Research Article

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Early infection events highlight the limited transmissibility of hepatitis C virus *in vitro*

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Background & Aims: Hepatitis C virus (HCV) poses a global health problem, with over 170 million chronically infected individuals at risk of developing progressive liver disease. The ability of a virus to spread within a host is a key determinant of its persistence and virulence. HCV can transmit *in vitro* by cell-free particle diffusion or via contact(s) between infected and naïve hepatocytes. However, limited information is available on the relative efficiency of these routes, our aim is to develop physiologically relevant assays to quantify these processes.

Methods: We developed a single-cycle infection assay to measure HCV transmission rates.

Results: We compared HCV spread in proliferating and arrested cell systems and demonstrated a significant reduction in cell-to-cell infection of arrested target cells. Comparison of cell-free and cell-to-cell virus spread demonstrated relatively poor transmission rates, with 10–50 infected producer cells required to infect a single naïve target cell. We found HCV strain J6/JFH to be 10-fold more efficient at spreading via the cell-to-cell route than cell-free, whereas SA13/JFH and HK6/JFH strains showed comparable rates of infection via both routes. Importantly, the level of infectious virus released from cells did not predict the ability of a virus to spread *in vitro*, highlighting the importance of studying cell-associated viruses.

Conclusions: These studies demonstrate the relatively poor infectivity of HCV and highlight differences between strains in their efficiency and preferred route of transmission that may inform future therapeutic strategies that target virus entry.

Keywords: Hepatitis C virus; HCV; Transmission; Cell-to-cell; Synapse.
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Abbreviations: HCV, hepatitis C virus; mAb, monoclonal antibody; ROI, region of interest; SR-BI, scavenger receptor B1.

Introduction

Hepatitis C virus (HCV) evades host innate and adaptive immune responses to establish chronic infection [1]. The virus utilizes multiple strategies to evade immune recognition, including genetic escape via an error-prone RNA polymerase and perturbation of host immune signalling [2]. However, diffusion of viral particles through the vascular circulation exposes the virus to neutralizing antibodies and complement. Consequently, many viruses have evolved to transmit via direct cell-cell contacts, allowing the virus to evade adaptive immune responses (reviewed in [3,4]).

We [5,6] and others [7] reported that HCV can spread via cell-to-cell contacts, where transmission is dependent on the viral encoded glycoproteins and host cell receptors tetraspanin CD81, scavenger receptor B1 (SR-BI), and tight junction proteins claudin-1 and occludin [5]. However, the nature of cell contacts required for viral transmission, along with the prevalence and efficiency of early cell-to-cell transmission events, remains largely unexplored. Several different virus families have been shown to transmit via cell-cell contacts: HSV induces a direct cell-fusion event that leads to cytoplasmic mixing between naive and infected cells; HIV and HTLVI induce the formation of a virological synapse between cells, whilst MLV utilizes filopodia to transmit from one cell to another [3,4]. All of these mechanisms may be relevant to HCV transmission, where hepatocytes are known to form multiple adhesive contacts. Several reports have documented that HIV cell-to-cell transmission is significantly more efficient than cell-free infection [8–10], however, this information is currently lacking for HCV.

To address these issues, we studied the early cellular events that define HCV transmission. Development of our previously published co-culture system [5] enabled us to monitor single-cycle infection events and to quantify cell-to-cell and cell-free transmission rates. These studies highlight the relatively poor infectivity of HCV *in vitro*, where between 10 and 50 infected cells were required to infect a single naïve target cell. We observed differences in the ability of diverse genotypes to spread via cell-to-cell and cell-free routes, with HCV J6/JFH showing a ten-fold higher rate of transmission via cell-to-cell contacts compared to cell-free infection. In contrast, HCV SA13/JFH and HK6A/JFH showed comparable rates of infection via both routes. We

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observed significantly higher levels of infectious extracellular J6/JFH virus harvested from infected cells than predicted by the frequency of cell-free transmission events, suggesting differences in virus stability under the two experimental conditions. We found that adherens-like contacts between cells were required to initiate HCV cell-to-cell transmission and these junctions limit the efficacy of anti-viral antibodies to neutralize HCV transmission. Finally, we demonstrate that SR-BI localization and expression at filopodia may limit viral spread and provide an ideal target for therapeutic intervention [11].

Materials and methods

Cell lines, antibodies and viruses

HuH-7 and HuH-7.5 cells were propagated in Dulbecco's modified Eagle medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% non-essential amino acids. HuH-7.5 cells were transduced to overexpress AcGFP-CD81 or SR-BI-eGFP [12]. Cell proliferation was measured in an MTS assay (Promega). Monoclonal antibody (mAb) anti-CD81 2s131 was generated by immunizing mice with full-length CD81 [13], anti-SR-BI was a gift from Pfizer Ltd and ITX5601 was provided by iTherX. Immunoglobulin was isolated from the sera of normal and chronically infected HCV patients by protein G-conjugated Sepharose beads as previously reported [5]. Chimeric JFH-1 viruses expressing diverse structural proteins were previously reported [14], with the major strains under study representing genotypes 2 (J6/JFH), 5 (SA13/JFH), and 6 (HK6A/JFH).

Virus transmission assay

HCV infected cells were labelled with the fluorescent cell tracker CMFDA and co-cultured with an equal number of naïve HuH-7 cells at 2.5×10^4 cells/cm^2 [5]. Extracellular virus was neutralized by treating cultures with anti-HCV Ig or control Ig (150 μg/ml) and antibody efficacy confirmed by analysing the culture media after 24 h co-culture for the level(s) of infectious virus. Cells were harvested at defined time points and infected target cells identified by staining for HCV NS5A and flow cytometry. Cell-to-cell transmission is defined as the frequency of infection events in the presence of neutralizing anti-HCV-Ig, and subtraction of this value from the total number of infected cells provides an estimate of cell-free infection (Supplementary Fig. 1).

Live confocal microscopy

HuH-7.5 cells expressing AcGFP-CD81 or SR-BI-eGFP were seeded at 2.5×10^4 cells/cm^2 in phenol-red free media and imaged for 2 h on a Zeiss 780 laser scanning confocal microscope (100× 1.4NA objective), collecting 0.42 μM optical sections at 0.5 s/frame through the cell. Images were acquired with minimum laser power and microscope settings optimized for the highest signal to noise ratio whilst controlling for crosstalk. Protein expression was quantified by measuring arbitrary fluorescent units (AFU) compared to background fluorescence (area without cells) and was considered positive when the signal was 2× the background plus 3 standard deviations. Twenty regions of interest (ROI) from a total of 20 cells were scanned and 3D volumetric images obtained using the surface-volume function of Imaris 7.4 (Bitplane Scientific Software).

Results

HCV transmission kinetics

HCV has been reported to spread via cell-to-cell contacts [5–7], however, the majority of reports analysed infection over 48–72 h time periods, where division of infected target cells can complicate data interpretation. To address this, HCV strain SA13/JFH infected cells were labelled with CMFDA and co-cultured with naive HuH-7.5 target cells that were either actively proliferating or growth-arrested via irradiation (10,000 rad) or mitomycin C (10 μM) treatment. We confirmed that irradiation and mitomycin C treatments arrested hepatoma proliferation (Fig. 1A). Infections were allowed to proceed in the presence or absence of polyclonal anti-HCV Ig to neutralize extracellular infectious virus as depicted in Supplementary Fig. 1. We noted a significant increase in the frequency of cell-to-cell transmission events between 24 h and 48 h in proliferating cells (41–91%). In contrast, arrested target cells showed comparable cell-to-cell transmission frequencies at both time points, demonstrating that the division of infected target cells can be misinterpreted as neutralizing antibody-resistant infection (Fig. 1B).

We noted a significant reduction in the number of HCV infected arrested cells, most likely reflecting their reduced capacity to support HCV RNA replication [15]. However, the frequency of cell-to-cell transmission following a 24 h co-culture with non-arrested and arrested cells was comparable (Fig. 1B), demonstrating that 24 h is the optimal time to study virus transmission. We confirmed that diverse JFH chimeric viruses could transmit via the cell-to-cell route following a 24 h co-culture with non-arrested target cells. While the number of newly infected cells is a useful indicator of spreading infection, to compare transmission between viral genotypes, one needs to consider the number of HCV infected producer cells in each culture. We therefore calculated the number of newly infected targets/10^5 infected producers (Fig. 1C), demonstrating the relatively low transmissibility of HCV, where between 10–50 infected producers were required to infect a single naïve target cell.

To measure the rate of HCV transmission, we established a single-cycle assay where CMFDA-labelled infected producer cells were seeded with naive HuH-7.5 targets before adding neutralizing anti-CD81 mAb at specified times. The cells are cultured for an additional 16 h to allow viral proteins to accumulate in the newly infected target cells, for flow cytometric detection of NS5A (Fig. 1D). Addition of anti-CD81, immediately after co-culturing infected and naïve cells, inhibited viral spread as previously reported [5], validating the use of this reagent to quantify single-cycle early infection events. Newly infected targets were detected within the first hour of co-culturing producer cells infected with all three chimeric JFH-1 strains, showing a time-dependent increase in *de novo* infection events, and confirming the relatively poor transmissibility of these viruses *in vitro* (Fig. 1E).

Cell-to-cell transmission has been reported to be more efficient than cell-free infection for many viruses, including HIV [4,8]. To ascertain whether this mode of transmission is more efficient than cell-free HCV infection, we used the single-cycle co-culture assay to quantify viral spread in the presence or absence of neutralizing antibodies that ablate extracellular particle infectivity. For all viruses, cell-to-cell transmission was detectable within 1 h of co-culturing producer and target cells, whereas cell-free infection was significantly delayed (Fig. 2A). Since hepatoma cells grow as an adherent monolayer, they are removed from their culture vessel with 1% trypsin, to enable the co-culture of infected and naïve target cells. We confirmed that trypsinizing and replating the cells had no effect on infectious virus secretion, validating our earlier conclusion that differences exist between cell-to-cell and cell-free infection kinetics. Comparable early-stage kinetics were observed with RIG-I competent HuH-7 and HuH-7.5 target cells (Supplementary Fig. 2). The accumulation of newly infected cells during the first