Negative-strand HCV RNA Was Not Detected in Bone Marrow Cells of Patients with HCV Infection

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Summary: To determine whether hepatitis C virus (HCV) replicates in bone marrow, we investigated positive- and negative-strand HCV RNA in bone marrow cells and fluids, and sera from patients with HCV infection. The study population consisted of 15 patients positive for antibodies to HCV (anti-HCV). Positive- and negative-strands HCV RNA were detected using highly strand-specific \( rTth \) reverse transcription-polymerase chain reaction (\( rTth \) RT-PCR) followed by Southern blotting analysis. Positive-strand HCV RNA was detected in 12 (80%) serum samples, in 13 (86.7%) bone marrow fluid specimens, and in 6 (40.0%) bone marrow cell samples. Negative-strand HCV RNA was detected in 9 (60.0%) serum samples, 11 (91.7%) fluid specimens, while it was not detected in bone marrow cells. The absence of negative-strand HCV RNA in bone marrow cells suggested that HCV does not replicate in these cells. Negative-strand HCV RNA detected in serum and bone marrow fluid samples may have been due to contamination with circulating HCV RNA from hepatocytes.

Key words hepatitis C virus (HCV), bone marrow cells, positive- and negative-strand HCV RNA

INTRODUCTION

Hepatitis C virus (HCV) is the major etiological agent responsible for parenterally transmitted non-A, non-B hepatitis [1,2]. HCV is a small enveloped, positive-strand RNA virus, which appears to have a similar genetic organization to the pesti- or flaviviruses [3]. The viral genome is comprised of a single-strand positive-sense RNA that is translated into a large polyprotein, which is post-translationally processed to yield the structural and nonstructural viral proteins [4, 5]. As HCV appears to replicate via a negative-strand RNA intermediate, active replication in infected cells is demonstrated by the detection of negative-strand complementary to the genomic RNA [4]. Thus, detection of negative-strand HCV RNA in infected cells is considered a marker of active viral replication. The presence of HCV negative-strand RNA has not only been reported in liver but also in serum [6,7], peripheral blood mononuclear cells (PBMC) [8-10], and bone marrow cells [11]. On the other hand, different studies have recently indicated that the previous strand-specific methods to detect negative-strand HCV RNA lack strand specificity [12-15]. In this study, to investigate whether HCV can replicate in bone marrow cells in patients with hepatitis C, both positive- and negative-strands of RNA in bone marrow cells, fluids, and serum from anti-HCV positive patients were investigated, using highly specific \( rTth \) RT PCR [14, 15].

MATERIALS AND METHODS

Subjects

Subjects were 15 patients positive for anti-HCV admitted to our institute between April, 1993, and August, 1994. Table 1 shows the clinical background of the subjects including diagnosis, platelets, white
TABLE 1.
Clinical background of the patients and results of RT-PCR analysis for both positive- and negative-strand HCV RNA

<table>
<thead>
<tr>
<th>Patients No.</th>
<th>Age (yrs)/Sex</th>
<th>Diagnosis</th>
<th>PLT (10^3/mm^3)/WBC (10^3/mm^3)</th>
<th>IFN therapy</th>
<th>Time of bone-marrow puncture</th>
<th>Serum Po Ne</th>
<th>Bone marrow fluids Po Ne</th>
<th>Bone marrow cells Po Ne</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>40/M</td>
<td>CPH</td>
<td>5.9/8300</td>
<td>+</td>
<td>Before IFN</td>
<td>10^3</td>
<td>10^4</td>
<td>10^4</td>
</tr>
<tr>
<td>2</td>
<td>19/F</td>
<td>CAH</td>
<td>7.1/5700</td>
<td>+</td>
<td>Before IFN</td>
<td>10^2</td>
<td>10^3</td>
<td>10^4</td>
</tr>
<tr>
<td>3</td>
<td>34/F</td>
<td>LC</td>
<td>5.9/1500</td>
<td>+</td>
<td>Before IFN</td>
<td>10^3 10^2</td>
<td>10^3 10^2</td>
<td>10^3 10^2</td>
</tr>
<tr>
<td>4</td>
<td>40/F</td>
<td>CAH</td>
<td>6.3/3500</td>
<td>+</td>
<td>During IFN</td>
<td>10^2 1</td>
<td>10^3 10^2</td>
<td>10^2</td>
</tr>
<tr>
<td>5</td>
<td>62/F</td>
<td>CAH</td>
<td>13.2/2600</td>
<td>+</td>
<td>During IFN</td>
<td>10^3 1</td>
<td>10^4 10^2</td>
<td>10^2</td>
</tr>
<tr>
<td>6</td>
<td>65/F</td>
<td>CAH</td>
<td>8.0/3600</td>
<td>+</td>
<td>During IFN</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
<td>36/M</td>
<td>CAH</td>
<td>10.3/3100</td>
<td>+</td>
<td>During IFN</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>8</td>
<td>67/M</td>
<td>LC</td>
<td>11.4/4800</td>
<td>+</td>
<td>During IFN</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>63/F</td>
<td>LC</td>
<td>5.3/2000</td>
<td>+</td>
<td>During IFN</td>
<td>10^3</td>
<td>10^4</td>
<td>10^4</td>
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<tr>
<td>10</td>
<td>51/M</td>
<td>LC</td>
<td>3.3/2300</td>
<td>—</td>
<td>At admission</td>
<td>10^3 10^3</td>
<td>10^3 10^3</td>
<td>10^2</td>
</tr>
<tr>
<td>11</td>
<td>60/M</td>
<td>LC with HCC</td>
<td>11.5/3700</td>
<td>—</td>
<td>At admission</td>
<td>10^4 10^5</td>
<td>10^4 10^5</td>
<td>10^2</td>
</tr>
<tr>
<td>12</td>
<td>78/M</td>
<td>LC with HCC</td>
<td>9.8/4300</td>
<td>—</td>
<td>At admission</td>
<td>10^4 10^5</td>
<td>10^4 1 10^5</td>
<td>10^2</td>
</tr>
<tr>
<td>13</td>
<td>62/F</td>
<td>LC with HCC</td>
<td>11.4/3200</td>
<td>—</td>
<td>At admission</td>
<td>10^4 10^3</td>
<td>10^3 10^2</td>
<td>10^4</td>
</tr>
<tr>
<td>14</td>
<td>65/F</td>
<td>LC with HCC</td>
<td>8.0/3600</td>
<td>—</td>
<td>At admission</td>
<td>10^5 10^2</td>
<td>10^4 10^2</td>
<td>10^2</td>
</tr>
<tr>
<td>15</td>
<td>68/F</td>
<td>LC with HCC</td>
<td>5.3/1700</td>
<td>—</td>
<td>At admission</td>
<td>10^3 10^3</td>
<td>10^4 10^4</td>
<td>—</td>
</tr>
</tbody>
</table>

Mean Po HCV RNA titer (serum: fluids: cells) 4.0 x 10^7 : 5.6 x 10^7 : 2.1 x 10^7
Mean Ne HCV RNA titer (serum: fluids: cells) 3.4 x 10^7 : 1.0 x 10^7 : 0

M: male; F: female; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis; LC: liver cirrhosis; HCC: hepatocellular carcinoma; PLT: platelets; IFN: interferon; Po: positive-strand HCV RNA; Ne: negative-strand HCV RNA; N.S.: not significant

Mean HCV RNA titer (serum: fluids: cells) 8.4 x 10^7 : 5.6 x 10^7 : 2.1 x 10^7

N.S.: P < 0.001

M: male; F: female; CPH: chronic persistent hepatitis; CAH: chronic active hepatitis; LC: liver cirrhosis; HCC: hepatocellular carcinoma; PLT: platelets; IFN: interferon; Po: positive-strand HCV RNA; Ne: negative-strand HCV RNA; N.S.: not significant

Subjects consisted of 6 men and 9 women with a mean age 54 ± 16 years (range 19-78). One had chronic persistent hepatitis (CPH), 5 had chronic active hepatitis (CAH), 4 had liver cirrhosis (LC), and 5 had liver cirrhosis with hepatocellular carcinoma (LC with HCC). None were positive for HBsAg except Pt 1. Nine patients (Pt 1-9) were treated with IFN. In the 3 (Pt 1-3) treated patients, bone marrow punctures were performed before treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively). In the remaining 6 (Pt 4-9) treated patients, punctures were done after treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively). In the remaining 6 (Pt 4-9) treated patients, punctures were done after treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively). In the remaining 6 (Pt 4-9) treated patients, punctures were done after treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively). In the remaining 6 (Pt 4-9) treated patients, punctures were done after treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively). In the remaining 6 (Pt 4-9) treated patients, punctures were done after treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively). In the remaining 6 (Pt 4-9) treated patients, punctures were done after treatment because of marked reductions in platelets (5.9 x 10^4, 7.1 x 10^4, 5.9 x 10^4, respectively).

Bone marrow puncture sites, method and specimen handling

Bone marrow puncture was performed on the sternum at the level of the 2nd-3rd ribs. Directly after collection, the samples were divided for leucocyte and megakaryocyte counts, bone marrow cell classification, and HCV RNA detection. Bone marrow for HCV RNA detection was transferred to EDTA-treated tubes followed by separation (2500 rpm/10 min, 4°C) with cooled PBS and washed; this procedure was repeated three times. The bone marrow cells (over 1.0 x 10^5 cells/µl) and fluids were stored at −40°C until HCV RNA measurement.

Strand-specific RT-PCR

Aliquots of 100 µl of serum, bone marrow fluid and cells were diluted 10 fold with PBS, and RNA was extracted using the acid guanidium isothiocyanate phenol-chloroform method (Isogen-LS, Nippon Gene Corp., Toyama, Japan), precipitated with isopropanol and resuspended in 20 µl of RNase-free H2O. To detect HCV RNA by a semi-quantitative method, the RNA preparations were diluted 10 fold serially from which 10 µl aliquots were used for RT-PCR. Primers for RT-PCR (sense primer; HC-1: [5'-CACCCCCCCTGGGAGAACTACTCTGC-3'] from nt. 21-45) and antisense primer; HC-2: [5'-ATGGTGCAACGGTCAGAGC CTCC-3'] from...
NEGATIVE-STRAND HCV RNA IN BONE MARROW CELLS

nt. 302-326) were designed based on the highly conserved 5′ untranslated region (5′UTR) of HCV. The presence of positive- and negative-strand HCV RNA in serum, and bone marrow (fluids and cells) was detected by the so-called strand-specific RT-PCR method [6]. Thus, HC-2 was used as a cDNA primer to specifically prime positive-strand RNA, and HC-1 was used as a cDNA primer to specifically prime negative-strand RNA. Several studies have indicated that the standard method for detection of positive- and negative-strand RNA lacks sufficient strand specificity [12-15]. Therefore, in this study, RT-PCR was performed with a modified GeneAmp® Thermostable rTth Reverse Transcriptase RNA PCR Kit (Perkin-Elmer Cetus, Tokyo, Japan) as described previously [12-15]. Aliquots of 10 μl of each extracted RNA were overlain with mineral oil, heated at 90°C for 1 min, the temperature was lowered to 70°C, and 10 μl of preheated cDNA reaction mixture was added. The reaction mixture consisted of 10 mM Tris (pH 8.3), 90 mM KCL, 1mM MnCl₂, 200 μM each deoxynucleotide triphosphate, 50 ng of cDNA primer, and 5U of rTth. Then, annealing temperature was lowered to 60°C for 2 min, and increased to 70°C for 15 min for the cDNA reaction. To chelate the Mn²⁺ and inactivate the RT activity of rTth, the temperature was held at 70°C until addition of 40 μl of preheated buffer consisting of 10% (V/V) glycerol, 20 mM Tris (pH 8.3), 200 mM KCl, 0.1% (W/V) Tween® 20, and 1.5 mM EGTA (ethylene glycol-bis [β-aminoethyl ether]-N, N', N', N'-tetraacetic acid). Reaction tubes were held at 70°C until addition of 40 μl of preheated PCR mixture (50 ng of each opposite outside primer in 3.75 mM MgCl₂). The PCR profile consisted of the following 3-step reactions. Step 1: 94°C, 5 min; 55°C, 1 min; and 72°C, 2 min, 1 cycle; step 2: 94°C, 1 min; 55°C, 1 min; and 72°C, 2 min, 35 cycles; step 3: 72°C, 2 min, 1 cycle. The PCR products were analyzed by 1.2% agarose gel electrophoresis and ethidium bromide staining. These products were used to confirm the 306 bp band and estimate the dilution levels at which this band disappeared. Following electrophoresis, the PCR product was transferred from the gels onto nitrocellulose filters followed by hybridization with a 32P-labeled probe (5'-AGAGCCATAGTGCTGCGGAACCGGTGAGTACTCCGGA-3' from nt. 117-155) and autoradiography. Southern blotting was employed for all last diluted samples for which PCR products could not detected by ethidium bromide staining the PCR products. The sensitivity of single step PCR with 32P-labeled probes was reported to be equivalent to that of nested PCR, which is considered the reference assay [16]. Thus, the sensitivity of our RT-PCR assay may also be equivalent to that of nested PCR.

Serum collection and serological tests

To compare HCV RNA levels in the bone marrow and peripheral blood, serum was collected at the time of bone marrow puncture, and stored at -20°C until HCV RNA assay. HBsAg was examined by enzyme immunoassay (EIA; Mizuho Medy Co., Ltd., Tosu, Japan) and anti-HCV was determined by second-generation passive hemagglutination assay (PHA 2nd Generation, DAINABOT Co., Ltd., Tokyo, Japan).

Statistical analysis

Data are expressed as means ± SD. Statistical analyses were performed using the Mann-Whitney U test; P< 0.05 was defined as significant.

RESULTS

Results of detection of HCV RNA (Table 1)

Positive-strand HCV RNA was detected in the sera of 12 (80.0%) patients and negative-strand HCV RNA was detected in that of 9 (60.0%) patients. Neither positive- nor negative-strand HCV RNA was detected in sera of 3 patients (Pt 6, 7, 8). Positive-strand HCV RNA was detected in the bone marrow fluids of 13 (53.3%) patients, while negative-strand HCV RNA was detected in that of 11 (42.7%) patients. Positive-strand RNA was detected in the bone marrow cells of 6 (40.0%) patients, while negative-strand was not detected in any of the subjects. HCV RNA positive-strand titer in serum (mean, 8.4×10³) was significantly greater than that in bone marrow cells (mean, 2.1×10¹) (p<0.001), while it was not significantly greater than that in bone marrow fluid (mean, 5.6×10³). HCV RNA negative-strand titers in serum (mean, 3.4×10²) were significantly greater than those in bone marrow cells (mean, 0) (p<0.051), while it was almost equivalent to that in bone marrow fluid (mean, 1.0×10²).

DISCUSSION

Whether extrahepatic replication of HCV in bone marrow cells occurs is an important question with regard to the transmission and pathogenesis of this virus. As HCV has a similar genetic organization to
the pesti- or flaviviruses, detection of negative-strand HCV RNA in infected cells is considered a marker of active viral replication [5,6,17]. Several investigators have reported the detection of negative-strand HCV RNA not only in serum, liver, and PBMC but also in bone marrow cells [11]. However, other researchers suggested that negative-strand HCV RNA detected in extrahepatic sites may be due to contamination with plasma and/or adherence of circulating virus because methods for detecting negative-strand HCV RNA lack sufficient strand specificity [12-15]. Therefore, we examined both strands of HCV RNA using highly strand-specific \( Tth \) RT PCR [14,15]. Negative-strand HCV RNA was detected in serum and bone marrow fluids samples, while it was not found in any bone marrow cell samples. Both positive- and negative-strand HCV RNA detected in serum and bone marrow fluids samples might have been due to contamination and/or adherence of circulating HCV RNA. To detect HCV RNA more precisely, another important point is sensitivity. In this study, we used single-step PCR with a \( ^{32}P \)-labeled probes. It was reported that the sensitivity of this method was equivalent to that of nested PCR, which is considered the reference assay [16]. Thus, the sensitivity of our RT-PCR assay may also have been equal to that of nested PCR. From these observations, we conclude that HCV infection in bone marrow cells may be transient rather than replicative.

In this study, we semi-quantitatively examined both positive- and negative-strand HCV RNAs in serum and bone marrow (fluids and cells). Titers of both HCV RNAs in serum were almost equivalent to those of in bone marrow fluid, but were significantly greater than those in bone marrow cells. Although the number of subjects examined was limited and it is questionable whether we can correctly compare HCV RNA levels in different tissues (serum and bone marrow fluids or serum and bone marrow cells), these findings also suggest that both positive- and negative-strand HCV RNA detected in bone marrow fluid and positive-strand HCV RNA detected in bone marrow cell samples might have been due to contamination and/or adherence of circulating HCV RNA. In chronic hepatitis C patients, pretreatment serum hepatitis C virus RNA levels are one of the main prognostic factors of sustained response to IFN therapy [18,19], while this does not always predict the occurrence of a sustained response. Some researchers reported that the frequent relapses observed after cessation of treatment with IFN suggested persistence of HCV in the liver and/or extrahepatic cells such as PBMC [20]. In the present study, in 6 (Pt 4-9) treated patients, punctures were performed during IFN treatment. Interestingly, in Pt 7, only positive-strand HCV RNA was detected in bone marrow fluid, while both positive- and negative-strand HCV RNA had disappeared from serum and bone marrow cells. We could not explain why only positive-strand HCV RNA was detected in bone marrow fluid, but this result suggests a relationship between relapse and existence and/or replication of HCV in extrahepatic sites.

To clarify the potential for extrahepatic existence and/or replication of HCV in abdominal lymph nodes, we investigated both positive- and negative-strand HCV RNA in abdominal lymph node mononuclear cells; however, negative-strand HCV RNA was not detected [21]. We also reported a relationship between lichen planus and HCV infection [22,23]. Further investigation is necessary to determine whether HCV RNA exists and/or replicate in such lichen planus tissue.

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