Cross-Reactivity of Anti-CagA Antibodies With Vascular Wall Antigens
Possible Pathogenic Link Between Helicobacter pylori Infection and Atherosclerosis

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Background—Helicobacter pylori-CagA positive strains have been shown to be associated with atherosclerosis. However, the pathogenesis is still undetermined. The aim of this study was to determine whether anti-CagA antibodies cross-react with antigens of normal and atherosclerotic arteries.

Methods and Results—Eight umbilical cord sections, 14 atherosclerotic artery sections, and 10 gastrointestinal tract sections were examined by immunohistochemistry using polyclonal anti-CagA antibodies. Five atherosclerotic and 3 normal artery samples were also lysed in ice-cold lysis buffer containing protease inhibitors and were immunoprecipitated using the same antibodies. Anti-CagA antibodies reacted with cytoplasm and nuclei of smooth muscle cells in umbilical cord and atherosclerotic vessel sections, cytoplasm of fibroblasts-like cells in intimal atherosclerotic plaques, and the cell membranes of endothelial cells. Anti-CagA antibodies also specifically immunoprecipitated 2 high molecular weight antigens of 160 and 180 kDa from both normal and atherosclerotic artery lysates.

Conclusions—Anti-CagA antibodies cross-react with antigens of both normal and atherosclerotic blood vessels. We speculate that the binding of anti-CagA antibodies to those antigens in injured arteries could influence the progression of atherosclerosis in CagA-positive H pylori-infected patients. (Circulation. 2002;106:430-434.)

Key Words: atherosclerosis ■ infection ■ antibodies
_H. pylori_ has been proposed to increase the levels of lipids and fibrinogen in response to a low-grade persistent inflammatory stimulation and to raise anti-heat shock protein 65 (hsp65) antibodies, a known marker of atherosclerosis.\(^{18-21}\) Some strains of _H. pylori_ express the cytotoxin-associated gene-A (cag A), which encodes for a hydrophilic, surface-exposed protein named CagA.\(^{22}\) The size of the protein may vary between 116 and 140 kDa in different strains. _H. pylori_ strains expressing CagA and carrying the _cag_ pathogenicity island induce an inflammatory response in the gastric mucosa greater than that induced by strains lacking the pathogenicity island.\(^{23}\) CagA-positive strains have been found to be significantly more prevalent among patients with IHD than in controls,\(^{23}\) suggesting that the enhanced immunologic response evoked by such strains may influence plaque formation and/or activation. Despite the plausibility of this hypothesis, however, the exact mechanisms by which CagA-positive strains would contribute to the progression of atherosclerosis have not been elucidated.

Autoimmune responses have been shown to participate in both the initiation and progression of atherosclerosis.\(^{12-15}\) We hypothesized that antigenic mimicry between _H. pylori_ antigens and structural elements of blood vessels could participate in this process. Therefore, we designed a study aimed at determining whether antibodies against CagA cross-reacted with antigens of normal and atherosclerotic arteries, providing a possible pathogenic link with atherosclerosis.

**Methods**

### Vascular, Gastrointestinal, and Other Tissues

Fresh segments of atherosclerotic arteries (5 samples) and normal arteries (3 samples) were collected from 8 patients, either during autopsies performed shortly after death or from surgically amputated extremities. Formalin-fixed paraffin-embedded sections of umbilical cord (8 samples) and atherosclerotic tibial arteries (14 samples) were retrieved from the files of the Baylor College of Medicine Department of Pathology. Paraffin-embedded sections of stomach infected with CagA-positive _H. pylori_ strains (6 samples), colon (4 samples), liver (10 samples), and skin (4 samples) were also used as positive and negative controls for the immunohistochemical studies. All the procedures followed were in accordance with institutional guidelines.

### Immunohistochemistry

Immunostaining was performed on 5-μm formalin-fixed paraffin-embedded sections by a standard avidin-biotin detection system (Dako Autostainer, Dako Corporation). Briefly, tissue sections were mounted onto slides, deparaffinized in xylene, and rehydrated in graded ethanol solutions. Antigen retrieval was done by steaming the samples for 20 minutes. After washing with PBS, sections were incubated in 10% goat serum for 20 minutes to reduce non-specific antibody binding. Sections were then incubated with the primary mouse polyclonal anti-CagA antibodies (Acambis Inc, Cambridge, Mass) at a 1:300 dilution. Sections were then incubated with biotinylated rabbit anti-mouse IgG for 10 minutes, washed 3 times with PBS, treated with streptavidin-peroxidase reagent for 10 minutes, and washed again with PBS. Gastric sections from patients with _H. pylori_ infection that were previously shown to express CagA antigens by Western blot analysis were used as the positive control. Negative controls were stained without primary antibody. To confirm the specificity of the immunoreactivity, all immunostaining was repeated after incubation of the primary antibody with purified CagA antigen (Acambis Inc, Cambridge, Mass).

**Results**

### Immunohistochemistry

Anti-CagA antibodies showed immunoreactivity with both umbilical cord and atherosclerotic artery antigens. Specifically, they recognized nuclei (and to a lesser extent, the cytoplasm) of smooth muscle cells, as well as nuclei and cytoplasm of endothelial cells in all umbilical cord sections (Figure 1). Furthermore, in all atherosclerotic vessel sections, anti-CagA antibodies strongly reacted with smooth-muscle cells, the cytoplasm of fibroblasts-like cells in intimal atheromatous plaques, and endothelial cells, including the endothelium in re-canalized segments of vessel (Figure 2). No immunoreaction was observed after incubation of the arterial sections with the primary antibody previously treated with the purified CagA antigen (Figure 3A and 3B).

Anti-CagA antibodies intensely stained CagA-positive _H. pylori_ organisms in the stomach (Figure 4A); however the
same antibodies did not exhibit immunoreactivity with any gastrointestinal mucosal or submucosal structures, including small vessels and smooth-muscle cells. No immunoreactivity was observed between anti-CagA antibodies and small vessels of either liver or skin. This suggests that CagA cross-reacts only with vascular peptides in large- and medium-size arteries.

**Immunoprecipitation**

Anti-CagA antibodies immunoreacted with all artery samples. Specifically, anti-CagA antibodies recognized 2 vascular antigens of 160 K and 180 K from both normal and atherosclerotic artery lysates, as revealed by silver staining. The same antigens were not precipitated by normal mouse IgG, confirming the specificity of the reaction. Bacterial lysates immunoprecipitated with anti-CagA antibodies consistently showed a band at approximately 130 K.

**Discussion**

Atherosclerosis is the principal cause of death in Western societies, but its pathogenesis remains to be elucidated. Many patients develop atherosclerosis in the absence of conventionally recognized risk factors, a finding which suggests that unrecognized mechanisms may also be determinant in the pathogenesis of the disease. To explain such discrepancies, several authors have proposed additional risk factors for atherosclerosis, including *H pylori* infection.

*H pylori* infection has been associated with anti-hsp65 production and with increased fibrinogen and serum lipids, but inconsistent results have been reported by different studies. Recently, cross-reactivity between rabbit-raised hyperimmune anti-*H pylori* serum and antigens from atherosclerotic carotid arteries has been demonstrated. In this study, however, the interpretation of the results was limited by the absence of inclusion of normal arteries and the lack of identification of the bacterial antigens mimicking vascular peptides.
Our demonstration that anti-CagA antibodies are capable of reacting with both bacterial CagA and proteins present in the wall of medium- and large-size arteries provides evidence of molecular mimicry between CagA and vascular antigens. Specifically, we found that anti-CagA antibodies, which have been shown to be increased in patients with IHD,12 exhibited immunohistochemical cross-reaction with antigens expressed by cells involved in the atherogenic process, such as vascular smooth muscle cells, fibroblasts-like cells in intimal atherosclerotic plaques, and cell membrane of endothelial cells. To evaluate the specificity of the immunohistochemical reactivity, we also performed immunoprecipitation of artery lysates using the same anti-CagA antibodies used for immunohistochemistry. Our results showed that these antibodies immunoprecipitated 2 vascular antigens with molecular weights of approximately 160 K and 180 K, from both normal and atherosclerotic artery lysates, respectively, whereas the same antigens were not precipitated by normal mouse IgG.

Anti-CagA antibodies precipitated CagA and vascular antigens at different molecular weights: 120 K for CagA and 160-180 K for arterial proteins. This finding indicates that the immunoprecipitated proteins extracted from vessels are structural vascular components different from the CagA protein. Therefore, it seems unlikely that the reactivity detected in vessels and their extracts would represent bacterial CagA deposited within vascular walls. Recently, some studies reported the visualization of H pylori genomic material28 and H pylori organisms within atherosclerotic plaques.29 In the latter study, the detection of organisms was performed by immunohistochemical methods using specific anti-H pylori antibodies.29 The authors speculated that H pylori infection might affect atherosclerosis through a direct colonization of arterial walls. In our view, several points militate against the plausibility of this hypothesis. First, the published figures29 do not show specific morphological characteristics that would allow an observer to independently identify H pylori. Second, there was no evidence, until now, of H pylori’s ability to spread from its gastric niche via the blood circulation. Third, the presence of genomic material of H pylori within the arterial wall of atherosclerotic arteries could be related to a non-specific deposition of circulating DNA fragments, rather than to the presence of intact H pylori organisms inside plaques. Finally, other studies that specifically addressed this issue failed to confirm these findings.30

Our study does not establish whether anti-CagA antibodies would recognize the mimicking vascular antigens in normal arteries in vivo. It is tempting, however, to speculate that such phenomena may happen in injured arteries. Antigens normally made inaccessible by the endothelial integrity could become exposed to circulating antibodies after the initiation of the atherogenic process damages the arterial wall. Anti-CagA antibodies could then bind the exposed vascular antigens and further contribute to the activation of inflammatory cells within lesions. Production of cytokines and other inflammatory mediators by activated macrophages and fibroblasts31–33 could then lead to the destabilization of atherosclerotic plaques, possibly triggering ischemic events.

In conclusion, this study provides experimental evidence of molecular mimicry between the CagA antigen and vascular wall peptides. This finding yields biological plausibility to the theory that H pylori infection may play a role in the pathogenesis of atherosclerosis. The verification of these hypotheses and the elucidation of the mechanisms involved will require further studies, ideally using combined animal models of atherosclerosis and H pylori infection.

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