Occult Hepatitis C Virus Infection among Hemodialysis Patients

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Hepatitis C virus (HCV) belongs to the Flaviviridae family, and its genome is a single-stranded RNA molecule (genomic HCV-RNA).1 Virus replication involves the synthesis of a complementary RNA (antigenomic HCV-RNA) that acts as a template for production of genomic HCV-RNA.2 Despite screening of blood products for anti-HCV and implementation of precaution measures, HCV infection is still a major problem in hemodialysis (HD) units.3,4 Routine serologic controls performed biannually (including qualitative PCR technology) and testing at least every 2 mo for alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (γ-GTP) levels are important for monitoring viral hepatitis transmission within HD units.5,6 When ALT values are >28 IU/L (upper normal limit for hemodialysis patients)7–9 and/or γ-GTP levels are increased (>43 IU/L), viral markers (hepatitis B surface antigen and DNA, anti-HCV, and HCV-RNA) and nonviral causes of liver disease (e.g., autoimmunity, toxic hepatitis, metabolic disorders) are studied to identify the cause of the elevated liver enzymes; however, the cause of liver disease cannot be established in approximately 2% of HD patients (personal observation).

It was reported that up to 57% of patients with normal renal function but with abnormal values of liver enzymes of unknown cause have an occult HCV infection (presence of HCV-RNA in the liver or peripheral blood mononuclear cells) in the absence of both serum HCV-RNA and anti-HCV antibodies has not been investigated in hemodialysis patients. In this study, real-time PCR and in situ hybridization was used to test for the presence of genomic and antigenomic HCV-RNA in peripheral blood mononuclear cells of 109 hemodialysis patients with abnormal levels of liver enzymes. Occult HCV infection, determined by the presence of genomic HCV-RNA, was found in 45% of the patients; 53% of these patients had ongoing HCV replication, indicated by the presence of antigenomic HCV-RNA. Patients with occult HCV infection had spent a significantly longer time on hemodialysis and had significantly higher mean alanine aminotransferase levels during the 6 mo before study entry. Logistic regression analysis revealed that mortality was associated with age >60 yr (odds ratio 3.30; 95% confidence interval 1.05 to 10.33) and the presence of occult HCV infection (odds ratio 3.84; 95% confidence interval 1.29 to 11.43). In conclusion, the prevalence of occult HCV infection is high among hemodialysis patients with persistently abnormal values of liver enzymes of unknown cause. The clinical significance of occult HCV infection in these patients requires further study.

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RNA in PBMC is an alternative procedure when a liver biopsy is not available.

Until now, the presence of occult HCV infection in HD units has not been studied. This work aimed to (1) study the existence of occult HCV infection by testing for genomic HCV-RNA in PBMC of hemodialysis patients with abnormal levels of liver function tests of unknown cause and (2) determine whether the virus was replicating in these cells by detecting the antigenomic HCV-RNA.

A total of 109 HD patients (71 men, 38 women) who were repeatedly anti-HCV and serum HCV-RNA negative and had abnormal liver function tests of unknown cause for 23.8 ± 24.5 mo were included in the study. Their mean age was 61.7 ± 14.9 yr. The mean duration on maintenance HD was 51.5 ± 49.7 mo, and 59 (54%) patients had received blood transfusions. On study entry, mean ALT and γ-GTP levels were, respectively, 34.1 ± 21.2 and 99.3 ± 82.0 IU/L, whereas during the 6 mo before the beginning of the study, mean ALT was 25.7 ± 15.1 IU/L and mean γ-GTP was 80.0 ± 69.5 IU/L.

Genomic HCV-RNA was found by strand-specific real-time PCR in PBMC of 49 (45%) of 109 patients, indicating that they had an occult HCV infection. Viral RNA was not detected in negative controls, and the results of HCV-RNA detection performed by different operators on different days were identical in all cases. Mean load ± SEM of the genomic HCV-RNA was \(6.6 \times 10^4\) ± 7.8 \(\times 10^3\) copies of HCV-RNA per μg of total RNA. Presence or absence of genomic HCV-RNA was confirmed by fluorescence in situ hybridization.

Genotyping of the HCV-RNA isolated from PBMC showed that the 49 positive patients had HCV genotype 1. Sequence analysis of the HCV core region from six randomly selected cases confirmed that HCV isolates belonged to genotype 1b. Phylogenetic analysis demonstrated that the genetic distances of the clones within patients were lower than among patients (Table 1), except in patient 5 because one of his clones segregated separately from the others (Figure 1). The phylogenetic tree showed that the clones segregated separately in the six patients, indicating that no cross-contamination among samples occurred (Figure 1).

Regarding HCV replication in PBMC, antigenomic HCV-RNA was found by strand-specific real-time PCR in 26 (53%) of 49 patients with occult HCV (mean load ± SEM of 7.3 \(\times 10^3\) ± 1.2 \(\times 10^3\) copies per μg of total RNA). This ongoing viral replication was confirmed by fluorescence in situ hybridization.

No differences were found in demographic or clinical features between patients with and without occult HCV infection on study entry, except that the occult HCV infection group showed a longer duration of hemodialysis (63.9 ± 62.1 versus 41.4 ± 34.1 mo; \(P = 0.03\)). During the 6 mo before inclusion into the study, the mean ALT level (four to six values per patient) was significantly higher (\(P = 0.04\)) in patients with occult HCV infection (29.0 ± 15.5 IU/L) than in the negative ones (23.1 ± 14.4 IU/L), but no difference was found in the mean γ-GTP level. Within the group of occult HCV infection, patients with presence of replicating virus (as detected by antigenomic HCV-RNA) did not differ in any demographic or laboratory features from those without replication.

After entry into the study, patients with and without occult HCV infection were followed for 12.9 ± 14.4 and 15.8 ± 15.1 mo, respectively. All remaining anti-HCV and serum HCV-RNA negative with abnormal ALT and/or γ-GTP values. In occult HCV infection, 39% (19 of 45) of the patients died during the follow-up period, a percentage significantly higher than that of patients without occult HCV (12 [20%] of 60; \(P = 0.031\)). In a binary logistic regression analysis, it was found that mortality was associated with age >60 yr (odds ratio [OR] 3.30; 95% confidence interval [CI] 1.05 to 10.33; \(P = 0.04\)) and with the presence of occult HCV infection (OR 3.84; 95% CI 1.29 to 11.43; \(P = 0.015\)). None of the deaths was attributed to liver disease, but deceased patients with occult HCV infection had significantly higher ALT levels than those without occult HCV (26.8 ± 14.0 versus 16.7 ± 8.0 IU/L; \(P = 0.032\)). In addition, deceased patients with occult HCV infection were significantly younger than the negative ones (61.4 ± 14.6 versus 74.1 ± 3.3 yr; \(P = 0.002\)). Among alive patients, 11 (seven patients with and four without occult HCV infection) received a kidney transplant; the remaining patients continued on HD. Within the group of occult HCV, no differences were found between patients with viral replication in PBMC and those without it. One of the patients with occult HCV, who was placed on the renal transplantation waiting list, underwent a liver biopsy for histologic assessment and to confirm occult HCV infection. By strand-specific real-time PCR and by in situ hybridization, it was demonstrated that HCV was infecting and replicating in the liver cells. Liver histology revealed cholestasis, but necroinflammation or fibrosis was not

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detected. In another patient with occult HCV, liver cirrhosis was visualized during a laparoscopy procedure.

Patients who have normal renal function but abnormal liver function tests and are anti-HCV and serum HCV-RNA negative may have an occult HCV infection in liver and in PBMC. In this work, we detected the

**Figure 1.** Neighbor-Joining tree constructed with the HCV core nucleotide sequences of the clones of the six randomly selected patients and those corresponding to the various HCV genotypes. Bootstrap values ≥70, obtained after 1000 replicates of the data sheet, are shown in the nodes of the tree.
presence of the genomic HCV-RNA in PBMC of a high percentage (45\%) of anti-HCV– and serum HCV-RNA–negative HD patients with abnormal ALT and/or γ-GTP values, indicating that they have an occult HCV infection. Furthermore, antigenomic HCV-RNA was detected in 53\% of these HD patients with occult HCV, showing that HCV is replicating in PBMC and suggesting that these patients could be potentially infectious; therefore, because universal precautions may not be strictly followed when patients present abnormal liver function tests of unknown cause, occult HCV infection could play a role in HCV spread within HD units.

According to the original report of occult HCV infection,\textsuperscript{10} all HD patients with HCV-RNA in PBMC must also have viral RNA in liver. This could be verified in the patient who had occult HCV infection and underwent a liver biopsy. Nevertheless, it should be stressed that detection of HCV-RNA in PBMC does not identify all cases with occult HCV, so some of the HD patients without viral RNA in PBMC could have an occult HCV infection in liver; however, liver biopsy is not routinely recommended for HD patients except in a subset of cases (e.g., in renal transplant candidates, before starting antiviral therapy).

Duration of HD was significantly longer in patients with occult HCV infection, whereas antecedents of blood transfusion did not differ between groups. This finding suggests the possible role of nosocomial transmission in the spread of occult HCV, as reported in HD units for classical HCV infection.\textsuperscript{1,2,11}

During the follow-up study, it was found that mortality was significantly associated with age and with the presence of occult HCV infection. None of the deaths could be attributed to liver disease; however, that deceased patients with occult HCV had significantly higher ALT levels than deceased uninfected patients and the finding of liver cirrhosis in one of the two patients who had occult HCV infection and for whom liver histology was available suggest that occult HCV infection may contribute to the worse outcome of these patients.

In summary, a high percentage of HD patients who have abnormal values of liver enzymes and are anti-HCV and serum HCV-RNA negative present an occult HCV infection. Further studies involving a larger number of patients with a much longer observation period are required to elucidate the natural history and clinical implications of occult HCV infection in HD patients.

CONCISE METHODS

Ten HD units from different Spanish regions participated in the enrollment of patients under stable substitutive HD therapy. All virologic assays were performed in a single center. The inclusion criteria were (1) documented ALT levels >28 IU/L (normal limit for HD patients)\textsuperscript{3–7} and/or abnormal γ-GTP levels (>43 IU/L) for >6 mo before entry into the study; (2) Persistently negative serologic markers of HIV (anti-HIV), hepatitis B virus (surface antigen and DNA), and HCV (anti-HCV and HCV-RNA); and (3) exclusion of all other causes of liver diseases (e.g., autoimmune, toxic hepatitis, metabolic disorders). The study was approved by the coordinating center’s ethics committee and conducted according to the Declaration of Helsinki. Inclusion criteria were fulfilled by 109 hemodialysis patients who gave their consent to participate in the study. Serum and PBMC samples were collected from all patients on the study entry day. Anti–HCV (INNOTEST HCV Ab IV, Innogenetics, Ghent, Belgium) and HCV-RNA were tested again in all of these serum samples, confirming that all patients included were negative for these HCV serologic markers. ALT and γ-GTP levels were also measured.

PBMC samples were isolated from fresh heparinized venous blood by gradient centrifugation. Cells were divided into two aliquots: One was stored at −20°C in RNA-Later (Ambion, Austin, TX) until its use for detection of genomic and antigenomic HCV-RNA and for HCV genotyping by strand-specific quantitative real-time PCR. The other aliquot was processed for detection of both HCV-RNA strands by fluorescence in situ hybridization. PBMC from 10 healthy volunteers who had normal liver function tests and were repeatedly HCV-RNA negative in PBMC were used as negative controls.

Detection of Genomic and Antigenomic HCV-RNA and HCV Genotyping

Total RNA was isolated from 250 μl of serum with TRIzol LS Reagent (Invitrogen, Carlsbad, CA) and from PBMC using the SV Total RNA Isolation System (Promega, Madison, WI). After precipitation, RNA pellets were dissolved in diethyl-pyrocarbonate–treated water, and total amount of RNA isolated from PBMC was determined by spectrophotometry. The total RNA extracted from serum or 0.5 μg of total RNA from PBMC was used for the detection of the 5’ noncoding region of genomic and antigenomic HCV-RNA by strand-specific quantitative real-time PCR. Synthesis of the corresponding cDNA was performed at high temperature using the thermostable enzyme Tth (Applied Biosystems, Foster City, CA) as described previously.\textsuperscript{12} Real-time PCR using fluorescence resonance energy transfer probes was run in a Light Cycler (Roche Molecular Biochemicals, Indianapolis, IN) with 2 μl of cDNA in a final volume of 20 μl, using the LightCycler FastStart DNA Master HybProbe Kit (Roche Molecular Biochemicals). Primers and conditions of this strand-specific real-time PCR have been reported.\textsuperscript{13} The fluorescence resonance energy transfer probes used were described by Bullock et al.\textsuperscript{14}These allow discrimination between HCV genotypes 1, 2, 3, and 4 by melting-curve analysis. Quantification of both HCV-RNA strands was performed using two standard curves constructed with 10-fold dilutions of synthetic genomic and antigenomic HCV-RNA (from 3.2 × 10\(^{8}\) to 0.32 copies). Quantification assay linearity ranged from 3.2 to 3.2 × 10\(^{8}\) copies of genomic or antigenomic HCV-RNA per reaction. This assay was capable of detecting 3.2 molecules of the correct strand while unspecifically detecting 10\(^{7}\) to 10\(^{8}\) copies of the incorrect strand.\textsuperscript{13}

For ensuring the specificity of the results, HCV-RNA detection was performed blindly on different days by two different operators. In addition to PBMC from the 10 healthy anti-HCV negative patients, total RNA isolated from HepG2 cells and blanks were used as negative controls. All procedures were per-
formed following the recommendations of Kwock and Higuchi.15

Phylogenetic Analysis
For discarding cross-contamination among positive samples, the HCV core region was amplified from total RNA isolated from PBMC of six randomly selected patients by reverse transcriptase–PCR as described previously.10 The 302-bp core PCR products were cloned into the pCR II TOPO vector (Invitrogen), and a minimum of four clones from each patient were automatically sequenced. Sequences were aligned with core sequences corresponding to all HCV genotypes retrieved from GenBank, using the ClustalX 1.81 program.16 Phylogenetic and molecular evolutionary analyses were conducted using MEGA 2.1.17 Genetic distances were estimated using the Kimura 2 parameter method, and SE of the distances were computed by bootstrap method (1000 replicates). Phylogenetic tree was constructed with the Neighbor-Joining method, and its statistical significance was tested by bootstrap method (1000 replicates).

Detection of the Genomic and Antigenomic HCV-RNA in PBMC by Fluorescence In Situ Hybridization
Genomic HCV-RNA was detected using a complementary RNA probe obtained by in vitro transcription of the pCS’NCR plasmid (which contains the complete 5’ noncoding region of the HCV genome) in the presence of digoxigenin-11-UTP (Roche Molecular Biochemicals). Detection of the antigenic HCV-RNA was done with a complementary digoxigenin-labeled RNA probe spanning 390 nucleotides of the HCV core region, obtained by in vitro transcription of the pCore plasmid. Hybridization conditions for the in situ detection of both HCV-RNA strands were as described previously.18

Statistical Analysis
Statistical analysis was conducted with SPSS 15.0 (SPSS, Chicago, IL). Continuous variables were expressed as means ± SD except where indicated. Means were compared with the t test according to the homogeneity of variances. Categorical variables were expressed as percentages and were compared with the χ² test. Demographic and clinical data of the patients (gender, age, antecedents of blood transfusions, duration of hemodialysis, chemotherapy, and ALT and γ-GTP levels) and the presence or absence of occult HCV infection were included in a binary logistic regression analysis to identify independent factors that were associated with mortality.

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DISCLOSURES
None.

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