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Hepatitis C virus infection of neuroepithelioma cell lines

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Abstract

Background & Aims—Hepatitis C virus (HCV) establishes chronic infections in 3% of the world's population. Infection leads to progressive liver disease; hepatocytes are the major site of viral replication in vivo. However, chronic infection is associated with a variety of extrahepatic syndromes, including central nervous system (CNS) abnormalities. We therefore screened a series of neural and brain-derived cell lines for their ability to support HCV entry and replication.

Methods—We used a panel of neural-derived cell lines, HCV pseudoparticles (HCVpp), and an infectious, HCV JFH-1 cell-culture system (HCVcc) to assess viral tropism.

Results—Two independently derived neuroepithelioma cell lines (SK-N-MC and SK-PN-DW) permitted HCVpp entry. In contrast, several neuroblastoma, glioma, and astrocytoma cell lines were refractory to HCVpp infection. HCVcc infected the neuroepithelioma cell lines and established a productive infection. Permissive neuroepithelioma cells expressed CD81, scavenger receptor BI (SR-BI), and the tight junction proteins Claudin-1 (CLDN1) and occludin, whereas non-permissive neural cell lines lacked CLDN1 and in some cases SR-BI. HCVpp infection of the neuroepithelioma cells was neutralized by antibodies to CD81, SR-BI, CLDN1 and HCV E2.

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Furthermore, anti-CD81, interferon and the anti-NS3 protease inhibitor VX-950 significantly reduced HCVcc infection of neuroepithelioma and hepatoma cells.

Conclusions—Neuroepithelioma-derived cell lines express functional receptors that support HCV entry at comparable levels to that of hepatoma cells. HCV infection in vitro is not restricted to hepatic-derived cells, so HCV might infect cells of the CNS in vivo.

Keywords

OC4N; neurotropism; brain; therapy; replicon; Huh-7; VX-950

Introduction

Hepatitis C virus (HCV) is an enveloped positive-strand RNA virus, classified in the Hepacivirus genus of the Flaviviridae family. Worldwide, approximately 170 million individuals are persistently infected with HCV that leads to a progressive liver disease including hepatic fibrosis, cirrhosis and hepatocellular carcinoma. Infection is associated with a variety of extrahepatic syndromes, including cryoglobulinemia, glomerulonephritis and central nervous system (CNS) abnormalities¹.

While HCV is primarily a hepatotropic virus, genomic viral RNA has been detected in peripheral blood mononuclear cells (PBMCs), cerebrospinal fluid and the brain of chronically infected patients with neuropathological abnormalities (reviewed in²⁻⁴). Discriminating RNA association from true HCV replication is technically challenging and multiple artefacts complicate the detection and quantification of replicative intermediate minus-strand HCV RNA. Sequence analysis of PBMC/brain and liver derived HCV sequences demonstrate tissue specific differences, suggesting independent evolution at different anatomical sites⁵⁻⁸. Moreover, CNS derived HCV 5'-untranslated region sequences were reported to have reduced translation efficiency in some cell lines compared with sequences derived from the serum and liver⁶, supporting a model of viral adaptation in the brain. To date, the majority of reports studying HCV replication have utilized hepatoma-derived cells. Limited progress has been made to assess HCV replication in other cell types, largely attributable to a lack of efficient cell culture systems and small animal models to study sites of HCV replication in vivo. Kato and colleagues reported HCV JFH-1 sub-genomic replication in two non-hepatocyte derived cell lines: HeLa cells established from human cervical carcinoma and 293 cells derived from human embryonic kidney, demonstrating that HCV replication can occur in non hepatic cells⁹. Earlier reports demonstrated low level replication of sub-genomic replicons in non-hepatic cell lines^{10,11}, lending further support to the model that HCV replication is not restricted to cells of hepatic origin.

Viruses initiate infection by attaching to molecules or receptors at the cell surface, whose expression can define cellular or tissue permissivity. The availability of pseudoparticles bearing HCV E1E2 glycoproteins (HCVpp) and the recently reported JFH-1 strain of HCV that replicates and assembles infectious particles in cell culture (HCVcc) have enabled considerable advances in our understanding of the receptors involved in HCV internalization. HCVpp have been reported to show a restricted tropism for hepatocyte derived cell lines, suggesting that HCV glycoprotein-receptor interactions play a major role in defining viral tropism for the liver¹²⁻¹⁴. Recent evidence shows a number of host cell molecules to be important for HCV entry: tetraspanin CD81, scavenger receptor class B member I (SR-BI) and the tight junction proteins Claudin-1 (CLDN1) and occludin (reviewed in¹⁵). RNA profiling and protein expression studies demonstrate that the HCV entry factors are expressed in a variety of tissues, albeit with an increased expression in the

liver^{16,17}. Reports of cognitive defects and nuclear magnetic resonance abnormalities in HCV infected patients¹⁸⁻²¹ led us to screen a series of neural and brain-derived cell lines for their ability to support HCV entry and replication. We found two independently derived neuroepithelioma cell lines which support HCVpp infection. HCVpp infection was neutralized by antibodies specific to CD81, SR-BI, CLDN-1 and HCV E2, demonstrating a common entry pathway for infection of neuroepithelioma and hepatoma cell lines. Furthermore, both neuroepithelioma cell lines support HCVcc infection, albeit at a 130-330 fold reduced infectious titer compared to the permissive Huh-7.5 hepatoma cell line. In summary, these data demonstrate that HCV infection in vitro is not restricted to cells of hepatocyte origin and raises the possibility of HCV infecting cells of the CNS.

Materials and Methods

Cells and reagents

SK-N-MC, MC-IXC, SK-PN-DW, SK-N-SH, 1321N1, NP2, U87 and SH-SH5Y cells were all obtained from ATCC (Table 1). MC-IXC is a twice cloned subline of SK-N-MC²². Huh-7.5²³ and 293T cells were provided by C. Rice, Rockefeller University. Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1% nonessential amino acids and 1% penicillin/streptomycin (Invitrogen). MC-IXC cells were maintained in DMEM/F12 medium with the above supplements.

The primary antibodies used were: anti-NS5A 9E10 (C. Rice, Rockefeller University); anti-CD81 (2.131)²⁴; anti-SR-BI (V. Flores, Pfizer); anti-CLDN1 (Abnova and R&D); anti-CLDN1 polyclonal sera²⁵; anti-occludin (Invitrogen); anti-ZO-1 (Invitrogen) and anti-E2 (9/27, 11/27 and 3/11)¹². Immunoglobulin from normal healthy volunteers and chronically HCV infected donors was purified by Protein-G affinity chromatography, as previously reported²⁶. Fluorescent labelled secondary antibodies, Alexa Fluor 488 anti-mouse, anti human, anti-rat and anti-rabbit IgG were obtained from Invitrogen.

Flow cytometric analysis of receptor expression

For CD81, SR-BI and CLDN1 staining, 2×10^5 cells were incubated in PBS containing 1% BSA and 0.01% sodium azide (PBA) for 20 minutes at 37°C. For occludin staining, cells were fixed with 1% paraformaldehyde and permeabilized with PBS containing 1% BSA and 1% saponin. 2µg receptor-specific antibodies or irrelevant IgG controls were incubated with cells in PBA for 30 minutes at 37°C, and unbound antibody removed by washing. Secondary anti-rabbit, mouse, human or rat Alexa-488 conjugated antibodies were incubated for a further 30 minutes at 37°C and the cells washed and fixed in 1% paraformaldehyde. Antibody binding was assessed by flow cytometry using a FACSCalibur (BD Biosciences) and data analyzed with FlowJo software (TreeStar, Ashland, OR).

Laser scanning confocal microscopy

The various cell lines were plated on collagen coated chamber slides (Fisher Scientific, UK) and fixed with ice-cold methanol (CLDN1, Occludin) or 3% paraformaldehyde (CD81) 24h post seeding. Primary antibodies were applied for 1h at room temperature. After washing twice with PBS, anti-mouse, rabbit or rat Alexa Fluor 488 (Invitrogen, CA) secondary antibody was applied for 1h at room temperature. Cells were counterstained with 4',6-diamidino-2-phenylindole (DAPI) (Invitrogen) for nuclei visualization and mounted with ProLong Gold antifade (Invitrogen). Cells were viewed by laser-scanning confocal microscopy on a Zeiss META head confocal microscope with a 100× oil-immersion objective.

HCVpp genesis and infection

Pseudoparticles were generated by transfecting 293T cells with plasmids encoding a HIV provirus expressing luciferase and vesicular stomatitis virus G (VSV-G), a panel of HCV envelope glycoproteins (genotype 1)²⁷ or a no-envelope control, as previously reported¹². Supernatants were harvested at 48h post transfection, clarified, and filtered through a 0.45- μ m membrane. Virus-containing medium was added to target cells plated in 96 well plates seeded at 7.5×10^3 cells/cm² and incubated for 72h. At 72h post infection, cells were lysed with cell lysis buffer (Promega, Madison, WI) and luciferase activity measured for 10 s in a luminometer (Lumat LB 9507). Specific infectivity was calculated by expressing the HCV or VSV-G luciferase signal (relative light units, RLU) relative to the no envelope RLU value.

HCVcc genesis and infection

The plasmid encoding chimeric J6/JFH-1 (provided by T. Wakita, National Institute of Infectious Diseases, Japan and C. Rice, Rockefeller University, NY) was used to generate RNA as previously described²⁸. Briefly, RNA was transcribed using the RiboMAX express T7 kit (Promega) and electroporated into Huh-7.5 cells. Supernatants were collected at 72 and 96h post infection, pooled and stored at -80°C and their infectious titer for Huh-7.5 cells determined. Cells were fixed with ice-cold methanol and infected cells identified by staining for NS5A with mAb 9E10 and Alexa 488-conjugated anti-mouse IgG. Infection was quantified by enumerating NS5A-positive foci and infectivity defined as the number of focus forming units/ml (FFU/ml). 2×10^5 Huh-7.5, 293T, MC-IXC or SK-PN-DW cells were seeded per well of 12 well plate and infected with 1.6×10^4 FFU of J6/JFH for 8h, unbound virus was removed by three sequential washes and the cells incubated at 37°C. Infected cells were identified by the expression of NS5A or HCV RNA. Purified cellular RNA samples (Qiagen) were amplified for HCV RNA (Primer Design Ltd) in a single tube RT-PCR in accordance with the manufacturer's guidelines (CellsDirect kit, Invitrogen) and fluorescence monitored in a MxPro 3000 real time PCR machine (Stratagene). In all reactions the housekeeping gene GAPDH was included as an internal endogenous control for amplification efficiency and for RNA quantification (primer-limited endogenous control, ABI).

Neutralization of HCVpp infection

Huh-7.5, SK-N-MC, MC-IXC or SK-PN-DW cells were seeded in 96-well plates at 7.5×10^3 cells/cm². 24h post seeding, the cells were incubated with 5 μ g/ml anti-receptor or irrelevant IgG control monoclonal antibody (mAb). After 1h, HCV-H77pp, VSV-Gpp or No-envpp was added and incubated for 72h at 37°C. In addition, anti-E2 mAbs or HCV⁺ IgG was incubated with virus for 1h prior to infecting the appropriate target cells. At 72h post infection, cells were lysed with cell lysis buffer (Promega, Madison, WI) and luciferase activity measured for 10s in a luminometer (Lumat LB 9507). The percent neutralization was calculated relative to the irrelevant IgG control.

Statistical analysis

Results are expressed as the mean $\pm$ 1 standard deviation of the mean. Statistical analyses were performed using Students t-test in Prism 4.0 (GraphPad, San Diego, CA) with a P <0.05 being considered statistically significant.

Results

Neuroepithelioma cell lines are highly permissive for HCVpp entry

To assess the ability of HCVpp to infect cells of neural origin, a panel of neuroblastoma, glioma, astrocytoma and neuroepithelioma cell lines were incubated with HCVpp-H77 and control VSV-Gpp. The neuroepithelioma cell lines SK-N-MC, MC-IXC and SK-PN-DW were highly permissive for HCVpp entry, similar to Huh7.5, whereas the neuroblastoma, glioma and astrocytoma cells failed to support HCVpp infection (Fig.1). The non-permissive CLDN1-null human embryonic kidney 293T cell line was included as a control²⁹ (Fig.1). To assess lentiviral promoter activity in the various cell lines we evaluated the ability of pseudotypes expressing VSV-G to infect the various cell lines. All of the neuroepithelioma cell lines supported VSV-Gpp infection at comparable levels to Huh-7.5 hepatoma cells, however NP2, SH-SH5Y and 1321N1 cells demonstrated 10-20 fold reduced entry (Fig.1 A). Similar results were observed for MLVpp infection, suggesting a reduced efficiency of the HIV LTR promoter in these cell types. If one normalizes HCVpp infection of NP2, SH-SH5Y and 1321 cells relative to VSV-Gpp entry, these cell types remain refractory to HCVpp infection. In contrast, the SK-N-SH neuroblastoma cell line was non-permissive for VSV-Gpp (Fig.1 A) and MLVpp (specific infectivity of 2.3 ± 0.5) infection, rendering the HCVpp infection data difficult to interpret. In contrast, the inability of HCVpp to infect the U87 neuroblastoma cell line, which supports high level VSV-Gpp infection, confirms that these cells are refractory to HCV entry (Fig.1 A).

To investigate whether the neuroepithelioma cells support the entry of diverse HCVpp, we assessed the ability of pseudoparticles bearing E1E2 envelope glycoproteins cloned from several genotype 1a/b acutely HCV infected subjects to infect SK-N-MC, MC-IXC and SK-PN-DW cells (Fig.1B). All HCVpp tested infected the neuroepithelioma cell lines at comparable levels to Huh-7.5, demonstrating a 2-6 fold range in permissivity (Fig.1B). In conclusion, two independently derived neuroepithelioma cell lines, SK-N-MC and SK-PN-DW, support HCV entry at comparable levels to those observed with the Huh-7.5 hepatoma cell line, demonstrating that HCV glycoprotein-dependent pseudoparticle entry is not restricted to hepatocytes.

Neuroepithelioma cell lines express HCV entry factors

To ascertain whether these neural cell lines express the HCV receptor proteins, live cells were stained with antibodies specific for extracellular epitopes of CD81, SR-BI and CLDN1 or were fixed and permeabilized prior to staining with antibodies specific for the occludin C-terminal region (Fig.2). The permissive neuroepithelioma cell lines SK-N-MC, MC-IXC and SK-PN-DW expressed all four entry factors (Fig.2A), with mean fluorescence intensities (MFIs) within two-fold of Huh-7.5 cells (Fig.2B). In contrast, NP2 and SH-SH5Y cells express low levels of CD81, SR-BI and CLDN1 (Fig.2B). SK-N-SH express negligible levels of SR-BI and CLDN1 (Fig.2B). Whereas CLDN1 and Occludin MFIs were not significantly greater than the isotype control antibody for 1321N1 cells. Finally, U87 cells had no detectable CLDN1 expression (Fig.2B). To assess receptor localization, antibody stained cells were imaged by confocal microscopy (Table 2). In Huh-7.5, SK-N-MC, MC-IXC and SK-PN-DW cells, CD81, CLDN1 and Occludin localized to the plasma membrane of cellular junctions and showed minimal cytoplasmic staining (Fig.3 and Supplementary Fig.1). In contrast, Occludin localized to intracellular sites in all of the non-permissive cells that expressed detectable levels of Occludin by flow cytometry (NP2, SH-SH5Y, SK-N-SH and U87) (Table 2), as represented by U87 cells (Fig.3). In summary, neuroepithelioma cells express all of the essential HCV entry factors, most likely accounting for their permissivity to HCVpp infection.

Receptor dependency of HCVpp infection of neuroepithelioma cells

To investigate the receptor dependency of neuroepithelioma cell infection, we assessed the ability of antibodies specific to CD81, SR-BI and CLDN1 to inhibit HCVpp-H77 infection (Fig.4A). Anti-CD81 mAb inhibited HCVpp infection of Huh-7.5, SK-N-MC, MC-IXC and SK-PN-DW cells, confirming that infection was CD81 dependent. An anti-SRBI mAb significantly ($p<0.01$) reduced HCVpp infection of the neuroepithelioma cells but demonstrated a more modest effect on HCVpp infection of Huh-7.5 cells, as previously reported²⁶ (Fig.4A). Similarly, the anti-CLDN1 sera demonstrated a greater potency to inhibit HCVpp infection of the neuroepithelioma cells ($p<0.01$) than Huh-7.5 hepatoma cells ($p<0.05$) (Fig.4A). None of the antibodies had any effect on VSV-Gpp infection of hepatoma or neuroepithelioma target cells tested (data not shown).

To assess the glycoprotein dependency of HCVpp infection we investigated the ability of mAbs to specific E2 neutralizing epitopes and polyclonal IgG purified from chronic HCV infected subjects (HCV⁺ Ig) to modulate infection of neuroepithelioma and Huh-7.5 cells. All of the mAbs were used at a saturating concentration previously reported to neutralize HCVpp-H77 infection of Huh-7.5 cells¹². E2-specific mAbs and HCV⁺ Ig neutralized HCVpp infection of all target cells (Fig.4B) but had no effect on VSV-Gpp infection (data not shown). In summary, these data demonstrate a common pathway for HCVpp infection of neuroepithelioma and Huh-7.5 hepatoma cells that is dependent on HCV E2 and CD81, SR-BI and CLDN1 entry factors.

HCVcc infection of neuroepithelioma cells

Given the permissive nature of the neuroepithelioma cells to support HCVpp entry, we investigated their ability to support HCVcc J6/JFH infection by quantifying NS5A expression and HCV RNA levels. Since infection studies require extensive washing to remove input virus prior to extracting cellular RNA for qPCR HCV RNA analysis, we selected the MC-IXC sub-clone of SK-N-MC that are more resilient to the washing process. As a control, we included the non-permissive parental 293T cell line, as detailed in Table 1. All cells were incubated with J6/JFH for 12h and 72h and NS5A and HCV RNA levels quantified (Fig.5). Both neuroepithelioma cell lines express NS5A and HCV RNA, whereas there was no detectable antigen in 293T cells. The NS5A defined viral titer on MC-IXC and SK-PN-DW cells was 130-330 fold lower than Huh-7.5 cells (Fig.5A). There was no detectable HCV RNA 12h post infection, demonstrating a time dependent increase in HCV RNA levels (Fig.5B). Incubation of both neuroepithelioma cell lines and Huh-7.5 with Interferon α (100 IU/ml) and anti-NS3 VX-950 (10 μ g/ml) significantly inhibited J6/JFH replication (Fig.5B). To assess whether HCV infection persisted in the neuroepithelioma cells, HCV RNA levels were quantified at 48, 72 and 96h post infection. HCV RNA increased significantly in all cell lines tested between 48 and 72h post infection (Fig.5C), and persisted in the MC-IXC and SK-PN-DW cells. HCV RNA levels were significantly reduced by anti-CD81, confirming receptor-dependent infection (Fig.5C).

We were unable to detect infectious particle release from the HCV infected neuroepithelioma cells, however this is consistent with the low levels of infection observed in these cells compared to the highly permissive Huh-7 cell line. We noted a linear relationship between the levels of infectious HCV released from Huh-7.5 cells and multiplicity of infection (MOI) (Supplementary Fig.2). At the MOI achieved in SK-PN-DW and SK-N-MC (order of 0.003), we failed to detect infectious particle release from Huh-7.5 cells. In summary, these data demonstrate that neuroepithelioma cells replicate and maintain HCV RNA and protein expression over time, consistent with a productive infection.

Discussion

Our studies demonstrate that HCVpp can infect two independently derived neuroepithelioma cell lines, SK-N-MC and SK-PN-DW. HCVpp entry was inhibited by antibodies specific for CD81, SR-BI, CLDN1 and E2 glycoprotein, demonstrating a common entry pathway to that reported for hepatocytes and hepatoma derived cell lines (reviewed in^{30,31}). However, HCVpp failed to infect several other neuroblastoma cell lines, including U87 as previously reported^{13,14}. SK-N-MC were as permissive for HCVpp entry as the Huh-7.5 hepatoma cell line, demonstrating that HCVpp has a broader cell tropism than previously reported¹²⁻¹⁴. Indeed, we¹² and others^{13,14} reported that HCVpp have a restricted tropism for hepatocyte derived cells, concluding that glycoprotein-receptor interactions may in part define HCV tropism for the liver. A recent report showing that CD81, SR-BI, CLDN1 and occludin expression in Chinese Hamster Ovary (CHO) or murine NIH-3T3 cells conferred HCVpp entry, demonstrates that these four molecules constitute the minimal receptor requirements³². Importantly, mRNA and protein profiling data bases suggest that CD81, SR-BI, CLDN1 and occludin are expressed in a variety of epithelial and endothelial cells derived from various tissues^{16,17}, raising the possibility that other cell types may support HCV entry. Our data support this model and demonstrate the presence of functional receptor complexes in cells of brain origin.

SK-N-MC and the MC-IXC sub-clone represent a neuroepithelioma cell line derived from a metastatic tumor²². SK-PN-DW is an independent neuroepithelioma line derived in 1979 from a 17 year old Caucasian male³³. Both SK-N-MC and SK-PN-DW have been used extensively as model cell systems to study neuroepithelioma biology. A DNA microarray study identified SK-N-MC to express Ewing family tumour specific genes³⁴. Ewing sarcomas and primitive neuroectodermal tumours, including neuroepitheliomas, express a chimeric protein product of the gene fusion EWS-FLI1³⁵.

SK-N-MC, MC-IXC and SK-PN-DW expressed comparable levels of all entry factors to Huh-7.5 hepatoma cells (Fig.2), consistent with the ability of antibodies specific for CD81, SR-BI and CLDN1 to neutralize HCVpp infection (Fig.4). Several of the non-permissive glioma and neuroblastoma cells (NP2, SH-SH5Y and SK-N-SH) did not express detectable levels of SR-BI or CLDN1 (Fig.2). Although CLDN1 is essential for the formation of tight junction strands, additional transmembrane proteins (Occludin, tricellulin, junctional adhesion molecule) and scaffold proteins are required for the spatial and functional organization of tight junctions in polarized cells (reviewed in³⁶). Assessment of receptor localization by confocal microscopy demonstrated an intracellular localization of Occludin in NP2, SH-SH5Y, SK-N-SH and U87 cells (Table 2), that may reflect the low to undetectable levels of the associated tight junction CLDN1 protein. In contrast, Occludin, CLDN1 and CD81 localized to the plasma membrane in the permissive hepatoma and neuroepithelioma cells (Fig.3 and Supplementary Fig.1).

Viral tropism is likely to be defined at multiple levels of the lifecycle, including particle entry, RNA replication and assembly (reviewed in³⁷). The majority of studies demonstrate efficient HCV replication in hepatoma cell lines, which may reflect the presence of regulatory factors such as the liver specific micro RNA, miR122³⁸. However, HCV has been reported to replicate to low levels in non-hepatic cells^{9,10}, suggesting that additional cellular reservoirs may exist in vivo. Given our observation that MC-IXC and SK-PN-DW allow efficient HCV entry we assessed their ability to support HCVcc strain J6/JFH infection. Both neuroepithelioma cell lines support HCV replication that was significantly inhibited by anti-CD81, interferon α . It was interesting to note the reduced level(s) of HCV RNA/NS5A⁺ in MC-IXC and SK-PN-DW cells compared to Huh-7.5 (Fig.5), more akin to the low HCV RNA levels reported in HCV infected primary hepatocytes³¹. It is important to

note that Huh-7.5 cells have a mutation in the RIG-I dsRNA sensing pathway (reviewed in³⁹) which reduces their innate signalling responses and may contribute to their highly permissive nature. Such innate signalling responses are likely to be intact in the neuroepithelioma cells, that may impact on their permissivity to support HCV replication. Indeed, J6/JFH infects parental Huh-7 cells with 12-fold reduced titer compared to Huh-7.5 cells (data not shown), providing an additional comparison to the neuroepithelioma cell lines.

HCV RNA levels in the MC-IXC and SK-PN-DW cells increased significantly between 48h and 72h post infection and were maintained for the following 24h studied in the experiment. This is in contrast to our experience with a mutant J6/JFH bearing a deletion in NS5A (DelB) that blocks the assembly of infectious particles, whilst maintaining wild type levels of RNA replication⁴⁰. The frequency of DelB RNA and NS5A expressing cells declines rapidly between 48h and 96h following electroporation into Huh-7.5 cells (Joe Grove and Claire Brimacombe, unpublished data). In contrast, parental J6/JFH transmits to most cells in the culture and is maintained at 96h, as observed with the neuroepithelioma cells. In summary, these data are consistent with a productive replication cycle in the MC-IXC and SK-PN-DW cells that maintains the viral genome over time.

Our study is the first report that non-hepatocyte cell lines not engineered to express exogenous viral receptors support efficient HCVpp infection. The concept of extrahepatic HCV replication is not new and several reports have described the detection of HCV RNA with PBMCs, brain and CNS tissue (reviewed in²⁻⁴). Marukian and colleagues recently reported that primary resting or activated lymphocytes fail to support HCVpp entry or replication using the currently available JFH-1 strain of HCV⁴¹, suggesting that immune cells do not constitute active sites of virus replication in vivo. The limited expression of CLDN1 and Occludin tight junction proteins in T- and B-lymphocyte subsets may account for their inability to support productive HCV entry^{16,17}. Indeed, we recently demonstrated that JFH-1 HCVcc can associate with B-cells via SR-BI and the lectin molecule DC-SIGN, however, the virus failed to establish a productive infection in the B cells and required hepatoma cells to replicate and release progeny virus⁴².

Despite the many reports of cerebral dysfunction in chronic HCV infection and detection of viral RNA in autopsy brain tissue^{5,20,43-46}, there has been a paucity of in vitro studies to investigate HCV infection of neural derived cell lines. HCV belongs to the flaviviridae family, whose members include yellow fever, dengue and tick-borne encephalitis viruses that are well known to have neurotropic infectivity. Our data raise the possibility that neuronal cells may be susceptible to HCV infection and warrant further studies to investigate HCV strains from different host compartments to determine the full range of HCV tropism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

CNS	Central nervous system
CLDN1	claudin-1
FFU	Focus forming units
MFI	Mean Fluorescence Intensity
HCV	hepatitis C virus
HCVcc	cell culture-derived HCV
HCVpp	HCV pseudotype particles
PBMC	Peripheral blood mononuclear cells
RLU	relative light units
SR-BI	Scavenger receptor BI
VSV Gpp	vesicular stomatitis virus G pseudoparticles

Fig. 1. HCVpp infection of neuroepithelioma cells

(A) HCVpp-H77 (white) and VSV Gpp (grey) infection of control Huh-7.5 permissive hepatoma cells, non-permissive 293T and a range of neural and brain derived cells summarised in Table 1. (B) Infectivity of HCVpp bearing primary envelope strains cloned from four acute infected donors for Huh-7.5, 293T, SK-N-MC, MC-IXC and SK-PN-DW cells. Data is presented as the mean specific infectivity where the virus specific RLU is expressed relative to no env RLU $\pm$ SD. The dotted line represents the mean specific infectivity + 3 SD of HCVpp infection of 293T cells and provides a threshold for defining cellular permissivity to HCVpp entry. This data is representative of two independent experiments.

Fig.2. Neural cell expression of HCV entry factors

A panel of neuroepithelioma and neuroblastoma cells were screened for expression of HCV co-receptors CD81, SR-BI, CLDN1 and Occludin. Live cells were stained with antibodies specific for extracellular epitopes on CD81, SR-BI and CLDN1. The only available Occludin antibodies recognise intracellular epitopes and cells were stained following permeabilization with paraformaldehyde and saponin. Cells were analysed by flow cytometry and the percentage of receptor positive cells relative to an irrelevant control IgG determined, where the mean percentage for all cells tested staining with control IgG was 0.75% (A). The level of receptor expression per cell was determined by quantifying the mean fluorescence intensity (MFI) for each antibody, where the MFI for cell lines binding irrelevant control IgG was 4.3 (B). This data is representative of two independent experiments.

Fig.3. Viral receptor localization in hepatoma and neuroepithelioma cells

All of the cell lines listed in Table 1 were stained for CD81, CLDN1 and Occludin expression and imaged by laser scanning confocal microscopy. Receptor localization was classified as plasma membrane or intracellular. Selected images of receptor stained Huh-7.5, SK-N-MC, MC-IXC, SK-PN-DW and U87 are presented, where the scale bar represents 10 μ m. This data is representative of two independent staining experiments.

Fig.4. Receptor dependency of HCVpp infection of neuroepithelioma cells

(A) Huh-7.5, SK-N-MC, MC-IXC and SK-PN-DW cells were incubated with 5 μ g of neutralizing anti-receptor antibody specific for CD81, SR-BI and CLDN1 or a control irrelevant IgG for 1h at 37°C. Antibody treated cells were incubated with HCVpp-H77 for 72h and luciferase activity measured to assess infectivity. (B) HCVpp-H77 was incubated with neutralizing rat mAbs 9/27, 11/20 and 3/11 or HCV⁺ patient IgG for 1h at 37°C. The virus-antibody mixture was allowed to infect the various cell types and infectivity measured as detailed above. Data is presented as % neutralization where HCVpp infectivity in the presence of a receptor specific or E2 antibody is expressed relative to the control mAb or normal donor IgG, respectively. Statistical comparisons were made using student's t-test, where * $p < 0.05$ and ** $p < 0.01$. This data is representative of three independent experiments.

Fig.5. HCVcc infection of hepatoma and neuroepithelioma cells

HCVcc J6/JFH was allowed to infect Huh-7.5, MC-IXC, SK-PN-DW and 293T cells for 12h. Unbound virus was removed by washing and the infection measured 72h later by quantifying focus forming units/ml (FFU/ml) (A). Representative images of NS5A⁺ J6/JFH infected Huh-7.5, MC-IXC and SK-PN-DW cells denoting comparable levels of NS5A expression, where the scale bar represents 10 μ m. (B) Cellular RNA was extracted from uninfected (UF) and J6/JFH infected cells after 12h and 72h in the presence or absence of anti-NS3 VX-950 (10 μ g/ml) or Interferon- α (IFN- α , 100 IU/ml) and the level of HCV RNA copies assessed by qPCR. (C) J6/JFH was allowed to infect Huh-7.5, MC-IXC, SK-PN-DW and 293T for 12h, unbound virus removed by washing and the cells lysed for RNA purification after 48h (white), 72h (light grey) and 96h (dark grey). HCV RNA levels significantly increased between 48 and 72h post infection for all cell types tested. Cells were treated with anti-CD81 2s131 at 5 μ g/ml and HCV RNA copies assessed after 96h (black). Statistical comparisons were made using student's t-test, where * $p < 0.05$. This data is representative of two independent experiments.

Table 1

Cell line	Origin	Reference
Huh-7.5	Hepatoma	Blight et al ²³
293T	Embryonic Kidney	DuBridge et al ⁴⁷
SK-N-MC	Neuroepithelioma	Biedler et al ²²
MC-IXC	Neuroepithelioma	Biedler et al ²²
SK-PN-DW	Neuroepithelioma	Schneider et al ³³
NP2	Glioma	Soda et al ⁴⁸
SH-SH5Y	Neuroblastoma	Biedler et al ²²
SK-N-SH	Neuroblastoma	Biedler et al ²²
1321N1	Astrocytoma	Meeker and Harden ⁴⁹
U87	Neuroblastoma	Ponten and Macintyre ⁵⁰

Table 2

Cell line	CD81	CLDN1	Occludin
Huh-7.5	PM/IC	PM	PM
293T	PM	-	PM
SK-N-MC	PM	PM	PM
MC-IXC	PM	PM/IC	PM/IC
SK-PN-DW	PM	PM	PM
NP2	-	-	IC
SH-SH5Y	-	-	IC
SK-N-SH	PM	-	IC
1321N1	PM/IC	-	-
U87	PM/IC	-	IC

Cells were fixed according to protocols listed in the material and methods and stained for CD81, CLDN1 and Occludin. Protein localization in five fields of view from 2 independent coverslips was assessed and defined as plasma membrane (PM) or intracellular (IC).