Hepatitis C Virus Infection of Neuroepithelioma Cell Lines

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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 nonpermissive 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. 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 levels comparable to those of hepatoma cells. HCV infection in vitro is not restricted to hepatocellular-derived cells, so HCV might infect cells of the CNS in vivo.

Keywords: OCLN; Neurotropism; Brain; Therapy; Repli-con; Huh-7; VX-950.

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) abnormalities.1

While HCV is primarily a hepatotropic virus, genomic viral RNA has been detected in peripheral blood mononuclear cells, cerebrospinal fluid, and the brain of chronically infected patients with neuropathological abnormalities (reviewed in references 2–4). 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 peripheral blood mononuclear cells/brain and liver-derived HCV sequences demonstrate tissue-specific differences, suggesting independent evolution at different anatomical sites.5–8 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 liver,6 supporting a model of viral adaptation in the brain. To date, a 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 subgenomic replication in 2 nonhepatocyte-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 nonhepatic cells.9 Earlier reports demonstrated low-level replication of subgenomic replicons in nonhepatic cell lines.10,11 Lending

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Abbreviations used in this paper: CNS, central nervous system; CLDN1, claudin-1; FFU, focus-forming units; HCV, hepatitis C virus; HCVcc, cell culture–derived HCV; HCVpp, HCV pseudotype particles; IgG, immunoglobulin G; mAb, monoclonal antibody; SR-BI, scavenger receptor BI; VSV-Gpp, vesicular stomatitis virus G pseudoparticles.

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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, the expression of which can define cellular or tissue permissivity. The availability of pseudoparticles bearing HCV E1E2 glycoproteins (HCVpp) and the recently reported JFH-1 strain of HCV, which 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 liver.\textsuperscript{12–14} Recent evidence shows a number of host cell molecules to be important for HCV entry: tetraspanin CD81, scavenger receptor class B member 1 (SR-BI), and the tight junction proteins, Claudin-1 (CLDN1) and occludin (reviewed in Burlone et al\textsuperscript{15}). 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.\textsuperscript{16,17} Reports of cognitive defects and nuclear magnetic resonance abnormalities in HCV-infected patients\textsuperscript{18–21} led us to screen a series of neural and brain-derived cell lines for their ability to support HCV entry and replication. We found 2 independently derived neuroepithelioma cell lines that support HCVpp infection. HCVpp infection was neutralized by antibodies specific to CD81, SR-BI, CLDN1, and HCV E2, demonstrating a common entry pathway for infection of neuroepithelioma and hepatoma cell lines. Furthermore, both neuroepithelioma cell lines support HCVpp infection, albeit at a 130- to 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 (Manassas, VA) (Table 1). MC-IXC is a twice-cloned subline of SK-N-MC.\textsuperscript{22} Huh-7.5\textsuperscript{23} and 293T cells were provided by C. Rice, Rockefeller University, New York, NY. Cells were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, and 1% penicillin/streptomycin (Invitrogen, Carlsbad, CA). MC-IXC cells were maintained in Dulbecco’s modified Eagle’s medium/F12 medium with the previously mentioned supplements.

The primary antibodies used were: anti-NS5A 9E10 (C. Rice); anti-CD81 (2.131)\textsuperscript{24}; anti-SR-BI (V. Flores, Pfizer, New York, NY); anti-CLDN1 (Abnova, Taipei, Taiwan, and R&D Systems, Minneapolis, MN); anti-CLDN1 polyclonal sera\textsuperscript{25}; anti-occludin (Invitrogen); anti-ZO-1 (Invitrogen); and anti-E2 (9/27, 11/27, and 3/11).\textsuperscript{12} Immunoglobulin from normal healthy volunteers and chronically HCV-infected donors was purified by Protein-G affinity chromatography, as reported previously.\textsuperscript{26} Fluorescent-labeled secondary antibodies, Alexa Fluor 488 anti-mouse, anti-human, anti-rat, and anti-rabbit immunoglobulin G (IgG) were obtained from Invitrogen.

Flow Cytometric Analysis of Receptor Expression

For CD81, SR-BI, and CLDN1 staining, 2 \times 10^6 cells were incubated in phosphate-buffered saline containing 1% bovine serum albumin and 0.01% sodium azide for 20 minutes at 37°C. For occludin staining, cells were fixed with 1% paraformaldehyde and permeabilized with phosphate-buffered saline containing 1% bovine serum albumin and 1% saponin. Two micrograms receptor-specific antibodies or irrelevant IgG controls were incubated with cells in sodium azide 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, Oxford, UK) 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, Loughborough, UK) and fixed with ice-cold methanol (CLDN1, Occludin) or 3% paraformaldehyde (CD81) 24 hours post-seeding. Primary antibodies were applied for 1 hour at room temperature. After washing twice with phosphate-buffered saline, antimouse, rabbit, or rat Alexa Fluor 488 (Invitrogen) secondary antibody was applied for 1 hour at room temperature. Cells were counterstained with 4’,6-diamidino-2-phenylindole (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.

<table>
<thead>
<tr>
<th>Table 1. Neurally Derived Cell Lines Available for This Study</th>
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<tr>
<td>Cell line</td>
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<tr>
<td>Huh-7.5</td>
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<td>293T</td>
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<td>SK-N-MC</td>
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<td>MC-IXC</td>
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<td>SK-PN-DW</td>
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<td>NP2</td>
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<td>SH-SH5Y</td>
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<td>SK-N-SH</td>
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<td>1321N1</td>
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**HCVpp Genesis and Infection**

Pseudoparticles were generated by transfecting 293T cells with plasmids encoding an HIV pro-virus—expressing luciferase and vesicular stomatitis virus G (VSV-G), a panel of HCV envelope glycoproteins (genotype 1) 27 or a no-envelope control, as reported previously.12 Supernatants were harvested at 48 hours post-transfection, clarified, and filtered through a 0.45-μm membrane. Virus-containing medium was added to target cells plated in 96-well plates seeded at 7.5 × 10³ cells/cm² and incubated for 72 hours. At 72 hours post-infection, cells were lysed with cell lysis buffer (Promega, Madison, WI) and luciferase activity measured for 10 seconds in a luminometer (Lumat LB 9507). Specific infectivity was calculated by expressing the HCV or VSV-G luciferase signal (relative light units) relative to the no envelope relative light unit 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) was used to generate RNA as described previously.28 Briefly, RNA was transcribed using the RibomAX express T7 kit (Promega) and electroporated into Huh-7.5 cells. Supernatants were collected at 72 and 96 hours post-infection, pooled and stored at −80°C, and their infectious titer for Huh-7.5 cells was determined. Cells were fixed with ice-cold methanol and infected cells identified by staining for NS5A with monoclonal antibody (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 per mL (FFU/mL). The 2 × 10⁵ Huh-7.5, 293T, MC-IXC, or SK-PN-DW cells were seeded per well of a 12-well plate and infected with 1.6 × 10⁴ FFU of J6/JFH for 8 hours, unbound virus was removed by 3 sequential washes and the cells were incubated at 37°C. Infected cells were identified by the expression of NS5A or HCV RNA. Purified cellular RNA samples (Qiagen, Hamburg, Germany) were amplified for HCV RNA (Primer Design Ltd, Southampton, UK) in a single-tube reverse-transcription polymerase chain reaction in accordance with manufacturer’s guidelines (CellsDirect kit; Invitrogen) and fluorescence monitored in an MxPro 3000 real-time polymerase chain reaction machine (Stratagene, La Jolla, CA). In all reactions the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase was included as an internal endogenous control for amplification efficiency and for RNA quantification (primer-limited endogenous control; ABI, Carlsbad, CA).

**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 × 10³ cells/cm². Twenty-four hours post-seeding, cells were incubated with 5 μg/mL anti-receptor or irrelevant IgG control mAb. After 1 hour, HCV-H77pp, VSV-Gpp, or No-envpp was added and incubated for 72 hours at 37°C. In addition, anti-E2 mAbs or HCV* IgG was incubated with virus for 1 hour prior to infecting the appropriate target cells. At 72 hours post-infection, cells were lysed with cell lysis buffer (Promega), and luciferase activity was measured for 10 seconds in a luminometer (Lumat LB 9507). The percent neutralization was calculated relative to the irrelevant IgG control.

**Statistical Analysis**

Results are expressed as mean ± standard deviation of the mean. Statistical analyses were performed using Student t test in Prism 4.0 (GraphPad, San Diego, CA) with a P < .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 (Figure 1). The nonpermissive CLDN1-null human embryonic kidney 293T cell line was included as a control29 (Figure 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- to 20-fold reduced entry (Figure 1A). 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 nonpermissive for VSV-Gpp (Figure 1A) 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 (Figure 1A).

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 (Figure 1B). All HCVpp tested infected the neuroepithelioma cell lines at comparable levels to Huh-7.5, demonstrating a 2- to 6-fold range in permissivity (Figure
In conclusion, 2 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 (Figure 2). The permissive neuroepithelioma cell lines SK-N-MC, MC-IXC, and SK-PN-DW expressed all 4 entry factors (Figure 2A), with mean fluorescence intensities within 2-fold of Huh-7.5 cells (Figure 2B). In contrast, NP2 and SH-SH5Y cells express low levels of CD81, SR-BI, and CLDN1 (Figure 2B). SK-N-SH express negligible levels of SR-BI and CLDN1 (Figure 2B), whereas CLDN1 and occludin mean fluorescence intensities were not significantly greater than the isotype control antibody for 1321N1 cells. Finally, U87 cells had no detectable CLDN1 expression (Figure 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 (Figure 3 and Supplementary Figure 1). In contrast, occludin localized to intracellular sites in all of the nonpermissive 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 (Figure 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 (Figure 4A). Anti-CD81 mAb inhibited HCVpp infection of 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 (Figure 3 and Supplementary Figure 1). In contrast, occludin localized to intracellular sites in all of the nonpermissive 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 (Figure 3). In summary, neuroepithelioma cells express all of the essential HCV entry factors, most likely accounting for their permissivity to HCVpp infection.
cells \((P < .05)\) (Figure 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 cells.\(^{12}\) E2-specific mAbs and HCV\(^+\) Ig neutralized HCVpp infection of all target cells (Figure 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.

### HCV 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. Because infection studies require extensive washing to remove input virus prior to extracting cellular RNA for quantitative polymerase chain reaction HCV RNA analysis, we selected the MC-IXC subclone 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.

### Table 2. HCV Entry Factor Expression and Localization in Neuroepithelioma Cell Lines

<table>
<thead>
<tr>
<th>Cell line</th>
<th>CD81</th>
<th>CLDN1</th>
<th>Occludin</th>
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<tr>
<td>Huh-7.5</td>
<td>PM/IC</td>
<td>PM</td>
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<tr>
<td>293T</td>
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<td>MC-IXC</td>
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<td>PM/IC</td>
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<td>SK-PN-DW</td>
<td>PM</td>
<td>PM</td>
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<tr>
<td>NP2</td>
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<td>—</td>
<td>IC</td>
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<td>SH-SH5Y</td>
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<td>SK-N-SH</td>
<td>PM</td>
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<tr>
<td>1321N1</td>
<td>PM/IC</td>
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<td>U87</td>
<td>PM/IC</td>
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NOTE. Cells were fixed according to protocols listed in the material and methods and stained for CD81, CLDN1, and Occludin. Protein localization in 5 fields of view from 2 independent coverslips was assessed and defined as plasma membrane (PM) or intracellular (IC).
All cells were incubated with J6/JFH for 12 hours and 72 hours, and NS5A and HCV RNA levels were quantified (Figure 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- to 330-fold lower than Huh-7.5 cells (Figure 5A). There was no detectable HCV RNA 12 hours post-infection, demonstrating a time-dependent increase in HCV RNA levels (Figure 5B). Incubation of both neuroepithelioma cell lines and Huh-7.5 with interferon-α (100 IU/mL) and anti-NS3 VX-950 (10 μg/mL) significantly inhibited J6/JFH replication (Figure 5B). To assess whether HCV infection persisted in the neuroepithelioma cells, HCV RNA levels were quantified at 48, 72, and 96 hours post-infection. HCV RNA increased significantly in all cell lines tested between 48 and 72 hours post-infection (Figure 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 (Figure 5C).

Figure 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 μm. These data are representative of 2 independent staining experiments.
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 the multiplicity of infection (Supplementary Figure 2). At the multiplicity of infection 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 2 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 references 12–14). However, HCVpp failed to infect several other neuroblastoma cell lines, including U87 as reported previously.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 reported previously.12–14 Indeed, we12 and others13,14 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 or murine NIH-3T3 cells conferred HCVpp entry, demonstrates that these 4 molecules constitute the minimal receptor requirements.32 Importantly, messenger RNA and protein profiling databases 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 subclone represent a neuroepithelioma cell line derived from a metastatic tumor.32 SK-PN-DW is an independent neuroepithelioma line derived in 1979 from a 17-year-old white male.33 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 tumor-specific genes.34 Ewing sarcomas and primitive neuroectodermal tumors, including neuroepitheliomas, express a chimeric protein product of the gene fusion EWS-FLI1.35

SK-N-MC, MC-IXC, and SK-PN-DW expressed comparable levels of all entry factors to Huh-7.5 hepatoma cells (Figure 2), consistent with the ability of antibodies specific for CD81, SR-BI, and CLDN1 to neutralize HCVpp infection (Figure 4). Several of the nonpermissive glioma and neuroblastoma cells (NP2, SH-SH5Y, and SK-N-SH) did not express detectable levels of SR-BI or CLDN1 (Figure 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 in Piontek et al136). 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), which 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 (Figure 3 and Supplementary Figure 1). Viral tropism is likely to be defined at multiple levels of the lifecycle, including particle entry, RNA replication, and assembly (reviewed in Kaul et al37). A majority of studies demonstrate efficient HCV replication in hepatoma cell lines, which may reflect the presence of regulatory factors, such as the liver-specific microRNA, miR122.38 However, HCV has been reported to replicate to low levels in nonhepatic cells9,10 suggesting that additional cellular reservoirs may exist in vivo. Given our observation that MC-IJC 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/H9251. It was interesting to note the reduced level(s) of HCV RNA/NSSA+/H11001 in MC-IJC and SK-PN-DW cells compared to Huh-7.5 (Figure 5), more
akin to the low HCV RNA levels reported in HCV-infected primary hepatocytes. It is important to note that Huh-7.5 cells have a mutation in the RIG-I dsRNA sensing pathway (reviewed in Horner et al), which reduces their innate signaling responses and may contribute to their highly permissive nature. Such innate signaling responses are likely to be intact in the neuroepithelioma cells, which 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-I-XC and SK-PN-DW cells increased significantly between 48 hours and 72 hours post-infection and were maintained for the next 24 hours 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, while maintaining wild-type levels of RNA replication. The frequency of DelB RNA and NS5A-expressing cells declines rapidly between 48 and 96 hours after electroporation into Huh-7.5 cells (Joe Grove and Claire Brimacon, unpublished data). In contrast, parental J6/JFH transmits to most cells in the culture and is maintained at 96 hours, as observed with the neuroepithelioma cell lines. In summary, these data are consistent with a productive replication cycle in the MC-I-XC and SK-PN-DW cells that maintain the viral genome over time.

Our study is the first report that nonhepatocyte cell lines not engineered to express exogenous viral receptors support efficient HCVpp infection. The concept of extra-hepatic HCV replication is not new, and several reports have described the detection of HCV RNA with peripheral blood mononuclear cells, brain, and CNS tissue (reviewed in references 2–4). 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 HCV, 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. Indeed, we recently demonstrated that JFH-1 HCVcc can associate with B cells via SR-B1 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 virus.

Despite the many reports of cerebral dysfunction in chronic HCV infection and detection of viral RNA in autopsy brain tissue, 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

Note: To access the supplementary material accompanying this article, visit the online version of Gastroenterology at www.gastrojournal.org, and at doi: 10.1053/j.gastro.2010.06.008.

References


Supplementary Figure 1. Viral receptor localization in nonpermissive neuroepithelioma cells. 1321N1, SK-N-SH, and NP-2 cell lines were stained for CD81, claudin-1 (CLDN1), and occludin expression and imaged by laser scanning confocal microscopy. Scale bar represents 10 μm. These data are representative of 2 independent staining experiments.
Supplementary Figure 2. Relationship between extracellular infectious hepatitis C virus and multiplicity of infection. Increasing amounts of J6/JFH were incubated with Huh-7.5 cells for 4 hours, unbound virus was removed by washing, and the cells were incubated for 72 hours. The extracellular medium was collected and assessed for levels of infectious virus by back titration on Huh-7.5 cells. A linear relationship was observed between the multiplicity of infection and extracellular infectious particles, where the 95% confidence limits are annotated with a dashed line.