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

Atherosclerotic vascular disease, the major cause of mortality and morbidity in industrialized countries, is a chronic multifactorial disorder.1 Ischemic heart disease (IHD), cerebrovascular disorders, and gangrene of the extremities are among the most common and devastating consequences of this condition.2 Epidemiological studies have linked atherosclerosis to hypertension, cigarette smoking, diabetes, hyperlipidemia, hypercoagulability, and hyperhomocystinemia.3 Nevertheless, completely explains the occurrence and progression of the disease. Other factors are likely to be involved.4–8

Both humoral and cellular immune reactions participate in atherogenesis.9–12 Fibroblasts and macrophages are an active component of atherosclerotic plaques, where they play a crucial role in accumulating lipids. Immunoglobulin complement deposits and activated CD4+ T cells have also been associated with atherosclerotic plaques.9–10 These latter findings have marked the beginning of a new era in the field of atherosclerosis research. The emphasis has shifted from the traditional view of atherogenesis as an essentially mechanical process (the “response to injury hypothesis”) to the “inflammatory hypothesis.” According to this view, both the initiation and progression of atherosclerosis, including its thrombotic and thromboembolic complications, may be linked to an inflammatory process taking place within the arterial wall and the atherosclerotic lesion. The activation of inflammatory cells could promote plaque irregularity and rupture, eliciting ischemic events.9–11 Although the mechanisms of activation of inflammatory cells within atherosclerotic lesions have not been elucidated, there is evidence for a role of autoimmunity as well as infections,13–15 particularly those caused by Cytomegalovirus, herpes simplex, Chlamydia pneumoniae, and, most recently, Helicobacter pylori.

Since 1994, more than 30 studies have been published reporting an association between H pylori infection and ischaemic heart disease (IHD), the most common clinical manifestation of atherosclerosis.16,17 The majority of these studies were seroepidemiological, on the basis of the detection of antibodies against H pylori in patients with IHD. 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. Some strains of H pylori express the cytotoxin-associated gene-A (cag A), which encodes for a hydrophilic, surface-exposed protein named CagA. 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. CagA-positive strains have been found to be significantly more prevalent among patients with IHD than in controls, 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. 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 tissue sections with the primary antibody previously treated with the negative controls for the immunohistochemical studies. All the procedures followed were in accordance with institutional guidelines.

Immunoprecipitation

Five fresh atherosclerotic samples and 3 normal artery samples were lysed in an equal volume of ice-cold PBS buffer containing 1% nonylphenol polyoxyethylene 40 (NP-40), 100 mmol/L Na2VO4, 5 mmol/L EGTA, 10 mmol/L NaF, 207, 100 mmol/L phenylmethylsulfonyl fluoride (PMSF), and 2 μg/mL each of leupeptin, pepstatin, and aprotinin. Samples were then incubated on ice for 30 minutes, cleared of insoluble debris by centrifugation at 13 000g for 20 minutes at 4°C, and diluted with PBS to obtain a final concentration of NP-40 of 0.5%. Immunoprecipitation was performed by incubating the lysates overnight at 4°C with the same mouse-raised polyclonal anti-CagA antibodies used for immunohistochemistry (Acambis Inc, Cambridge, Mass) or with normal mouse IgG as control (Santa Cruz Biotechnology, Santa Cruz, Calif), followed by incubation with 100 μL protein A/G Agarose (Santa Cruz Biotechnology) for 1 hour at the same conditions. After 3 washes with PBS buffer containing protease inhibitors, precipitated proteins were separated by SDS-PAGE and revealed by silver staining.

To determine the molecular weights of the CagA present in bacterial strains isolated from patients with gastric cancer, we also immunoprecipitated bacterial lysates with anti-CagA antibodies (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 atherosclerotic 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 (Figure 4B). 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 (Figure 5). 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.

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**Figure 2.** Anti-CagA antibodies reacted with endothelium (EC), smooth muscle cells (SMC), and plaque fibroblasts (FB) in an atherosclerotic artery.

**Figure 3.** Umbilical cord arteries. A, Negative control (absence of primary antibody). Similarly, no reactivity was found after primary antibodies were incubated with purified CagA antigen (B). EC indicates endothelial cells; SMC, smooth muscle cells.

**Figure 4.** A, Anti-CagA antibodies stained CagA-positive *H pylori* organisms, visible within the gastric pit and in the mucous layer adjacent to the gastric surface. B, Low-power photomicrograph of the same gastric biopsy specimen depicted in A. No structures (including small vessels and smooth-muscle cells) showed immunoreactivity when stained with anti-CagA antibodies. The reactivity seen in inflammatory cells is non-specific; it is common to virtually all immunoperoxidase-based immunostains and is due to reactivity with secondary reagents.
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, 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 material and H pylori organisms within atherosclerotic plaques. In the latter study, the detection of organisms was performed by immunohistochemical methods using specific anti-H pylori antibodies. 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 figures 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.

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 fibroblasts 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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References


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