

Detection of Hepatitis C Virus Sequences in Brain Tissue Obtained in Recurrent Hepatitis C After Liver Transplantation

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Patients with chronic hepatitis C frequently report tiredness, easy fatigability, and depression. The aim of this study is to determine whether hepatitis C virus (HCV) replication could be found in brain tissue in patients with hepatitis C and depression. We report two patients with recurrent hepatitis C after liver transplantation who also developed severe depression. One patient died of multiorgan failure and the other, septicemia caused by *Staphylococcus aureus*. Both patients had evidence of severe hepatitis C recurrence with features of cholestatic fibrosing hepatitis. We were able to study samples of their central nervous system obtained at autopsy for evidence of HCV replication. The presence of HCV RNA–negative strand, which is the viral replicative form, was determined by strand-specific Tth-based reverse-transcriptase polymerase chain reaction. Viral sequences were compared by means of single-strand conformation polymorphism and direct sequencing. HCV RNA–negative strands were found in subcortical white matter from one patient and cerebral cortex from the other patient. HCV RNA–negative strands amplified from brain tissue differed by several nucleotide substitutions from serum consensus sequences in the 5' untranslated region. These findings support the concept of HCV neuroinvasion, and we speculate that it may provide a biological substrate to neuropsychiatric disorders observed in patients with chronic hepatitis C. The exact lineage of cells permissive for HCV replication and the possible interaction between viral replication and cerebral function that may lead to depression remain to be elucidated. (*Liver Transpl* 2002;8:1014-1019.)

Patients with chronic hepatitis C frequently report tiredness, easy fatigability, and depression. Dwight et al¹ reported that 28% of patients with chronic hep-

atitis C had current depressive disorders. They concluded that disability and fatigue were related more closely to severity of depression than to progression of liver disease. More recently, Forton et al² addressed the hypothesis that hepatitis C virus (HCV) itself may affect cerebral function and provide the substrate for the chronic fatigue syndrome frequently associated with HCV infection. The investigators used proton magnetic resonance spectroscopy and showed elevations in choline-creatine ratios in the basal ganglia and white matter of patients with histologically mild hepatitis C that were not present in healthy volunteers and patients with hepatitis B. This elevated ratio was unrelated to hepatic encephalopathy or a history of intravenous drug abuse. The investigators suggested that a biological process underlies extrahepatic symptoms in chronic HCV infection, although they did not speculate on its nature. Furthermore, because HCV has been found to replicate in lymphoid cells and monocyte/macrophages³⁻⁵ and these cells can enter the central nervous system (CNS),⁶ a possibility remains that psychosomatic syndromes observed in patients with HCV infection are related to viral replication in the CNS.

We studied two patients with recurrent hepatitis C after cadaveric liver transplantation for end-stage liver disease caused by HCV infection who presented a clinical picture of profound depression. These patients subsequently died, and we were able to study samples of their CNS for evidence of HCV replication.

Patients and Methods

Case 1

A 56-year-old man underwent cadaveric liver transplantation December 26, 2000, for decompensated cirrhosis caused by chronic hepatitis C and complicated by hepatocellular carcinoma.

Soon after transplantation, the patient developed evidence of recurrent hepatitis C, for which he was placed on interferon alfa and ribavirin therapy 1 month after the diagnosis was established. His clinical course was characterized by severe depression that preceded antiviral therapy and continued to worsen despite antiviral therapy discontinuation. The patient

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Supported in part by grant no. RO1 DA13760-01 from The National Institutes of Health (J.R.).

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1527-6465/02/0811-0005\$35.00/0

doi:10.1053/jlts.2002.36393

also presented with anorexia, profound weight loss, and renal insufficiency.

The patient was hospitalized April 4, 2001, for management of profound depression, anorexia, and massive ascites. The patient required dialysis therapy for the management of renal failure; he also developed spontaneous bacterial peritonitis secondary to *Streptococcus viridans*. His clinical course progressively deteriorated, with acute respiratory distress syndrome and renal failure, until he died May 8, 2001.

Autopsy examination showed the allograft liver with centrilobular congestion and necrosis, acute and organizing diffuse alveolar damage with acute bronchopneumonia, acute pancreatitis, congested kidneys with features of membranous glomerulonephritis, ascites, and pleural effusion. Brain examination showed no evidence of either gross or microscopic disease; there was no evidence of infarct, vasculitis, or viro-pathic changes. There was no evidence of recurrence of hepatocellular carcinoma.

Depression was the most noticeable and important symptom in the patient and presented soon after transplantation. He was initially treated with sertraline, 50 mg/d orally, January 8, 2001. Therapy was switched to olanzapine, 10 mg/d orally, January 18, 2001. Subsequently, therapy was switched to citalopram, 20 mg/d orally, February 26, 2001, and the patient remained on that medication until his death. There was no noticeable improvement of his depression despite changing medications.

His liver tests showed serum aminotransferase levels between three and five times the upper limit of normal, and total bilirubin levels progressively increased to a peak value of 18.6 mg/dL before his admission. Prothrombin time, initially within normal range, became progressively prolonged before his death.

The patient underwent two liver biopsies; January 25, 2001, and March 2, 2001. The initial biopsy showed early recurrence, and the second biopsy, lobular portal hepatitis with focal hepatic necrosis and cholestasis. There also was significant portal fibrosis. These findings were compatible with fibrosing cholestatic hepatitis.

Case 2

A 46-year-old man underwent cadaveric liver transplantation March 3, 2001, for decompensated cirrhosis caused by chronic HCV infection and chronic alcoholism. The patient had undergone transjugular intrahepatic portosystemic shunt placement for management of variceal bleeding caused by portal hypertension before transplantation.

The patient was discharged March 29, 2001, and readmitted March 30, 2001, with fever, right pleural effusion, and pneumonia at the right-lung base. He also showed progressive elevation of serum aminotransferase and alkaline phosphatase levels. Endoscopic retrograde cholangiopancreatography was performed and showed a dilated anastomotic stricture. Because of progressive serum aminotransferase level elevation, a liver biopsy was performed and showed recurrent hepatitis C.

Combination therapy with interferon and ribavirin was initiated April 11, 2001.

Depression, a predominant symptom, also was present since transplantation, associated with anorexia and exacerbated with antiviral therapy. The patient was placed on therapy with oral citalopram, 20 mg/d, May 2, 2001, without noticeable improvement.

His clinical course was complicated by sepsis caused by *Staphylococcus aureus*. The patient became progressively encephalopathic and developed renal and hepatic insufficiency. He died May 26, 2001.

The autopsy examination showed no evidence of biliary duct stricture, and the anastomosis appeared without abnormalities. There was evidence of recurrent chronic hepatitis caused by HCV, with a marked fibrosing cholestatic pattern. There were multiple microabscesses in the anterior left ventricle and epimyocardium of the left ventricle, midbrain, and basal ganglia, consistent with disseminated *S aureus*. A 2.5-cm necrotic *Coccidioides immitis* granuloma was present in the left-lung upper lobe; this condition had been treated with fluconazole before transplantation. Recurrent renal insufficiency was present without focal lesions in the kidneys.

There were pyogenic abscesses with gram-positive cocci surrounding sections in the left upper quadrant abdominal fascia along the liver transplantation scar. This site was believed to be the source of *S aureus* septicemia. There also was Alzheimer's type II gliosis in the basal ganglia, midbrain, and border zone cortex.

Biological Samples

Brain-tissue samples were obtained during routine autopsies conducted 24 hours after death and stored at -80°C until analysis. Samples were collected of subcortical white matter and cerebral cortex from the frontal region and brain stem. In addition, cerebellum tissue from patient 1 also was available for analysis. Analyzed tissues were homogenized, and RNA was extracted by means of a modified guanidinium thiocyanate-phenol/chloroform technique using a commercially available kit (RNAzol; Gibco BRL, Grand Island, NY). One and 5 μg of total RNA (determined by spectrophotometry) were used for reverse-transcriptase polymerase chain reaction (RT-PCR). In the case of serum, the amount of extracted RNA loaded into the reaction corresponded to 30 μL .

Strand-Specific RT-PCR

Strand specificity of RT-PCR for the detection of HCV RNA-negative strands was ascertained by conducting complementary DNA synthesis at a high temperature using the thermostable enzyme Tth. Sensitivity and strand specificity of this reaction were established by using synthetic RNA as templates. A detailed description of our strand-specific assay and sequence of primers used was published previously.^{7,8} In brief, complementary DNA was generated in 20 μL of reaction mixture containing 50 pmol/L of sense primer, 1 \times RT buffer (Perkin Elmer, Foster City, CA), 1 mmol/L of MnCl_2 , 200 $\mu\text{mol/L}$ of (each) dNTP, and 5 U of Tth (Perkin Elmer).

After 20 minutes at 65°C, Mn²⁺ was chelated with 8 µL of 10 × egtazic acid chelating buffer (Perkin Elmer), 50 pmol/L of antisense primer was added, volume was adjusted to 100 µL, and MgCl₂ concentration was adjusted to 2.2 mmol/L. Amplification was performed in the Perkin Elmer GenAmp PCR System 9600 thermocycler as follows: initial denaturing for 1 minute at 94°C and 50 cycles of 94°C for 15 seconds, 58°C for 30 seconds, and 72°C for 30 seconds, followed by a final extension at 72°C for 7 minutes. One microliter of this reaction was added to the second nested PCR and amplified for another 35 cycles. Because nested protocols may be prone to carryover contamination, all positive reactions subsequently were verified in an independent experiment by Southern hybridization with a phosphorus 32–labeled internal oligoprobe using first round RT-PCR product.

The strand-specific assay was capable of detecting approximately 100 genomic equivalent (Eq) molecules of the correct strand, whereas nonspecifically detecting 10⁸ genomic Eq of the incorrect strand. The addition of 1 to 5 µg of total cellular RNA extracted from human tissues would reduce the sensitivity of the reaction by no more than one log, whereas the specificity of the assay was not affected.

Standard RT-PCR

Moloney Murine leukemia virus (MMLV) RT-based detection of HCV has been described in detail previously.⁷ Appropriate measures, described elsewhere,^{7,8} were used to prevent and detect contamination.

Analysis of HCV Quasispecies

The analysis was conducted on the stable 5′ untranslated region (5′ UTR) because a small number of expected viral variants within quasispecies allows for reliable comparison, and we previously found that variations in this region may correlate with extrahepatic replication.^{7,9,10} For the purpose of sequence comparison, nested protocols were used to maximize the yield of PCR product. Amplification of 5′ UTR was conducted using RT-PCR assay, as previously described.⁷

HCV quasispecies were compared using the single-strand conformation polymorphism (SSCP) assay as described elsewhere,⁷ with minor modifications. In brief, PCR products were purified with a DNA-binding resin system (Wizard PCR; Promega, Madison, WI) and resuspended in 50 µL of water. Next, 2 to 4 µL of purified product was diluted in 15 µL of low ionic-strength solution (10% saccharose, 0.5% bromophenol blue, and 0.5% xylene cyanol), denatured by heating at 97°C for 3 minutes, immediately cooled on ice, and subjected to nondenaturing 8% polyacrylamide gel electrophoresis in 1 × Tris-borate-EDTA buffer with 400 V applied for 5 to 6 hours at a constant temperature of 25°C. Bands were visualized using silver staining (Silver Stain; Promega). This assay enables the detection of minor variants representing 3% to 5% of the entire population.⁷

All analyzed products were sequenced directly in both directions using the Perkin-Elmer ABI 377 automatic

sequencer. HCV genotypes were determined by direct sequencing of the NS5 region.

Genotypes of infecting HCV strains were determined by means of direct sequencing of the NS5 region as described previously.¹¹

Results

Both patients were positive for the presence of HCV RNA in serum and every sample of brain tissue analyzed. HCV RNA titers, determined by analyzing 10-fold serial dilutions of RNA template, ranged from 10² to 10⁴ genomic Eq/µg of total RNA. Both patients were found to be infected with genotype 1a, and HCV-RNA loads in their serum were greater than 850,000 IU/mL. Using a strand-specific Tth-based assay, viral-negative strand was detected in subcortical white matter and cerebral cortex in patients 1 and 2, respectively. These reactions were unlikely to represent false-positive results because nonspecific detection of the incorrect strand might be expected when the latter is present in a high number, at least 10⁸ genomic Eq/reaction. However, the approximate concentration of HCV RNA in brain samples containing viral-negative strand was only 10⁴ genomic Eq/µg of total RNA.

In the next step, viral sequences amplified from different parts of brain were compared by means of SSCP with one another and with respective viral sequences amplified from serum. As seen in Figure 1, band patterns representing viral-positive strands were identical in each patient, except for those derived from gray matter in patient 1, which were fewer in number. However, because dominant bands in the latter sample were identical to all other samples in this patient, the observed lower complexity most likely was caused by sampling differences related to stochastic phenomena. Analysis of HCV RNA–negative strands showed they were different from positive strands. The discrepancy between brain-derived positive and negative HCV RNA strands can be explained because in large part, the former represent serum-derived contamination, whereas the latter represent indigenous replicating virus.

Direct sequencing confirmed the presence of identical master sequences in serum and brain tissue with an indiscernible SSCP band pattern. However, master sequences of HCV RNA–negative strand recovered from cerebral cortex in both patients differed by several nucleotide substitutions from serum consensus sequences (Fig. 2). Thus, in both patients, HCV RNA–negative strands amplified from brain tissue were different from those circulating in serum.

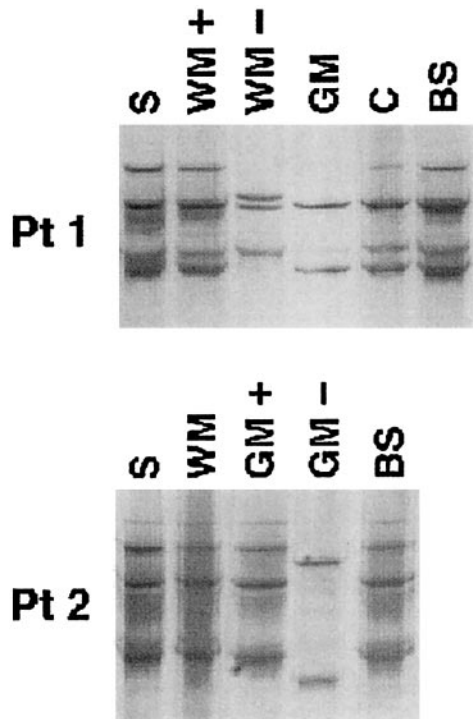


Figure 1. Analysis by SSCP of 5' UTR HCV sequences amplified from serum (S) and various autopsy brain tissue samples from two patients who died after orthotopic liver transplantation. The following brain tissue samples were examined: subcortical white matter (WM), gray matter (GM), cerebellum (C), and brain stem (BS). HCV RNA-negative strands were detected in subcortical white matter in patient 1 (Pt1) and cerebral cortex in patient 2 (Pt2). The presence of identical and dissimilar viral sequences in analyzed samples was verified by direct sequencing. (WM pos, subcortical white matter, HCV-positive strand; WM neg, subcortical white matter, HCV-negative strand; GM pos, gray matter, HCV-positive strand; GM neg, gray matter, HCV-negative strand.)

Discussion

In a recent study, we reported the detection of HCV RNA-negative strands in brain tissue in three of six studied patients.¹² In two of these patients, serum- and brain-derived viral sequences were different and classified as belonging to different genotypes. These results suggested at least an occasional presence of HCV replication in the CNS.

In the current study, we found the presence of viral replicative forms in CNS in two patients who also developed severe depression in the course of recurrent hepatitis C after liver transplantation. To the best of our knowledge, this is the first attempt to detect HCV replicative intermediaries in brain tissue in patients who also have psychiatric symptoms.

In a recently published study, we showed the common presence of HCV replication in blood macrophages/monocytes in human immunodeficiency virus (HIV)-infected subjects.⁹ Because brain microglial cells are tissue-resident macrophages of blood monocytic origin,¹³ we postulate that they also could support HCV replication. Our findings support this hypothesis because HCV replicative forms were detected in the CNS of both studied subjects, although the lineage of infected cells was not established.

A possible mechanism of neuroinvasion could be related to trafficking of infected cells of monocyte/macrophage lineage through the blood-brain barrier, similar to that postulated for HIV type 1 (HIV-1) infection.^{14,15} It could be speculated that there subsequently would be secondary spread of HCV to permissive cells, such as resident microglial cells. Replication in the CNS could be facilitated by the immunosuppression program of the patients. This possibility is supported by the demonstration of HCV replication in hematopoietic cells inoculated into severe combined immunodeficiency mice.¹⁶

Moreover, whereas HCV RNA-negative strand rarely is detected in peripheral-blood mononuclear cells from healthy subjects,^{17,18} it commonly is found in HIV-coinfected patients or liver transplant recipients.^{9,19}

We speculate that HCV infection of the CNS may provide the biological substrate for neuropsychiatric syndromes associated with this viral infection. HIV-1 infection may provide a paradigm to HCV infection of the CNS; HIV-1 can infect macrophages and microglia, but not neurons, although neurons die by apoptosis.^{20,21} The postulated pathway is through release of macrophage, microglial, and astrocyte beta-chemokines.²¹ Although HCV infection of microglia has not been shown directly, this cell compartment is likely to be permissive to HCV replication in the CNS.

Because HIV-1 dementia is a well-defined clinical entity and its underlying pathophysiological characteristics are being clarified, HCV-associated neuropsychiatric syndromes²² are less well defined, and their underlying mechanism is unclear. Our observations and those of Forton et al² are beginning to define a biological substrate that may provide deeper understanding of the interaction between HCV and the CNS.

In conclusion, we found evidence of HCV replication in CNS autopsy samples in two transplant recipients with recurrent hepatitis C and severe depression. We speculate that the depressive syndrome in these patients may have HCV viral replication in the brain as an organic substrate. The exact lineage of cells permis-



Figure 2. Nucleotide sequence alignment of 5' UTR fragment of HCV recovered from serum (S) and brain tissue samples from patients 1 (Pt 1) and 2 (Pt 2). Sequences are compared with the prototype sequence published by Choo et al²³ shown on the top line. (S, sequence identity; +, positive strand; -, negative strand; WM, subcortical white matter; GM, gray matter; C, cerebellum, BS, brain stem.)

sive for HCV replication and the possible interaction between replicating virus and cerebral function that may lead to depression remain to be elucidated.

Acknowledgment

The authors thank Lane M. Romanick for expert assistance and preparation of this manuscript.

References

- Dwight MM, Kowdley KV, Russo JE, Ciechanowski PS, Larson AM, Katon WJ. Depression, fatigue, and functional disability in patients with chronic hepatitis C. *J Psychosom Res* 2000;49:311-317.
- Forton DM, Allsop JM, Main J, Foster GR, Thomas HC, Taylor-Robinson SD. Evidence for a cerebral effect of the hepatitis C virus. *Lancet* 2001;358:38-39.
- Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J. The presence of active hepatitis C virus replication in lymphoid tissue in patients coinfecting with human immunodeficiency virus type 1. *J Infect Dis* 1998;178:1189-1192.
- Sansonno D, Iacobelli AR, Cornacchiulo V, Iodice G, Dammaco F. Detection of hepatitis C virus (HCV) proteins by immunofluorescence and HCV RNA genomic sequences by non-isotopic in situ hybridization in bone marrow and peripheral blood mononuclear cells of chronically HCV-infected patients. *Clin Exp Immunol* 1996;103:414-421.
- Bain C, Fatmi A, Zoulim F, Zarski JP, Trepo C, Inchauspe G. Impaired allostimulatory function of dendritic cells in chronic hepatitis C infection. *Gastroenterology* 2001;120:512-524.
- Hickey WF. Leukocyte traffic in the central nervous system: The participants and their roles. *Semin Immunol* 1999;11:125-137.
- Laskus T, Radkowski M, Wang LF, Jang SJ, Vargas H, Rakela J. Hepatitis C virus quasispecies in patients infected with HIV-1: Correlation with extrahepatic viral replication. *Virology* 1998;248:164-171.
- Laskus T, Radkowski M, Wang LF, Vargas H, Rakela J. Lack of evidence for hepatitis G virus replication in the livers of patients coinfecting with hepatitis C and G viruses. *J Virol* 1997;71:7804-7806.
- Laskus T, Radkowski M, Piasek A, Nowicki M, Horban A, Cianciara J, Rakela J. Hepatitis C virus in lymphoid cells of patients coinfecting with human immunodeficiency virus type 1: Evidence of active replication in monocytes/macrophages and lymphocytes. *J Infect Dis* 2000;181:442-448.
- Laskus T, Radkowski M, Wang LF, Nowicki M, Rakela J. Uneven distribution of hepatitis C virus quasispecies in tissues from subjects with end-stage liver disease: Confounding effect of viral adsorption and mounting evidence for the presence of low-level extrahepatic replication. *J Virol* 2000;74:1014-1017.
- Laskus T, Wang LF, Radkowski M, Vargas H, Nowicki M, Wilkinson J, Rakela J. Exposure of hepatitis C virus (HCV) RNA-positive recipients to HCV RNA-positive blood donors results in rapid predominance of a single donor strain and exclusion and/or suppression of the recipient strain. *J Virol* 2001;75:2059-2066.

12. Radkowski M, Wilkinson J, Nowicki M, Adair D, Vargas H, Ingui C, et al. Search for hepatitis C virus negative strand and analysis of viral sequences in the central nervous system: Evidence of replication. *J Virol* 2001;76:600-608.
13. Davis EJ, Foster TD, Thomas WE. Cellular forms and functions of brain microglia. *Brain Res Bull* 1994;34:73-78.
14. Price RW, Sidtis J, Rosenblum M. The AIDS dementia complex: Some current questions. *Ann Neurol* 1988;23(suppl):S27-S33.
15. Zheng J, Gendelman HE. The HIV-1 associated dementia complex: A metabolic encephalopathy fueled by viral replication in mononuclear phagocytes. *Curr Opin Neurol* 1997;10:319-325.
16. Bronowicki JP, Lorient MA, Thiers V, Grignon Y, Zignego AL, Brechot C. Hepatitis C virus persistence in human hematopoietic cells injected into SCID mice. *Hepatology* 1998;28:211-218.
17. Lanford RE, Chavez D, Chisari FV, Sureau C. Lack of detection of negative-strand hepatitis C virus RNA in peripheral blood mononuclear cells and other extrahepatic tissues by the highly strand-specific rTth reverse transcriptase PCR. *J Virol* 1995;69:8079-8083.
18. Mellor J, Haydon G, Blair C, Livingstone W, Simmonds P. Low level or absent *in vivo* replication of hepatitis C virus and hepatitis G virus/GB virus C in peripheral blood mononuclear cells. *J Gen Virol* 1998;79:705-714.
19. Radkowski M, Wang LF, Vargas HE, Rakela J, Laskus T. Detection of hepatitis C virus replication in peripheral blood mononuclear cells after orthotopic liver transplantation. *Transplantation* 1998;66:664-666.
20. Kaul M, Garden GA, Lipton SA. Pathways to neuronal injury and apoptosis in HIV-associated dementia. *Nature* 2001;410:988-994.
21. Persidsky Y, Zheng J, Miller D, Gendelman HE. Mononuclear phagocytes mediate blood-brain barrier compromise and neuronal injury during HIV-1-associated dementia. *J Leukoc Biol* 2000;68:413-422.
22. Dieperink E, Willenbring M, Ho SB. Neuropsychiatric symptoms associated with hepatitis C and interferon alpha: A review. *Am J Psychiatry* 2000;157:867-876.
23. Choo QL, Richman KH, Han JH, Berger K, Lee C, Dong C, et al. Genetic organization and diversity of the hepatitis C virus. *Proc Natl Acad Sci U S A* 1991;88:2451-2455.