

Newly Discovered Hepatitis C Virus Minicores Circulate in Human Blood

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Hepatitis C virus (HCV) is one of the most prevalent causes of chronic blood-borne infections worldwide. Despite developments of highly effective treatments, most infected individuals are unaware of their infection. Approximately 75% of infections are in low- and middle-income countries; therefore, continuing research in HCV molecular virology and the development of vaccines and affordable diagnostics is required to reduce the global burden. Various intracellular forms of the HCV nucleocapsid (core) protein are produced in cell culture; these comprise the conventional p21 core and the newly discovered shorter isoforms (minicores). Minicores lack the N-terminus of p21 core. This study was conducted to determine if minicores are secreted in cell culture and more importantly if they circulate in the blood of individuals infected with HCV. We also developed a new monoclonal antibody that detects minicores targeting a C-terminal region common to p21 core and minicores. Direct evidence of minicores requires western blot analysis to distinguish the detection of p21 core from minicores. However, the sensitivity for western blot detection of HCV proteins from blood is nil without their prior purification/enrichment from blood. Therefore, we developed a purification method based on a heparin/Mn²⁺ precipitation of apolipoprotein B-containing lipoproteins because HCV is thought to circulate as a hybrid lipoviral particle. Minicores are secreted in culture when cells are grown in the presence of human serum. The heparin/Mn²⁺ precipitate from HCV-infected cell culture supernatants and from the blood of 4 patients with high-titer genotype-1 HCV contained minicores. *Conclusion:* Minicores are major newly discovered HCV proteins that are secreted and circulate in blood during natural infections. Minicore proteins have translational potential as targets in diagnostic assays and in vaccine development. (*Hepatology Communications* 2018;2:21-28)

Introduction

Hepatitis C virus (HCV) chronically infects about 150 million people worldwide, and 75% of cases are in low- and middle-income countries (LMICs).⁽¹⁾ Although effective antiviral drug treatments are available, most HCV-infected individuals are unaware of their infection and remain undiagnosed. With the high prevalence of cases in LMICs along with ongoing new infections and high pharmaceutical costs, additional interventions may be needed to bring the global HCV epidemic under control. As a

result, there is continued interest in HCV molecular virology, virus-host interactions, and the development of vaccines and affordable diagnostics.

In 2009, we discovered that HCV expresses a family of previously unknown proteins derived from the *core* gene.⁽²⁾ These newly discovered core isoforms, referred to as “minicores,” have the C-terminal portion of the mature p21 nucleocapsid (core) protein but lack the N-terminus. The estimated sizes of two prominent minicore proteins, 70 and 91, suggest that they begin at amino acids 70 and 91, respectively. Of interest, mutations in positions 70 and 91 are associated with

Abbreviations: apoB, apolipoprotein B; DIPE, diisopropyl ether; FBS, fetal bovine serum; HCV, hepatitis C virus; HS, human serum; LDS, lithium dodecyl sulfate; LMIC, low- and middle-income countries; LVP, lipoviral particle; mAb, monoclonal antibody; MWCO, molecular weight cutoff; VLDL, very low density lipoprotein.

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an increased risk of hepatocellular carcinoma, insulin resistance, and failure on interferon-based treatments,⁽³⁻⁸⁾ suggesting that minicores may affect clinical outcomes.

Since our initial discovery of intracellular minicores in cell culture, we have explored the possibility of extracellular secreted minicores in culture and in patients. HCV circulates in blood as a hybrid lipoviral particle (LVP) containing lipoproteins,⁽⁹⁻¹⁵⁾ HCV RNA, and the viral structural proteins p21 core, envelope 1, and envelope 2. We and others have shown that culturing Huh-7 or Huh-7.5 cells in media containing 2% adult human serum (HS) differentiated these hepatoma cells into cells that are more hepatocyte-like with an up-regulation of hepatocyte-specific genes^(8,16); furthermore, the secretion of very low density lipoprotein (VLDL) was restored, which is absent when these cells are cultured with fetal bovine serum (FBS).⁽¹⁶⁾ We examined culture supernatants of HCV-infected, HS-cultured, and FBS-cultured Huh-7.5 cells for secreted minicores. Secreted minicores were more abundantly detected from the HS-cultured cells. The culture supernatants were ultrafiltered through a membrane with a 100-kDa molecular weight cutoff (MWCO). Minicores and p21 core were found solely in the postultrafiltration retentate, suggesting that minicores were part of a larger complex because individual minicore isoforms have molecular weights ranging only between 6-14 kDa. These findings prompted us to develop methods to determine whether minicores circulate in blood during natural HCV infection.

Blood and serum contain large quantities of albumin, globulins, and other proteins. Unfractionated, this high concentration of host proteins makes it difficult to detect and characterize viral proteins; however, there are many well-established methods for fractionating serum. Heparin in the presence of Mn^{+2} precipitates apolipoprotein B (apoB)-associated lipoproteins, including low density lipoprotein, intermediate density

lipoprotein, and VLDL.⁽¹⁷⁾ Nielsen and colleagues used heparin/ Mn^{+2} precipitation to successfully isolate HCV proteins from a highly infected liver (5×10^9 IU/gram)⁽¹⁸⁾; however, they failed to detect HCV proteins from matched high-titer serum (6×10^8 IU/mL). Although they were unable to detect viral proteins in blood by western blot analysis, their findings suggested that heparin/ Mn^{+2} precipitation might be a useful first step in the purification of blood-borne HCV proteins. We tested our *in vitro* HS-culture supernatants with heparin/ Mn^{+2} precipitation and found that minicores were isolated in the lipoprotein pellet.

An obstacle for the study of HCV minicores in general is the lack of a publicly shared or commercially available antibody to detect them. The N-terminal portion of the core protein is more immunogenic than the C-terminal portion. Notably, most anti-core antibodies produced by patients during natural infections⁽¹⁹⁾ and all commercially available anti-core antibodies have epitopes in the N-terminus. These antibodies do not recognize minicores because they lack the N-terminus of the conventional p21 core.

We developed a monoclonal antibody that detects HCV minicores targeting C-terminal epitopes common to p21 core and minicores. We showed that minicores are secreted in a cell culture system, and we developed a purification method to isolate HCV core and minicore proteins from blood.

Materials and Methods

STUDY POPULATION

Hepatologists and other providers at the Icahn School of Medicine at Mount Sinai recruited patients. Written informed consent (GCO #03-0425) was obtained, and up to 16 mL of blood was collected and serum was prepared. Data on age, sex, HCV genotype, viral load, and other medical conditions were extracted

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from medical records. The study was conducted in compliance with the Icahn School of Medicine at Mount Sinai Institutional Review Board and the Helsinki accord.

DEVELOPMENT OF A MOUSE MONOCLONAL ANTIBODY THAT DETECTS MINICORES

A peptide with amino acids 104-121 of the HCV core protein was used for immunization. The peptide was conjugated to keyhole limpet hemocyanin at the C-terminus through an added cysteine. Hybridomas were initially screened by enzyme-linked immunosorbent assay using the peptide. Hybridoma supernatants and ascites generated from enzyme-linked immunosorbent assay-positive clones were western blot screened for core/minicore detection. One hybridoma clone with an exceptionally strong signal was identified. The antibody isotype was determined to be immunoglobulin G1. Antibodies were purified with protein G sepharose. Our newly developed antibody was named Neo4.

IMMUNOFLUORESCENT CELL STAINING

Cells grown on cover slips were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS; 15 minutes, 25°C), washed 2 times with PBS with Tween 20 (PBST; 0.1% Tween 20 in PBS), permeabilized with ice-cold methanol (20 minutes, -20°C), and then washed 3 times with PBST. Cells were blocked with PBS containing 5% goat serum and 1% FBS (1 hour, 25°C). Neo4 antibody was incubated at 5 µg/mL in PBS containing 1% FBS (1 hour, 25°C) and washed 3 times with PBST. Secondary anti-mouse immunoglobulin G1 was incubated (1 hour, 25°C), washed 3 times, and stained with 4',6-diamidino-2-phenylindole (DAPI) (5 minutes, 25°C). Stained cells were viewed by confocal microscopy.

CELL CULTURE STUDIES

Huh-7.5 cells cultured in 10% FBS media (Dulbecco's modified Eagle's medium 11995-065; Life Technologies) were infected with a Con1/JFH (genotypes 1b/2a) chimeric virus (APP144; Apath LLC)⁽⁸⁾ at a multiplicity of infection of 0.07. After 2 days, the culture was split 1:3 to allow for viral spread. Three days after this, the medium was changed to either a 2% FBS or 2% HS medium and cultured for an additional

3 days. Cell lysates were lysed directly in culture dishes after three washes with Dulbecco's PBS with 2 times lithium dodecyl sulfate (LDS) NuPage sample buffer (Life Technologies) containing 4% LDS and 10% 2-mercaptoethanol. Cell culture supernatants were collected and processed by either centrifugal ultrafiltration with a 100-kDa MWCO membrane (Amicon Ultra; Millipore) or by heparin/Mn⁺² precipitation; retentates after ultrafiltration were subsequently dialyzed 3 times with PBS by diafiltration. Heparin/Mn⁺² precipitation proceeded as described below. After washing the precipitate, the heparin/Mn⁺² pellet was resuspended in 2X LDS buffer.

ISOLATION AND CHARACTERIZATION OF MINICORES AND P21 CORE IN BLOOD

An equal volume of 50 mM Tris (pH 7.3) and 150 mM NaCl was added to 3.5 mL of serum and passed through a 0.45-µm syringe filter to remove aggregates. ApoB-associated lipoproteins were then precipitated by mixing an equal volume of heparin/Mn⁺² solution containing 60 mM Tris (pH 7.3), 110 mM MnCl₂·4H₂O, 154 mM NaCl, and 400 United States Pharmacopoeia (USP)/mL heparin (H3149; Sigma). The solution was incubated for 1 hour on ice in the dark. The precipitate was recovered by centrifugation at 3,000g (30 minutes, 4°C) and washed with gentle resuspension 3 times with 3.5 mL of an ice-cold solution containing 50 mM Tris (pH 7.3), 55 mM MnCl₂·4H₂O, 154 mM NaCl, and 200 USP/mL heparin. Heparin/Mn⁺² was then removed as follows: manganese was removed from the heparin/Mn² pellet by resuspension in 2.0 or 2.5 mL of 10% NaHCO₃, which precipitates an insoluble Mn(HCO₃)₂ that is removed by centrifugation at 1,500g (15 minutes, 4°C). Heparin was removed by dialyzing 3 times (in 24 hours) with 1 L of 5% BaCl₂ in 20 mM Tris (pH 7) in a Slide-A-Lyzer Cassette G2 (Thermo Scientific) with a 20,000 MWCO. The supernatant was collected from the dialysis unit, and the heparin-BaCl₂ insoluble complex was removed by centrifugation at 1,500g (15 minutes, 4°C). Excess BaCl₂ was removed from the supernatant by dialyzing 3 times (in 24 hours, 4°C) with 1 L of buffer consisting of 20 mM Tris (pH 7.0), 0.15 M NaCl, and 1 mM ethylene diamine tetraacetic acid. The supernatant was collected from the dialysis unit and stored at -80°C. After thawing, the supernatant was concentrated using a 15-mL Amicon Ultra

centrifugal filtration unit with a 100-kDa MWCO (3,200g; 90 minutes, 4°C). Concentrated volumes ranged between 130 μ L and 210 μ L. Finally, samples were delipidated to allow for loading onto a western blot gel. Samples were delipidated using an extraction method previously described for whole serum and plasma, which partitions proteins into an aqueous phase and lipids into an organic phase using a mixture of butanol and diisopropyl ether (DIPE) (butanol:DIPE at 40:60 volume per volume).⁽²⁰⁾ We modified the original protocol by adding a 1X volume of the 40:60 butanol:DIPE mixture to our sample rather than a 2X volume because the 2X volume can greatly dehydrate the aqueous phase. Samples were briefly vortexed. The lipid-containing organic and protein-containing aqueous phases were separated by centrifugation at 400g (2 minutes, 25°C). The lower aqueous phase was immediately collected, placed on ice for 1 hour, and then stored at -80°C. Incubation of the protein-containing aqueous phase on ice for 1 hour followed by freeze thawing led to the formation of a precipitate that was pelleted by gentle centrifugation at 1,000g (30 seconds, 4°C). The pellet was solubilized in a solution containing 8 M urea and 1% sodium dodecyl sulfate, sonicated, adjusted with NuPage sample buffer, heated, and loaded onto NuPAGE gels. This pellet contained core and minicore proteins; these were not detected in the supernatant. After electrophoresis, gels were processed for western blot analysis. Protease inhibitors, Complete- ethylene diamine tetraacetic acid free (Roche), and 1 mM phenylmethylsulfonyl fluoride were used throughout the purification procedure.

WESTERN BLOT ANALYSIS

Samples were electrophoresed in 10% NuPAGE Bis-Tris gels (Life Technologies), and the proteins were transferred to 0.2- μ M pore size polyvinylidene difluoride membranes. For the cell culture studies, Neo4 monoclonal antibody (mAb) and C7-50 mAb were used at a concentration of 2 μ g/mL. For patient studies, antibodies targeting the C-terminal portion of p21 core and used to detect minicores were a combination of Neo4 at a concentration of 2 μ g/mL mixed with mAb1 at 1 μ g/mL.⁽²⁾ The antibody targeting the N-terminal portion of core is clone C7-50.

QUANTIFICATION OF HCV RNA

We used QIAamp Viral RNA mini kit (Qiagen) to purify RNA from serums, heparin/Mn⁺² pellets, and

supernatants. For heparin/Mn⁺² pellets and supernatants, heparin was removed from the purified RNAs by treating with heparinase I (Sigma).⁽²¹⁾ Reverse transcription of RNA was performed using SuperScript III First-Strand Synthesis (Invitrogen) and random hexamers. Reverse-transcription reaction products were used for quantitative-polymerase chain reaction using the LightCycler 480 SYBR Green I Master kit and LightCycler 480 (Roche).

Results

We developed a mouse monoclonal antibody, termed Neo4, that detects minicores. A peptide corresponding to amino acids 104-121 of core was used for immunization. An antibody recognizing this region would detect both core and minicores (Fig. 1A). The Neo4 antibody was first evaluated by immunofluorescent staining of mock- or HCV-infected cultures (Fig. 1B). Neo4-specific staining was detected only in the HCV-infected culture and not in the mock-infected culture.

To evaluate minicore secretion in culture, HCV-infected Huh-7.5 cells were cultured in either 2% FBS- or 2% HS-supplemented media. Both the intracellular cell lysates and the extracellular cell culture supernatants were analyzed by western blot using the Neo4 antibody. Mock-infected culture samples served as a control for nonspecific antibody reactivity to host proteins. We detected 70 and 91 minicores along with p21 core in the cell lysates of HCV-infected cultures grown in either FBS or HS media (Fig. 1C). Another minicore with a molecular weight higher than the 70/91 minicores was also detected in the lysates. The amounts of each core-isoform (p21 core, 70 and 91 minicore) were similar between FBS and HS cell lysates.

The cell culture supernatants were processed either by concentration with an ultrafiltration membrane having a 100-kDa MWCO or by heparin/Mn⁺² precipitation of apoB-containing particles (Fig. 1C). Ultrafiltration retentates and heparin/Mn⁺² pellets were analyzed for core-isoforms. Only p21 core and a trace amount of 70 minicore were detected from infected FBS culture supernatants. In contrast, 70 and 91 minicores along with p21 core were readily detected from supernatants of HCV-infected HS cultures. A duplicate blot with heparin/Mn⁺² pelleted samples was also probed with a commercially available antibody, C7-50 (epitope 21-40) that targets the

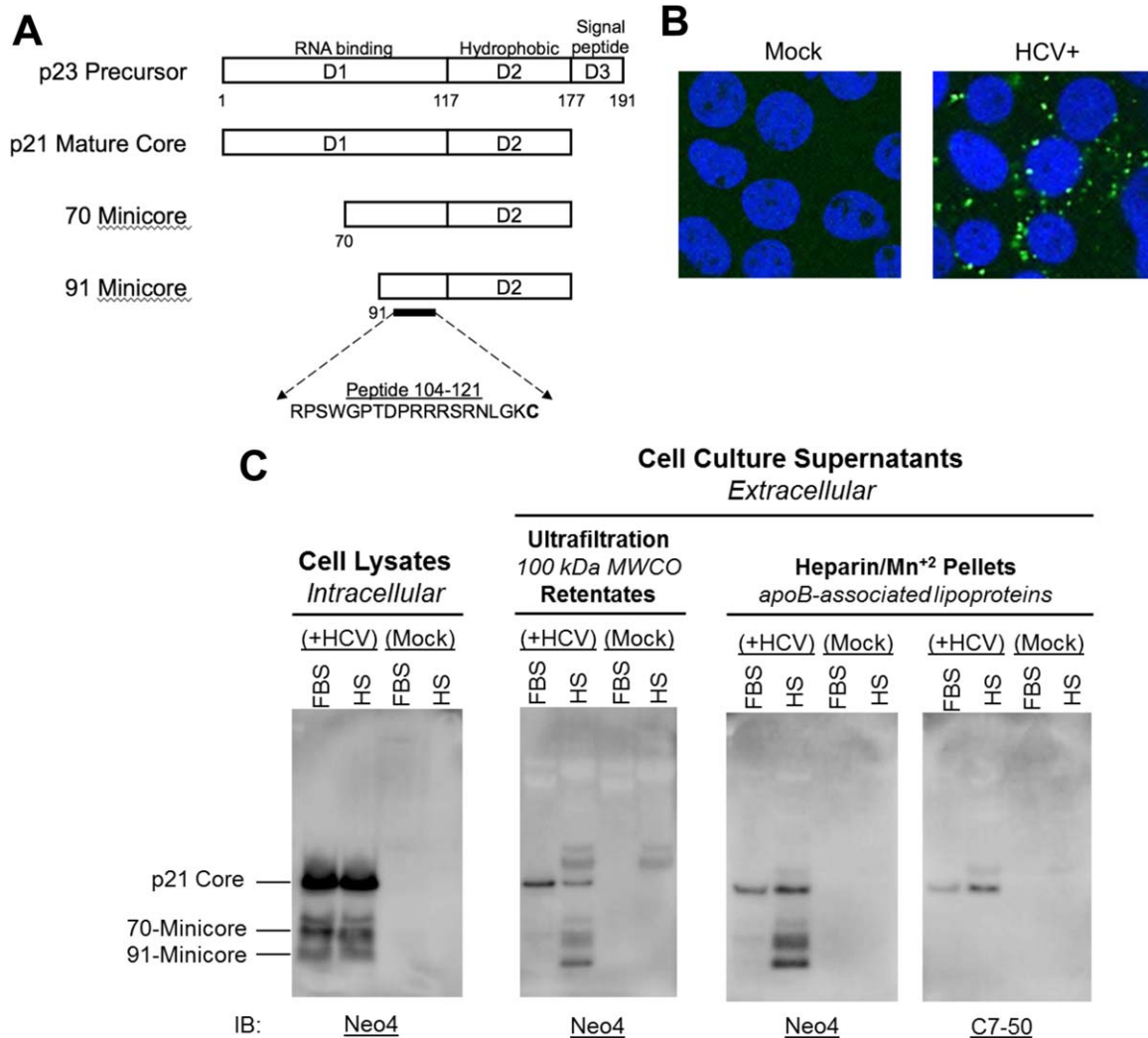


FIG. 1. Minicores secreted in cell culture. (A) Both p21 mature core and minicore proteins contain the hydrophobic domain D2, which interacts with lipids. Minicores contain only part of domain D1, the RNA binding domain. Peptide 104-121 was used to generate a monoclonal antibody, Neo4, capable of detecting minicores and p21 core. (B) Neo4-specific immunofluorescent staining of HCV-infected cells. Mock-infected cells served as a control. Nuclei were stained with DAPI. Cells were viewed by confocal microscopy using a 63 \times oil immersion objective. (C) Minicores detected by western blot of cell lysates (intracellular) and cell culture supernatants (extracellular) of HCV-infected Huh-7.5 cells cultured in FBS- or HS-supplemented media. Mock-infected cells served as a control. Cell culture supernatants were processed by ultrafiltration through a 100-kDa MWCO membrane or by heparin/Mn⁺² precipitation. Ultrafiltration retentates and heparin/Mn⁺² pellets were examined. Western blots were probed with antibody Neo4. A duplicate western blot with heparin/Mn⁺² samples was probed with antibody C7-50 (epitope 21-40) directed to the N-terminus of p21core.

N-terminus of p21 core. As expected, only p21 core was detected. By western blot, the sensitivity of the Neo4 mAb is roughly similar to the commercial C7-50 mAb.

We obtained blood from 4 patients with high-titer HCV and 2 noninfected volunteers (Table 1; P1-P4, N1, and N2) to determine if minicores circulate *in vivo*. All 4 patients infected with HCV were male and had an HCV viral load over 20 million IU/mL. Patients with a high HCV viral load were selected

because they were expected to have relatively high blood levels of HCV proteins.

To enrich for HCV RNA and protein, 3.5 mL of serum was reacted with heparin/Mn⁺² under conditions that precipitate apoB-associated lipoproteins (Fig. 2A).⁽⁹⁾ The heparin/Mn⁺²-enriched pellet contained an average 87.9% (\pm 10.7%) of the HCV RNA in serum (Fig. 2B). Prior to western blot analysis, the heparin, Mn⁺², and lipids were removed as outlined in

TABLE 1. CLINICAL CHARACTERISTICS OF PATIENTS

Patient ID	HCV Viral Load (IU/mL)	HCV Genotype	Age (Years)	Sex	ALT (U/L)	Other
P1	7.46×10^7	1b	49	M	149	HIV co-infected
P2	2.91×10^7	1b	63	M	29	5 years after kidney transplant
P3	1.23×10^7	1a	67	M	39	-
P4	$>1.0 \times 10^{8*}$	1a	60	M	212	1 year after OLT and B cell lymphoma
N1	-	-	55	M	NA	Healthy control
N2	-	-	65	F	NA	Healthy control
L1	3.24×10^6	1a	60	M	89	-
L2	2.21×10^6	1a	75	M	33	-
L3	1.21×10^3	1b	38	M	22	On SOF/RBV, still viremic
L4	0	1a	28	F	NA	IFN/RBV/TVR 3 months + IFN/RBV 6 months

*Value was above the upper limit of detection of the assay.

Abbreviations: ALT, alanine aminotransferase; HIV, human immunodeficiency virus; IFN, interferon; OLT, orthotopic liver transplantation; SOF, sofosbuvir; RBV, ribavirin; TVR, telaprevir.

Fig. 2A. Briefly, the heparin/ Mn^{+2} pellet was resuspended in $NaHCO_3$, solubilizing the macromolecules and precipitating $Mn(HCO_3)_2$. $BaCl_2$ was then used to precipitate and remove heparin. ApoB-associated lipoprotein complexes were then concentrated by ultrafiltration with membranes having a 100-kDa MWCO. Lipids were removed using organic solvents (butanol and diisopropyl ether), which resulted in the precipitation of core-isoforms. The isoforms were analyzed by western blotting (Fig. 2C,D).

Minicores were detected in the blood of all 4 patients with high-titer HCV (Fig. 2C). The relative amounts of p21 core, 70 minicore, and 91 minicore varied from patient to patient. Patient samples P1, P2, and P4 had prominent p21 core bands. Interestingly, P1, P2, and P4 were immune compromised as a result of human immunodeficiency virus/HCV co-infection or immunosuppressive drugs, while P3 had no immunologic deficiency (Table 1). P1, P2, and P3 had prominent 70 minicore bands, and P4 had a prominent 91 minicore band. These differences may be due to experiment-to-experiment technical variation and/or biological variation. Further investigation is needed to determine if there is any significance to these differences. A cell culture lysate of HCV-infected Huh-7.5 cells provided molecular weight markers of the core protein isoforms (Fig. 2C,D, lane C). To confirm the identity of the core-isoform bands, duplicate western blots of sample P4 were probed with antibodies specific for either the N- or C-terminal portion of the core protein (Fig. 2D), allowing detection of p21 core only (N-terminus mAb) or p21 plus the 70 and 91 minicores (C-terminus mAbs).

Minicores and p21 core were not detected from 3 patients with low-titer HCV, most likely because they

are below the limit of detection of our assay (Table 1; L1-L3). Patient L3 was actively on HCV treatment when the blood sample was obtained. The core-isoforms were also not detected from a patient after HCV elimination with telaprevir-based triple therapy (Table 1; L4).

Discussion

We provide evidence that minicores can be secreted in cell culture. Of note is the observation that while intracellular core-isoforms were expressed similarly between HS and FBS culture conditions, it was with HS supplementation that minicore secretion was greatly promoted (Fig. 1C). This may be due to either an HS-dependent restoration of the VLDL pathway in Huh-7.5 cells and/or due to the absence from FBS of fetal growth factors, which alters cellular gene expression. Interestingly, infected-Huh-7.5 cells cultured with adult bovine serum secrete the core-isoforms at identical levels as seen for HS-grown cells (data not shown). Further investigations will be needed to elucidate the mechanism of minicore secretion. Our data indicate that minicores are associated with apoB-containing particles, as indicated by their heparin/ Mn^{+2} precipitability. They may be incorporated within infectious HCV LVPs that also contain p21 core or may be incorporated into a distinct population of LVPs that lack p21 core, infectious or not.

A limitation to this present work is our inability to distinguish between distinct LVP populations because our new Neo4 antibody recognizes both p21 core and minicores. Despite this, our development of the Neo4 antibody provides an available tool for the general

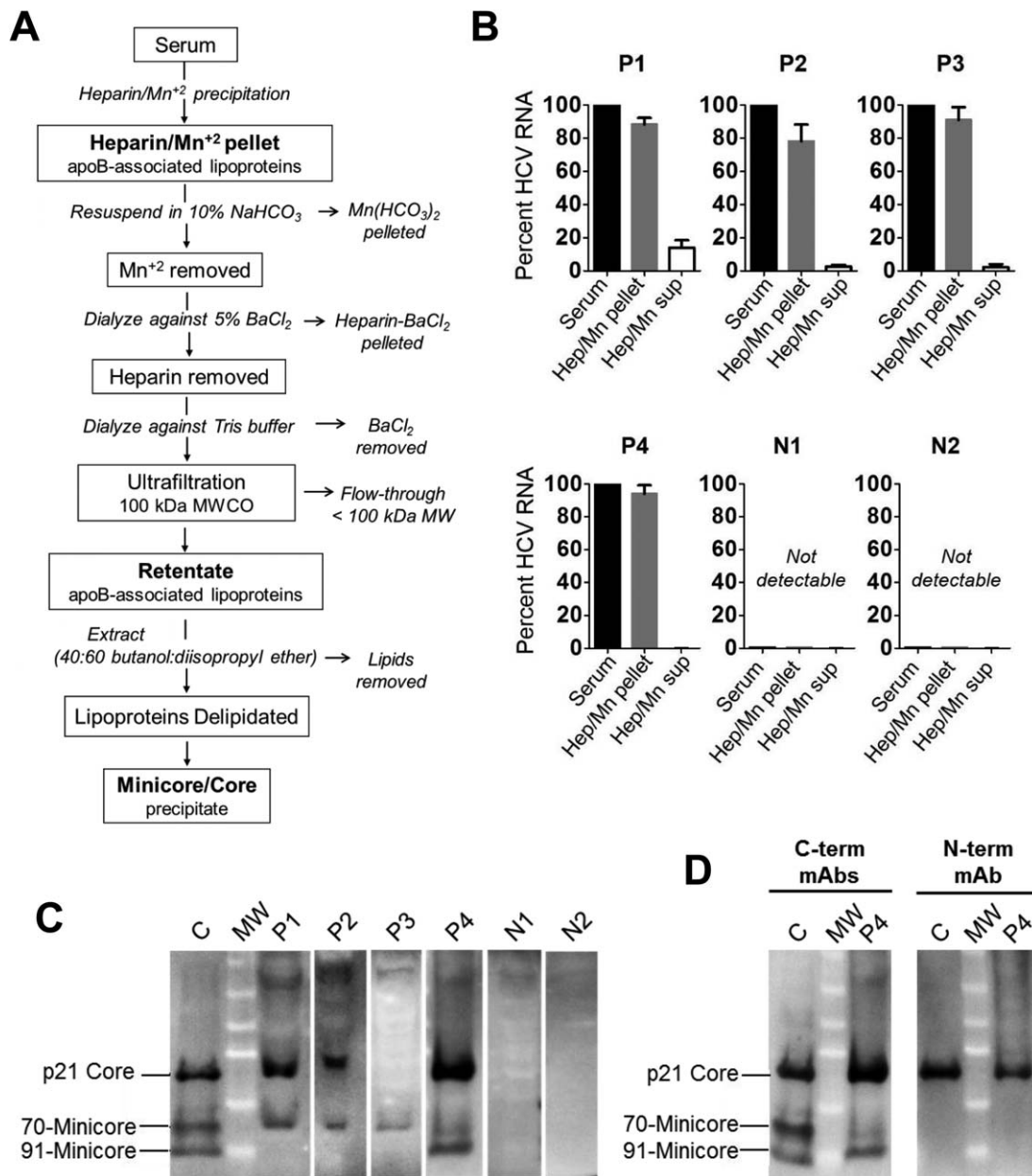


FIG. 2. Minicores circulate in blood. (A) Purification scheme isolating core and minicores. (B) HCV RNA was enriched in the heparin/ Mn^{+2} pellet fraction. Error bars represent standard error of technical triplicates. (C) Minicores were detected by western blot in 4 patients with HCV (lanes P1-P4) and not in 2 normal controls (lanes N1, N2). Cell culture lysate of infected cells served as a positive control (lane C). (D) Duplicate western blots of sample P4 probed with antibodies directed either to the C-terminal or N-terminal portion of the core protein confirmed the identity of minicores. Abbreviation: MW, molecular weight; sup, supernatant; term, terminus.

research community to study HCV minicores. Interestingly, it has been reported that the majority of circulating HCV LVPs in patients are subviral particles that are apoB and envelope positive but lack p21 core and the HCV genome.⁽²²⁾ Minicores lack portions of the RNA binding domain present at the N-terminus of p21 core. One possibility is that these abundantly

circulating, genome-negative, subviral particles that lack p21 core may include minicores.

This study more importantly provides the first direct evidence that HCV-expressed nonclassical proteins (minicores) are present in blood during natural infections. The presence of minicores in blood suggests that they enhance viral transmission and/or pathogenesis/

persistence. Minicores were detected in 4 patients with a high HCV viral load. Our inability to detect either p21 core or minicores in the patients with a lower HCV viral load is probably because the HCV proteins are below our assay's detection limit.

Minicore proteins are novel viral antigens to target in detection assays. The detection of minicores in addition to p21 core has the potential to increase the sensitivity of HCV core antigen assays that are currently in commercial use⁽²³⁾ and possibly of future point-of-care antigen tests that can be deployed more globally, especially in LMICs. It has been suggested that point-of-care HCV core antigen tests can potentially replace HCV nucleic acid tests as a more cost-effective way to screen for HCV infection and to assess treatment outcomes in LMICs.^(1,24) Furthermore, if minicores enhance infectivity, they may be useful targets in vaccine development.

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