Heme Oxygenase-1 Is Expressed in Carotid Atherosclerotic Plaques Infected by *Helicobacter pylori* and Is More Prevalent in Asymptomatic Subjects

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**Background and Purpose**—It is not well established what are the features, if any, that distinguish symptomatic from asymptomatic carotid atherosclerotic plaques. Inducible heme oxygenase-1 (HO-1) is a component of cellular defense mechanisms against oxidative stress. We aimed to assess the presence of *Helicobacter pylori* (*H pylori*) and the expression of HO-1 in carotid atherosclerotic plaques of patients with and without prior neurologic symptoms attributable to the operated artery.

**Methods**—We examined 25 symptomatic and 23 asymptomatic carotid atherosclerotic plaques removed during endarterectomy and 7 normal carotid arteries obtained at autopsy. We investigated the presence of *H pylori* DNA in the vessel wall and performed immunohistochemical detection of HO-1.

**Results**—*H pylori* DNA was present in 28 plaques and HO-1 was expressed in 30 plaques. HO-1 was found in 27 *H pylori*-positive specimens but in only 3 *H pylori*-negative specimens (*P*<0.001). All 7 normal carotid arteries were negative for both *H pylori* and HO-1. Although 82% of asymptomatic specimens were positive for *H pylori* and 87% for HO-1, only 36% of symptomatic specimens were positive for both *H pylori* and HO-1 (*P*<0.01).

**Conclusions**—This study suggests a strong association between *H pylori* infection and expression of HO-1 in carotid atherosclerotic plaques. There was a substantial prevalence of these features in specimens obtained from asymptomatic subjects. *(Stroke. 2005;36:000-000.)*

**Key Words:** atherosclerosis ■ carotid arteries ■ infection ■ inflammation ■ stroke

The prognosis of equally severe symptomatic and asymptomatic carotid stenoses is quite dissimilar, suggesting differences in plaque composition.1 Nevertheless, studies to find triggers of plaque rupture in carotid arteries have been largely unsuccessful. It is as yet unclear how a stable asymptomatic plaque becomes an unstable symptomatic lesion. Most prior research has attempted to establish characteristics present in symptomatic plaques that may influence their behavior. Surface ulceration, plaque rupture, fibrous cap thinning, cap infiltration by large numbers of macrophages and T cells, and smooth muscle cell (SMC) apoptosis are more often found in specimens from symptomatic patients.1 High levels of the proteolytic enzymes matrix metalloproteinases have been demonstrated at the site of the inflammatory infiltrate in the fibrous cap of symptomatic patients.2 One report documented increased intracellular adhesion molecule-1 (ICAM-1) expression in the stenotic region of symptomatic plaques.3 However, other investigators were unable to reproduce these findings.4 Conversely, whether there are systemic or local factors that may promote plaque stabilization and maintain an asymptomatic condition is so far uncertain.

Several investigations have established an association between systemic infections and occurrence of coronary disease and stroke.5-9 Infectious agents have also been found in atherosclerotic plaques in diverse territories, including carotid arteries.10,11 However, the clinical significance of microorganisms resident within the arterial wall has not been entirely elucidated. It is possible that the effects of local infection are quite dissimilar to those of systemic infections. Although infectious agents have been associated to the initiation and progression of the atherosclerotic process, there is scant data regarding the relationship between the presence of microorganisms and occurrence of neurologic events in the territory of an affected cerebral artery. So far, it is unknown whether...
plaque infection leads to changes affecting its susceptibility to rupture and occurrence of symptoms. The presence of *Chlamydia pneumoniae* in carotid atherosclerotic plaques appears to be unrelated to prior symptoms. Furthermore, the existence of microorganisms is not a universal phenomenon, and it is conceivable that they do not participate in the initial steps of the atherosclerotic process but reach the plaque at some point during disease progression.

Seropositivity for *Helicobacter pylori* (*H pylori*) has been postulated to be an independent risk factor for vascular events, including ischemic stroke, especially when the more virulent Cag A strain is found.13–15 *H pylori* can elicit oxidative stress during host colonization in experimental models16 and is one of the microorganisms involved in the concept of “infectious burden.”17 The bacillus is present in a substantial number of carotid atherosclerotic lesions and is associated with features of inflammatory cell response.11,18

Heme oxygenase (HO) is a rate-limiting enzyme that catalyzes the degradation of heme into equimolar amounts of biliverdin, iron, and carbon monoxide (CO).19 These resulting reaction products appear to mediate cytoprotection against several insults related to oxidative stress.20 Three isoforms of HO have been identified: HO-1, HO-2, and HO-3. Only HO-1 is inducible and its synthesis is elicited by a variety of stimuli, including infections, heavy metals, ultraviolet irradiation, fever, inflammatory cytokines, and oxidized low-density lipoprotein (LDL).20,21 HO-1 is postulated to be a component of cellular defense mechanisms against oxidative stress-mediated injury and belongs to a family of cytoprotective genes of the vascular wall.19 Such protective effect of HO-1 can be explained by several mechanisms. HO-catalyzed CO release has potent antiinflammatory and antiapoptotic effects and may promote oxidation of fatty acids through inhibition of cytochrome P-450.20,22 HO-1 induction modulates vascular tone through the production of CO under “stressful” conditions, and this gas plays a major antiproliferative role, mediated by cGMP, inhibiting the synthesis of growth factors from vascular cells.23 Furthermore, CO blocks SMC growth and modulates the expression of platelet-derived growth factor-β.19,20,23 Recent in vitro and in vivo studies have demonstrated that bilirubin exhibits potent antioxidant properties, preventing the oxidative damage triggered by a wide range of oxidant-related stimuli.24 Bilirubin can act as a strong peroxyl radical scavenger and is capable of inhibiting LDL oxidation and monocyte chemotaxis induced by oxidized LDL, and the adhesion of neutrophils elicited by ischemia/reperfusion.22 Ferritin is a cytoprotective molecule with antioxidant effects based on its capacity for free iron chelation.20 It is expressed in atherosclerotic lesions and can be induced by oxidized LDL.25–27 The enzyme may promote reendothelialization of blood vessels at sites of vascular injury through stimulation of endothelial cell proliferation.28 HO-1 deficiency in humans is associated with endothelial damage.29 These potential beneficial effects of HO-1 expression make it a likely candidate to be considered a factor that promotes stabilization of atherosclerotic plaques.

The objective of the present study was to advance further in the investigation of features that may affect clinical stability of carotid plaques. We aimed to assess HO-1 expression in atherosclerotic specimens obtained at carotid endarterectomy from patients with and without prior neurologic events in the operated territory (ie, symptomatic and asymptomatic plaques). We also investigated the relationship among HO-1 expression, detection of *H pylori* in the vessel, and the presence/absence of neurologic symptoms.

**Materials and Methods**

**Subjects**

The investigation was approved by the institutional ethics committee and subjects gave written informed consent. A detailed medical history was obtained for every patient followed by physical and neurologic examinations. Every subject was evaluated before surgery by a neurologist who also reviewed ancillary tests. Percent stenosis was calculated using standardized criteria by duplex ultrasonography, magnetic resonance angiography, and/or digital subtraction angiography. Every subject had stenosis ≥70%. According to NASCET criteria, we considered symptomatic plaques those from patients who had experienced a transient ischemic attack or stroke in the territory of the operated artery within the last 180 days.30 These criteria were chosen because they allow identification of subjects at high risk of future cerebrovascular events secondary to carotid disease, suggesting instability of the lesion.

**Carotid Plaques**

We studied 48 atherosclerotic plaques obtained during carotid endarterectomy and 7 carotid arteries of autopsy material from subjects without carotid athrotherosclerosis. Samples were obtained between March 21, 1997, and December 30, 2002. 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 hematoxylin and eosin, elastica van Gieson, periodic acid-Schiff, Giemsa, and immunohistochemical staining.

**Immunohistochemistry**

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; Novoecastra), and CD34 at 1:100 (clone QBEnd/10 at 1:100; BioGenex). 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. Endothelial preservation was assessed by immunohistochemical detection (avidin–biotin method) for CD34, CD31, and factor VIII–von Willebrand factor.

**H pylori Detection**

High-molecular-weight DNA was isolated from formalin-fixed paraffin-embedded tissue according to standard procedures following a polymerase chain reaction (PCR) technique.31,32 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 min, followed by 35 cycles of amplification consisting of 95°C for 1 min, 55°C for 1 min, 72°C for 1 min, and a final extension cycle of 72°C for 7 min. 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 microbiologic cultures were used as positive controls.

Heme Oxygenase-1 Expression

After paraffin removal and hydration, sections were rinsed in phosphate-buffered saline (PBS) and incubated in blocking buffer (PBS containing 1% bovine serum albumin and 2% normal goat serum) for 1 hour at room temperature. They were then pretreated by microwave heating for antigen retrieval. Slides were incubated with HO-1 monoclonal antibody (StressGen Biotechnologies) used at 1:1,000 dilution in TTBS–1% bovine serum albumin. Incubation was done during 1 hour at room temperature and antibody concentration was 1.2 mg/mL. Slides were washed in PBS and successively exposed to a biotinylated goat antiserum to mouse IgG (Dako) diluted 1:500, and a complex of streptavidin–biotin–alkaline phosphatase (Dako). Localization of phosphatase alkaline was revealed by using Fast Red substrate solution (Dako). Three experiments were performed to assess the specificity of the immunostaining: first, preincubation of the primary antibody with recombinant HO-1 protein in a 1:10 molar ratio; second, by replacement of the primary antibody with a control isotype antibody; and lastly by omitting the primary antibody.33

HO-1 immunocytochemical expression was evaluated under a light microscope and assessed semiquantitatively as −, negative; +, weak; ++, moderate; and ++++, intense as described previously.34

Statistical Methods

Mean and standard deviations for continuous variables and frequency for dichotomous variables were calculated. The Fisher’s exact test (2-tailed) or chi-square test was used to examine association among asymptomatic specimens (58%). HO-1 was found in 27 of 28 H pylori-positive specimens and in 3 of 20 H pylori-negative specimens (P<0.001, chi-squared test). The 7 normal carotid arteries were negative for both HO-1 and H pylori. We found a strong association among asymptomatic plaques, HO-1 expression, and H pylori presence (Figure 2). Although 19 of 23 asymptomatic specimens (83%) were positive for H pylori and 20 of 23 (87%) for HO-1, only 9 of 25 symptomatic plaques (36%) were positive for both H pylori and HO-1 (P<0.01; Fisher exact test, 2-tailed). Interestingly, the patient who was operated on both carotid arteries had H pylori and HO-1 on the asymptomatic plaque alone. Only 1 of 9 symptomatic patients positive for both H pylori and HO-1 had a stroke, whereas the remaining 8 subjects had transient ischemic attacks. The association among H pylori infection, HO-1 expression and absence of prior neurologic symptoms was still significant in a multivariate logistic approach, including age, gender, and vascular risk factors and considering H pylori infection and HO-1 expression as independent factors (P<0.01).

HO-1 expression was weak in 0 plaques, moderate in 8 plaques, and intense in 22 plaques. The results were similar when only the 22 plaques with intense HO-1 expression were analyzed. Twenty-one of these specimens were H pylori-positive, and 17 of them had been obtained from asymptomatic subjects. HO-1 expression was more frequent in specimens from hypercholesterolemic subjects. Of 27 plaques from hypercholesterolemic patients, 20 (74%) were positive for HO-1, whereas 10 of 21 (48%) normocholesterolemic patients had plaques that were positive for HO-1 (P<0.05). However, there was no association between hypercholesterolemia and either H pylori presence or prior symptoms.

Presence of ulceration, thrombosis, and other pathologic features were routinely assessed. There was no correlation between pathologic criteria of plaque stability and H pylori presence or HO-1 expression.

Discussion

This study demonstrated expression of HO-1 in a substantial number of human carotid atherosclerotic plaques. The en-
zyme was more prevalent in specimens obtained from carotid arteries of asymptomatic subjects. Also, most symptomatic plaques with HO-1 expression were from subjects who had sustained transient neurologic symptoms but not fully developed strokes. Prior evidence, together with these results, supports the emerging concept of a possible beneficial vascular role of the enzyme. HO-1 expression has been shown to provide protection against several noxious stimuli, including infections.35 HO-1 in atherosclerotic lesions can ameliorate oxidative stress through biologic actions of its reaction products, CO, ferritin, and biliverdin. HO-1 may inhibit inflammatory processes in the vessel wall, potentially acting as a vascular protector that stabilizes the atherogenic process.36

The present data confirmed that H pylori infection is common in carotid plaques. A prior study did not find differences in the rate of H pylori infection between symptomatic and asymptomatic subjects. It is possible that a larger sample size and more strict criteria to define prior symptoms in the present investigation accounted for the discordance. To the best of our knowledge, this is the first evidence of a strong association between HO-1 expression and presence of H pylori. Although intriguing, our findings do not prove a causal relationship among local H pylori infection, HO-1 expression, plaque stabilization, and absence of symptoms. Further research is needed to establish if H pylori infection of the atherosclerotic plaque may promote oxidative stress and induce a protective reaction. The role of other infections of the vascular wall and their potential to induce HO-1 expression are unknown. We cannot rule out that other factors are...
responsibility for expression of HO-1 in these subjects. HO-1 induction by oxidized phospholipids has been reported in previous studies. We found an association between hypercholesterolemia and HO-1 expression. However, hypercholesterolemia was not related to *H pylori* infection and presence/absence of prior symptoms.

In summary, our study demonstrates that HO-1 expression is highly prevalent in asymptomatic plaques. Therefore, HO-1 may be a novel, clinically relevant therapeutic target in vascular disease. A potential role of *H pylori* in oxidative stress-mediated injury and a subsequent defense reaction represented by HO-1 expression deserves further investigation.

**Conclusions**

We assessed the presence of *H pylori* and the expression of HO-1 in 25 symptomatic and 23 asymptomatic carotid plaques. *H pylori* was found in 28 plaques. HO-1 was expressed in 27 of 28 *H pylori*-positive plaques but only in 3 *H pylori*-negative specimens. Although 83% of asymptomatic plaques were positive for *H pylori* and 87% were positive for HO-1, only 36% of specimens from symptomatic patients were positive for *H pylori* and HO-1. This study demonstrates a strong association among *H pylori* infection, HO-1 expression, and absence of symptoms in subjects with carotid atherosclerosis.

**References**

Is Heme Oxygenase-1 a Causal Player for Plaque Stability?

Inflammatory mechanisms play an important role in all stages of the atherosclerotic process. Various studies implicate that certain infectious agents represent candidates that trigger these inflammatory responses. An association of viral infection with atherosclerosis was first reported in the 1970s, when experimental infection of germ-free chickens with an avian herpes virus was found to produce arterial disease. Although several infectious pathogens have been detected within the atherosclerotic plaque, including Chlamydia pneumoniae, Cytomegalovirus, and Helicobacter pylori, the precise role of these pathogens in causing atherosclerosis or in aggravating the atherosclerotic process remains to be established.

In addition, a pathogen resident in an atherosclerotic vessel wall may be just an “innocent bystander” rather than a causally relevant agent, and atherosclerotic arteries might be simply more susceptible to infections. Although multiple seroepidemiological studies could demonstrate associations between atherosclerosis and antibodies against different pathogens, other studies did not. More important, recent secondary prevention studies failed to prevent cardiovascular events by administering antibiotics. Because several types of pathogens may contribute to the multifaceted process of atherosclerosis, it seems to be unlikely that a single microbe causes atherosclerosis. Instead, the total pathogen burden of infection at various sites may affect atherosclerosis progression.

Various mechanisms and hypotheses have been proposed to explain possible interactions between pathogen agents and the atherosclerotic vessel wall. These include induction of specific antibodies or alteration of circulating cytokines, acute phase proteins, and white blood cells. Results from several studies also point to a significant role of immune responses contributing to atherogenesis. One of the auto antigens discussed as a possible immune target is the heat shock protein (HSP), which is synthesized in cells exposed to inflammation, infection, or oxidative stress. HSP has been detected on endothelial cells, macrophages, and smooth muscle cells located in atherosclerotic plaques. Bacterial pathogens encoding for HSP within atherosclerotic lesions can be induced by myobacterial HSP in vitro. Some authors suggest that bacterial infections trigger the formation of antibodies against bacterial HSPs, which might cross-react with human HSPs within endothelial cells, thereby provoking endothelial dysfunction as the first step in the atherosclerotic process.

H pylori is a Gram-negative microaerophilic bacterium that colonizes the gastric mucosa of ~50% of all adults. It represents the major cause of chronic gastritis and peptic ulcer disease. Several investigators could show association between H pylori seropositivity and manifestations of atherosclerosis in different vascular beds, whereas other did not. H pylori has been identified within atherosclerotic plaques, and it is one of the pathogens included into the concept of pathogen burden. Recent publication demonstrates an association between H pylori and endothelial dysfunction. In experiments, an extract of H pylori has been reported to induce a disturbance of proliferation and apoptosis and to decrease the viability of cultured endothelial cells from the stomach. In addition, a cross-reactivity of H pylori anti-Cag antibodies with antigens of normal and atherosclerotic blood vessels has been shown. Moreover, H pylori infection with chronic gastritis causes malabsorption of folate and vitamins B12, followed by hyperhomocysteinemia, which is discussed as cardiovascular risk factor.

In recent years, there has been much interest in the role of oxygen-derived free radicals and the subsequent oxidation of low-density lipoprotein (LDL) to oxidized LDL in the pathogenesis of atherosclerosis. The body has evolved a complex defense strategy to minimize the damaging effect of various oxidants, and central to this defense are the antioxidant enzymes of the blood. H pylori infection with chronic gastritis causes malabsorption of folate and vitamins B12, followed by hyperhomocysteinemia, which is discussed as cardiovascular risk factor.
have been identified. Thus, an upregulation of HO-1 may point to a high degree of oxidative stress within vascular tissue or an atherosclerotic plaque and theoretically may stabilize a plaque via its antioxidant mechanisms.

In this issue of Stroke, Ameriso et al28 examined in neurologically symptomatic and asymptomatic patients plaques obtained during endarterectomy and 7 normal carotid arteries that were obtained at autopsy. The authors found that H pylori–positive specimens were present in 28 plaques, and HO-1 was expressed in 30 plaques. All 7 normal carotid arteries were negative for H pylori and HO-1. Although 82% of asymptomatic patients were positive for H pylori and 87% for HO-1, only 36% of symptomatic patients were positive for both. Therefore, the authors demonstrate that HO-1 expression is highly prevalent in asymptomatic plaques, indicating that HO-1 may indeed be able to stabilize plaques. In addition, these findings indicate that HO-1 represents a clinically relevant therapeutic target in vascular disease. It also provides indirect evidence that H pylori–induced damage may be mediated to a large extent by stimulation of the production of reactive oxygen species.

How does a H pylori infection induce oxidative stress? Oxidative damage of the stomach is one of the pathogenetic factors in chronic gastric infection with H pylori.29 H pylori is able to induce polymorphonuclear and mononuclear cells that produce large amounts of reactive oxygen species via activation of the NADPH oxidase, which could cause DNA damage to the adjacent cells, leading to gastric cancer development. In addition, H pylori induces the activation of NO pathway in macrophages and gastric endothelial cells. In the presence of reactive oxygen species such as superoxide, NO may react with superoxide to form to the highly reactive peroxynitrite.29 Peroxynitrite has been shown to induce chronic inflammation associated with point mutations and DNA damage.29 Over that, it causes endothelial dysfunction via uncoupling of the endothelial NO synthase and by causing tyrosine nitration of the prostacyclin synthase, ultimately leading to a cessation of endothelial NO and prostacyclin formation, respectively.30,31 Several antioxidative enzymes have been shown to be upregulated in the H pylori–infected gastric mucosa, including catalase, endogenous peroxidase, and superoxide dismutase in the gastric mucosa. Recently, upregulation of HO-1 has also been described as possible adaptive response to protect mucosa from oxidative injury in patients with H pylori–positive gastritis.32 Therefore, it is reasonable to conclude that similar mechanism exists in an atherosclerotic lesion in response to a local H pylori infection.

At present, H pylori is discussed to be less involved in plaque initiation rather than plaque progression and destabilization, followed by cardiovascular events including myocardial infarction and stroke. It has been suggested that chronic infections can induce a procoagulant state and thereby destabilize atherosclerotic plaques.33 In addition, it has been postulated that a persistent inflammatory response that accompanies chronic low-grade infections such as Chlamydia pneumoniae or H pylori contribute to atherosclerosis progression by increasing the concentrations of acute phase reactants such as C-reactive protein or fibrinogen. This could be due either to a direct action to plaques or secondary to remote signaling processes induced by these inflammatory mediators. The molecular mimicry between bacterial pathogens and human molecules may contribute to the activation of inflammation, too.3,35 Unfortunately, in the present study, neither systemic nor local markers of inflammation have been measured to prove this concept.

In conclusion, the article by Ameriso et al indicates that H pylori infection might contribute to plaque destabilization, all of which may be secondary to the induction of oxidative stress within the plaque. The simultaneously observed high prevalence of HO-1 expression in neurologically asymptomatic patients may indeed indicate that this enzyme plays a crucial role in stabilizing plaques infected with H pylori. Future research should focus on mechanisms of how H pylori is able to cause upregulation of HO-1 and whether targeting of HO-1 by drugs leading to an upregulation of this enzyme is indeed leading to a stabilization of the plaques and subsequently to fewer cardiovascular events.

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