H pylori infection and systemic antibodies to CagA and heat shock protein 60 in patients with coronary heart disease

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Abstract

AIM: To determine the overall prevalence of H pylori and CagA positive H pylori infection and the prevalence of other bacterial and viral causes of chronic infection in patients with coronary heart disease (CHD), and the potential role of anti-heat-shock protein 60 (Hsp60) antibody response to these proteins in increasing the risk of CHD development.

METHODS: Eighty patients with CHD and 160 controls were employed. We also compared the levels of anti-heat-shock protein 60 (Hsp60) antibodies in the two groups. The H pylori infection and the CagA status were determined serologically, using commercially available enzyme-linked immunosorbent assays (ELISA), and a Western blotting method developed in our laboratory. Systemic antibodies to Hsp60 were determined by a sandwich ELISA, using a polyclonal antibody to Hsp60 to sensitise polystyrene plates and a commercially available human Hsp60 as an antigen.

RESULTS: The overall prevalence of H pylori infection was 78.7% (n = 63) in patients and 76.2% (n = 122) in controls (P = 0.07). Patients infected by CagA-positive (CagA+) H pylori strains were 71.4% (n = 45) vs 52.4% of infected controls (P = 0.030, OR = 2.27). Systemic levels of IgG to Hsp60 were increased in H pylori-negative patients compared with uninfected controls (P < 0.001) and CagA-positive infected patients compared with CagA-positive infected controls (P = 0.007).

CONCLUSION: CagA positive H pylori infection may concur to the development of CHD; high levels of anti-Hsp60 antibodies may constitute a marker and/or a concomitant pathogenic factor of the disease.

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Key words: H pylori; Coronary heart disease; CagA protein; Heat shock protein 60; Antibody response

INTRODUCTION

Atherosclerosis-related diseases -particularly coronary heart disease (CHD)- are a leading cause of death and disability in most developed countries. Many epidemiological studies have shown a strong relationship between CHD and chronic bacterial and viral infections, suggesting a primary role of inflammatory diseases in the pathogenesis of vascular cardiac disorders[1,2]. Infectious agents may cause a spectrum of systemic effects and induce atherosclerosis in several different ways. For instance, by increasing the production of circulating cytokines (interleukin-1 [IL-1] and interleukin-6 [IL-6]), through the generation of acute-phase reactants (white blood cells and C reactive protein) and the stimulation of immune-mediated responses, such as the production of antibodies targeted to the invading pathogens, etc[3].

Several authors[4] have also reported that infections might stimulate smooth muscle cell proliferation and migration and lipid accumulation; apoptosis of endothelial cells can be inhibited and many procoagulant effects could be produced[4].

H pylori infection is one of the most widely spread in-
fectious diseases in human\textsuperscript{[9]}. This microorganism infects half the world population and causes chronic gastritis. The disease usually lasts for the entire host’s life and constitutes a main risk determinant of peptic ulcer and gastric neoplasia\textsuperscript{[6,7]}. The infection elicits a chronic humoral and cellular inflammatory response, stimulates an increase of polymorphs and basophils\textsuperscript{[8]} and elevates the local and systemic concentrations of vasoactive cytokines\textsuperscript{[9]}, whose effects may not be confined to the digestive tract\textsuperscript{[10]}. Recent epidemiological surveys have indicated that \textit{H pylori} infection may be associated with atherosclerotic vascular diseases\textsuperscript{[11-13]}, although it is still disputed whether this infection increases the risk of CHD\textsuperscript{[14-18]}. Some studies have shown an increased risk of CHD in patients with a systemic immune response to heat shock proteins (Hsps)\textsuperscript{[19]}. Hsps are families of highly conserved proteins that share wide homologies of sequence among different species, ranging from bacteria to human beings\textsuperscript{[16,17]}. They are induced or up-regulated in cells exposed to sudden elevations in temperature, but are also synthesized in large numbers when cells are exposed to stressful stimuli such as inflammation, infections, mechanical stress, hypoxia and oxidizing agents\textsuperscript{[14,15,19,20]}. They play a fundamental role in the growth of bacteria at all temperatures and their production could represent an essential mechanism of cell protection against different noxae\textsuperscript{[17,16,19]}. \textit{H pylori} produces two main Hsps, a groES-like HspA with a mass of 13 kDa, and a groEL-like HspB with a mass of 54-60 kDa\textsuperscript{[19-21]}. Both proteins stimulate a specific systemic antibody response and, due to the high sequence homology of Hsps, it is highly possible that they can trigger an autoimmune response directed against the bacterial proteins and also to human tissues expressing Hsps, including vascular endothelial cells\textsuperscript{[20,21]}. The aim of the present study was to determine the prevalence of anti-Hsp antibodies in patients with CHD and controls and to identify the potential role of an antibody response to these proteins in increasing the risk of CHD development. We tested serum samples for the overall prevalence of \textit{H pylori} and CagA positive \textit{H pylori} infection, and for antibodies to the other bacterial and viral causes of chronic infection that are recognised determinants of CHD risk development. Our results suggest that CagA positive \textit{H pylori} infection may concur to the development of CHD and that high levels of anti-Hsp antibodies may constitute a marker and/or a pathogenic factor of the disease.

**MATERIALS AND METHODS**

**Patients and controls**

We studied 80 consecutive patients with stable angina; their mean age was 65 years (range 45 to 75 years). Patients were admitted to this Institute for evaluation by clinical history, physical examinations, heart echography, and basal and exercise ECGs. Patients were enrolled if they showed signs or symptoms of angina at exercise ECG; an ST segment depression more than 2 mm was considered positive. As control, we enrolled 160 age- and gender-matched patients, who came from the same socio-economic background and were hospitalised in the same Institute for diseases other than CHD, vascular diseases, dyspeptic and liver disorders, hematological diseases, and thyroid abnormalities. Their mean age was 64.5 years (range 43 to 75 years). Patients and controls had not taken antibiotics potentially active against \textit{H pylori} in the last three months. Both patients and controls gave their written informed consent.

**Determination of \textit{H pylori} infection and CagA status**

The \textit{H pylori} infectious status was determined serologically using a commercially available enzyme-linked immunosorbent assay with a sensitivity and specificity of 96% ca. (Helicobacter pylori IgG, Diessel, Monteriggioni, Siena, Italy). \textit{H pylori} infectious status was confirmed by Western blotting (WB). WB was also used to detect antibodies to \textit{H pylori} CagA. Briefly, a whole cell suspension of \textit{H pylori} CCUG 17874 (a CagA-positive and cytotoxic strain) was denatured in Laemmli’s buffer at 100°C for 5 min and electrophoresed in a 10% polyacrylamide gel with sodium dodecyl sulphate. The resolved proteins were transferred electrophoretically onto nitrocellulose membranes, and the free sites were saturated with 3% skim milk in phosphate buffered saline (PBS) pH 7.4 containing 0.1% Triton X (PMT). Afterward, strips were cut and immunoblotted with serum samples diluted 1:100 in PMT for immunoglobulin G (IgG). After overnight incubation at room temperatures, strips were washed three times with PMT, and a peroxidase labelled antibody to human IgG, diluted in PMT 1:2000 (Sigma Che. Co., Milan), was added and incubated at room temperatures for 90 min. Strips were washed three times with PMT, once with PBS-Triton X, and twice with Tris buffer 0.05 mol/L pH 6.8. The reaction was visualised by addition of the substrate (H2O2 in a solution of 4-chloro-1-naphthol in Tris buffer 0.05 M pH 6.8). The reaction was stopped with water. The presence of more than six bands of reaction indicated an infection. As positive controls, anti-CagA and anti-Hsp rabbit polyclonal antibodies (kindly given by R. Rappuoli, Novartis, Siena) were used.

**Determination of anti-Hsp60 antibodies**

Antibodies to Hsp60 were determined by an ELISA, using a commercially available human Hsp60 (Sigma Che. Co., Milan, Italy). In preliminary tests, we determined the working concentrations of Hsp60 with the aid of a pool of human serum samples, which contained antibodies to \textit{H pylori} HspB (54-60 kDa), as detected on WB. Briefly, we sensitised each well of polystyrene microtiter plates with 150 μL of an anti-polyclonal \textit{H pylori} HspB antibody raised in rabbits, diluted 1:50 in PBS pH 7.4. After one hour of incubation at 37°C, we washed the plates three times with PBS containing 0.05% Tween 20 (PBST) and 2% bovine serum albumin (BSA). Then, we added to each well 2.5 μg of Hsp contained in 100 μL of PBS-BSA (this amount of Hsp was determined in preliminary tests). After one hour of incubation at 37°C and three washes with PBS-TBSA, we added 100 μL of each serum samples, both from patients and controls, diluted 1:50 in PBS-BSA. Plates were incubated at 37°C for one hour, then they were washed...
Infection, herpes simplex virus and Epstein-Barr virus antibodies (Table 1). We found that the majority of both patients and controls had a similar mean age of 64.5 years (range 43 to 75 years). The overall prevalence of \textit{H pylori} infection was 78.7\% (\(n = 63\)) in patients and 76.2\% (\(n = 122\)) in controls (\(P = 0.07\)).

**Determination of CagA status in infected patients and controls**

Recent studies have shown that infection by strains that express CagA protein induces increased levels of local and systemic cytokines that could contribute to the damage of the cardiovascular system. We therefore determined the seroprevalence of CagA seropositivity in patients and controls. Patients infected by CagA-positive (CagA\(^+\)) \textit{H pylori} strains were 71.4\% (\(n = 45\)) vs 52.4\% of infected controls (\(P = 0.030\), OR = 2.27; 95\% CI 1.0-5.1) (Figure 1).

**Prevalence of infections by pathogens other than \textit{H pylori}**

Since it is well-known that many pathogens could contribute to the genesis of a chronic systemic inflammatory status, we determined the seroprevalence of the most common infectious agents that might increase the risk of CHD. We found that the majority of both patients and controls were seropositive for \textit{C. pneumoniae}, cytomegalovirus, \textit{herpes simplex} virus and Epstein-Barr virus, while 46.2\% of patients and 38.7\% of controls had anti-\textit{M. pneumoniae} antibodies. No statistically significant difference was found in the prevalence of infections by the different pathogens in patients and controls (data not shown).

**Determination of anti-Hsp60 antibodies in patients and controls**

Hsps are a family of well-conserved proteins and Hsp60, in particular, is widely shared by \textit{H pylori} and eukaryotic cells. As antibodies to Hsps are found at high titers in cardiovascular disorders, we compared the levels of anti-Hsp60 antibodies in patients and controls. Levels of antibodies to Hsp60 were significantly increased in \textit{H pylori}-negative (\(Hp^\text{-}\)) patients, compared with those in \textit{H pylori}-negative controls (341.5 ± 159.6 vs 197.6 ± 44.4; \(P < 0.001\), 95\% CI 66.4-221.3) (Figure 2); levels of antibodies to Hsp60 in CagA\(^+\) patients were higher than in CagA\(^-\) controls (418.8 ± 144.2 vs 317.2 ± 175.6; \(P = 0.007\), 95\% CI 28.8-174.3), but were not significantly higher than in \(Hp^\text{-}/CagA^+\) patients (350.2 ± 169.1; \(P = 0.110\)) and in \(Hp^\text{-}\) patients (341.5 ± 159.6; \(P = 0.072\)) (Figure 2). Levels of antibodies to Hsp60 in CagA\(^+\) controls (317.2 ± 175.6)
HspB may support such observa-

tions. However, such an observation is still questioned.
atherosclerotic vascular disease is due to an autoimmune reaction to endothelial cells that express high levels of Hsp in response to stressful stimuli, like oxidized LDL, free radicals, local infections, cytokines or hemodynamic stress. In the present study, we confirmed that the increased anti-Hsp60 immune response observed in patients cannot be attributed to chronic infections by pathogens other than H. pylori, since their prevalence in patients was similar to that in controls. On the contrary, the infection by CagA+ H. pylori strains increased the levels of anti-Hsp60 antibodies both in patients and controls; however, although in patients the difference was not statistically significant, in controls such a difference was significant, suggesting that a relationship between chronic H. pylori infection and development of antibodies to Hsp60 cannot be excluded. Another explanation could consist in the possibility that the inflammatory response triggered by the infection, together with putative toxic substances secreted by bacteria, alters the epithelial Hsp to such a degree that the patient’s immune system loses the immune tolerance to self-chaperon and starts producing autoantibodies that cross-react with H. pylori Hsps. Latif 

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