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POINT OF VIEW

Role of tight junctions in hepatitis C virus infection

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INTRODUCTION

Hepatitis C virus (HCV) infection becomes chronic in most patients, not being precisely understood the mechanisms that determine the insufficient immune response aimed at eradicating the virus. In the infected liver, complex physiopathogenic mechanisms are activated with the purpose of clearing HCV and repairing the damaged tissue. Failure of both processes favors infection persistence and liver disease progression.

HCV STRUCTURE

HCV is an enveloped virus that belongs to the *Flaviviridae* family, whose natural tropism is restricted to humans and chimpanzees (1). The HCV genome, a 9.6 kilobase positive-strand RNA molecule, codes for a ~3000 aminoacid polyprotein that is processed by viral and cellular proteases to generate ten mature proteins, including three structural proteins –the capsid protein (core) and two envelope glycoproteins (E1 and E2)–, the p7 protein and the non-structural proteins (NS2, NS3, NS4A, NS4B, NS5A and NS5B) (2). To date, the three-dimensional (3D) structure of HCV structural proteins has not been resolved, and high-resolution images of the viral particle have not been obtained. However, using transmission electron microscopy, it has been observed that the virus is formed by a nucleocapsid, which contains the viral genome compacted by the core protein, surrounded by a lipid bilayer where envelope proteins E1 and E2 are anchored (3, 4). E1 and E2 are type-I transmembrane proteins with an N-terminal ectodomain and a short C-terminal transmembrane domain, and are key for HCV entry into the hepatocyte (2,4). During E1 and E2 synthesis within the infected cell, ectodomains are located in the lumen of the endoplasmic reticulum and transmembrane domains are anchored to this organelle's membrane, where they mediate the formation of non-covalent heterodimers between both proteins (5). However, it has been suggested that on the viral surface E1 and E2 form covalent complexes stabilized by disulfide bonds (6). Their ectodomains are highly glycosylated, which is important for their correct folding and the viral entry into the host cell (7), as well as its capacity to evade the immune system by masking immunogenic epitopes (8).

In the serum of patients, HCV is found as a heterogeneous population of viral particles with densities ranging from 1.03 to 1.34 g/ml (9). Within this range, viral RNA has been identified in association with different kinds of particles, including envelope-free nucleocapsids (10), exosomes (vesicles containing viral RNA, envelope proteins, and CD81) (11) and lipoviroparticles (12,13). The latter represent around 40% of viral RNA in plasma (14), and are formed by the viral nucleocapsid, the envelope proteins, and triglyceride-rich lipoproteins containing apolipoproteins ApoB and ApoE (12,13,15), which are responsible for their low density. In fact, HCV particle density has been shown to be highly dynamic *in vivo*, and to depend on plasma triglyceride-rich lipoprotein levels (15,16). Although HCV and lipoproteins have been suggested to potentially become associated outside the cell, with both particles originating independently from each other (16), multiple studies have shown that viral morphogenesis is closely related to the biosynthesis of very low density lipoproteins (VLDL) (17-22). In addition, intracellular HCV precursors present higher densities than extracellular virions (23). These data suggest that during their assem-

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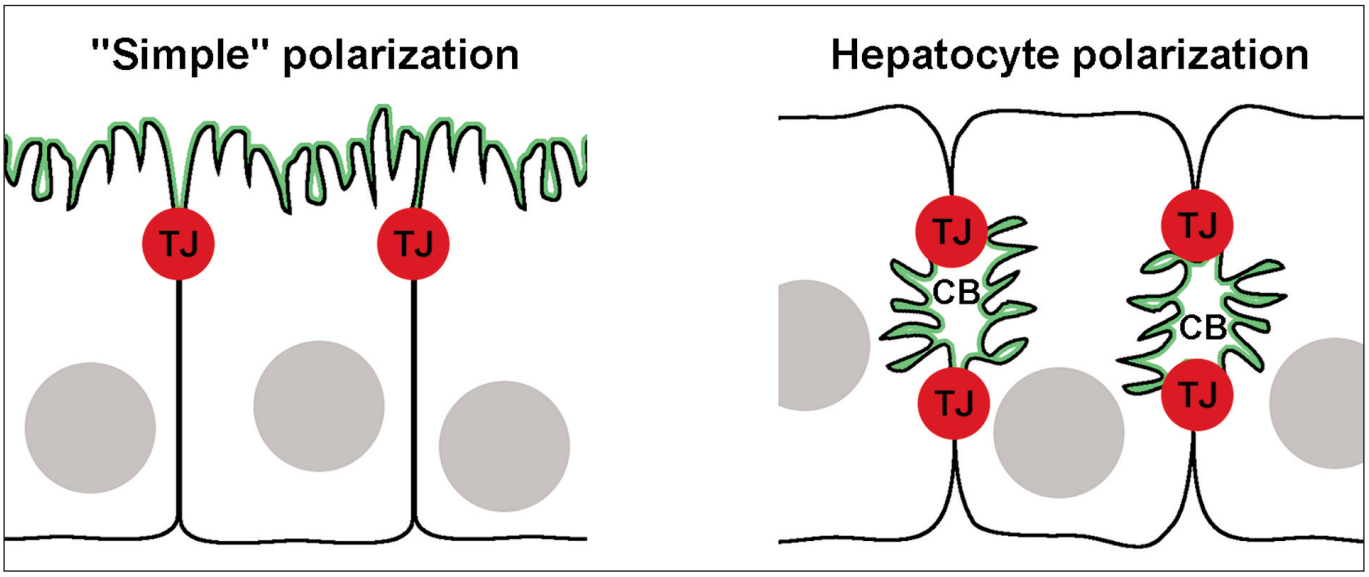

Fig. 1. A comparative scheme of "simple" epithelial polarization and hepatocyte polarization. TJs (red) represent a barrier between the cell membrane's apical domain (green) and basolateral domain. BC, bile canaliculus.

bly viral particles associate with lipoproteins along the VLDL maturation and secretion pathway.

An inversely proportional relationship has been shown between viral particle density and infectivity both *in vitro* and *in vivo* (24-28). In addition, HCV infection may be inhibited *in vitro* by using antibodies against ApoB (12,24,29) and ApoE (12,18,24,30), as well as by blocking or reducing the expression of the low density lipoprotein receptor (LDL-R) (12,24,26,31). Similarly, VLDL and LDL have been shown to block HCV infection, possibly by competing for LDL-R binding sites thus preventing the effective binding of viral particles to cells (12,31). Finally, lipoviroparticle delipidation by lipoprotein lipase has been described to inhibit HCV infection *in vitro* (32). Overall, these observations suggest that lipoproteins present in the viral particle play a relevant role in the process of HCV entry into the host cell. In addition, they might be implicated in viral protection against the immune system by masking the envelope protein epitopes that are recognized by neutralizing antibodies.

TIGHT JUNCTIONS: MAINTAINING CELL POLARITY

Tight junctions (TJs) are contact areas between adjacent cells where the intercellular space is sealed by a web of fibers integrated within the plasma membrane of the involved cells (33). These structures form a selective paracellular barrier that restricts the passage of solutes through the cell monolayer, which is of crucial importance for the maintenance of separate compartments within the organism (34). Furthermore, they maintain cell polarity by preventing lipids and proteins from freely diffusing between the basolateral and apical domains in the plasma membrane (34). At the molecular level, TJs are complexes constituted by integral membrane proteins such as claudin-1 and occludin, which form homotypical and heterotypical contacts with adjacent cells and interact via their intracellular domains with cytoplasmic proteins providing anchorage for the actin cytoskeleton (35,36). These adaptor proteins can also interact with one another, which suggests the presence of a TJ-related macromolecular network (36). Overall, all these interactions regulate TJ function and play a role in the transduction of signals involved in the control of key cell functions, including cell proliferation and differentiation (35,36).

Most epithelial cells have a so-called "simple" polarity. The plasma membrane in these cells has an apical domain, localized at the cell apex and oriented towards the lumen of the organ to which they belong, and a basolateral domain comprising the rest of the membrane (37) (Fig. 1). In contrast, hepatocytes have several apical and basolateral domains. The apical poles of adjacent hepatocytes form a continuous network of bile canaliculi into which bile is secreted, whereas basolateral domains are in contact with sinusoidal blood (37). Appropriate liver functioning depends on the maintenance of this highly polarized phenotype, which in turn depends on TJs. Hepatocyte TJs constitute an intercellular barrier between blood and bile, and allow a correct intramembrane distribution of the various transporters involved in bile secretion (37).

TJs AS ENTRYWAYS FOR VIRUSES

Both the morphlogical appearance and biochemical composition of the multimeric TJ complexes have led to con-

sider these junctions as rigid, static structures for a long time. One can hardly imagine that a structure with these characteristics may function as a viral receptor. However, recent studies have revealed that TJs are dynamic systems both at the structural and molecular level, which is indeed important for a correct development of their functions (38,39). TJs have been shown to be areas with very active membrane recycling and a high number of endocytic processes, thus representing a strategic site for proteins involved in vesicular traffic and intracellular signaling (40-42). Furthermore, TJs seal the epithelium by raising a barrier between organ lumina and inner layers, which are in this way separated from the outer environment. Thus, TJs provide a first line of defense that precludes most microbes from entering the body. However, some bacteria and viruses have developed specific strategies to utilize and/or modify TJs in order to enter a host (40,43). For instance, rotaviruses have been shown to gain access into the body from the intestine lumen using a mechanism by which they alter TJs, open the paracellular barrier between intestinal cells, and leave the integrins at the basolateral domain exposed, which then serve as viral receptors (44). Other studies have revealed that CAR, a TJ-associated protein, interacts with and acts as a receptor for coxsackie viruses, swine vesicular disease virus, and a number of adenoviruses (40), and that reoviruses and feline calicivirus use JAM-A, another TJ protein, as cell receptor (45,46).

TJs AND HCV: A CLOSE RELATIONSHIP

TJ-associated proteins as HCV co-receptors

Although some studies have suggested that HCV can replicate outside the liver (47), hepatocytes are in fact the primary virus target. The first step HCV takes to enter a hepatocyte is its binding to the cell surface heparan sulfate glycosaminoglycan (48-50) and LDL-R (24,31). Once the virus contacts the hepatocyte a number of proteins in the cell membrane play a role in viral internalization, including tetraspanin CD81, the scavenger receptor class B type I (SR-BI), and the TJ-associated proteins claudin-1 and occludin (51) (Fig. 2). Recently, the epidermal growth factor receptor and Niemann-Pick C1-like 1 cholesterol absorption receptor have been shown to also play a role in HCV entry into cells (52,53).

In contrast to CD81 and SR-BI, no direct interaction between claudin-1 and HCV has been demonstrated as yet (54,55). However, two amino acids in the first extracellular loop of claudin-1 have been shown to be essential for its role as a receptor (54), which suggests the presence of some sort of extracellular binding with the virus. Furthermore, an interaction between claudin-1 and the viral envelope proteins E1 and E2 has been demonstrated following overexpression in cell lines (56). Under such conditions, an interaction between claudin-1 and CD81 has also been observed both in the plasma membrane and intracellular vesicles expressing early endosome markers (56), which suggests a potential coordinated endocytosis of both proteins. CD81-claudin-1 interaction has also been shown in other experimental systems (57-60), where its inhibition by blocking antibodies or directed mutagenesis –which targets key amino acids involved in their interaction– precludes an effective binding of the virus to the cell surface, thus preventing HCV infection (55,60). In addition, by using blocking antibodies in different infection stages, claudin-1 has been shown to be involved in an entry phase closely related to CD81 (55), thus reinforcing the notion that both proteins may work together during the entry process. Since the association of claudin-1 with CD81 occurs primarily in the basolateral domain of the plasma membrane (60), and a claudin-1 mutant lacking the cytoplasmic C-terminal end needed for its inclusion in TJs (61) can function as a HCV co-receptor, viral entry has been suggested to take place outside the TJ area (54). Overall, all these data suggest that the role of claudin-1 during viral entry is closely related to that of CD81 and occurs in membrane areas not involved in intercellular junctions.

Despite the fact that HCV infection requires the presence of TJ-associated proteins, the role of TJs themselves during viral entry is controversial. Although some authors suggest that TJ integrity is necessary for HCV infection (62-64), studies performed in polarized cells question this hypothesis (58,65,66) and posit that TJs act as a barrier to restrict infection. On the other hand, vascular endothelial growth factor (VEGF) has been shown to induce cell depolarization and favor HCV infection (66). However, VEGF treatment also promotes the intracellular localization of occludin (66,67), possibly as a result of its endocytosis from TJs. Hence, these data do not allow to discriminate whether VEGF-induced increased infection may result from cell polarization loss itself or the induction of occludin internalization, which might mediate viral endocytosis. Anyway, the mechanism by which occludin plays a role in HCV infection remains unknown, and its analysis has been challenged by a lack of useful tools such as an antibody against occludin to block viral entry. In addition to the aforementioned potential coordinated internalization of the viral particle and occludin, other alternative hypotheses may be readily conceivable to explain the role of occludin in HCV infection. As with claudin-1, no direct association of occludin with HCV envelope proteins has been demonstrated (68). This might mean that, similarly to the viral particle-CD81-claudin-1 complex, the interaction of occludin with another viral coreceptor (X) may be necessary for the formation of a viral particle-X-occludin complex to mediate HCV entry into cells. One possibility is that occludin may direct this complex to a plasma membrane area where virion endocytosis would take place. This area may be defined either by the presence of a third co-receptor necessary for viral internalization or by a dynamic domain with a high number of endocytic processes such as TJs (39). In fact, this sort of lateral transport before viral entry into cells has already been described for multiple

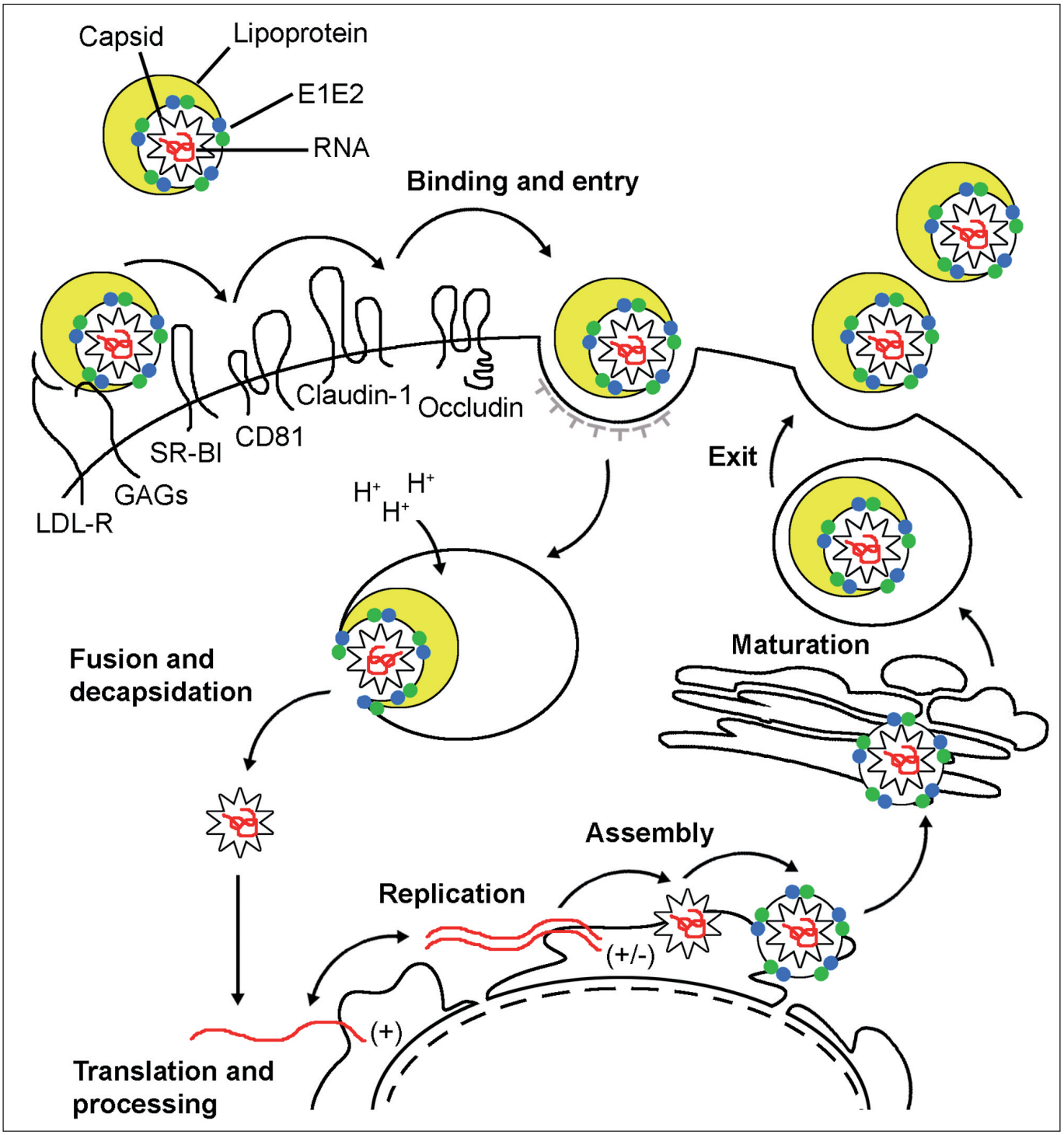

Fig. 2. HCV cycle. A viral particle interacts with anchorage factors and cell co-receptors, and is internalized via clathrin-mediated endocytosis. Early endosome acidification leads to fusion with the viral envelope. Following decapsidation, the positive polarity (+) genomic RNA is translated into a single polyprotein that is then processed by celllular and viral proteases. Viral offspring assembly and maturation follows replication, and new viruses exit the cell via its vesicular secretion system. GAG: glycosaminoglycans; E1, E2: viral envelope proteins.

viruses (69). Another possibility would be that the inclusion of occludin within the viral entry macromolecular complex may facilitate a conformational change in some host cell factor or HCV envelope proteins in such a way as to allow virion endocytosis or fusion with the endosomal membrane. Notably, cell-cell fusion experiments have revealed

that occludin plays a role in membrane fusion that is dependent on HCV envelope proteins (70). Interestingly, this phenomenon has also been described for CD81 and claudin-1 (54,71), which may indicate the presence of a coordinated set of proteins favoring fusion during viral entry. In this regard, protein gp120 present at the human immunodeficiency virus envelope undergoes a conformational change by interacting with CD4, present in the target cell membrane, which enables its further association with co-receptors CXCR4 and CCR5, as well as the fusion between viral and cell membranes (72).

HCV-induced TJ changes

Hepatocyte polarity maintenance is crucial for bile synthesis and secretion into bile canaliculi. The effective separation between hepatocyte apical and basolateral domains is essential for this process as it allows the appropriate localization of the various transporters and the formation of osmotic gradients necessary for bile secretion (73). TJs play a key role in the preservation of hepatocyte polarity, and seal bile canaliculi thus blocking the contact between blood and bile. Both in patient samples and different experimental models, the alteration of TJ structural and functional integrity has been shown to be closely related to the development of cholestasis, a condition characterized by a partial or complete interruption of bile flow (74-76). HCV induces TJ disruption and cell polarity loss in various *in vitro* systems (66,77,78), which may explain the cholestatic hepatitis that develops in some patients with relapsing HCV following liver transplant, where viral load is particularly high because of immunosuppressors (79). Similarly to this hypothesis, some bacteria and viruses have been shown to alter TJ structure and function in their target cells inducing varying adverse events in the different organs involved, thus giving rise to gastrointestinal or respiratory diseases (40,43). On the other hand, a relationship between TJ disassembly and liver carcinoma development and progression has been established (80-83); this finding, and the fact that TJ disruption may affect signaling pathways involved in cell proliferation and differentiation (35), may influence HCV-associated hepatocarcinoma development.

Besides damaging liver functioning, TJ disruption may play a role in the viral cycle favoring viral dissemination and survival. Similarly to adenovirus and rotavirus (44,84), HCV might well induce TJ disruption to ease viral spreading or access to viral receptors. In fact, VEGF-mediated cell polarity and TJs integrity changes have been shown to facilitate HCV infection (66), although the mechanism through which this happens remains unknown. Similarly, the intracellular interaction of occludin with HCV envelope protein E2 (77) may play a role in viral exit from infected cells since, taking advantage of occludin transport towards the plasma membrane, virions might position themselves in areas suitable for exocytosis.

THE IMPORTANCE OF CELL POLARITY MAINTENANCE FOR THE STUDY OF HCV *IN VITRO*

A detailed understanding of HCV infection mechanisms is necessary in order to design efficient antiviral strategies, with a particular focus on the way host cell factors are involved. Targeting these might overcome the issue entailed by the virus' high genetic variability, which prevents effective immune response and enables viral adaptation to treatments through the emergence of resistant strains (51). In the last few years the development of tools for the study of HCV cycle *in vitro* has allowed a highly significant advance in the identification of cell factors involved in different infection stages. Nevertheless, the interpretation of findings should be cautious since data obtained are markedly different depending on the experimental system employed. Such discrepancies may stem from the fact that hepatocytes present very special characteristics that are not preserved in most *in vitro* systems. Thus, Huh-7 cells and several related clones, the primary source of HCV *in vitro*, lack the typical hepatocyte polarity (37,77) and are unable to secrete authentic VLDLs (27). Given the relationship established between polarization and lipoprotein secretion (85,86), and the existing connection between VLDL biogenesis and HCV assembly (17-19,23,87), it is reasonable to surmise that deficient polarization of source cells may entail the generation of viral particles with a composition differing from that seen when coming from polarized hepatocytes. As a matter of fact, it has been demonstrated that, in contrast to highly polarized cells Caco-2 and HepG2, the Huh-7 cell line is unable to secrete HCV envelope proteins complexed with apolipoprotein ApoB (20). Hence, the different composition and/or disposition of lipoproteins in viral particles either generated from Huh-7 cells or isolated from patient sera may account for the diverging findings obtained with both systems, particularly concerning LDL-R dependence for infectivity (24,31,88-90).

In addition to the potential alterations of the infective particle depending on the polarization extent of source cells, consideration should be given to the fact that infection mechanisms may also be linked to target cell polarization. Regarding HCV this consideration is particularly relevant since claudin-1 and occludin, which are TJ-associated proteins, act as viral receptors. On the other hand, the fact that intercellular HCV transmission may occur by direct transfer from an infected cell to adjacent cells using an as yet unclear mechanism that might operate with no release of viral particles to the extracellular environment must be highlighted (91-93). In this respect, cell polarization extent may also somehow influence this type of viral spread via cell-cell junction modulation.

Despite the current controversy regarding the potential role of cell polarity in viral entry, it seems logical that HCV-producing cell polarization, lipoprotein synthesis and secretion, virion composition, and the mechanisms of viral entry into polarized hepatocytes may be closely interrelated.

Therefore, to obtain accurate conclusions, the entire HCV cycle should be studied in a context where these factors mimic hepatocytes and viral particles as present in patient serum as much as possible. The use of primary human hepatocytes (PHHs) is considered adequate for the study of HCV infection *in vitro* since, apart from being susceptible to infection, these cells may be maintained in a highly polarized, differentiated state (14). In addition, new infective viral particles can be produced from infected PHHs (27), and the system is thus valid to study the complete viral cycle. However, intrinsic limitations to the use of primary human cells, such as restricted availability or high inter-sample heterogeneity, render the use of PHHs difficult as a routine system for the study of HCV. This is why experimental *in vitro* systems based on cell lines are necessary, but their characteristics should also mimic those of PHHs as much as possible. The HepG2 cell line with ectopic expression of CD81 has been used as a model for the study of HCV infection in a hepatocyte polarity setting (66, 58), although its limited susceptibility to infection challenges its use for the study of virus assembly and secretion. On the other hand, the complete viral cycle has been reproduced in bioreactor-grown 3D cultures (94-96). These studies, while showing an increased expression of specific differentiation genes in 3D cultures as compared to bidimensional traditional cultures, have not shown a polarization extent similar to that of hepatocytes in the former. On the other hand, the use of Matrigel (a commercial product consisting of a mix of extracellular matrix proteins) for the culture of PHHs and hepatocyte-derived cell lines has been employed extensively to maintain cell polarity, differentiation, and functionality *in vitro* (97). Recently, 3D cultures of Matrigel-embedded Huh-7 cells, in addition to acquiring structural and functional polarity feature of hepatocytes, were shown to be susceptible to HCV infection and to produce new infective viral particles with similar titers to those obtained in standard bidimensional cultures (98). On the one hand, these cultures allow an analysis of cell requirements for infection to occur in a hepatocyte-like polarity setting. On the other hand, they enable the study of viral effects on the host cell under conditions similar to those of hepatocytes in their natural environment. Furthermore, this system may be used to characterize in detail viral particles from polarized cells. The latter utility may be of great interest as, when compared to viral particles generated by bidimensional cultures, viruses obtained from 3D-cultured Huh-7 cells present lower density and increased specific infectivity (98). It should be highlighted that these characteristics are similar to those of viruses from HCV-inoculated PHHs (27) and present in the serum of patients (12,13, 26,99) or infected animals (28).

To conclude, all these data suggest that both the structural and functional properties of virions may depend on the characteristics of their source cells. Thus, the mechanisms and molecular requirements of infection may differ according to the origin of viral particles, which would be particularly relevant when selecting experimental systems suitable for the assessment of antiviral compound effectiveness and when designing new therapeutic strategies against the chronic, progressive liver disease associated to HCV infection.

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