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Exosome-associated hepatitis C virus in cell cultures and patient plasma

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ABSTRACT

Hepatitis C virus (HCV) infects its target cells in the form of cell-free viruses and through cell–cell contact. Here we report that HCV is associated with exosomes. Using highly purified exosomes and transmission electron microscopic imaging, we demonstrated that HCV occurred in both exosome-free and exosome-associated forms. Exosome-associated HCV was infectious and resistant to neutralization by an anti-HCV neutralizing antibody. There were more exosome-associated HCV than exosome-free HCV detected in the plasma of HCV-infected patients. These results suggest exosome-associated HCV as an alternative form for HCV infection and transmission.

1. Introduction

HCV is an enveloped RNA virus with a diameter of 50–65 nm [1,2]. Exosomes are extracellular vesicles of about 30–100 nm and play important roles in intercellular communications via delivery of cargos such as proteins and RNAs [3–6]. Several studies suggest potential roles of exosomes and their biogenesis pathways in HCV infection. CD81, one of the exosome markers and also one of the HCV entry receptors, regulates the secretion of HCV envelope proteins in the form of exosomes [7]. Endosomal sorting complex responsible for transport (ESCRT)-0 components are involved in HCV budding [8]. In addition, HCV RNA is detected in exosomes and is capable of eliciting innate immune response in dendritic cells [9]. Moreover, a recent cryo-electron microscopic study reveals that a small fraction of cell culture-produced HCV particles are surrounded by an additional layer of "envelope", although the biological nature of this extra "envelope" is not defined [10]. Thus, in this study, we took advantage of a widely established centrifugation-based exosome purification strategy and characterized the relationship between HCV and exosomes in both the cell-culture setting and HCV patient plasma.

2. Materials and methods

2.1. Cells and reagents

Huh7.5.1 were obtained from Dr. Charles Rice's laboratory of Rockefeller University, New York and maintained in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin/L-glutamine. The sources of antibodies used in the study were: mouse anti-HCV core (clone C7-50, Santa Cruz, CA), mouse anti-β actin (A1978, Sigma, St. Louis, MO), mouse anti-Hsp 70 (clone 3A3, Santa Cruz), goat anti-E2 (Meridian Life Science Inc, Memphis, TN), rabbit anti-CD63 (Systems Biosciences, Mountain View, CA), goat anti-rabbit IgG-HRP (Systems Biosciences), goat anti-rabbit IgG-Alexa555 (Invitrogen), sheep anti-mouse IgG-HRP (Sigma), and donkey anti-goat IgG-HRP (Sigma).

2.2. HCV production, titration and infection

HCV viral stock was prepared and titrated with foci-forming assay as previously described [11]. Briefly, 10-fold serially diluted stock was added to Huh7.5.1 and the medium was changed after two hours. Immunostaining against HCV core was performed at 72 h post infection. The number of foci formed at the highest dilution was used to calculate the virus titer, which was expressed as focus-forming units per milliliter (FFU/ml). The titers of the JFH1 viral stock were usually in a range of 10^4 to 10^6 FFU/ml. Unless stated otherwise, HCV infection was performed by incubation of viral

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stock with target cells for 2 h at 37 °C at a multiplicity of infection of 0.1.

2.3. Preparation of bovine exosome-depleted medium and bovine exosome-depleted HCV

Bovine exosomes were depleted from complete culture medium (DMEM supplemented with 10% FBS and 1% penicillin/streptomycin/L-glutamine) by ultracentrifugation at 4 °C and 100,000*g* overnight (~16 h). The supernatants were collected and if needed, sterilized by passing through 0.22 μm filter and stored at 4 °C as bovine exosome-depleted medium. For the production of bovine exosome-depleted HCV, Huh7.5.1 was inoculated with JFH1 viral stock and cultured for 3–4 days. Culture medium was then changed to bovine-exosome depleted medium; the cells were continued to culture for 24 h and the culture supernatants were collected and saved as bovine exosome-depleted HCV.

2.4. Exosome and HCV purification by Opti-prep gradient ultracentrifugation

Huh7.5.1 culture supernatants (30–400 ml) were removed of cell debris by centrifugation at 3000*g* for 10 min, passed through a 0.22 μm filter, and concentrated by ultracentrifugation at 250,000*g* and 4 °C for 90 min in a SW55Ti rotor or 130,000*g* and 4 °C for 90 min in a SW28 rotor to obtain the exosome pellet. Meanwhile, a 5 ml 6–24% Opti-prep gradient was prepared with the Hoefer SG50 gradient maker. Specifically, 3 ml each of 2.4% and 24% iodixanol working solutions were prepared from the Opti-prep density gradient medium (60% w/ v iodixanol in water) with the Opti-prep diluent (235 mM KCl, 12 mM $MgCl_2$, 25 mM $CaCl_2$, 30 mM EGTA, 150 mM Hepes-NaOH, pH 7.0). The two 3 ml working solutions were loaded into the two chambers of the gradient maker, respectively. A 5 ml 6–24% iodixanol continuous gradient was then generated with the gradient maker according to manufacturer's instructions. The exosome pellet was suspended in 500 μl PBS, loaded onto the Opti-prep gradient and centrifuged at 250,000*g* and 4 °C for 2 h in the SW55Ti rotor. Eleven fractions (500 μl each) were collected from top to bottom; an aliquot of each fraction was subjected to AchE assay, Western blotting, qRT-PCR, or foci formation assay.

2.5. HCV patient plasma samples

Plasma from chronic hepatitis C patients were collected in heparinized vials (15 U heparin/ml) and frozen immediately in −70 °C. All plasma samples were treated with Heparinase I (6 U/140 μl plasma) with the presence of RNase inhibitor (20 U/140 μl plasma) at 30 °C for 2–3 h before any further processing or assays.

2.6. Acetylcholinesterase (AchE) assay

AchE activity was measured as described previously [12]. Briefly, 5 μl culture supernatant or iodixanol fraction was mixed with 12.5 μl 10 mM acetylthiocholine and 82.5 μl PBS, and then 100 μl 0.1 mM DTBNBA/PBS in a 96-well plate. The reaction was incubated at room temperature or 37 °C for 5–30 min until a yellow color appeared, at which point the absorbance of the reaction at 415 nm was taken using a multi-well spectrophotometer (Bio-Rad).

2.7. Western blotting

Cell lysates were prepared using a RIPA buffer (50 mM Tris–HCl, pH 8.0, 0.5% NP40, 2 mM EDTA, 137 mM NaCl, and 10% glycerol) containing protease inhibitor cocktail (Roche, Indianapolis, IN) and PMSF. Protein concentration of the lysates was determined with the Bio-Rad DC Protein Assay (Bio-Rad Laboratories). Proteins (20–100 μg) were separated on a 12% polyacrylamide–SDS gel. The proteins were transferred onto nitrocellulose membranes and probed with appropriate primary antibodies (1:200 for mouse α-HCV core/Hsp70, 1:500 for goat α-HCV E2, 1:1000 for rabbit α-CD63, and 1:2000 for mouse α-β-actin) and secondary antibodies (1:2000). The protein bands were visualized by either adding homemade enhanced chemiluminescence reagents (for HRP-conjugated secondary antibodies) and imaging, or direct imaging for fluorescence intensity (for Alexa dyes-conjugated secondary antibodies) with a Bio-Rad ChemiDot MD system. For Western blotting of exosome and/or virus fractions from Optiprep gradient centrifugation, the fraction was diluted to 1.4 ml with PBS and centrifuged at 4 °C and 20,000*g* for 90 min; the resulting pellet was suspended and boiled in 20 μl 4X SDS–PAGE sample buffer (8% SDS, 0.4 M DTT, 0.25 M Tris. HCl, pH 6.8, 40% glycerol and 0.1% bromophenol blue). Alternatively, proteins in those fractions were precipitated by TCA as described [13] and the resulting pellets were suspended in the same SDS–PAGE sample buffer, followed by Western blotting as described above.

2.8. RNA extraction and qRT-PCR

For the detection of cell-culture produced HCV, total RNA was isolated from Optiprep gradient fractions (5–50 μl) using TRizol LS reagent according to the manufacturer's instructions (Invitrogen). The isolated RNA was used for qRT-PCR as previously described [11]. For the detection of patient plasma-derived HCV, if the sample was more than 140 μl, it was first concentrated to 140 μl with Amicon Ultracel-100 K centrifugal filter device by centrifugation at 4000*g* and 4 °C for 25 min. Total RNA was then extracted from the samples with the QIAamp Viral RNA Mini Kit (QIAGEN) and eluted with 60 μl AVE buffer. The isolated RNA was used for qRT-PCR as described above except that the reverse primer was changed to: 5′-CAC TCG CAA GCA CCC TAT CA-3′.

2.9. Electron microscopic imaging

Exosomes and HCV were produced, purified, concentrated and fractionated as described above. Fractionated samples were diluted 9-fold with PBS, concentrated by centrifugation at 4 °C and 130,000*g* for 90 min, and suspended in 40 μl PBS. An aliquot of the sample was fixed by addition of an equal volume of 8% paraformaldehyde/PBS. The fixed sample was then embedded, sectioned, negatively stained, and examined by electron microscopic imaging at Robert P. Apkarian Integrated Microscopy Center of Emory University School of Medicine.

2.10. Data analysis

Where appropriate, values were expressed as mean ± SD of triplicate experiments. All data were representative of multiple independent experiments.

3. Results and discussion

3.1. HCV infection did not alter exosome secretion in hepatocytes

To understand the relationships between HCV and exosomes, we first determined whether HCV infection affected exosome secretion. We infected Huh7.5.1 cells with cell culture-produced HCV JFH1 and monitored exosome secretion in the culture supernatants. HCV infection was confirmed by immunoblotting for HCV core expression in the cells (Fig. 1A). Exosome secretion was

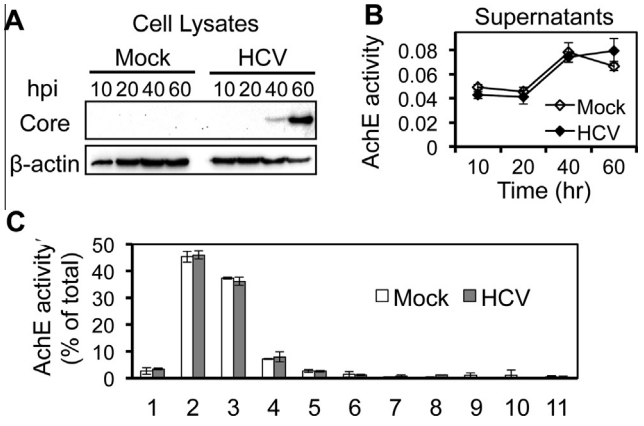

Fig. 1. HCV infection does not affect exosome production. (A and B) Huh7.5.1 cells were infected with HCV for 2 h, removed of unbound viruses through extensive PBS wash, and then added with the complete culture medium. Conditioned medium (Mock) was used as a control. Cells were cultured for indicated lengths of time and then harvested for Western blotting analysis (A), while the cell culture supernatants were collected for the AchE activity assay (B). (C) The culture supernatants were collected on day 3 post infection, removed of cell debris by centrifugation at 3000*g* for 10 min, passed through a 0.22 μm filter, and concentrated by ultracentrifugation at 250,000*g* and 4 °C for 90 min. The pellet was suspended in 500 μl PBS, loaded onto a 5 ml 6–18% iodixanol gradient and fractionated by ultracentrifugation at 250,000*g* and 4 °C for 90 min in a SW55Ti rotor. Eleven fractions (500 μl each) were collected from top to bottom of the gradient; 15 μl of each fraction was used for AchE assay; data were expressed as % of total AchE activity in the gradient. The data were mean ± SD from triplicate and representative of two independent experiments.

determined by measuring the activity of acetylcholinesterase (AchE), an exosome marker [12,14], as previously described [12]. No apparent differences of exosome secretion were detected between HCV-infected cells and mock-infected control through 60 h post HCV infection (Fig. 1B). We next determined whether HCV infection affected the size distribution of exosomes. Concentrated exosomes were prepared from the culture supernatants of Mock- or HCV-infected Huh7.5.1 and then fractionated through an iodixanol density gradient by ultracentrifugation. AchE activity assay of each fraction showed similar distribution patterns of exosomes from Mock- and HCV-infected cells (Fig. 1C). These results indicate that HCV infection does not result in significant changes in exosome secretion from hepatocytes.

3.2. Association of HCV with exosomes

We then took advantage of the well-established three-step centrifugation-based exosome purification strategy [12] to determine the relationship between exosomes and HCV. This strategy includes the last step of 6–24% iodixanol density gradient ultracentrifugation, followed by fractionations. The AchE activity of each fraction exhibited a typical pattern of exosome distribution (bar graph, Fig. 2A). In addition to the AchE activity assay, HCV RNA was isolated from each fraction and quantified using qRT-PCR. In contrast to the single peak of exosomes, HCV RNA distribution exhibited two distinct peaks: one minor one that was co-fractionated with exosomes (fraction 2–4, line graph, Fig. 2A), and one major one that was absent of exosomes (fraction 7–10, line graph, Fig. 2A). Western blotting of all fractions was performed against two other exosome markers Hsp70 and CD63 as well as HCV E2 and Core proteins. As expected, Hsp70 and CD63 were only detected in the exosome fractions (Fig. 2B). Consistent with the data of the HCV RNA qRT-PCR (Fig. 2A), HCV E2 and Core proteins were detected in both exosome fractions (fraction 2–4, Fig. 2B) and exosome-free fractions (fraction 7–10, Fig. 2B). Quantitative analyses of the Western blots indicated that about 10% out of total HCV RNA, core protein and E2 protein were detected in exosome fractions. We also purified exosomes using other published exosome purification protocols including use of PEG 8000 exosome/HCV precipitation, or a vertical rotor for gradient ultracentrifuge and determined HCV association with exosomes. Similar results were obtained (data not shown). It was noted that the E2 protein in exosome fractions appeared to be 20 KD larger than the one of the expected size detected in exosome-free HCV alone fractions. This

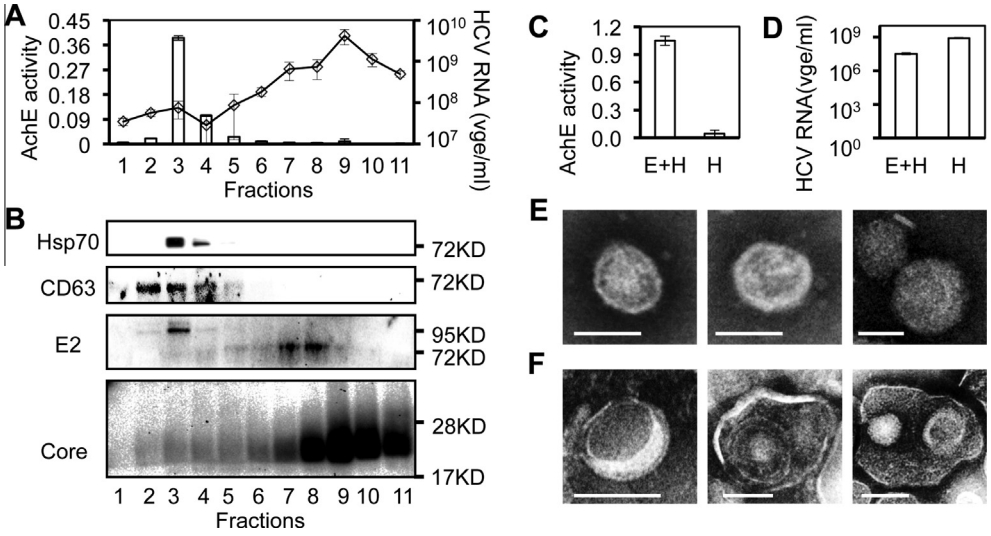

Fig. 2. Detection of exosome-associated HCV. Huh7.5.1 were infected with HCV, the culture supernatants were produced, processed and fractionated as described above except that a 6–24% iodixanol gradient was centrifuged for 2 h to accommodate the density of HCV. Eleven fractions, each with 500 μl, were collected from top to bottom of the gradient; aliquots of each fraction were used for the AchE activity assay (bar graph, A), RNA isolation for qRT-PCR (line graph, B, expressed as viral genome equivalent (vge) per ml of iodixanol gradient), or protein isolation for Western blotting (B). (C–F) Fractions 2–4 were pooled as exosome-associated HCV fractions (E+H), while fractions 7–10 were pooled as exosome-free HCV fractions (H). These two pooled fractions were subjected to the AchE activity assay (C), RNA isolation, followed by qRT-PCR for HCV RNA (D), or embedding, sectioning, negative staining, and TEM imaging (E, F). (E) H sample, scale bar = 50 nm. F, E+H sample, scale bar = 100 nm. The data were mean ± SD from triplicate and representative of three independent experiments.

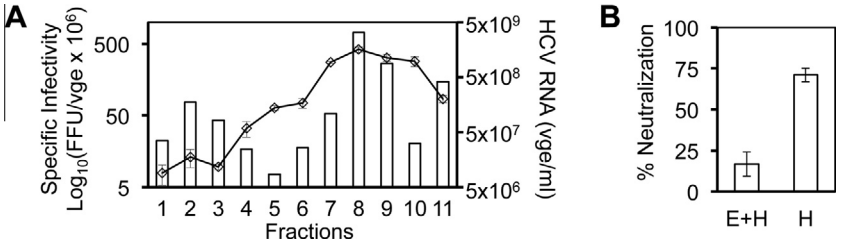

Fig. 3. The specific infectivity and resistance to neutralizing antibody of exosome-associated HCV. Huh7.5.1 were infected with HCV, the culture supernatants were produced, processed and fractionated as described above. Aliquots of each fraction were assayed for the HCV infectivity by serial dilution followed by foci formation assay (bar graph, A) and qRT-PCR for HCV RNA (line graph, A). The specific infectivity was calculated as focus formation units (FFU) per vge, i.e., FFU/vge. The rest of each fraction was pooled as described in Fig. 2 to obtain E+H and H samples. The foci formation assay was performed with these two samples in the presence of 5 μg/ml human anti HCV E2 neutralizing antibody CBH-5 or a control antibody R04 (B). The % neutralization was calculated as (foci number in CBH-5 treatment/foci number in R04 treatment) %. The data were mean ± SD from triplicate and representative of two independent experiments.

is likely due to additional post-translational modifications such as glycosylation that might have occurred to the exosome form of E2 [7,15].

To understand the nature of the association of HCV with exosome, we then performed transmission electron microscopic imaging for exosome-associated and exosome-free HCV purified as above. After gradient ultracentrifugation, we combined exosome fractions (fraction 2–4, Fig. 2A) and exosome-free fractions (fraction 7–10, Fig. 2A), which were designated as E+H and H, respectively. The AchE activity assay (Fig. 2C) of these two pooled fractions confirmed the successful separation of exosome-associated fractions from exosome-free fractions. HCV RNA quantitation detected HCV RNA in both pooled fractions (Fig. 2D). The E+H and H samples were then processed for transmission electron microscopic imaging (TEM). The exosome-free H sample contained only HCV virions that were mostly 50–65 nm but occasionally up to 100 nm in size (Fig. 2E). In contrast, the E+H sample contained exosomes of 100 nm or larger, where one or more HCV virions were detected inside (Fig. 2F). Taken together, these TEM images provide direct evidence that HCV virions reside inside a fraction of exosomes.

3.3. Exosome-associated HCV was infectious and resistant to neutralization by anti-HCV neutralizing antibodies

To determine whether exosome-associated HCV would be infectious, we performed the HCV focus formation assay for all ultracentrifugation fractions obtained from cell-culture produced HCV as described previously [11]. Consistent with the above findings, HCV RNA exhibited a minor exosome-associated peak and a major exosome-free peak (line graph, Fig. 3A). Meanwhile, the focus formation assay showed two peaks of the number of focus formation (bar graph, Fig. 3A), expressed as the specific infectivity, focus formation units per viral genome equivalent (FFU/vge), corresponding to those two peaks of the HCV RNA. The specific infectivity of the exosome-associated HCV appeared to be about 10-fold lower than that of the exosome-free HCV. These results suggest that exosome-associated HCV was infectious but exhibited a lower specific infectivity than the exosome-free HCV. The lower specific infectivity of the exosome-associated HCV may be due to the spatial hindrance of the larger E2 (Fig. 2B), the presence of HCV RNA-containing exosomes [9], and low affinity of the associated HCV to target cells. To determine whether exosome-associated HCV is resistant to neutralization by anti-HCV neutralizing antibody, we performed a neutralization assay with the anti-HCV neutralizing antibody CBH-5 that has been shown to be very efficient in blocking cell-free HCV infection [16,17]. We obtained exosome-associated (E+H) and exosome-free HCV (H) as described above and performed the focus formation assay in the presence of CBH-5 or its isotope-matched control antibody R04. As expected, CBH-5 neutralized 71% of the infectivity of H sample, but only neutralized 17% of E+H sample's infectivity (Fig. 3B). Taken together, exosome-associated HCV was infectious to hepatocytes and more resistant to neutralization by anti-HCV neutralizing antibody than exosome-free HCV.

3.4. Exosome-associated HCV in HCV-positive patient plasma

To ensure that the HCV association with exosomes is not an artifact of the cell-culture produced HCV, we performed similar experiments with HCV-positive patient plasma. Plasma from five chronic hepatitis C patients (P1 to P5, all HCV genotype 1a) were subjected to the same exosome purification procedures. Similarly, the exosomes from all five patient plasma samples exhibited a similar pattern of the exosome distribution (Fig. 4A). We then combined exosome fractions (E+H) and exosome-free fractions (H) as described above and analyzed their HCV RNA levels. The AchE activity assay of these two combined samples was performed to ensure the separation (Fig. 4B). HCV RNA quantitation showed that HCV RNA was also detected in exosomes (Fig. 4C, E+H) derived from HCV-positive patient plasma, and that the HCV RNA level in the exosomes was 3–20-fold higher than that in exosome-free fractions (Fig. 4C and H). Taken together, these results strongly suggest that HCV occurs in the form of exosomes *in vivo*.

Exosome-associated HCV infection could conceivably serve as a new route of HCV infection and transmission, which adds another layer of complexity to HCV transmission and pathogenesis. HCV establishes chronic infection in about 80% of infected people [18], suggesting its high capability to evade the immune system. Neutralizing antibodies often fail to control the chronic HCV infection, albeit they are generated in those patients [19]. A recent report was published to demonstrate that exosome-mediated HCV infection is more resistant to three out of eight patient-derived neutralizing sera than cell-free virus infection [20], suggesting a role of exosome-mediated transmission in HCV immune evasion. However, this study only used the cell-culture produced HCV and the exosome preparations that did not involve gradient ultracentrifugation or other exosome purification methods such as immunocapturing [21], which likely enables incomplete separation of the exosomes from cell-free HCV. In the current study, we demonstrated HCV-exosome association by both the density gradient fractionation and the TEM. We showed the presence of exosome-associated HCV in both cell-culture produced HCV and HCV-positive patient plasma. We also demonstrated that even though exosome-associated HCV was less infectious than exosome-free HCV, they were resistant to neutralization by anti-HCV neutralizing antibody. The molecular mechanisms of exosome-associated HCV infection and its contribution to HCV pathogenesis

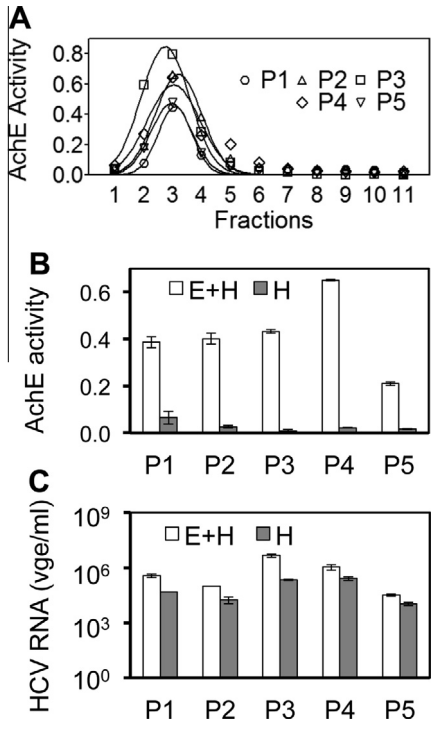

Fig. 4. Co-fractionation of exosomes with HCV in HCV-positive patient plasma. Heparinase I-treated HCV-positive patient plasma (140 μl from each of five patient plasma, P1 to P5) were diluted with PBS to 500 μl and fractionated on a 6–24% iodixanol gradient as described above. Aliquots of each fraction were used for the AchE activity assay (A, data were fit to Gaussian distribution). Then the rest of each fraction was pooled as described in Fig. 3 to obtain E+H and H samples. These two samples were subjected to the AchE activity assay (B) or RNA isolation, followed by qRT-PCR for HCV RNA (C, expressed as vge per ml of plasma). Viral titers in the plasma samples were in the range of 2.1 × 105 to 2.1 × 107 vge/ml as determined by qRT-PCR. The data were mean ± SD from triplicate.

merit further investigation. In addition, it will be of great interest to determine the receptor-dependency and tissue tropism of this new form of HCV and its possible contribution to the well documented but poorly understood extrahepatic manifestations of HCV in the lymph system [22–24] and the brain [25–28].

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