In Situ Detection of Hepatitis C Virus RNA in Salivary Glands

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Chronic hepatitis C virus (HCV) infection has been associated with several extrahepatic manifestations, among these, to diseases with oral manifestations such as Sjögren's syndrome or sialadenitis. HCV-RNA has been detected in saliva and in salivary glands from patients with sialadenitis by polymerase chain reaction. However, morphological evidence of HCV replication in salivary gland cells is needed to support a role for HCV in causing sialadenitis or Sjögren's syndrome. We have used *in situ* hybridization and immunohistochemistry to analyze the presence of HCV-RNA of sense and antisense polarity and HCV core antigen, respectively, in salivary gland biopsies from 19 patients with chronic sialadenitis or Sjögren's syndrome (eight anti-HCV-positive; 11 anti-HCV-negative). HCV-RNA of both positive and negative polarity as well as HCV core antigen were detected in the epithelial cells of the salivary gland biopsies from all of the anti-HCV-positive patients but in none of the anti-HCV-negative cases. The percentage of HCV-infected cells ranged from 25 to 48.8% in the patients studied. In conclusion, we have shown that HCV infects and replicates in the epithelial cells from salivary glands of patients with Sjögren's syndrome or chronic sialadenitis. However, its implication in the pathogenesis of these diseases deserves future research. (Am J Pathol 2001, 158:259–264)

Hepatitis C virus (HCV), which is a small, enveloped virus belonging to a new genus within the *Flaviridae* family of viruses,¹ is the causative agent of most cases of post-transfusional non-A, non-C hepatitis.² Its genome consists of a single-stranded RNA molecule of positive polarity of ~9.4 kb in length³ and it is thought to replicate through a RNA molecule of negative polarity.⁴

HCV causes acute and chronic liver disease and the chronic HCV infection is associated with the development

of liver cirrhosis and hepatocellular carcinoma.⁵ Chronic HCV infection is also associated with several extrahepatic manifestations.^{6–8} Among these diseases, HCV infection has been related to diseases with oral manifestations such as lichen planus, Sjögren's syndrome, or chronic lymphocytic sialadenitis.^{9–11}

Sjögren's syndrome is an autoimmune disease characterized by a lymphocytic infiltration of lachrymal and salivary glands.¹² Although its etiology is unknown, viral infections have been implicated in the development of this disease.^{13,14} Epidemiological studies have shown that up to 40% of Sjögren's syndrome patients may have anti-HCV antibodies.^{15,16} Furthermore, patients with chronic HCV infection frequently have a mild form of lymphocytic sialadenitis that is histologically similar to the primary Sjögren's syndrome but which is not associated with other clinical features of the Sjögren's syndrome.¹⁷

Whether the replication of HCV in salivary glands is the cause of the sialadenitis or not is currently unknown, however, the fact that HCV RNA has been detected by reverse-transcriptase-polymerase chain reaction (RT-PCR) in saliva as well as in the total RNA isolated from salivary glands^{18,19} favors this hypothesis. Nevertheless, blood contamination of the saliva and the salivary glands may be the reason that HCV RNA is detected in both sites and thus, morphological evidence of HCV replication in salivary gland cells is needed to support a role for the virus in causing sialadenitis.

For this reason, in the present study we have used *in situ* hybridization and immunohistochemistry to analyze the presence and replication of HCV in salivary gland biopsies from 19 patients with sialadenitis or Sjögren's syndrome.

Patients and Methods

The presence of HCV was analyzed in serum samples and in paraffin-embedded salivary gland biopsies from 19 patients (18 females, one male; mean age, 57.2 ± 15.3 years) affected by xerostomia. Salivary gland biopsies were obtained for the histological diagnosis and after that the remaining tissue was used for the *in situ* hybrid-

Supported by the Fundación para el Estudio de las Hepatitis Virales. N. O. M. is a research fellow of the Fundación Conchita Rábago.

Accepted for publication September 22, 2000.

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ization analysis. Six patients had Sjögren's syndrome whereas the remaining 13 patients had chronic sialadenitis according to clinical and histopathological criteria. Eight of the patients (one Sjögren's syndrome and seven chronic sialadenitis) were anti-HCV-positive when the salivary gland biopsies were obtained and all of them had abnormal alanine aminotransferase levels (88.25 ± 25.06 IU/L). The remaining 11 patients had no serum markers of HCV infection and had normal alanine aminotransferase levels. None of the patients had hepatitis A, B, or D markers.

This study was approved by the Ethics Committee of our hospital and a written informed consent according to the legal requirements was obtained from each patient participating in the study.

Serological Markers

Anti-HCV was determined by a third generation enzyme-linked immunoassay (INNOTEST HCV Ab III; Innogenetics, Heiden, Germany) and confirmed by a recombinant immunoblot assay (INNO-LIA HCV Ab III, Innogenetics). Hepatitis B virus surface antigen (HBsAg), anti-hepatitis D virus antibody (anti-HDV), and IgM anti-hepatitis A virus antibody (anti-HAV) were tested by commercial enzyme immunoassays (Roche Diagnostic Systems, Basel, Switzerland).

HCV RNA Detection in Saliva Samples

Saliva samples for the detection of HCV-RNA were available from four of the eight anti-HCV-positive patients and from five of the 11 anti-HCV-negative patients.

Total RNA from saliva samples was isolated using Trizol (Life Technologies, Inc., Grand Island, NY) following the manufacturer's instructions. HCV-RNA was detected by RT-PCR using primers deduced from the 5' noncoding of the HCV genome as described.²⁰

Quantitation of the HCV RNA in Serum and HCV Genotyping

HCV RNA presence and titers were determined by using the Amplicor Monitor Test kit (HCV Monitor Quantitative Assay, Roche Diagnostic Systems) following the manufacturer's instructions.

HCV genotypes were determined by a line probe assay (INNO-LIPA HCV II; Innogenetics NV, Zwijnaarde, Belgium).

In Situ Hybridization

A PCR product of 340 bp corresponding to the complete 5'-noncoding region (5'NCR) of the HCV genome was directly cloned into the pCR II-TOPO vector (Invitrogen, San Diego, CA) to generate the plasmid pC5'NCR. In this plasmid, the cDNA insert was flanked by the SP6 (5' to the cDNA insert) and T7 (3' to the insert) RNA promoters. The pC5'NCR plasmid was digested with *Hind*III or *Xba*I and the linearized plasmid was transcribed *in vitro* with

SP6 (*Xba*I linearized plasmid) or T7 (*Hind*III linearized plasmid) RNA polymerases (Boehringer Mannheim Biochemicals, Indianapolis, IN) in the presence of digoxigenin 11-UTP (Boehringer Mannheim Biochemicals) to generate probes of sense and antisense polarity, respectively.

Consecutive sections of the salivary gland biopsies were hybridized with the labeled RNA probes of antisense and sense polarity to detect HCV RNA of sense and antisense polarity, respectively. Paraffin-embedded salivary gland sections (4 mm) were dewaxed in xylol and rehydrated through a series of ethanol dilutions. After digestion with proteinase K (1 mg/ml) at 37°C for 7 minutes, the sections were postfixed in a freshly prepared solution of 4% paraformaldehyde in 0.1 mol/L phosphate buffer at pH 7.0 and acetylated in 0.5% (v/v) acetic anhydride in 0.1 mol/L triethanolamine (pH 8.0) for 20 minutes at room temperature. The slides were rinsed in 2× standard saline citrate (SSC) (20× SSC: 3 mol/L NaCl, 0.3 mol/L sodium citrate) and quickly dehydrated in ethanol. After that, 20 μ L of hybridization mixture consisting of 50% deionized formamide, 0.1 mol/L phosphate buffer (pH 7.0), 4× SSC, yeast tRNA (500 mg/ml), 10% dextran sulfate, and 5 ng of heat-denatured labeled probe were placed on each slide, sealed with rubber solution, and sections and probe were denatured again together at 80°C for 10 minutes. The slides were incubated at 50°C for 16 hours. After hybridization, slides were washed for 1 hour at 50°C with 3× SSC, digested with RNase A (20 mg/ml), and rinsed in 1.5× SSC and 0.75× SSC at 50°C for 1 hour each. The digoxigenin-labeled hybrids were detected with a digoxigenin antibody-alkaline phosphatase conjugate and an enzyme substrate chromogen (nitroblue tetrazolium-5-bromo-4-chloro-3-indolylphosphate) according to the manufacturer's instructions (Dig Nucleic Acid Detection kit; Boehringer Mannheim Biochemicals). The slides were counterstained with 0.2% safranin in 5% ethanol.

The signal specificity was assessed by: 1) digestion of the sections with RNase A (0.2 mg/ml) (Sigma Chemical Co., St. Louis, MO) and DNase I (20 U/ml) (Sigma Chemical Co.) for 2 hours at 37°C before the *in situ* hybridization; 2) competitive experiments using an excess of unlabeled HCV probe or an excess of an unrelated unlabeled probe [a 360-bp fragment of the chloranfenicol acetyl transferase (CAT) gene, generated by PCR and cloned in the pCRII-TOPO vector]; and 3) omission of the probe in the hybridization mixture.

Visualization of the *in situ* hybridization signals was performed using a Nikon Eclipse E-400 light microscope (Nikon Co. Tokyo, Japan) using $\times 20$, $\times 40$, and $\times 100$ objectives. The images were captured with a high-resolution monochrome CCD camera (DIC-N; Wared Precision Instruments, Cambridge, UK).

The percentage of infected cells was determined by visual inspections, counting at least 20 different acini structures from each salivary gland biopsy.

Dot Blot Hybridization

To test the hybridization specificity of the sense and antisense probes with their complementary HCV-RNA, a

Table 1. Biochemical and Virological Data of the HCV-Infected Patients Included in the Study

Patient no.	ALT levels (IU/l)	Serum HCV viremia (genome copies/ml)	No. of HCV+ acini/total acini	% of HCV+ cells
1	147	4.3×10^5	19/20	33.3
2	73	1.4×10^6	21/23	25
3	74	7.6×10^5	20/20	32
4	75	5.1×10^5	18/20	33.5
5	72	8.6×10^5	19/20	47.6
6	82	2.3×10^5	19/20	48.8
7	89	1.3×10^3	20/20	36
8	94	7.4×10^5	19/20	40.6

dot blot hybridization was performed. For that purpose the p5'NCR plasmid was transcribed *in vitro* with SP6 and T7 RNA polymerases to generate synthetic HCV-RNA of sense and antisense polarity, respectively. Serial dilutions (from 10 ng to 1 pg) of each synthetic RNA were denatured by treatment with 6 mol/L deionized glyoxal at 50°C for 20 minutes, spotted onto two nitrocellulose membranes, and fixed by UV irradiation. After deglyoxylation by treatment with 20 mmol/L Tris, pH 8.0, the filters were prehybridized for 1 hour at 50°C in a solution consisting of 50% deionized formamide, 5× SSC, 5× Denhardt's solution, 0.1% sodium dodecyl sulfate, and 50 µg/ml of yeast t-RNA. Hybridization was performed at 50°C for 6 hours in the same prehybridization solution to which dextran sulfate (10%) and 5 ng of the corresponding digoxigenin-labeled probe were added. After hybridization, the filters were washed two times at room temperature (5 minutes each) with 2× SSC, 0.1% sodium dodecyl sulfate, followed by two more washes at 50°C (15 minutes each) with 0.1× SSC, 0.1% sodium dodecyl sulfate. Finally, the hybrids were detected using the DIG Nucleic Acid Detection Kit (Boehringer Mannheim) following the manufacturer's instructions.

Immunohistochemistry

Immunohistochemical detection of the HCV core antigen in salivary gland sections was automatically performed (Tech Mate 500; DAKO, Glostrup, Denmark) with an anti-HCV core monoclonal antibody (Hepatitis C Virus Monotope; ViroStat, Portland, ME) at a 1/30 dilution after antigenic retrieval pressure cooking (3 minutes in 10 mmol/L citrate buffer, pH 6.0) using the DAKO Envision staining procedure and amino benzidine as the chromogen.

Histological Diagnosis

Histological diagnosis was performed in hematoxylin and eosin-stained salivary gland biopsies.

Statistical Analysis

The Spearman correlation was used for correlation and all statistical analyses were performed using the SPSS package (SPSS for Windows, release 6.0; SPSS Inc., Chicago, IL).

Results

HCV-RNA in Serum

All anti-HCV-positive patients had HCV RNA in serum at concentrations ranging from 1.3×10^3 to 2.3×10^6 genome copies per ml (Table 1). With respect to the viral genotype, all patients were infected by the HCV 1b genotype.

HCV-RNA in Saliva

HCV-RNA was detected in the saliva samples obtained from the four anti-HCV-positive patients but in none of the five anti-HCV-negative patients analyzed.

HCV-RNA Detection by *in Situ* Hybridization

Positive hybridization signals were observed in the salivary gland biopsies from the eight patients with HCV-RNA in serum but in none of the biopsies from the 11 patients without viral RNA in serum (Figure 1).

The specificity of the *in situ* hybridization technique was demonstrated by the results obtained from the specificity test. RNase treatment before the hybridization abolished the hybridization signals whereas DNase pretreatment did not change the hybridization pattern. Competitive hybridization with the excess of unlabeled HCV probe diminished the hybridization but the use of an unrelated unlabeled probe (CAT probe) as competitor did not change the hybridization pattern. Finally, no hybridization signals were observed when the probe was omitted in the hybridization mixture (Figure 2).

Regarding the cell types infected by HCV within the salivary glands, *in situ* hybridization signals were only observed in the epithelial cells of the acini structures in all of the salivary gland biopsies analyzed. In all cases, the majority of the acini structures presented at least one epithelial cell with hybridization signals. The intracellular location of the hybridization signals was restricted to the cytoplasm of the epithelial cells with no nuclear staining (Figure 1).

The mean percentage of HCV-infected epithelial cells was 37.1 ± 8.1 (range, 25 to 48.8%) in the patients studied.

With respect to the HCV replication in epithelial cells from the acini, hybridization signals were observed with

Figure 1. *In situ* hybridization in salivary gland biopsies from an anti-HCV-positive (a) and -negative (b) patients. Original magnification, $\times 400$. Intracellular localization of the hybridization signal (c). Original magnification, $\times 1,000$. Counterstained with safranin.

both positive and negative polarity probes in all of the HCV-positive patients. Furthermore, the majority of the positive cells observed when the HCV probe of positive polarity was used showed hybridization signals using the HCV probe of negative polarity, demonstrating the viral replication in these cells (Figure 3).

No statistical correlation was found between the serum levels of HCV RNA and the percentage of cells showing hybridization signals ($r = 0.14$; $P = 0.70$).

Figure 2. *In situ* hybridization of serial sections of the same salivary gland biopsy with (a) and without (b) predigestion with RNase and hybridized in the presence (c) or in the absence (d) of an excess of unlabeled probe. Original magnification, $\times 1,000$. Counterstained with safranin.

Dot Blot Hybridization

A dot blot hybridization was performed to rule out the possibility of unspecific hybridization between the sense probe and the HCV-RNA of genomic polarity and the antisense probe and the HCV-RNA of antigenomic polarity. As shown in Figure 4, no unspecific hybridization was detected.

Immunohistochemical Detection of HCV Core Protein in Salivary Gland Biopsies

HCV core antigen was detected by immunohistochemistry in the cytoplasm of the epithelial salivary gland cells from the anti-HCV-positive patients in a pattern similar to that observed for the HCV-RNA by *in situ* hybridization. The specificity of the detection was confirmed by the lack of signals in the salivary gland biopsies from the anti-HCV-negative patients (Figure 5).

Discussion

Chronic HCV infection is not only associated with the liver disease but also with the appearance of several extrahepatic manifestations.²¹ Among these manifestations, HCV infection has been linked to the Sjögren's syndrome and lymphocytic sialadenitis (Sjögren's-like syndrome).¹⁵⁻¹⁷

Primary Sjögren's syndrome is a systemic autoimmune disease of unknown etiology characterized by a lymphocytic infiltration of lachrymal and salivary glands.¹² Early studies, using first generation enzyme-linked immunosorbent assay tests to detect anti-HCV antibodies, showed that anti-HCV was more prevalent in patients with Sjögren's syndrome than in the general population.²² More recent works using second generation enzyme-linked immunosorbent assay tests and PCR have shown that the prevalence of HCV infection among Sjögren's syndrome patients may vary between 0 to 40%, mostly

Figure 3. *In situ* detection of HCV-RNA of sense (a) and antisense (b) polarity. Original magnification, X400. Counterstained with safranin.

depending on the geographical area and the criteria to classify the patients.^{15,16,23,24} On the other hand, lymphocytic sialadenitis (Sjögren's-like syndrome) is a common feature in chronic HCV infection patients.¹⁷

Furthermore, the hypothesis that HCV is involved in these diseases is reinforced by the finding of HCV RNA detected by RT-PCR in saliva and in total RNA isolated from salivary glands.^{18,19} However, because of the high

Figure 5. Immunological detection of HCV core antigen in salivary gland biopsies from a HCV-RNA-positive (a) and -negative (b) patients. Arrows show the positive acini for the HCV core protein.

sensitivity of RT-PCR, it cannot be ruled out that the HCV RNA detected came from blood or serum contaminating these samples which would thus lead to a misinterpretation of the results. Thus, the need for morphological evidence of HCV infection of cells from the salivary glands to support a role for HCV in these diseases led us to use *in situ* hybridization and immunohistochemistry to analyze the presence and replication of HCV in salivary gland biopsies from anti-HCV-positive and -negative patients with Sjögren's syndrome or lymphocytic sialadenitis.

With respect to the detection of HCV RNA in salivary glands, we have found positive hybridization signals in the biopsies from all anti-HCV-positive patients but in none of the anti-HCV-negative cases. Furthermore, almost every acini examined in each biopsy showed at least one positive cell. The specificity of the *in situ* hybridization was demonstrated by the fact that no hybridization signals were detected in the salivary gland biopsies from the anti-HCV-negative patients and by the results obtained in the specificity experiments.

The finding of hybridization signals in the salivary glands of the patients with chronic HCV infection is in agreement with previous reports on the detection of HCV RNA by RT-PCR in this tissue,^{18,19} but it contrasts with the results reported by Taliani and colleagues²⁵ who did not

Figure 4. Dot blot hybridization of serial dilutions of synthetic HCV-RNA of genomic and antigenomic polarity with the HCV antisense (a) and sense (b) probes.

detect HCV RNA in epithelial cells isolated from salivary glands from patients with chronic HCV infection. However, it is important to note that none of the patients studied by Taliani and co-workers had sialadenitis whereas all of our patients had xerostomia and had a histologically demonstrated sialadenitis, which may explain the discrepancy in the results.

Regarding the cell types infected by the HCV, the hybridization signals were detected exclusively in the cytoplasm of the epithelial cells from the acini. The percentage of cells showing hybridization signals ranged from 25 to 44%. However, we found no correlation between the percentage of salivary gland epithelial cells showing hybridization signals and the serum HCV RNA concentration. This finding suggests that the HCV infection in salivary glands does not depend on the serum viremia levels.

On the other hand, positive hybridization signals were detected with probes of both sense and anti-sense polarity. Furthermore, by analyzing serial sections from the same biopsies we have demonstrated that the positive signals obtained with both probes were in the same cells, demonstrating that HCV replicates in epithelial cells from the salivary glands. It would be useful to semiquantitate the HCV-RNA of genomic and antigenomic polarity. However, in the present work it has not been possible to semiquantitate the intensity of the hybridization signals by digital image analysis because of technical reasons. Furthermore, as we do not have frozen salivary gland biopsies, we could not perform this analysis by semiquantitative PCR.

It may be argued that the detection of HCV-RNA of antisense polarity was because of the unspecific hybridization of the sense probe with the genomic HCV-RNA. However, the results obtained in the dot blot hybridization using synthetic target RNAs of both polarities showed that there was no unspecific hybridization, at least in the conditions used in this report. Furthermore, the detection by the immunohistochemistry of HCV core protein in salivary gland cells as well as the detection of HCV-RNA in the saliva samples from anti-HCV-positive patients support the notion that HCV replicates in salivary gland cells.

Finally, in relation with the possible pathogenic role of the HCV in Sjögren's syndrome or in chronic sialadenitis, we have not found any differences in the lesions between patients with and without HCV in the salivary glands. Furthermore, the HCV-infected cells did not show any difference with respect to the uninfected cells. All these findings suggest that HCV infection of salivary glands is not necessary or sufficient to cause these diseases.

In conclusion, in this report we have demonstrated that HCV infects and replicates in epithelial cells from salivary glands of patients with Sjögren's syndrome or chronic sialadenitis. However, its implication in the pathogenesis of these diseases deserves future research.

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