Role of *Helicobacter pylori* specific heat shock protein-60 antibodies in the aetiology of coronary artery disease

Mahadev D. Dixit, Kishore G. Bhat, Aruneshwari Dayal

Department of CardioVascular and Thoracic Surgery, KLES Heart Foundation, KLES Dr. Prabhakar Kore Hospital; Medical Research Centre and Jawaharlal Nehru Medical College, Nehru Nagar, India

Abstract

The role of chronic infections in causing coronary artery disease (CAD) has been investigated for the past several years. Among them, the role of *Helicobacter pylori* has stimulated keen interest. Though initial results were conflicting, there are growing data to support the role of *H. pylori* in CAD. The main mechanism of endothelial damage is hypothesized to be through molecular mimicry involving heat shock proteins. This study was designed to determine the prevalence of *H. pylori* and cytotoxin associated gene A (cagA) positive *H. pylori* infection in patients undergoing coronary artery bypass grafting (CABG) and the potential role of anti-*H. pylori* specific heat shock protein-60 (Hp-HSP-60) antibody response in these patients, for cardiac events. One hundred patients undergoing CABG and 100 controls were studied. The *H. pylori* infection and cagA status were determined serologically by enzyme-linked immunosorbent assay (ELISA). Hp-HSP-60 Immunoglobulin G (IgG) antibodies were estimated by using an in house ELISA. Although there was no difference in the prevalence of *H. pylori* infection in patients and controls (74% vs 70%), 58% of patients were infected with cagA positive *H. pylori* compared to 36% of controls ($P=0.002$). Mean systemic levels of Hp-HSP-60 IgG were also higher in patients than in controls (27.9 vs 18.7, $P=0.0001$). These antibody levels were also significantly higher in *H. pylori* positive patients ($P=0.0001$). There was a strong correlation between Hp-HSP-60 antibody levels and occurrence of myocardial infarction ($P=0.003$). CagA positive *H. pylori* infection may be associated with the development of CAD. High levels of Hp-HSP-60 antibodies may constitute a marker and/or concomitant pathogenic factor of the disease.

Introduction

Several studies over the past two decades have suggested an association between various microbial infections with atherosclerosis and coronary artery disease (CAD).¹ Questions were raised about the biological meaning of such associations and the possible mechanism by which infections could contribute to the development and progression of atherosclerosis. One of the hypotheses is based on immune mediated mechanism through heat shock proteins (HSP).² These proteins are highly conserved in virtually all eukaryotes and prokaryotes and, because of the structural similarity between the microbial and human HSPs, immune reactants against microbial HSP may cross react with homologous host proteins in the form of molecular mimicry.³

Recently virulent strains of *H. pylori* that express cytotoxin associated gene A (cagA), have been shown to be significantly associated with CAD.^{4,5} Heat shock proteins are known to be expressed and secreted by *H. pylori* and by endogenous cells within atherosclerotic plaques.⁶ Given that *H. pylori* infection has been shown to result in Immunoglobulin G (IgG) antibodies against HSP-60, it has been stated that this may promote the development of atherosclerosis through interaction of *H. pylori* immune reactants and host HSP-60.⁷

The main objectives of the present work were: i) to study the association between virulent *H. pylori* strains and CAD; ii) to estimate the levels of IgG antibodies against Hp specific HSP-60 (*H. pylori* specific heat shock protein-60) in patients with CAD and healthy subjects; and iii) to compare the prevalence of virulent *H. pylori* infection and Hp-HSP-60 antibody titers with various risk factors and cardiac events.

Materials and Methods

The present study was conducted in the Department of Cardiovascular and Thoracic surgery, Heart Foundation, KLE's Dr. Prabhakar Kore Hospital and Medical Research Centre, Belgaum, India, during the period from June 2009 to August 2010. The study included one hundred patients undergoing coronary artery bypass and grafting (CABG), both males and females between 35 and 77 years of age, and an equal number of age and sex matched healthy subjects who had attended the medical outpatient department for a routine health check up and were found to be free of conventional cardiac risk factors and CAD (negative tread mill test). The study was approved by the Hospital Institutional

Correspondence: Mahadev D. Dixit, KLES Heart Foundation, KLES Dr. Prabhakar Kore Hospital and Medical Research Centre, Nehru Nagar, Belgaum, Karnataka, 590010, India.
Tel: +91.831.2473777 - Fax: +91.831.2476570.
E-mail: drmdixit@gmail.com

Key words: *H. pylori*, cagA, heat shock protein-60, coronary artery bypass grafting, myocardial infarction.

Contributions: MDD, collection of clinical data, study design and statistical analysis; performance of relevant tests; KGB, study design and statistical analysis, performance of Lab tests; AD, collection of clinical data and study design.

Conflict of interests: the authors report no potential conflict of interests.

Received for publication: 4 August 2011.

Revision received: 4 May 2012.

Accepted for publication: 5 May 2012.

This work is licensed under a Creative Commons Attribution NonCommercial 3.0 License (CC BY-NC 3.0).

©Copyright M.D. Dixit et al., 2012

Licensee PAGEPress, Italy

Microbiology Research 2012; 3:e15

doi:10.4081/mr.2012.e15

Review and Ethics Committee. Informed consent was obtained from each participant before enrolment.

All study subjects underwent a thorough clinical examination and a detailed history was collected and recorded. This included the presence of various risk factors such as hypertension, hyperlipidemia, diabetes mellitus and smoking. Body mass index (BMI) was calculated and recorded for each subject. Previous history of myocardial infection (MI), presence or absence of angina, number of blocked vessels, and all other relevant clinical findings were carefully noted for each patient.

A 10 mL blood sample was taken from each participant and the serum obtained was frozen in aliquots at -80°C and thawed only when specific tests were performed. Commercially available enzyme-linked immunosorbent assay (ELISA) kits were used to determine the levels of serum IgG antibodies to *H. pylori*, EUROIMMUN, and IgG antibodies to cagA gene product of *H. pylori*, RADIM. The manufacturer's guidelines were strictly adhered to when performing the assays and interpreting the results of the test. A value of 22 relative units/mL was considered the upper limit of normal range for *H. pylori* specific IgG levels and 15 relative units/mL for cagA specific IgG antibodies. Other relevant tests, including fasting blood glucose and total lipid profile (total cholesterol, HDL, LDL, and triglycerides) were carried out

for each participant.

Standard strain of *H. pylori* (ATCC 43504) was used for extraction of HSP-60 for subsequent studies and preparation of the in house ELISA. Briefly, the lyophilized culture was revived by inoculating into trypticase soy broth and then plating on selective medium to obtain bacterial growth. After confirming the growth to be free of contamination, harvested bacterial cells were killed and cell membranes ruptured by repeated freezing and thawing.⁸ The soluble protein suspension of *H. pylori* was then subjected to discontinuous polyacrylamide gel electrophoresis with 10% resolving gel and 1% sodium dodecyl sulphate. The bands of HSP-60 were identified, cut from the gel, eluted and concentrated by dialysis.^{8,9} The Hp-HSP-60 antigen obtained by the above mentioned procedure was used for preparation of plates for Indirect ELISA.¹⁰ A 96-well microtiter plate was coated with Hp-HSP-60 protein (20 µg/50 µL/well) in 0.1 M carbonate-bicarbonate buffer (pH9.6) at 4°C overnight. After washing thoroughly with wash buffer (PBS with 0.1% Tween-20), the wells were blocked with 200 µL of blocking buffer (PBS containing 1% BSA) for 2 h at room temperature (RT). The plates were again washed, sealed and stored at -20°C till used. To perform the ELISA, plates were brought to RT and wells were incubated with 100 µL of 100-fold diluted sera for 2 h at RT. The wells were then incubated with horse radish peroxidase labelled secondary antibody for 1 h. Extensive washing between steps was performed with wash buffer. Color was developed with 100 µL of TMB (tetramethylbenzidine) as substrate. The reaction was terminated after 15 min of incubation with 0.2N H₂SO₄ and optical density at 450 nm was measured. Recombinant HSP-60 (R & D systems) was used as a standard for preparation and standardization of the procedure. Calibration was performed against *H. pylori* specific total IgG and the values were expressed as relative units/mL.

Statistical analysis

Differences between groups were tested by χ^2 for categorical variables. Logistical regression analysis was performed for adjusted age, sex, cardiovascular risk factors (smoking, hyperlipidemia, hypertension, diabetes mellitus and BMI) in a multivariate model. Confidence intervals at the 95% level were calculated for the odds ratios. $P < 0.05$ was considered significant.

Results

The study included one hundred patients undergoing CABG who were admitted to the

Heart Foundation of KLE's Dr Prabhakar Kore Hospital & Medical Research Centre, Belgaum, India, for treatment. There were 80 men and 20 women aged 35-77 years (mean 58.9 ± 8.85). We also included one hundred healthy subjects without any history of conventional cardiac risk factors made up of 79 men and 21 women aged 37-70 years (mean 58.7 ± 4.99).

Among the patients, 55% gave a history of diabetes mellitus, 65% had hypertension and 11% were smokers. The mean BMI was 23.6 ± 3.61 which was similar to that of controls. The mean values of cholesterol, LDL and triglycerides were much higher in patients than in control subjects. There was also a significant difference in the fasting glucose levels with 55% of patients having levels of more than 110 mg/dL compared to 25% of controls ($P = 0.0001$) (Table 1).

When we assessed the presence of complications in the patients, it was seen that 88% of them had history of angina (11% stable angina, 77% unstable angina). Similarly, 28% of patients had a previous history of MI and 67% had triple vessel disease and 81% had left ven-

tricular ejection fraction of 60% or under.

Estimation of *H. pylori* specific IgG antibodies showed that 70% of controls and 74% of patients had positive titers ($P = 0.529$). On the other hand, estimation of IgG antibodies to cagA gene product of *H. pylori* revealed that there was a significant difference in the positivity rate (36% controls *vs.* 58% patients, $P = 0.002$) (Figure 1).

We also analyzed the distribution pattern of *H. pylori* and cag A IgG positivity (Figure 2) and the results were highly significant with 50% of patients showing positive results for both *H. pylori* and cag A IgG in comparison to 34% of controls. Cag A positivity (8% of patients *vs.* 2% controls) was the only other significant finding.

Estimation of Hp-HSP-60 antibodies showed that there was a significant difference in the mean values between patients and controls (27.9 ± 11.90 *vs.* 18.7 ± 6.65 , $P = 0.000$). Furthermore, these antibody titres were arbitrarily divided into 5 categories (<10 , 10.1-19.9, 20-29.9, 30-39.9, >40 RU/mL) and the seropositivity between patients and healthy

Table 1. Distribution of risk factors in patients and controls.

Features	Patients	Controls	P
Age	58.9 ± 8.85	58.7 ± 4.99	
Males	79	80	
D. Mellitus	55%	-	
Hypertension	65%	-	
Smoking	11%		
Body mass index	23.6 ± 3.61	24.2 ± 2.78	0.149
Fasting Glucose	125.2 ± 49.49	104.1 ± 12.82	0.0001
Cholesterol	155.8 ± 30.31	138.9 ± 32.86	0.0001
HDL	43.16 ± 6.71	37.7 ± 6.94	0.390
LDL	85.6 ± 23.16	70.5 ± 27.23	0.0001
Triglycerides	166.1 ± 79.67	143.4 ± 67.68	0.001

Table 2. Multivariate analysis of risk factors.

Risk factor	Unadjusted			Adjusted		
	OR	P	95% CI	OR	P	95%CI
<i>H. Pylori</i>	1.22	0.529	0.65-2.26			
CagA+ve	2.45	0.002	1.39-4.34	1.64	0.178	0.79-3.37
Anti HSP-60	14.33	0.0001	5.36-38.29	14.03	0.0001	4.29-45.94
FBS (>110)	3.24	0.0001	1.80-5.83	2.19	0.035	1.06-4.56
BMI (>25)	0.80	0.454	0.44-5.83			
Cholesterol	1.49	0.672	0.23-9.69			
HDL	1.08	0.854	0.48-2.38			
LDL	0.14	0.0001	0.05-0.39	0.14	0.0001	0.05-0.36
Triglycerides	3.05	0.007	1.35-6.92	3.08	0.005	1.39-6.79

OR, odds ratio; CI, confidence interval; HSP-60, heat shock protein-60; FBS-Fasting Blood Sugar; BMI, body mass index; CagA, cytotoxin associated gene A.

subjects in each category were compared to the distribution pattern of *H. pylori* and *cagA* seropositivity. It was observed that a significantly higher number of patients who were positive for either only *H. pylori* ($P=0.45$) or for both *H. pylori* and *cagA* ($P=0.0001$) had much higher titers of Hp-HSP-60 antibodies (Figure 3). An attempt was also made to correlate the prevalence of *H. pylori*, *cagA* seropositivity and Hp-HSP-60 antibody titers with various cardiac events including MI, angina, left ventricular dysfunction and number of blocked vessels. The findings showed that even though these various cardiac events occurred more frequently in patients with *cagA* seropositivity, the results were not significant. The most notable factor was the strong correlation between the occurrence of MI in patients with higher titers of HpHSP-60 antibodies ($P=0.003$) (Figure 4).

Logistical regression analysis was performed in a multivariate model on the results. The analysis showed that *cagA* seropositivity was highly significant before adjustment ($P=0.002$). But after appropriate adjustments

were made for age, sex and conventional risk factors, the significance became weaker, even though the 95% CI continued to be quite relevant (Table 2). On the other hand, presence of HpHSP-60 antibody titers was highly significant even after adjustment for risk factors ($P=0.0001$). Among various risk factors studied, mean levels of fasting blood sugar, LDL and triglycerides were highly significant by multivariate analysis (Table 2).

Discussion

Chronic infections caused by various microbes are considered to be a potential risk factor for the development and progression of CAD. One such organism is *H. pylori* whose probable role in causing endothelial inflammation and subsequent damage has stimulated keen interest in the medical community in recent years.^{11,12} It has been suggested that infection with *H. pylori* influences the development of atherosclerotic changes in coronary

arteries postulating a damaging influence of these microorganisms or their products on coronary endothelium.⁶

A number of studies have been conducted in the last one and half decades to correlate the association between *H. pylori* infection and CAD. Initially, the studies were confined only to determine *H. pylori* seroprevalence and the results were conflicting and controversial.¹³ This prompted researchers to look for better markers that could clarify the controversy and the immunodominant virulence marker *cagA* was chosen for this purpose. Several studies have shown positive association of virulent *H. pylori* strains with CAD.^{13,14}

The exact mechanism by which *H. pylori* causes endothelial damage is not clear and several different hypotheses have been suggested. Among them, a popular postulate is based on the immunological pathway in which HSPs may act as targets for autoimmune reactions. According to this theory, gastric infection with *H. pylori* activates immune mechanisms due to cross-reacting antibodies of *H. pylori* specific HSP with endothelial HSP of

Figure 1. Prevalence of *H. pylori* and cytotoxin associated gene A, Immunoglobulin G seropositivity in controls and patients.

Figure 3. Distribution pattern of *H. pylori* specific heat shock protein-60 antibody levels in *H. pylori* and cytotoxin associated gene A infection.

Figure 2. Distribution of combination patterns of *H. pylori* and cytotoxin associated gene A seropositivity in controls and patients.

Figure 4. Distribution of *H. pylori* specific heat shock protein-60 antibodies in patients with and without myocardial infarction.

human origin.^{7,15}

Heat shock proteins are a class of intracellularly located functionally related proteins. Their expression is increased in response to environmental stress such as exposure to inflammation, infection and oxidizing agents and they then present on the cell surface.^{16,17} It has been postulated that surface expression of these usually intracellular molecules make them appear like cryptic antigens and the human immune system recognizes them as foreign.¹⁸ The fact that HSPs are highly conserved in all eukaryotes and prokaryotes, led to the hypothesis that immune reactants against microbial HSP may cross-react with homologous host proteins in a form of molecular mimicry.^{3,17} Heat shock proteins are known to be expressed and/or secreted by a range of pathogens including *H. pylori* and by endogenous cells such as macrophages, smooth muscle cells and cells lining the vessel wall.⁶ Among various HSP families, autoimmune responses targeted to HSP-60 play a role in the pathogenesis of atherosclerosis as suggested by several observations that include: i) increased expression of human HSP 60 on endothelial cells in atherosclerotic lesions; and ii) correlation of the severity of CAD with the titers of anti-human HSP 60 antibodies.¹⁸

To our knowledge, so far there have been no reports of such studies being conducted in India even though the prevalence of *H. pylori* infection is quite high.¹⁹ In the present study, the incidence of *H. pylori* seropositivity was quite high (70% in controls versus 74% in patients) and there was no significant difference between the two groups studied. Similar findings have been reported by several other workers.^{20,21} On the other hand, there was a marked difference in the prevalence of cagA positive strains between patients and control subjects. Even after adjustment for confounding factors, the association remained weakly significant (OR 1.64, 95% CI 0.79-3.368). These findings are similar to the observations made by several other authors who have analyzed various studies.^{4,5} Most of these studies have supported an association between *H. pylori* cagA positive strains and ischemic stroke and CAD.

The most notable finding in our study was the strong association between Hp-HSP-60 antibody levels and CAD. Not only were the mean levels high, but also the antibody titers were significantly high in a greater number of patients in comparison to controls. Furthermore, the higher antibody titers correlated strongly with *H. pylori* and cagA seropositivity. The association remained highly significant by multivariate analysis, even after adjustment for all the confounding risk factors (OR 14.03, 95% CI 4.29-45.94, P=0.0001). Another important observation was the detec-

tion of higher titers of Hp-HSP-60 antibodies in patients with MI that clearly indicates the importance of these antibodies in the occurrence of cardiac events. A similar type of a study was conducted by Lenzi *et al.*²² in patients with stable angina who showed a strong correlation between Hp-HSP-60 antibodies and CHD.

Several studies have confirmed the association between high antibody titers against HSP-60 and CAD.^{23,24} Many groups have subsequently verified the atherogenic role of antibodies against HSP-60s.²⁵⁻²⁷ Antibodies against HSP-60s have now been well established as risk factors for CAD prevalence, incidence and mortality.^{7,12}

To date, there have been very few studies conducted on the role of Hp-HSP-60 in atherogenesis and those few have shown a positive association between antibodies to Hp-HSP-60 and CAD.^{7,12,22} In fact, our work highlights the role likely to be played by *H. pylori* infection and the possible underlying mechanism in the development and progression of CAD.

Conclusions

In conclusion, it appears that virulent strains of *H. pylori* have a higher prevalence in patients with CAD and *H. pylori* infection stimulates antibody production against Hp-HSP-60 which may exacerbate the condition by molecular mimicry. However, further prospective studies with larger sample sizes are needed to confirm these findings.

References

1. Fong IW. Emerging relations between infectious diseases and coronary artery disease and atherosclerosis. CMAJ 2000; 163:49-56.
2. Epstein SE. The multiple mechanisms by which infection may contribute to atherosclerosis development and course. Circ Res 2002;90:2-4.
3. Pokley AG. Heat shock proteins, inflammation and cardio-vascular disease. Circ 2002;105:1012-7.
4. Pietroiusti A, Diomed M, Silvestrini M, et al. Cytotoxin-associated gene A positive Helicobacter pylori strains are associated with atherosclerotic stroke. Circ 2002;106: 580-4.
5. Kowalski M, Pawlik M, Konturek JW, Konturek SJ. Helicobacter pylori infection in coronary artery disease. J Physiol Pharmacol 2006;57:101-11.
6. Wick G, Knoflach, Xu Q. Autoimmune and

inflammatory mechanisms in atherosclerosis. Ann Rev Immunol 2004;22:361-403.

7. Ayada K, Yokota K, Kobayashi K, et al. Chronic infections and atherosclerosis. Clin Rev Allerg Immunol 2009;37:44-8.
8. Gobert AP, Bambou JC, Werts C, et al. Helicobacter pylori heat shock protein 60 mediates interleukin-6 production by macrophages via a toll-like-receptor (TLR)-2, TLR-4 and myeloid differentiation factor 88 independent mechanism. J Biol Chem 2004;279:245-50.
9. Andrew SM, Titus JA, Zumstein L. Dialysis and concentration of protein solutions. Current Protocols Immunol 1997;320:A.3 H.1-A.3H.5.
10. Crawther JR. Practical exercises in the ELISA guidebook - 2nd Ed. Totowa, New Jersey: Humana Press Inc; 2009. pp 149-224.
11. Zhang S, Guo Y, Ma Y, Teng Y. Cytotoxin-associated-gene-A seropositive virulent strains of Helicobacter pylori and atherosclerotic diseases: a systematic review. Chin Med J 2008;121:946-51.
12. Okada T, Ayada K, Usui S, et al. Antibodies against heat shock protein 60 derived from Helicobacter pylori: diagnostic implications in cardiovascular disease. J Autoimmune 2007;29:106-15.
13. Pasceri V, Cammarota G, Patti G, et al. association of virulent Helicobacter pylori strains with ischemic heart disease. Circ 1998;97:1675-9.
14. Singh RK, McMahon AD, Patel H, et al. Prospective analysis of the association of infection with cagA bearing strains of Helicobacter pylori and coronary artery disease. Heart 2002;88:43-6.
15. Birnie DH, Holme ER, McKay IC, et al. Association between antibodies to heat shock protein-65 and coronary atherosclerosis: possible mechanisms of action of Helicobacter pylori and other bacterial infections in increasing cardiovascular risk. Eur Heart J 1998;19:387-94.
16. Benjamin V, McMillan DR. Stress (heat shock) proteins: molecular chaperones in cardiovascular biology and disease. Circ Res 1998;83:117-32.
17. Xu Q. Role of heat shock proteins in atherosclerosis. Arterioscler Thromb Vasc Biol 2002;22:1547-59.
18. Epstein SE, Zhu J, Najafi AH, Burnett MS. Insights into the role of infection in atherogenesis and in plaque rupture. Circ 2009;119:3133-41.
19. Chaudhary A, Rajasekhar D, Latheef SA, Subramanian G. Seropositivity of Chlamydia pneumoniae and Helicobacter pylori among coronary heart disease patients and normal individuals in a South Indian population. Indian Heart J 2003; 55:384.

20. Wald NJ, Law MR, Morris JK, Bagnall AM. *Helicobacter pylori* infection and mortality from ischemic heart disease: negative result from a large prospective study. *BMJ* 1997;315:1199-201
21. Folsom AR, Neito JF, Sorlie P, et al. *Helicobacter pylori* seropositivity and coronary heart disease incidence. *Circulation* 1998;98:845-50.
22. Lenzi C, Palazzuoli A, Giordano N, et al. *Helicobacter pylori* infection and systemic antibodies to cagA and heat shock protein 60 in patients with coronary heart disease. *World J Gastroenterol* 2006;48:7815-20.
23. Zhu J, Quyyumi AA, Rott D, et al. Antibodies to human heat shock protein-60 are associated with the presence and severity of coronary artery disease: evidence for an autoimmune component of atherogenesis. *Circ* 2001;103:1071-5.
24. Huittinen T, Leinonen M, Tenkanen L, et al. Autoimmunity to human heat shock protein-60, *Chlamydia pneumoniae* infection and inflammation in predicting coronary risk. *Arterioscler Thromb Vasc Biol* 2002;22:431-7.
25. Zhang X, He M, Cheng L, et al. Elevated heat shock protein-60 levels are associated with higher risk of coronary heart disease in Chinese. *Circ* 2008;118:2687-93.
26. Zhang X, He M, Cheng L, et al. Joint effects of antibody to Heat shock protein-60, hypertension and diabetes on risk of coronary heart disease in Chinese. *Clin Chem* 2008;54:1046-52.
27. Grundtman C, Kreutmayer JB, Almanzar G, et al. Heat shock protein-60 and Immune inflammatory responses in atherosclerosis. *Arterioscler Thromb Vasc Biol* 2011;31:960-8.

Non-commercial use only