotting using mouse anti-apoE mAb (ab8226; Abcam), while virion E2(FLAG) was detected by anti-FLAG M2 mAb (Sigma-Aldrich). Quantification of apoE, E2(FLAG), and actin protein expression was analyzed using ImageJ software (National Institutes of Health, Bethesda, MD).

Quantification of Hepatitis C Virus Particle Buoyant Density Distribution

VL:JFH1 HCVcc virus was concentrated 10-fold using a Vivaspin column (GE Healthcare, Little Chalfont, UK). Density distributions of infectious HCVcc were determined by overlaying 0.5 mL culture media on a 5-mL, 4%–40% iodixanol step gradient, and ultracentrifuging samples for 16 hours at 40,000 rpm on a SW-55 rotor (Beckman Coulter, Brea, CA). Six hundred and twenty-five microliter fractions were carefully harvested from the top of each tube, and density was determined by weighing 0.5 mL of each fraction. Infectivity of each fraction was quantified by luciferase activity or by tissue culture infectious dose 50%.
Hepatitis C Virus Cell-to-Cell Transmission

HCV cell-to-cell transmission was assessed as described previously. Producer Huh7.5.1 cells were electroporated with Jc1 RNA or VL:JFH1 RNA. Medium was exchanged 4 hours and again 24 hours post electroporation, and naïve target Huh7.5 green fluorescent protein cells were added concomitantly with mouse nAb AP33 (10\(\mu\)g/mL) to block cell-free transmission. For analysis of the role of virus and host cell factors, cells were incubated with 10\(\mu\)g/mL of either control IgG, anti-CD81 (QV-6A8-F2-C4), or anti-apoE (1D7) antibodies. Seventy-two hours post electroporation, cells were fixed with paraformaldehyde, stained with a human anti-E2 (AR3B) antibody and analyzed via flow cytometry.

Immunoprecipitation, RNA Extraction, and Reverse Transcription Quantitative Polymerase Chain Reaction

VL/JFH1 HCVcc virus was concentrated 10-fold from cell supernatants using a Vivaspin column (GE Healthcare) and immunoprecipitations were performed using Protein A/G PLUS-agarose beads according to the manufacturer instructions (Santa Cruz Biotechnology, Santa Cruz, CA). HCV RNA from immunoprecipitated viruses was isolated using RNeasy extraction (Qiagen, Valencia, CA) and quantified using reverse transcription quantitative PCR as described previously. E2-apoE co-immunoprecipitation using lysates of Huh7/LunetCD81H cells stably expressing ApoEWT or ApoEHA and transfected with VL/JFH1 or the 447 mutant were performed as described elsewhere. Immunoprecipitation of apoE from Huh7.5.1 cells was performed as described previously.

Statistics

Datasets were analyzed using the Mann–Whitney test.

Results

Because apoE has been shown to be part of the HCV infectious virion mediating viral attachment, we investigated whether particle-associated apoE influences viral evasion from host nAbs. To experimentally probe this question, we used 3 different HCV strains comprising genotypes 1a, 1b, and 2a. We first studied HCV strain JFH1 carrying the structural genes of a patient-derived viral variant termed VL. We had previously shown that this genotype 1b variant is selected during liver graft infection due to envelope-mediated enhanced viral entry and broad escape from patient-derived

Figure 5. ApoE depletion in HCV producer cells does not modulate the buoyant density profile of virions. Huh7.5.1 cells were either electroporated with VL/JFH1 RNA or Luc-Jc1 alone (black squares and black circles, respectively), or co-electroporated with either scrambled siRNA (siCtrl) (dark gray squares and dark gray circles) or siRNA targeting apoE mRNA (siApoE) (light gray squares and light gray circles). HCVcc produced from these cells were fractionated using 4%–40% iodixanol density gradient ultracentrifugation. Each fraction was assayed for infectivity by (A) end-point dilution assay (log10 tissue culture infectious dose 50%/mL) for VL/JFH1 or (B) quantification of luciferase activity (log10 RLU) for Luc-Jc1. Mean ± SEM from 3 experiments are shown. The light gray circles on the density plot show the mean density for each fraction and the error bars indicate the SD of the density of the respective fraction from 6 independent experiments. ApoE knockdown was confirmed 72 hours post-electroporation by immunoblotting (lower panels); actin was detected as loading control.
autologous and heterologous nAbs.\textsuperscript{5,6} Thus, it represents a prototype variant that may be largely refractory to immunopreventive approaches or vaccines. We first generated VL:JFH1 HCVcc by cotransfecting HCV RNA alone, with a scrambled nucleotide control (siCtrl), or with siRNA that targets apoE expression (siApoE). Similar to the range reported previously,\textsuperscript{24,27,28} silencing of apoE expression in Huh7.5.1 producer cells resulted in a marked (50\%–70\%) and significant decrease ($P < .001$) in infectivity of viruses relative to viruses with unchanged apoE expression. Exposure of VL:JFH1 HCVcc produced from apoE-depleted cells to multiple patient sera with chronic HCV infection modified these HCVcc to be highly sensitive to nAbs (Figure 1A). Depending on the patient serum and the dilution used, viruses produced from apoE-depleted cells were 4–17 times more sensitive to neutralization by patient sera than viruses from

**Figure 6.** ApoE and cell-to-cell transmission of HCV. (A) Interference of cell-to-cell transmission of Jc1 and VL:JFH1 by anti-apoE antibody 1D7, nonspecific IgG as a negative control (Ctrl), or anti-CD81 antibody as a positive control was tested. For cell-to-cell transmission, green fluorescent protein–expressing naïve target cells were co-cultured with cells containing HCV with neutralizing mouse anti-E2 antibody AP33. Representative results of fluorescence-activated cell sorting analysis are shown. The quantity of positively stained cells for HCV E2 (y-axis), and green fluorescent protein–labeled target cells (x-axis) are represented. (B) Histograms of cell-to-cell transmission for Jc1 and VL:JFH1 summarized from 3 independent experiments performed in duplicate are shown: Values represent percentage of cell-to-cell transmission relative to control. (C) The capacity of antibodies to inhibit infection was tested by inoculation of naïve Huh7.5.1 cells with HCV in the presence of antibodies specified below the plot. Values were normalized to those obtained with nonspecific IgG (Ctrl). Mean ± SD from 3 experiments are shown. *$P < .05$. 
control cells with unchanged apoE expression (Figure 1A). To corroborate these findings, we studied the phenotype of 2 additional variants, H77R2a expressing the structural genes of the HCV strain H77 (genotype 1a) and J6:JFH1 prototype Luc-Jc1 expressing the structural genes of the genotype 2 HCV strain J6. Similar to findings obtained for VL:JFH1 viruses, apoE depletion resulted in enhanced sensitivity to neutralization by patient sera (Figure 1B and C).
Figure 7. Envelope glycoprotein E2 residue 447 and apoE-HCV interactions. (A) Residue 447 is relevant for inhibition of HCV infection by anti-apoE antibodies. VL:JFH1 and 447 variant HCVcc were incubated with control IgG (Ctrl) or 1D7 antibody prior to infection of Huh7.5.1 cells. Infectivity was assessed by tissue culture infectious dose 50%. Results are expressed as percentage infectivity relative to Ctrl. Mean ± SEM from 4 experiments are shown. *P < .05. (B) Residue 447 modulates immunoprecipitation of virions by anti-apoE antibodies. VL:JFH1 and 447 mutants HCVcc were immunoprecipitated using non-specific mouse IgG (Ctrl), or 3 different anti-apoE antibodies (1D7, 3H1, 6C5). HCV RNA was extracted from precipitates and quantified by reverse transcription quantitative polymerase chain reaction (RT-qPCR). Results are expressed as fold increase of HCV RNA immunoprecipitated relative to control IgG. Means ± SEMs from 2 experiments are shown. The viral input was quantified by immunoblot evaluating the amount of core protein in the culture media (lower panel). (C) ApoE depletion in HCV producer cells impairs immunoprecipitation of virions by anti-apoE antibody. After immunoprecipitation of virions in HCV producer cells using control IgG or anti-apoE antibody (1D7), HCV RNA was quantified by RT-qPCR. Results are expressed as normalized HCV RNA. Mean ± SEM from three experiments are shown. *P < .01. (D) Intracellular interaction of apoE and E2. Huh7-derived cells stably expressing ApoEWT or ApoEHA were electroporated with RNA genomes of VL:JFH1 or the F447L mutant and lysed 72 hours later. Immunoprecipitation was performed with a hemagglutinin-specific antibody and captured complexes were analyzed by immunoblotting using anti-E2 antibodies. To determine capture efficiency, 0.5% of total cell lysates used for immunoprecipitation were analyzed in parallel (input). One representative immunoblot is shown. (E) ApoE and E2 associations in purified virions of wild-type and mutant viruses. Virions were immunopurified from supernatants of HCV producer cells using an anti-FLAG antibody. Virion-associated proteins were analyzed by immunoblots using FLAG and apoE-specific antibodies. Results of 2 independent purifications and immunoblots are shown.
apoE association. If apoE protects HCV glycoproteins from nAbs directly, then our observations regarding the nAb-sensitivity of HCV generated from apoE knockdown cells will not be mediated by a marked difference in buoyant density distribution. To test this hypothesis, we analyzed the buoyant density distribution of infected particles using isopycnic density gradient ultracentrifugation. Interestingly, the density distribution of VL:JFH1 and Luc-Jc1 was unchanged after apoE knockdown, though infectivity was diminished approximately equally in each density fraction in both VL:JFH1 and Luc-Jc1 viruses (Figure 5A and B). These data suggest that apoE knockdown does not modulate HCV-TRL association, and apoE directly associated with the virus accounts for nAb evasion.

Cell-to-cell transmission is another mechanism to avoid nAbs. To investigate whether apoE association is relevant in cell-to-cell transmission, we used an anti-apoE antibody (1D7) that binds the low-density lipoprotein receptor–binding domain of apoE and inhibits HCV infection (Figure 7A). Jc1 demonstrated an approximately 3-fold higher capacity to spread by direct cell-to-cell transmission than VL:JFH1 in this assay (Figure 6A). While 1D7 did not affect cell-to-cell spread, despite its capacity to strongly inhibit extracellular transmission, antibodies recognizing HCV entry factor CD81 inhibited 70%–80% of cell-to-cell transmission of both Jc1 and VL:JFH1 (Figure 6A, B, and C). These data indicate that cell-cell transmission is not mediated by apoE domains targeted by anti-apoE antibody 1D7.

Recently, 2 studies demonstrated that apoE interacts with HCV E2. To define E2 domains relevant for association with apoE and neutralization escape, we took advantage of a mutation identified in variant A (VA), a pre-transplant variant that was selected against in the same transplant recipient that contained the predominant VL variant. Indeed, replacing phenylalanine with the leucine encoded in VA rendered the virus both sensitive to neutralization and less dependent on CD81 for cell entry. To determine whether this residue is involved in the differential utilization of apoE, we used the previously described mutant restoring neutralization escape (F447L), as well as an additional mutant where phenylalanine is replaced by alanine (F447A), a smaller amino acid residue than leucine. Interestingly, the wild-type VL:JFH1 escape variant was efficiently neutralized by anti-apoE 1D7, while the F447L and F447A mutants were at least 3-fold less sensitive to neutralization by this antibody, indicating that the wild-type virus is more dependent on apoE for infection (Figure 7A). In contrast, 2 other apoE-specific antibodies (3H1 and 6C5) that bind the N- and C-terminal regions of apoE, respectively, did not inhibit HCV infection to the same degree (data not shown) despite their capacity to immunoprecipitate apoE from cell lysates (Supplementary Figure 5). To determine if this modulation might be due to altered apoE association with virus particles, we immunoprecipitated VL:JFH1 with either nonspecific mouse IgG or with each of the three apoE-specific antibodies 1D7, 3H1, or 6C5. Interestingly, all 3 anti-apoE antibodies were at least 2.5-fold more efficient at precipitating the VL:JFH1 than the 447 mutants (Figure 7B), suggesting a more robust association of the escape variant with apoE or a different E2-apoE conformation with different exposure of these epitopes on this variant. The functional relevance of apoE for immunoprecipitation of virions was confirmed by less efficient immunoprecipitation of HCV RNA by anti-apoE using VL:JFH1 and Jc1 viruses produced in apoE-depleted cells (Figure 7C and data not shown).

To investigate whether the difference in sensitivity and pull-down of VL:JFH1 447 mutants by anti-apoE antibodies was due to an altered association of apoE and virion E2 or mediated by a different conformation of the apoE-E2 complex, we performed co-immunoprecipitation analyses in cell lysates of HCV producer cells. We observed similar intra-cellular apoE-E2 binding in wild-type and mutant viruses (Figure 7D). Next, we determined the amount of apoE incorporated into virions by using immunopurified E2FLAG-tagged viruses. We found that FLAG-tagged wild-type and F447L mutant viruses contained similar levels of apoE (Figure 7E). These data indicate that residue 447 alters the conformation of the virion E2-apoE complex without changing apoE content of the particles. Collectively, our data indicate that both the level (Figures 1–3), as well as the conformation (Figure 7) of virion apoE are relevant for the evasion from nAbs.

**Discussion**

Here we present experimental evidence indicating that apoE levels in HCV-producing cells determine HCV’s capacity to avoid the effect of nAbs through modifying incorporation of apoE into the viral particle. These results were obtained using structural genes from 3 different viral strains from genotypes 1a, 1b, and 2a, sera from multiple patients as well as patient-derived purified IgG and mAbs, indicating that utilization of the host factor apoE as a mechanism to escape from nAbs is pan-genotypic. While the utilization of apoE is consistently employed, it was most prominently observed in both genotype 1 variants H77 and VL, a clinically derived variant previously characterized as effective at nAb escape, highlighting that apoE “shielding” may be employed by the most difficult to neutralize variants. These differences of neutralization profiles between H77 or VL:JFH1 and Jc1 may be partially explained by a more efficient depletion of apoE in VL:JFH1 compared to Jc1 as shown in Figure 3. Alternatively, these results may point to genotype- or isolate-dependent differences in utilization of apoE and neutralization escape. Finally, the differences may be due to different cross-reactivity of the patient sera used.

We further confirmed the role of apoE through observing altered apoE:E2 ratios on purified virions after silencing (Figure 3) and trans-complementing apoE expression after knockdown, which rescued HCV’s capacity to avoid inhibition of HCV entry by nAbs present in serum of chronically infected patients (Figure 2A). However, down-regulation of apoE expression did not affect HCV-TRL association (Figure 5), indicating that the apoE-mediated mechanism of nAb escape is distinct from the previously described role of TRL in escape.
Using an anti-apoE antibody that blocks extracellular transmission, we did not detect a role of apoE in cell-to-cell transmission of Jc1, VL:JFH1, as well as the 2 VL:JFH1 variants (Supplementary Figure 6). This is consistent with the report from Barretto et al.33 Engineering nonhepatic cells to produce HCV through ectopic apoE expression can confer cell-to-cell transmission capacity, albeit to a limited degree relative to the levels observed in the current study.34 We thus cannot exclude that epitopes not recognized by the utilized anti-apoE antibody play a role in cell-to-cell transmission or that this antibody may have limited access to the virus during this process.

In variants that were selected during liver transplantation, replacement of phenylalanine at E2 residue 447 appeared to alter the immunoprecipitation of virions by anti-apoE antibodies (Figure 7B). Given the comparable level of intracellular E2-apoE association (Figure 7D) and apoE incorporation into virions (Figure 7E) of the wild-type and the mutant, mutations of E2 residue 447 appear to induce conformational changes in the E2-apoE complex affecting the sensitivity to neutralization by both apoE- and E2-specific nAbs (Figures 1, 2, and 7). Collectively our data indicate that virion apoE levels (Figures 1–3) and conformation (Figure 7) are relevant for the evasion from nAbs.

ApoE plays a key role in viral attachment to heparan sulfate proteoglycans,25,35 whereas subsequent steps of viral entry require E2-SR-BI and E2-CD81 interactions (reviewed in Zeisel et al.36). Thus, dissociation of apoE and/or alteration of the apoE-E2 complex might be required for envelope-SR-BI and CD81 interactions occurring post binding. Supporting this model, our results demonstrate that conformational human mAbs CBH-23 and HC-1, which impair HCV entry by interfering with E2–CD81 interactions and act at a post-binding step,37 more effectively neutralize apoE-depleted HCV.

HCV association with very-low-density lipoprotein during viral production was hypothesized to block nAbs-envelope glycoprotein binding and confer viral resistance to nAbs. Our findings point to apoE shielding of E2 from neutralization, independently from association with lipoproteins as measured by density distribution. This new role of apoE is consistent with our previous observations showing that nonhepatic cell lines lacking very-low-density lipoprotein—producing components and engineered to express apoE can sustain the entire HCV life cycle and produce viruses with buoyant density profiles similar to virus produced in hepatoma cells.38

Collectively, we demonstrate that apart from lipoprotein association, apoE mediates escape from nAbs. This finding reveals a novel strategy contributing to HCV's remarkable capacity to establish chronic infection. According to the core crystal structure of E2,38,39 residue 447 that appears to alter the conformation of the E2-apoE complex resides on the periphery of a nonstructured E2 region and, thus, would be an ideal candidate for association with exposed apoE regions. This finding might be relevant for vaccine design and we assume that immunogens that mimic epitopes at the E2/apoE interface might help to achieve a broadly neutralizing humoral immune response.

### Supplementary Material

Note: To access the supplementary material accompanying this article, visit the online version of *Gastroenterology* at www.gastrojournal.org, and at http://dx.doi.org/10.1053/j.gastro.2015.09.014.

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Supplementary Figure 1. Increased nAb sensitivity of apoE-depleted HCV is not due to decreased virus quantity or the ratio of infectious virions and nAbs. Huh7.5.1 cells were electroporated with Luc-Jc1 RNA alone, or co-electroporated with either scrambled siRNA (siCtrl) or siRNA targeting apoE mRNA (siApoE). Two experiments using different HCV viral titers (high viral titer vs low viral titer) were performed to study the impact of virus quantity on the sensitivity to patient serum. Results of neutralization assay of HCV produced from these cells using serum from a patient without HCV infection (Ctrl) or indicated dilution of serum from a patient with genotype 1 HCV infection was determined by quantification of luciferase activity (log_{10} RLU). Mean ± SEM of both experiments performed in triplicate are shown.
Supplementary Figure 2. Adjustment of input virus titers of viruses produced from control and apoE-depleted cells confirms association of virion apoE content and sensitivity to antibody-mediated neutralization. Huh7.5.1 cells were electroporated with H77R2a (A–C) or Luc-Jc1 (D–F) RNA alone, or co-electroporated with either scrambled siRNA (siCtrl) or siRNA targeting apoE mRNA (siApoE). (A, D) ApoE depletion in producer cells was confirmed 72 hours post electroporation by immunoblotting of cell lysates; actin was detected as loading control (right panels). (B, E) Titers of viruses produced in supernatants of transfected Huh7.5.1 cells (72 hours post electroporation) were determined by tissue culture infectious dose 50% (TCID50) using infection of naïve Huh7.5.1 for 72 hours as described in the Materials and Methods. After quantification of viral titers, titers were adjusted by dilution in cell culture medium. Titers of viruses from apoE-depleted cells served as the reference. Viral titers before and after dilution are shown as TCID50 of a representative titration analysis. (C, F) Neutralization experiments using viruses with adjusted titers shown in (B, E) were performed using sera from 4 different HCV-infected patients (1, 2, 3 or 4) as described in Figure 1. Means ± SDs from one representative experiment performed in duplicate are shown. Results are expressed as fold increase of neutralization relative to control serum.
Supplementary Figure 3. Inhibition of viruses produced from apoE silenced producer cells by anti-apoE or anti-CD81 antibodies. Huh7.5.1 cells were electroporated with VL:JFH1 alone, or co-electroporated with either scrambled siRNA (siCtrl) or siRNA targeting apoE mRNA (siApoE). To assess the role of CD81, cells were pretreated with 10 μg/mL anti-CD81 antibody for 30 minutes before HCVcc infection of Huh7.5.1 cells. To assess the role of apoE, HCVcc were preincubated with 10 μg/mL anti-apoE antibody (1D7) for 1 hour before infection of Huh7.5.1 cells. Infection was determined by end-point dilution assay and expressed as percentage of infection relative to control antibodies. Mean ± SEM from 4 independent experiments are shown. *P < .05.

Supplementary Figure 4. Silencing of apoE expression in HCV producer cells has no effect on apoB expression and vice versa. Huh7.5.1 cells were electroporated with Luc-Jc1 RNA alone, or co-electroporated with either scrambled siRNA (siCtrl) or siRNA targeting either apoE mRNA (siApoE) or apoB mRNA (siApoB). After 72 hours, apoB and apoE expression was quantified using immunoblots of cell lysates and anti-apoE and apoB specific antibodies; actin expression was analyzed as control. A representative immunoblot is shown.
Supplementary Figure 5. Immunoprecipitation of apoE using anti-apoE antibodies 1D7, 3H1 and 6C5 in Huh7.5.1 cell lysates. apoE was immunoprecipitated from cell lysates as described in Materials and Methods and apoE on immunoprecipitated beads was subjected to immunoblot using anti-apoE antibody (Abcam). Isotype IgG served as negative control for immunoprecipitation. Two independent experiments have been performed. A representative immunoblot is shown.

Supplementary Figure 6. Absent effect of anti-apoE antibody 1D7 on the cell-to-cell transmission of VL:JFH1 F447L and F447A mutants. Interference of cell-to-cell transmission of VL:JFH1 F447L and F447A by anti-apoE antibody 1D7, nonspecific IgG as a negative control (Ctrl), or anti-CD81 as a positive control was tested. For cell-to-cell transmission, green fluorescent protein-expressing naïve target cells were co-cultured with cells containing HCV with neutralizing antibody AP33. Histograms of cell-to-cell transmission summarized from 2 independent experiments performed in duplicate are shown. Values represent percentage of cell-to-cell transmission relative to control. P < .05.