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Hepatitis C virus: a new class of virus associated with particles derived from very low density lipoproteins

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Abstract

Hepatitis C virus (HCV) infects 3% of world population and is the leading cause of liver failure in the United States. A unique feature of HCV is that the viral particles are integral to very low density lipoprotein (VLDL)-derived lipoprotein particles. The virus is assembled into VLDL in hepatocytes, and released out of the cells together with VLDL. The virus then infects more hepatocytes by entering the cells through low density lipoprotein receptor, which mediates uptake of majorities of VLDL-derived lipoprotein particles. These observations suggest that HCV may belong to a novel class of viruses that is associated with VLDL. Understanding the relationship between HCV and VLDL metabolism may reveal new strategies to treat HCV infection.

Hepatitis C virus (HCV) infects more than 170 million people worldwide [1]. According to the Centers for Disease Control and Prevention, 4.1 million Americans are estimated to be infected by HCV, 3.2 million of whom become chronically infected. These individuals account for most cases of liver failure in the United States [2]. The most effective therapy for HCV infection involves inhibiting a HCV-encoded enzyme [3]. However, the HCV genome rapidly acquires mutations that render drug resistance owing to the low fidelity of the viral replication machinery [4]. Thus, these inhibitors must be combined with interferon in order to significantly improve treatment outcome of HCV infection. Because of the expense and severe side effects that accompany interferon treatment [5], the search for new strategies to treat HCV infection is merited.

HCV is a single-stranded positive-sense RNA virus of the Flaviviridae family [6]. The 9.6-kilobase HCV genome encodes a single polyprotein that is post-translationally processed into at least 10 structural and nonstructural (NS) proteins [7] (Figure 1). The amino-terminal one-third of the polyprotein encodes virion structural proteins: Core, E1 and E2. The remainder of the genome encodes NS proteins that are not found in viral particles but instead are required for replication and assembly of the virus. The NS3, NS4A, NS4B, NS5A and NS5B proteins, which are necessary and sufficient for replication of viral RNA [8], form a viral replication complex on endoplasmic reticulum (ER) membranes [9-12].

Association of HCV with very low density lipoprotein (VLDL)

An intriguing feature of HCV is that the viral particles are found in complex with VLDL, which plays an important role in transporting cholesterol and triglyceride from the liver to

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peripheral tissues [13]. VLDL contains a hydrophobic core of neutral lipids consisting of triglycerides and cholesteryl esters surrounded by a surface coat containing phospholipids, free cholesterol, and lipoproteins including apolipoprotein B (apoB) and apolipoprotein E (apoE) [14]. HCV particles isolated from the serum of virus-infected patients exhibited a density similar to that of VLDL [15-17]. Moreover, these particles were rich in triglyceride and contained apoB and apoE [15, 16]. Recently, Merz et al developed a strain of HCV in which the E2 protein was tagged with a FLAG epitope, and purified the HCV virion produced from the human hepatoma Huh7 cells through affinity purification with anti-FLAG [18]. The purified HCV virion appeared to contain more apoE than viral proteins at the surface of the particles [18]. Lipidomic analysis revealed that cholesteryl esters comprised almost half of the total lipid content in the affinity-purified viral particles [18]. In sharp contrast, the viral envelope derived from host cell membranes is predominantly composed of phospholipids. The triglyceride content in the purified viral particles were not determined owing to technical difficulty [18].

Electron microscopy (EM) analysis of HCV either isolated from patient serum or affinity-purified from the culture medium of virus-infected Huh7 cells revealed structures that contained lipid-rich cores resembling lipoprotein particles rather than the classical viral capsid structure [15, 18]. These observations suggest that the viral genome and capsid may be hidden within the hydrophobic core of VLDL. This structure may allow HCV to evade B cell/antibody-mediated immune surveillance during circulation, thereby providing a plausible explanation as to why the viral infection cannot be effectively prevented by vaccination. In contrast to these studies, EM analysis revealed structures resembling enveloped viral particles in a fraction of culture medium enriched in HCV infectivity [19]. Thus, HCV may exist as multiple forms, and more sophisticated EM analyses such as cryo-EM capable of visualizing structures within the hydrophobic cores of VLDL may be necessary to identify capsid structure of HCV.

Assembly of HCV-VLDL complex

The hepatic synthesis of VLDL requires generation of lipid droplets enriched in neutral lipids such as triglycerides and cholesteryl esters in the ER lumen [14]. These lipid droplets are produced by reactions catalyzed by microsomal triglyceride transfer protein (MTP) [20-22]. Although not formally demonstrated, apoE might also play an important role to generate these lipid droplets [23]. Upon fusion with apoB, these lipid droplets can be secreted out of cells as nascent VLDL through exocytosis [14]. In addition to generating lipid droplets in the ER lumen, MTP also stabilizes apoB during translation by transferring lipids to the nascent polypeptide chain of apoB [14, 22]. In the absence of this lipid transfer, the secretion of apoB is blocked and the protein is rapidly degraded in cells [24, 25]. VLDL secretion also requires hepatic synthesis of phosphatidylcholine (PC), the major phospholipid on the surface of the lipoprotein particles [26]. In human hepatoma Huh7 cells, long chain acyl-CoA synthetase 3 (ACSL3)-mediated PC synthesis is required for secretion of apoB [27].

Proteomic analysis of ER membrane vesicles containing HCV RNA and viral replication complex composed of viral proteins NS3-NS5B revealed that these vesicles were enriched in apoB, apoE, MTP and ACSL3 [12]. The reason for co-localization of the HCV replication and VLDL assembly appears to lie in a requirement for co-assembly and secretion of VLDL and HCV particles. Thus, secretion of HCV virion from virus-infected Huh7 cells was inhibited when cells were treated with pharmacological inhibitors of MTP [12, 28, 29]. Secretion of HCV was also inhibited in cells transfected with a siRNA targeting apoE [30] or ACSL3 [27]. The results regarding apoB are not consistent: knockdown of apoB was shown to block HCV secretion in two studies [12, 28] but had no effect on release of HCV virion in another study [29]. This discrepancy is most likely caused by the different HCV infection

system used in the studies. In the reports showing apoB was required for secretion of HCV, care was taken to ensure that HCV infection did not result in cellular apoptosis so that viral particles were only released through exocytosis. In contrast, the study showing the opposite result used a system known to cause apoptosis of virus-infected cells [31]. Consequently, intracellular infectious HCV particles containing NS5A were released into culture medium from dying cells [29]. Since apoB is not required to produce intracellular HCV particles [29], it is not surprising to observe apoB-independent production of infectious HCV particles in culture medium using this system.

A puzzling question regarding assembly of HCV-VLDL complex is how viral genome synthesized at cytosolic face of the ER is transported across the membrane bilayers to reach ER lumen where it is packaged into VLDL. A clue to the question may come from a unique property of HCV-encoded capsid core protein. HCV core protein contains two domains: an NH_2-terminal hydrophilic domain (D1) that binds viral RNA, and a COOH-terminal hydrophobic domain (D2) that interacts with neutral lipid [32, 33]. In HCV-infected Huh7 cells, the majority of core proteins are localized at the surface of cytosolic lipid droplets that are in contact with ER membranes containing the HCV replication complex [34], which is also the site for VLDL assembly [12]. Thus, HCV may replicate at an ER domain enriched in neutral lipids that can bud toward cytosol or lumen to form lipid droplets in both locations. A hypothetic model is proposed in Figure 2 to explain translocation of HCV capsid based on this localization. Core is targeted to cytosolic lipid droplets through its D2 domain, after it is cleaved from the viral polyprotein. The hydrophilic D1 domain is exposed to the cytosol, ready to accept viral RNA synthesized by the viral replication complex (Figure 2A). Once associated with viral RNA, core protein undergoes a conformational change so that hydrophilic residues that bind viral RNA are folded inside, whereas hydrophobic residues are exposed at the surface. This conformational change allows the core-RNA complex to become completely embedded in the hydrophobic core of lipid droplets (Figure 2B). The viral capsid-RNA complex then travels through the neutral lipid-rich ER membrane to reach lipid droplets in the ER lumen. The HCV-containing luminal lipid droplets then fuse with apoB, acquire two other lipoprotein-like viral structural proteins E1 and E2 [35], and are secreted out of the cells through exocytosis (Figure 2C).

The model shown in Figure 2 predicts that HCV capsids are able to enter the hydrophobic core of lipid droplets. Although there have not been many studies characterizing localization of cellular proteins in hydrophobic cores of lipid droplets, such localization was reported through EM analysis [36, 37]. Further studies are required to determine whether these host proteins facilitate translocation of HCV capsids. This model also predicts that HCV capsids travel across the membrane through neutral lipid-rich domain of the ER. ApoB has been reported to translocate from the ER lumen into the cytosol though a mechanism that involves lipid droplets [38]. This observation implies that lipid droplets across ER membranes are continuous and proteins may be able to transport across ER membranes through lipid droplet intermediates. More imaging and biochemical analyses will be required to further validate the model shown in Figure 2.

Requirement of LDL receptor (LDLR) for HCV entry

Once released out of cells, HCV enters more hepatocytes for a new round of infection. Several receptors for HCV entry have been identified based on their interaction with E2 [39], including CD81 and Scavenger receptor-B1 [40-44]. Proteins forming tight junction such as claudin-1 and occludin have also been implicated in HCV entry [45, 46]. However, all of these receptors appear to function at later stages of viral entry since they are not required for HCV to bind to the cell surface [45, 47]. This initial binding is at least partially mediated by LDLR, which plays a predominant role in acquiring VLDL-derived lipoprotein particles [48]. It has

been reported that cellular binding or uptake of HCV particles isolated from infected patients correlated with LDL receptor activity on cell surface [15, 49-51]. LDLR was also required for infectious entry of HCV virion produced from Huh7 cells, and this entry depended on the interaction between the receptor and apoE on the viral particles [52]. LDLR does not directly interact with viral proteins as the receptor was not required for entry of HCV pseudo particles, which were assembled by displaying HCV structural proteins E1 and E2 onto retroviral core particles that were not in complex with lipoproteins [53].

Mutations disrupting the function of the LDLR have been identified in human. These mutations produce autosomal dominant familial hypercholesterolemia, which affect 0.2% of world population [54]. Affected individuals have elevated plasma levels of LDL-cholesterol, which causes premature coronary atherosclerosis. However, during evolution when dietary cholesterol was scant, these mutations may not produce such a severe phenotype but may actually protect these individuals from infection of HCV or HCV-related virus. An analysis comparing the frequency of HCV infection in people expressing normal LDL receptor versus those affected by familial hypercholesterolemia will be needed to test the hypothesis. This hypothesis may also be tested with mice containing human liver grafts, an animal model successfully used to study HCV infection [55-57]. If this hypothesis is correct, mice grafted with human liver derived from patients affected by familial hypercholesterolemia are expected to resist the infection by HCV.

Treating HCV infection with drugs targeting VLDL metabolism

The dependency on VLDL for HCV life cycle offers opportunities to treat the viral infection with drugs targeting VLDL metabolism. Several MTP inhibitors have already been tested in clinical trials because of their ability to block VLDL secretion, thereby lowering the plasma levels of VLDL-triglycerides and LDL-cholesterol [58, 59]. Long-term treatment with MTP inhibitors led to the accumulation of fat in livers thus hampering the drugs to be approved for treatment of hypercholesterolemia, which may require life-long administration in the case of familial hypercholesterolemia [58, 59]. However, short-term treatment (up to several weeks) reduced the plasma level of VLDL with only minor adverse effects, which disappeared after drug removal [58]. Since the standard treatment for HCV infection with drugs targeting the viral enzymes lasts for only about 12 weeks, MTP inhibitors may be combined with these drugs to treat HCV infection. MTP inhibitors also have the advantage in that they target a host protein rather than viral proteins so they are less likely to face the drug-resistance problem caused by mutations in the viral genome.

Another drug that inhibits VLDL assembly is an antisense RNA drug targeting apoB [60]. Unlike MTP inhibitors, apoB antisense RNA lowered VLDL secretion in the absence of accumulation of fat in livers [61]. However, in cultured cells knockdown of apoB by siRNA was less potent than MTP inhibitors to inhibit HCV production [12, 28]. Thus, more studies are required to determine the efficacy of apoB antisense RNA on treatment of HCV infection *in vivo*.

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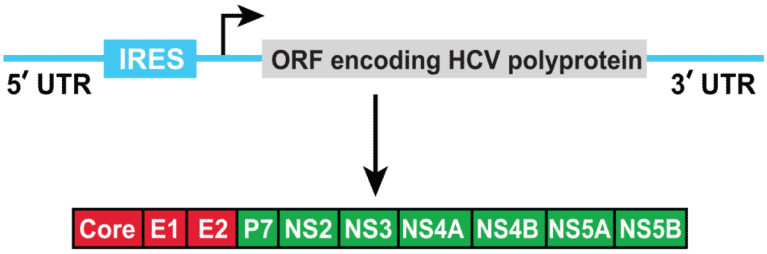

Figure 1.
Diagram of HCV genome. HCV genomic RNA contains 3′ and 5′-untranslated regions (UTR) that are required for viral replication. The 5′-UTR also contains an internal ribosomal entry site (IRES) that directs translation of a viral polyprotein, which is further proteolytically processed into 10 proteins. The viral structure and nonstructural protein are labeled in red and green, respectively.

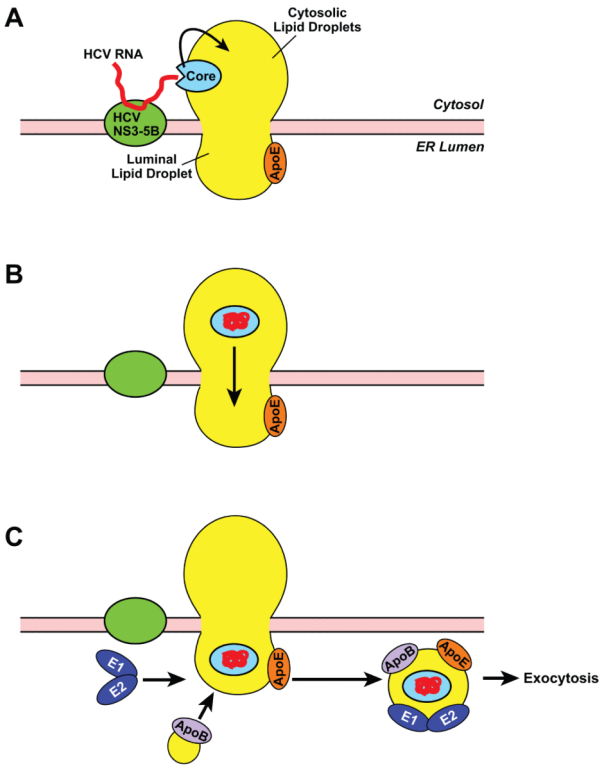

Figure 2.

A hypothetic model illustrating HCV assembly. HCV RNA is synthesized by HCV replication complex composed of viral proteins NS3-NS5B at the cytosolic face of the ER membranes. The viral RNA then binds to the core protein localized at the surface of the lipid droplets adjacent to the viral replication complex (**A**). Upon binding with viral RNA, core goes through a conformational change so that the core-RNA complex enters the core of cytosolic lipid droplets, allowing the complex to reach lipid droplets in the ER lumen by traveling through ER domains enriched in neutral lipids (**B**). The HCV-containing luminal lipid droplets fuse with apoB, acquire E1 and E2 heterodimer, and are secreted out of cells through exocytosis (**C**).