Detection of *Helicobacter pylori* in Human Carotid Atherosclerotic Plaques

Sebastián F. Ameriso, MD; Esteban A. Fridman, MD; Ramón C. Leiguarda, MD; Gustavo E. Sevlever, MD

**Background and Purpose**—Several lines of evidence point toward a relationship between infection and atherosclerotic vascular disease. Thus, infection and inflammation often precede ischemic neurological events. Transient alterations in coagulation and direct arterial invasion by certain microorganisms have been reported. *Helicobacter pylori* infection is the major cause of peptic ulcer disease and appears to be a risk factor for ischemic cerebrovascular disease. However, in contrast to other chronic infectious agents, *H pylori* has not been consistently isolated from atherosclerotic lesions.

**Methods**—We investigated the presence of *H pylori* in 38 atherosclerotic plaques obtained at carotid endarterectomy by using morphological and immunohistochemical techniques and a highly sensitive polymerase chain reaction method. We performed immunohistochemical detection of intercellular adhesion molecule-1, a marker related to inflammatory cell response. We also examined 7 carotid arteries obtained at autopsy from subjects without carotid atherosclerosis.

**Results**—*H pylori* DNA was found in 20 of 38 atherosclerotic plaques. Ten of the *H pylori* DNA–positive plaques also showed morphological and immunohistochemical evidence of *H pylori* infection. None of 7 normal carotid arteries was positive for *H pylori*. Intercellular adhesion molecule-1 was expressed in 75% of *H pylori*–positive plaques and in 22% of *H pylori*–negative plaques. The presence of the microorganism was associated with male sex but was independent of age, vascular risk factor profile, and prior neurological symptoms.

**Conclusions**—*H pylori* is present in a substantial number of carotid atherosclerotic lesions and is associated with features of inflammatory cell response. This study provides additional evidence of the relationship between *H pylori* infection and atherosclerotic disease. (*Stroke. 2001;32:385-391.)

**Key Words:** atherosclerosis ■ carotid artery diseases ■ *Helicobacter pylori* ■ infection ■ intercellular adhesion molecule-1

In recent years, numerous investigations have established an association between infectious and inflammatory processes and the occurrence of vascular disease.1-8 Expression of adhesion molecules in the endothelium and smooth muscle is a key component of the inflammatory response in atherosclerotic lesions.9 Increased endothelial inflammatory activity reflected by enhanced expression of intercellular adhesion molecule-1 (ICAM-1) and tumor necrosis factor-α is common in human carotid atherosclerotic plaques, and its role in the occurrence of neurological symptoms is under active research.9-12 Infectious processes may act through both systemic effects and direct arterial invasion.1 Infection by *Chlamydia pneumoniae*, cytomegalovirus, *Helicobacter pylori*, herpes simplex virus types 1 and 2, and hepatitis A virus appear to be more prevalent in individuals with systemic atherosclerosis.13-21 Acute infection frequently precedes myocardial and cerebral infarction, suggesting a role as an independent risk factor that may trigger the ischemic event.22-28 It is not unlikely that transient alterations in coagulation pathways and other as yet poorly understood processes mediate the association between infection and the onset of acute vascular episodes.26,28

Chronic infection may be implicated in the pathophysiological cascade leading to atherosclerosis.1,29 A direct role of microorganisms in the development of atherosclerosis is suggested by the presence of *C pneumoniae*, cytomegalovirus, and herpes simplex virus in diseased vessels.30-33 These agents can be found in atherosclerotic lesions of coronary and carotid arteries but appear to be absent in normal vessels. The role of these microorganisms in the initiation and progression of atherosclerosis and potential therapeutic implications are under investigation.

The Gram-negative curved bacillus *H pylori* colonizes the gastrointestinal system in approximately half of all adults and is associated with chronic gastritis, peptic ulcer disease, and...
gastrointestinal cancer. The relationship between this microorganism and other disorders is currently being debated. Several reports have implicated H. pylori infection in coronary artery disease, especially when more virulent strains are involved (ie, the Cag A strain). Seropositivity for H. pylori has been postulated to be an independent risk factor for ischemic stroke. Several potential mechanisms for the association are under research. Although serological evidence relates H. pylori infection with atherosclerotic disease, the bacterium has not yet been isolated from atherosclerotic lesions except for a single study published recently in abstract form.

The objective of the present study was to search for the presence of H. pylori in atherosclerotic plaques from patients undergoing carotid endarterectomy by use of immunohistochemical methods and a sensitive polymerase chain reaction (PCR) technique.

Subjects and Methods

The investigation was approved by the institutional ethics committee. We searched for the presence of H. pylori in 38 consecutive carotid artery specimens obtained during endarterectomy in patients with atherosclerotic lesions producing severe stenosis of the vessel. A detailed medical history was obtained for every subject; this was followed by physical and neurological examinations. Patients who had experienced a transient ischemic attack or stroke in the territory of the operated artery within the last 180 days were considered symptomatic. We also examined 7 carotid arteries of autopsy material from subjects without carotid atherosclerosis.

Specimens were fixed in 10% neutral buffered formalin and subsequently decalcified in formic acid when required. All samples were routinely processed, paraffin-embedded, and cut serially to expose coronal planes of the carotid artery and atheromatous plaque. Several sections were prepared from each specimen for the following: hematoxylin and eosin, elastica van Gieson, PAS, Giemsa, and immunohistochemical staining. Morphological evaluation included the investigation of inflammatory mononuclear cells and the identification of bacteria in the luminal or parietal area. The following antibodies and dilutions were used for immunohistochemistry: factor VIII–von Willebrand at 1:100 (polyclonal rabbit antibody, Dako Corp), CD31 at 1:50 (PECAM, clone 1A10, Novocastra), CD54 at 1:50 (ICAM-1, Dako Corp), CD34 at 1:100 (clone QBEnd/10 at 1:100, BioGenex), and H. pylori at 1:100 (polyclonal rabbit antibody NCL–HpP, Novocastra). After deparaffinization, sections were microwaved in 10 mmol/L sodium citrate buffer at pH 6.0 for 10 minutes and incubated with the antibodies. Sections incubated with normal mouse or rabbit IgG at the same dilutions served as negative controls. As a second step, biotinylated horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories Inc) was applied and detected by use of the ABC Elite kit (Vector Laboratories Inc) with diaminobenzidine as substrate. Formalin-fixed paraffin-embedded gastric biopsies with well-characterized H. pylori gastritis were used as positive controls. Endothelial preservation was assessed by immunohistochemical detection (avidin–biotin method) for CD34, CD31, and factor VIII–von Willebrand factor. Immunodetection for ICAM-1 was evaluated in cases disclosing endothelial markers.

High molecular weight DNA was isolated from formalin-fixed paraffin-embedded tissue according to Wright and Manos. A PCR technique reported by Lu et al was followed. Briefly, the PCR reaction used a set of primers that amplified the gluM gene between positions 784 and 1077, rendering a 294-bp amplification product. H. pylori DNA was amplified in a 50 μL reaction mixture containing 10 mmol/L Tris-HCl buffer, pH 8.4, 50 mmol/L KCl, 1.5 mmol/L MgCl2, 2.5 mmol/L of each deoxynucleoside triphosphate (Pharmacia/LKB), 1 mmol/L of each primer, and 2.5 U of Taq DNA polymerase (GIBCO-BRL). Reaction tubes were placed in a thermal cycler (PTC-200, MJ Research). Initial denaturing was carried out at 95°C for 5 minutes, followed by 35 cycles of amplification consisting of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute, and a final extension cycle of 72°C for 7 minutes. In a separate reaction tube, a second set of primers for the β-globin gene was incubated with the DNA template and served as a control to monitor the amplification ability of a single copy gene. PCR-amplified DNA was subjected to electrophoresis on a 2% agarose gel containing ethidium bromide. Samples from microbiological cultures were used as positive controls (Figure 1, lane 11).

We performed direct DNA sequencing of PCR products in 5 selected cases to confirm the bacterial origin of H. pylori DNA. PCR products were electrophoresed through 2% low-melting-point agarose gels, and expected fragments were localized and excised. DNA was extracted from melted gel slices by using a Wizard PCR Prep kit (Promega) according to the manufacturer’s instructions. The purified DNA was then sequenced directly by use of the ABI 373A DNA Sequencer with an ABI Taq Dye-Deoxy-Terminator Cycle Sequencing Kit (Perkin-Elmer Corp, Applied Biosystems Division). Signals were recorded and then analyzed by use of a Macintosh Quadra 650.

Statistical Methods

Mean and standard deviations for continuous variables and frequency for dichotomous variables were calculated. The Fisher exact probability test (2-tailed) or χ2 test was used to examine univariate association of categorical variables with H. pylori DNA presence and ICAM-1 expression (SAS System Software, version 6.12). The Cox logistic regression model was used for multivariate analysis (BMDP Statistical Software, version 7.0).

Results

We studied consecutive patients referred to our institution for carotid endarterectomy. We examined 38 atherosclerotic plaques from 38 subjects (29 men and 9 women) aged 67 ± 9 (mean ± SD) years. Hypertension was present in 25 patients; diabetes mellitus, in 10; smoking, in 14; hypercholesterolemia, in 22; and ischemic heart disease, in 13. None of the subjects had a history of chronic gastritis, peptic ulcer disease, or gastric cancer. Fifteen subjects had sustained symptoms consistent with ischemia in the territory of the operated artery (symptomatic patients). Demographic data for the patient population are provided in the Table. Sex, age, and vascular risk factor profile were similar for symptomatic and asymptomatic subjects.

H. pylori DNA was found in atherosclerotic plaques of 20 patients (53%) (Figure 1). Slender, curved, spiral microorganisms were detected on the endothelial surface as well as in subendothelial clefts in 10 of 20 H. pylori DNA–positive cases. Subendothelial inflammatory mononuclear cells were observed in these cases. The microorganisms were positively
identified as \textit{H pylori} by specific immunostaining (Figure 2).

None of the 7 carotid arteries without atherosclerotic lesions disclosed the presence of \textit{H pylori} by either PCR or immunostaining.

Nineteen of 38 atherosclerotic plaques (50\%) and none of 7 normal carotid arteries expressed ICAM-1 (Figure 3). Immunohistochemical evidence of ICAM-1 was associated with \textit{H pylori} DNA detection; ICAM-1 expression was present in 15 of 20 patients (75\%) with \textit{H pylori} DNA and in 4 of 18 patients (22\%) without \textit{H pylori} DNA ($P<0.01$).

Demographic and clinical features and ICAM-1 expression pattern in patients with \textit{H pylori}-positive and -negative plaques are depicted in the Table. No differences could be detected in age, vascular risk factor profile, or the presence of neurological symptoms between patients with or without \textit{H pylori} DNA or ICAM-1 expression in carotid plaques.

Multivariate logistic regression analysis demonstrated that \textit{H pylori} DNA detection was associated with the immunohistochemical presence of ICAM-1 (odds ratio 29.3, 95\% CI 2.84 to 302.00; $P<0.01$) and male sex (odds ratio 23.0, 95\% CI 1.58 to 333.00; $P<0.01$).

On the basis of DNA availability, 5 endarterectomy samples were selected for sequencing analysis. The 270-bp sequenced fragment showed no point mutation compared with the \textit{H pylori} strain J99 used as a control, demonstrating that the PCR 294-bp amplified fragment was specific for \textit{H pylori}.

\textbf{Discussion}

We found \textit{H pylori} DNA in 53\% of carotid atherosclerotic plaques. In half of these cases, the microorganism was also visualized in the lesion by use of specific immunostaining. \textit{H pylori} was not found in carotid arteries without atherosclerosis. ICAM-1 was expressed more often in plaques with \textit{H pylori} DNA. The presence of the bacillus was associated with male sex but not with age, prior neurological symptoms, or risk factor profile.

Our findings disagree with most prior work that failed to find \textit{H pylori} in vascular lesions. Malnick et al\textsuperscript{48} detected no \textit{H pylori} in carotid endarterectomy samples from 10 male patients. Blasi and colleagues\textsuperscript{49,50} examined material from

\begin{figure}
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\includegraphics[width=0.5\textwidth]{figure2}
\caption{Carotid atherosclerotic plaques (immunostaining for \textit{H pylori}, original magnification $\times 1000$). A, Immunodetection of the bacillus in subendothelial clefts. B, Immunodetection of the bacillus in the endothelial lumina.}
\end{figure}
surgical specimens of patients with aortic abdominal aneurysms but found no evidence of *H pylori* infection. We used a PCR protocol that amplified the gluM (ureC) gene, proven to be the most sensitive and specific gene for the detection of *H pylori* in gastric biopsies, compared with protocols that amplify other genes, such as 16S ribosomal RNA, the 26-kDa species-specific antigen gene, the ureA gene, and the random chromosome sequence.53 The sensitivity of this PCR method was assessed by 10-fold serial dilutions of 10 ng to 1 pg purified *H pylori* DNA. It detected up to 0.1 pg DNA corresponding to ϳ50 microorganisms. Malnick et al48 used the 26-kDa species-specific antigen reported to have poor sensitivity; Blasi and colleagues49,50 used the urease gene, which, in the same comparative study, was described to provide low sensitivity, which was probably due to sequence polymorphism. Using the urease A gene method, Akyön et al51 recently reported PCR detection of *H pylori* DNA in 19.5% of atherosclerotic plaques.

Our findings met the criteria proposed for diagnosis of *H pylori* infection in gastroduodenal diseases with specificities and predictive values for negative results >90%.54 Direct DNA sequencing of PCR products confirmed that the PCR 294-bp amplified fragment was specific for *H pylori*.

The present study did not establish the mechanism(s) by which *H pylori* colonizes the carotid lesions. Also, we did not determine whether the bacillus is transiently or permanently present at this site. Although none of the subjects had a diagnosis of chronic gastritis, peptic ulcer disease, or gastric cancer, we cannot completely rule out this possibility. Thus, the presence of *H pylori* could represent bacteremic seeding from a primary location in the gastrointestinal tract.

Infectious processes appear to be implicated in the occurrence of cerebrovascular disease.1,4,14,25–28,41 The exact nature of the association is not completely elucidated, and at least 3 different scenarios should be considered. First, acute infection may precipitate ischemic events, especially in subjects with vascular risk factors. This effect has been attributed, at least in part, to the transient imbalance of the coagulation pathway toward a prothrombotic status, and other putative mechanisms are under study.26–28 Second, chronic infection may be responsible for an increase in the atherosclerotic “load.” Numerous studies have reported an increased frequency of serological evidence of chronic infection in patients with cerebrovascular disease.4,14,41 These findings suggest that the presence of microorganisms in sites remote from the cerebral arteries may produce systemic alterations predisposing to the development or complication of atherosclerotic disease in cerebral vessels.1 Third, some microorganisms may participate in the atherosclerotic process by their actual presence on the vessel wall. Published studies have established that *C pneumoniae*, cytomegalovirus, and herpes simplex can be found in atherosclerotic lesions.30–33 The infectious process within the vessel wall may be responsible for the initiation, progression, and/or complication of the atherosclerotic plaque.1–3

Our findings support the last hypothesis and contribute to available evidence by demonstrating that *H pylori* may be present in human carotid atherosclerotic plaques. Furthermore, the higher frequency of ICAM-1 expression in *H pylori*-positive plaques suggests an association between

### Data for Patients With Positive and Negative *H pylori* DNA in Carotid Plaques

<table>
<thead>
<tr>
<th></th>
<th>Positive <em>H pylori</em> DNA</th>
<th>Negative <em>H pylori</em> DNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patients, N</td>
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<td>18</td>
</tr>
<tr>
<td>Males/females, n/n</td>
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<td>11/7</td>
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<td>Age (mean±SD), y</td>
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<td>Hypertension, n</td>
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<td>5</td>
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<tr>
<td>Ischemic heart disease, n</td>
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<td>6</td>
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<tr>
<td>Symptomatic/asymptomatic, n/n</td>
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<td>6/12</td>
</tr>
<tr>
<td>Endothelial expression of ICAM-1, n</td>
<td>15</td>
<td>4*</td>
</tr>
</tbody>
</table>

*P<0.01 Fisher exact probability test (2-tailed).
the presence of the bacillus and vessel inflammation. Interestingly, ICAM-1 is the predominant form among the cell adhesion molecules expressed in response to chronic *H pylori* gastric infection.55

We also found an association between male sex and *H pylori* DNA in carotid atherosclerotic plaques. The prevalence rate of *H pylori* seropositivity is similar in males and females.56,57 However, duodenal ulcer, gastric metaplasia, and stroke occur more often in males than in females,58–60 so that certain host or environmental factors may predispose men to a greater risk of developing gastrointestinal disease and atherosclerosis subsequent to *H pylori* infection.

In conclusion, *H pylori* is present in a substantial number of human carotid atherosclerotic lesions and is especially associated with those with inflammatory features. Although the present study fails to provide proof of a causal relation between *H pylori* infection and atherosclerosis, it adds to prior evidence suggesting a relationship between the bacillus and the pathogenesis of vascular disease. Further research may help to establish the role of *H pylori* infection in the occurrence of cerebrovascular disease and the potential for therapeutic intervention.

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References


References


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