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Short communication

Hepatitis C virus RNA localization in human carotid plaques

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ABSTRACT

Background: Hepatitis C virus (HCV) infection has certain characteristics that enable it to play an important role in atherosclerosis. Some studies report its association with an increased risk of carotid artery plaque.
Objectives: The aim of this study was to evaluate the presence of HCV genomic sequences and replicative intermediates in plaque tissues.
Study Design: A cohort of consecutive, prospectively recruited patients with HCV infection and chronic ischemic heart disease from the Cardiology, Vascular Surgery and Hepatology Units of a University Hospital in Florence, Italy, were studied.
Results: Positive-strand HCV RNA was detected in seven carotid plaque tissues from anti-HCV-positive patients and was not detected in the nine carotid plaque tissues obtained from anti-HCV-negative patients. In three patients, HCV RNA was found in carotid plaque and not in serum. HCV replicative intermediates were detected in three plaque samples. Direct sequencing of HCV RNA from the plaque and serum showed HCV genotypes 2 (five cases) and 1 (two cases).
Conclusions: The novel finding of HCV RNA sequences in plaque tissue strongly suggests an active local infection. This in turn makes it conceivable that the virus may exert local action in carotid atherosclerosis.

1. Background

After the first report of an association of viral infection and atherosclerosis in 1970,[1] evidence is accumulating that infections are involved in the development and progression of atherosclerosis, with special focus on carotid and coronary lesions.[2,3] The pathogenetic mechanisms may vary and are still poorly understood. Localization of the infective agent inside the plaque has been shown for most pathogens for whom a deeper evidence of such an association exists, suggesting the importance of local pathogenetic mechanism/s.[4–7] Available data suggest that the infection burden for atherosclerosis development and progression is mainly sustained by those infectious agents which possess particular tropism for cells of the vascular wall.[8] *Chlamydia pneumoniae* is capable of infecting vascular endothelial cells and smooth muscle cells, whereas cytomegalovirus and herpes virus foster the recruitment of monocyte/macrophages and T cells for atherosclerotic lesions.[9]

Hepatitis C virus (HCV) is responsible for both hepatic and extrahepatic diseases, due to the possibility of infecting not only hepatic, but also extrahepatic cells and the capability of persisting in the host.[10,11] Recently, the possibility of viral persistence in extrahepatic reservoirs after apparent HCV eradication has also been shown.[12–14]

Data supporting a possible role of HCV in atherosclerosis have been reported.[2,10,15–18] In particular, large cross-sectional studies in the general Japanese population showed an association between HCV infection and increased risk of carotid artery plaques.[19,20] More limited data are also available from European studies.[21,22] In a preliminary study, we found that the prevalence of intima–media thickening (>1 mm) in carotid arteries was significantly higher ($P < 0.001$) in 31 non-cirrhotic HCV-positive patients than in 120 age-matched HCV-negative controls, thus confirming previous observations. Using multivariate regression analysis, HCV infection remained an independent risk factor for carotid atherosclerosis.[23] HCV RNA was found in the plaque tissue from two patients.

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2. Objective

Based on these observations, the aim of the study was to prospectively evaluate the possibility of the presence of HCV infection in carotid plaque tissue.

3. Study design

3.1. Study population

Plaque and serum samples were obtained from 10 consecutive anti-HCV-positive patients with chronic ischemic heart disease and hemodynamic carotid stenosis who underwent percutaneous transluminal carotid angioplasty (Table 1) and from 9 age/sex matched anti-HCV-negative (control) patients. All patients had >80% carotid stenosis grade and were identified as candidates for carotid endoarterectomy according to the routine medical practices of the vascular Surgery Department.

The main demographic, clinical and virological characteristics of the patients are presented in Table 1. In most cases, patients were unaware of their anti-HCV positivity before the present study; in only three cases had this condition already been diagnosed 15, 8 and 11 years, before the study, respectively. The source of infection was in all cases unclear, but possibly related to uncontrolled medical or aesthetic procedures (i.e., dental care, colonoscopy, shaving with shared razors).

3.2. Experimental procedures

Plaque tissue samples were carefully washed in saline solution before being aliquoted and subjected to total RNA extraction. RNA isolation from plaque tissue was performed by Trizol reagent (Invitrogen Carlsbad, CA, USA) following the manufacturer's instructions. One microgram of total RNA was retro-transcribed in the presence of antisense primers for HCV 5′ non-coding region (5′NCR) and human GAPDH gene. HCV RNA sequences were detected by home-made qualitative nested RT-PCR (sensitivity: 1–5 HCV IU/mL)[24,25]; uracyl-N-glycosilase (1 U/sample) was added to the reaction mixture to avoid carryover of PCR products. GAPDH gene was amplified with sense (ACCACAGTCCATGCCATCAC) and antisense (TCCACCACCCTGTTGCTGTA) primers using the following protocol: denaturation step of 4 min at 95 °C followed by 30 cycles at 95 °C for 20 s, 55 °C for 25 s and 72 °C for 25 s.

Detection of the 5′ untranslated region (5′ UTR) HCV RNA-negative strands was performed by using a well standardized protocol based on cDNA synthesis at high temperature with the thermostable polymerase Tth with reverse transcriptase properties.[14,26] Both in case of positive- and negative-strand HCV RNA testing, several stringent controls of PCR specificity were systematically used to avoid the risk of contamination by amplification products from different tests, as previously described.[24] In addition, samples were blindly tested by three different laboratory technicians. Results were admitted when confirmed in at least two different experiments.

HCV RNA serum titers were evaluated by Real Time PCR (Cobas Taqman HCV, Roche Diagnostics GmbH, Mannheim, Germany).

The HCV genotype was determined by direct sequencing of PCR amplicons of the 5′NCR and core gene in plaque tissues and serum samples.[27,28] In serum samples, HCV genotyping was also performed using the Line Probe assay (INNO LiPA, Innogenetics, Gent, Belgium).

4. Results

HCV RNA sequences were detected in five serum samples (pts. 2, 6, 7, 9 and 10) and seven plaque tissue samples (pts. 3, 4, 5, 6, 7, 9 and

Table 1
Demographic, clinical and virological characteristics of 10 anti-HCV-positive patients from this study.

Patient no.	Age (years)	Gender (M/F)	CRP (mg/dL)	VES (mm/h)	ALT/AST (U/L)	Bilirubin (mg/dL)	Platelets (/mm^3)	Albumin (%)	Hyper tension	BMI	Smoking	Hyper cholesterolemia	Hyper triglyceridemia	Diabetes	Family history[a]	HCV genotype	HCV RNA		
																	Serum	Plaque	
																		Positive strand	Negative strand
1	74	M	1.9	14	11/26	1.1	247000	62.6	No	26.7	No	Yes	Yes	No	Yes	N.a.	Neg	Neg	Neg
2	78	M	0.5	N.d.	N.d.	N.d.	N.d.	N.d.	Yes	21	Pr.	No	N.d.	No	No	N.d.	Pos	Neg	Neg
3	84	F	3.2	23	6/11	0.50	317000	59.5	Yes	21.5	Yes	Yes	Yes	No	Yes	2	Neg	Pos	Neg
4	79	M	2.5	N.d.	9/19	0.77	264000	N.d.	Yes	N.d.	Yes	Yes	N.d.	No	Yes	2	Neg	Pos	Neg
5	80	M	3	30	19/19	0.59	226000	57.5	Yes	26.1	Pr.	No	No	No	No	2	Neg	Pos	Neg
6	68	M	4.5	N.d.	N.d.	N.d.	N.d.	N.d.	Yes	24.8	Yes	Yes	N.d.	No	N.d.	1b	Pos	Pos	Neg
7	79	M	1.6	53	30/30	0.52	247000	53.6	Yes	N.d.	Pr.	No	No	No	No	2	Pos	Pos	Pos
8	73	M	2.8	13	96/53	0.86	150000	55.7	No	N.d.	Pr.	No	No	No	Yes	N.a.	Neg	Neg	Neg
9	84	F	N.d.	25	45/44	0.30	158000	56.8	Yes	18.8	No	No	No	Yes	Yes	1b	Pos	Pos	Pos
10	73	M	3.1	N.d.	20/26	0.90	132000	61	Yes	30	Pr.	Yes	No	Yes	Yes	2	Pos	Pos	Neg

CRP: C reactive protein; ALT: alanine aminotransferase; normal value: 40 U/L; AST: aspartate aminotransferase; normal value: 40 U/L; Smoking: smoking habit; Pos: positive; Neg: negative; Pr.: previous; N.d.: not done; N.a.: not applicable.

[a] Family history of cardiovascular disease.

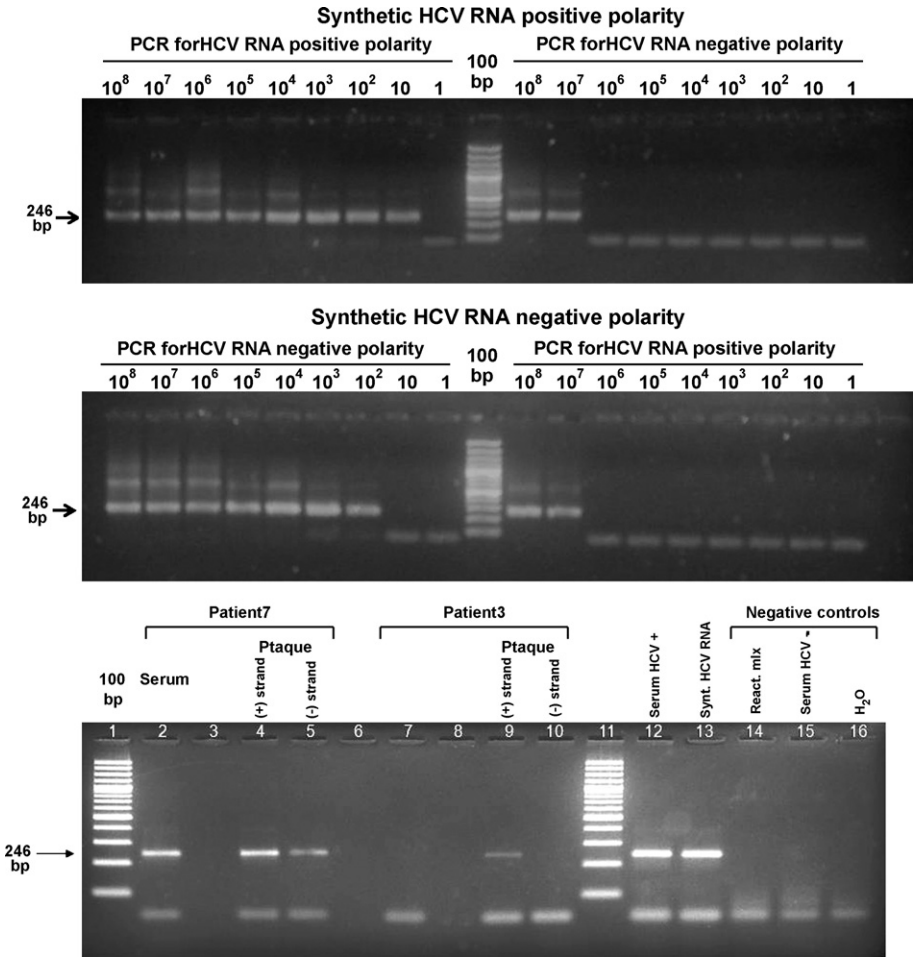

Fig. 1. (Panel A) Analysis of sensitivity and specificity of HCV strand-specific nested RT-PCR. Ten-fold dilutions (starting from 10^8 to 1 genomic equivalent/reaction – gen. eq.) of positive-strand (upper panel) and negative-strand (lower panel) synthetic HCV RNA were reverse transcribed with Tth polymerase in the presence of sense (for negative-strand detection) and antisense (for positive-strand detection) primers and then subjected to PCR amplification. The detection limit for positive-strand was 10 gen. eq./reaction, while for negative-strand was 10^2 gen. eq./reaction. The specificity of negative-strand detection was maintained in the presence of an excess of 10^6 gen. eq. of positive-strand HCV RNA. 100 bp: 100 base-pair DNA molecular weight marker. (Panel B) HCV RNA detection in serum and plaque tissue by nested RT-PCR. Lanes 1 and 11: 100 bp DNA molecular weight marker; lanes 2–10: PCR results from serum and plaque samples from two representative patients (from Table 1): patient #7 (lanes 2–5) showing HCV RNA sequences in both serum and plaque; patient #3 (lanes 7–10) in plaque tissue only. Lanes 12 and 13 (positive controls): serum sample from an HCV-positive patient and synthetic HCV RNA, respectively. Lanes 14–16 (negative controls): reaction mixture without cDNA, serum sample from an HCV-negative patient, distilled water instead of cDNA, respectively.

10) from anti-HCV-positive patients (Table 1 and Fig. 1). The mean viral load (detection of positive-strand HCV RNA) in serum was $7.79 \pm 4.21 \times 10^5$ IU/mL. No HCV RNA sequences were detected in anti-HCV-negative subjects (data not shown). HCV genotype analysis showed five out of seven typeable patients were infected by HCV genotype 2 (pts. 3, 4, 5, 7 and 10) and the remaining 2 by HCV genotype 1 (pts. 6 and 9). For one patient, genotyping techniques could not provide a definitive result (pt. 2, Table 1) probably due to the low HCV RNA concentration in the sample. Negative-strand HCV RNA (replicative intermediate) was detected in two out of seven HCV RNA-positive plaques (pts. 7 and 9; Table 1 and Fig. 1).

5. Discussion

Available data suggest that HCV is an independent risk factor for carotid atherosclerosis, even though the mechanism involved is unknown. The present study, for the first time, demonstrates the presence of HCV genomic sequences and replicative intermediates in plaque tissues, strongly suggesting the possibility of an active infection of the carotid plaque. The detection of HCV sequences in the sole plaque tissue in three patients further supports viral localization rather than contamination by circulating particles.

It is now widely accepted that infective agents contribute to the initiation and progression of the chronic immuno-mediated cell inflammation underlying atherosclerosis, through the inflammatory/immune response elicited in the host.[8] For a long time the prime culprit of atherogenesis was considered the antigen presentation to T lymphocytes of a fragment of macrophage “digested” oxidized low-density lipoproteins. However, infective agents can accelerate the occurrence of several key steps in the plaque formation, since they can promote endothelial dysfunction, potentiate the recruitment and activation of T lympho-monocytes and/or enhance the proliferation and migration of smooth muscle cells from tunica media to intima. Who is the culprit is still under debate.

In a preliminary study, we observed HCV infection was associated with asymptomatic carotid wall thickening in patients with chronic ischemic heart disease.[23] Unspecific markers of inflammation showed similar patterns in HCV-positive and HCV-negative patients, suggesting systemic inflammation did not play a major role in such an association.

The present study shows viral localization in plaques in the majority of HCV-seropositive patients, suggesting HCV could play

a role in carotid atherosclerosis through local action. This hypothesis is supported by several viral characteristics. For example, HCV particles are associated with circulating lipoproteins in the blood. It has been proposed that HCV enters target cells through the LDL receptor and/or the scavenger receptor B1.[15,16] Some HCV proteins can cause oxidative stress with increased local reactive oxygen species,[18] supporting the hypothesis that HCV could potentiate the oxidation of lipoprotein and, consequently, the atherogenetic process.[2] Additional viral characteristics may be involved, including an increased concentration of soluble intercellular adhesion molecules,[17] the appearance of anti-endothelial antibodies and the close association with vasculitis.[10] Concerning the distribution of HCV genotypes, it must be noted that, in our geographical area, the approximate prevalences for genotype 1 and 2 are 66 to >76% and 15 to 20%, respectively.[29] The predominance of HCV genotype 2 we observed in atherosclerotic patients is potentially interesting, as this genotype seems to be more closely associated with lipoproteins than other genotypes.[30] However, further studies involving larger populations are needed to confirm this hypothesis. The present study does not provide information about the mechanisms involved in the infection of plaque tissue. However, the prerogative of such a virus to be carried in serum by lipoproteins[15] as well as to infect peripheral blood mononuclear cells (PBMC)[31,32] could play a role. In fact, it is highly probable that infected cells could vehiculate the passage of HCV into the plaque. Specifically dedicated studies are ongoing in our laboratory to answer this question.

It is also noteworthy that persisting PBMC infection, even in serum HCV RNA-negative patients, has been previously demonstrated.[12–14] Accordingly, the detection of viral RNA in the plaque tissue of patients in the absence of detectable viremia (pts. 3–5; Table 1) points out the possibility of a compartmentalization of the virus in this district with pathogenetic consequences.

A correlation between viral load and the severity of atherosclerosis cannot be excluded. Further specifically addressed studies will clarify this interesting issue.

In conclusion, this study strongly suggests that HCV infection may be localized in plaque tissue. This in turn suggests a role of HCV in carotid atherogenesis. The clinical consequences of such a finding are potentially numerous and include the improvement of risk profiling and the assessment of new treatment strategies in the future.

Competing interests

None declared.

Ethical approval

Not required.

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