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

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Background—Antibodies to mycobacterial heat-shock protein (HSP) 65 have been reported to be associated with carotid artery thickening. We examined whether antibodies to human HSP60 are associated with the risk of coronary artery disease (CAD).

Methods and Results—Blood samples from 391 patients (62% men, mean age 57 years) being evaluated for CAD by coronary angiography were tested for IgG antibodies to human HSP60 by ELISA. We found that 75% of the study subjects had anti-HSP60 antibodies. The prevalence of CAD was increased in seropositive compared with seronegative patients (68% versus 49%, \( P = 0.0009 \)). Mean titers of HSP60 antibodies were higher in CAD patients than in non-CAD patients \( (P = 0.008) \). No association between HSP60 antibodies and infection or inflammation was found. Importantly, HSP60 antibodies were related to disease severity. The prevalence of HSP60 antibodies was 76%, 80%, and 85% in patients with 1-, 2-, and 3-vessel disease, compared with 64% in patients without CAD \( (P \text{ for trend} = 0.003) \). A similar association between increasing antibody titers and number of diseased vessels was also found \( (P = 0.03) \). Significant associations between antibodies to HSP60 and CAD severity persisted after adjustment for traditional risk factors by age, race, sex, smoking, diabetes, hypercholesterolemia, hypertension, and C-reactive protein levels. Adjusted OR for number of vessels diseased was 1.86 (95% CI 1.13 to 3.04).

Conclusions—This is the first study demonstrating a significant association between human HSP60 antibodies and both the presence and severity of CAD. (Circulation. 2001;103:1071-1075.)

Key Words: coronary disease • proteins • immunology

Increasing evidence suggests that autoimmunity contributes to atherogenesis and that heat-shock protein (HSP) may be one of the autoantigenic determinants.1–6 HSPs are highly conserved proteins synthesized in large amounts when cells are exposed to stressful stimuli such as inflammation, infection, and exposure to oxidizing agents. Of note, increased expression of human HSP60 has been observed on endothelial cells,2 macrophages,7 and smooth muscle cells8 in human atherosclerotic lesions. Antibodies to mycobacterial HSP65 have been reported to be associated with carotid artery thickening,4 and in one study with coronary heart disease,6 as determined by clinical assessment.

These associations are compatible with the concept that bacterial infection induces the development of antibodies (such as antibodies against mycobacterial HSP65), which then cross-react with human HSPs that are overexpressed on endothelial cells, thereby provoking an immune contribution to the development of atherosclerosis.9 Although there is extensive sequence homology between microbial and human HSPs, however, recent studies have demonstrated that cross-reactivity is at most only partial.10 These findings therefore raise questions as to whether anti–mycobacterial HSP65 antibodies are involved in immunopathological processes contributing to atherosclerosis or merely reflect other confounders, such as infection, which through multiple nonimmunological mechanisms may exert the primary influence on atherogenic processes.

Thus, previous studies demonstrating an association between atherosclerosis and anti–mycobacterial HSP65 antibodies leave unanswered 2 critical questions: whether there is in fact an immunopathological contribution to atherogenesis caused by infection through the mechanism of molecular mimicry (cross-reacting antibodies) and whether there is a true autoantigenic autoimmune process targeted to human HSP60 that is involved in atherogenesis. In the present study, we attempt to derive additional information about the latter question: whether there is an association between anti–human HSP60 antibodies and the risk of coronary artery disease.
disease (CAD). To increase the sensitivity and specificity of our end point, we assessed the presence or absence of CAD by coronary angiography, which also enabled us to examine the relation between anti-HSP60 antibodies and the severity of CAD.

**Methods**

**Patients**

Three hundred ninety-one individuals, under a National Institutes of Health (NIH) institutional review board-approved protocol, entered the study. The study cohort consisted of individuals with chest pain or with noninvasive tests compatible with myocardial ischemia who were referred for coronary angiography. We defined CAD as angiographic evidence of atherosclerosis (≥50% stenosis of ≥1 major coronary artery by coronary angiography). Patients with significant valvular heart disease or nonatherosclerotic cardiomyopathy were excluded. No patient admitted to the study had had a myocardial infarction within the previous 3 months.

**CAD Risk Factors**

Risk factors for CAD that were analyzed included age, race, male sex, cigarette smoking, diabetes, hypercholesterolemia, hypertension, C-reactive protein (CRP) levels, and seropositive status to human HSP60. Patients were asked to classify their race as white, black, or Asian. A history of past and current cigarette smoking of each patient was obtained. Patients who had stopped smoking ≥20 years ago and who were <30 years of age when they stopped smoking were considered not to have smoking as a risk factor. Patients were considered to have diabetes if they were taking insulin or oral hypoglycemic agents or had previously received such treatment or were currently using dietary modification to control the condition. Patients were considered to have hypercholesterolemia if they had a serum cholesterol value >240 mg/dL (6.2 mmol/L) or were receiving cholesterol-lowering treatment. Patients were considered to have hypertension if they had received the diagnosis with arterial pressure >140/90 mm Hg or were being treated with antihypertensive medications or dietary modification.

**Serum IgG Antibodies to Human HSP60**

Serum samples obtained from all study subjects were frozen at −80°C, and aliquots were thawed for specific tests. For ELISA of anti–human HSP60 IgG antibodies, 96-well microtiter plates were coated with 5 μg/mL recombinant human HSP60 (StressGen Biotechnologies Corp) in 100 μL carb/ bicarb buffer (pH 9.6) per well at 4°C overnight. After washing with wash buffer (Wampole) and blocking with 3% BSA in PBS at room temperature for 3 hours, plates were incubated with 100-μL serum samples diluted in serum diluent (Wampole) to 1 in 50 at room temperature for 1 hour. After a further wash, the plates were incubated with horseradish-peroxidase–conjugated goat anti-human IgG diluted 1 in 10 000 with PBS. After washing, 150 μL chromogen/substrate solution containing tetramethylbenzidine (Wampole) was added to wells. Absorbance at 450 nm was measured after 10 minutes after the stopping solution (Wampole) had been added. After correction for background absorbance, a serum sample was considered positive for antibodies against HSP60 if the optical density exceeded a prospectively defined cutoff value. This cutoff value was calculated from the negative and positive control absorbance values. The positive sample was further diluted to 1:200 and 1:400. The antibody titer was considered to be the dilution at which the positive serum sample was no longer reading positive. The OD value assigned to the antibody titer was that which was read from the 1:50 dilution.

**Detection of Serum CRP**

Serum CRP was measured by fluorescence polarization immunoassay technology (TDxFLEx analyzer, Abbott Laboratories). With this assay, 95% of healthy individuals (n=202) had a CRP level of ≤0.5 mg/dL, and 98% had levels ≤1.0 mg/dL in their sera. The between-run coefficient of variation of this assay (n=31) was 4.3% and 2.2% at mean levels of 1.10 and 2.94 mg/dL, respectively.

**Statistical Analysis**

Categorical data were analyzed by the χ² test or Fisher’s exact test for small samples. All tests were 2-sided. The dichotomous variables indicating the presence and severity of CAD were modeled as a function of other factors by multiple logistic regression. The odds ratio was used as a measure of the presence and severity of CAD in patients with a given risk factor compared with those without that factor or as a multiplicative factor for each unit increase in age or titer of HSP60 antibodies. The covariates considered were age, race, sex, smoking, diabetes, hypercholesterolemia, hypertension, and elevated CRP levels.

**Results**

**Characteristics of Patients**

A total of 391 subjects were studied. Men constituted 62% and whites 73% of the cohort. Their ages ranged from 30 to 82 years (mean 57.5 years, median 58.0 years). There were 248 (63%) with angiographic evidence of CAD. With the exceptions of smoking and hypertension, traditional CAD risk factors (age, male sex, diabetes, and hypercholesterolemia) and elevated CRP levels (≥0.5 mg/dL) were significantly associated with the risk of CAD by both univariate and multivariate analysis (Table 1).

**Anti-HSP60 Antibodies and CAD Risk**

In our study cohort, 292 of 391 (75%) had anti–HSP60 IgG antibodies with titers between 1 in 50 and 1 in ≥400 (mean 138). CAD prevalence was 68% in HSP60 seropositive and 49% in seronegative patients (P=0.0009). Mean titers of HSP60 antibodies were higher in CAD patients than in non-CAD patients (1:153±9.6 versus 1:112±11.5, P=0.008). Elevated titers of HSP60 antibodies were significantly associated with increased risk of CAD (Figure 1).

**Anti-HSP60 Antibodies and CAD Severity**

Importantly, increasing HSP60 antibody titers were related to severity of CAD (Figure 2). In addition, the prevalence of

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**TABLE 1. Association of Presence of CAD With Traditional Risk Factors**

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Univariate OR (95% CI)</th>
<th>P</th>
<th>Multivariate‡ OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (10 y)*</td>
<td>&lt;0.0001</td>
<td>2.6 (2.0–3.3)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Male sex</td>
<td>&lt;0.0001</td>
<td>3.4 (2.0–5.8)</td>
<td>&lt;0.0001</td>
<td></td>
</tr>
<tr>
<td>Race†</td>
<td>0.6165</td>
<td>0.7 (0.4–1.3)</td>
<td>0.2477</td>
<td></td>
</tr>
<tr>
<td>Smoking</td>
<td>0.0129</td>
<td>1.4 (0.8–2.3)</td>
<td>0.2411</td>
<td></td>
</tr>
<tr>
<td>Diabetes</td>
<td>&lt;0.0001</td>
<td>4.1 (1.9–9.2)</td>
<td>0.0005</td>
<td></td>
</tr>
<tr>
<td>↑ Cholesterol</td>
<td>0.0001</td>
<td>2.1 (1.2–3.5)</td>
<td>0.0073</td>
<td></td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.1840</td>
<td>0.7 (0.4–1.2)</td>
<td>0.1837</td>
<td></td>
</tr>
<tr>
<td>↑ CRP (&gt;0.5 mg/dL)</td>
<td>0.0157</td>
<td>1.7 (0.9–3.1)</td>
<td>0.0751</td>
<td></td>
</tr>
</tbody>
</table>

*In 10-year increments of age. †White vs nonwhite. ‡Adjusted covariates: age, sex, race, smoking, diabetes, hypercholesterolemia, hypertension, and elevated CRP levels.
HSP60 antibodies was 76%, 80%, and 85% in patients with 1-, 2-, and 3-vessel disease, compared with 64% in patients without CAD (P<0.003). Significant associations between antibodies to HSP60 and CAD severity persisted after adjustment for traditional CAD risk factors (age, race, sex, smoking, diabetes, hypertension, hypercholesterolemia, and elevated CRP levels). Adjusted odds ratios are shown in Table 2. A similar association between increasing antibody titers and number of diseased vessels was also found (P<0.03). Multiple logistic regression analysis demonstrated that high titers of anti-HSP60 antibodies in CAD patients predict the severity of disease (P<0.05).

**Anti-HSP60 Antibodies and CAD Risk Factors**

The association between HSP60 antibodies and CAD risk factors is presented in Table 3. The presence of anti-HSP60 antibodies was not associated with male sex, race, smoking, hypercholesterolemia, hypertension, or elevated CRP levels. Although HSP60 antibodies were significantly associated with diabetes, the increase of HSP60 antibodies in patients with CAD was independent of diabetes (Table 3). In addition, HSP60 antibodies were significantly associated with age on univariate analysis (mean age 58.3±0.7 years in the seropositive versus 55.1±1.2 years in the seronegative individuals), but not on multivariate analysis.

**Anti-HSP60 Antibodies and Pathogen Infection**

Associations of HSP60 antibodies with pathogen infections, including IgG seropositivity to cytomegalovirus, *Chlamydia pneumoniae*, *Helicobacter pylori*, hepatitis A virus, and herpes simplex virus types 1 and 2, were analyzed. No

### Table 2. Association of Severity of CAD* With CAD Risk Factors and Anti-HSP60 Antibodies

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Univariate P</th>
<th>Multivariate§ OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (10 y)†</td>
<td>&lt;0.0001</td>
<td>2.5 (2.0–3.1)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Male sex</td>
<td>&lt;0.0001</td>
<td>2.9 (1.8–4.4)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Race‡</td>
<td>0.9183</td>
<td>0.8 (0.5–1.3)</td>
<td>0.3445</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.0239</td>
<td>1.2 (0.8–1.8)</td>
<td>0.4661</td>
</tr>
<tr>
<td>Diabetes</td>
<td>&lt;0.0001</td>
<td>1.8 (1.1–3.1)</td>
<td>0.0192</td>
</tr>
<tr>
<td>↑ Cholesterol</td>
<td>0.0001</td>
<td>2.5 (1.6–3.7)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.2250</td>
<td>0.9 (0.6–1.3)</td>
<td>0.4898</td>
</tr>
<tr>
<td>↑ CRP (&gt;0.5 mg/dL)</td>
<td>0.0447</td>
<td>1.4 (0.9–2.3)</td>
<td>0.1477</td>
</tr>
<tr>
<td>HSP60 antibodies</td>
<td>0.0002</td>
<td>1.9 (1.1–3.0)</td>
<td>0.0142</td>
</tr>
</tbody>
</table>

*Increased number of vessels with ≥50% stenosis.
†In 10-year increments.
‡White vs nonwhite.
§Adjusted covariates: age, sex, race, smoking, diabetes, hypercholesterolemia, hypertension, elevated CRP levels, and HSP60 seropositivity.

### Table 3. Association of Presence of Anti-HSP60 Antibodies With Traditional Risk Factors and CAD

<table>
<thead>
<tr>
<th>Risk Factors</th>
<th>Univariate P</th>
<th>Multivariate‡ OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (10 y)*</td>
<td>0.0188</td>
<td>1.2 (0.9–1.5)</td>
<td>0.2061</td>
</tr>
<tr>
<td>Male sex</td>
<td>0.3654</td>
<td>1.0 (0.6–1.7)</td>
<td>0.9584</td>
</tr>
<tr>
<td>Race†</td>
<td>0.1850</td>
<td>0.6 (0.4–1.2)</td>
<td>0.1391</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.1696</td>
<td>1.4 (0.9–2.4)</td>
<td>0.1665</td>
</tr>
<tr>
<td>Diabetes</td>
<td>0.0025</td>
<td>2.6 (1.2–5.4)</td>
<td>0.0147</td>
</tr>
<tr>
<td>↑ Cholesterol</td>
<td>0.2099</td>
<td>1.2 (0.7–2.0)</td>
<td>0.5009</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.9573</td>
<td>0.8 (0.5–1.3)</td>
<td>0.3141</td>
</tr>
<tr>
<td>↑ CRP (&gt;0.5 mg/dL)</td>
<td>0.7054</td>
<td>0.7 (0.5–1.1)</td>
<td>0.0873</td>
</tr>
<tr>
<td>CAD</td>
<td>0.0008</td>
<td>1.9 (1.2–3.0)</td>
<td>0.0097</td>
</tr>
</tbody>
</table>

*In 10-year increments.
†White vs nonwhite.
‡Adjusted covariates: age, sex, race, smoking, diabetes, hypercholesterolemia, hypertension, elevated CRP levels, and CAD.
correlation between either the presence of HSP60 antibodies or the increase of antibody titers and these infectious agents was observed (all \( P > 0.05 \), data not shown).

**Discussion**

Multiple studies suggest that immune responses contribute to the development of atherosclerosis. These include the presence in atherosclerotic lesions of T lymphocytes, macrophages, HLA class II antigen presentation, immunoglobulin, complement, and cytokines that are involved in modulating immune responses. Although the antigens serving as immune targets are unknown, one of the major candidates is HSP. Although HSP is usually considered an intracellular protein, Wick and associates pointed out that the vascular wall is subject to various stresses that induce the expression of HSP60 and that marked overexpression may cause it to act as a “cryptic antigen,” inducing an autoimmune reaction and thereby contributing to the development of atherosclerosis. Furthermore, a possible pathogenic role of HSP in atherogenesis is suggested by observations in experimental models that atherosclerotic lesions can be induced by immunization of animals with mycobacterial HSP65.

Several seroepidemiological studies have been performed to test the hypothesis that HSPs serve as the antigenic stimulus. Studies have demonstrated that antibodies directed at the mycobacterial HSP65 are significantly elevated in subjects with carotid artery thickening and with coronary atherosclerosis. Furthermore, in a 5-year follow-up study, anti–mycobacterial HSP65 antibodies were associated with progressive carotid thickening in individuals with thickening at entry but were not predictive of the development of new lesions. Thus, the conclusion that an immune response to human HSP60 located in the vascular wall contributes to the atherogenic process derives from associations between atherosclerosis and the presence or absence of anti–mycobacterial HSP65 antibodies. The conclusion therefore necessarily depends on the assumption of cross-reactivity—that is, because HSP molecules are highly conserved, anti–mycobacterial HSP65 antibodies should cross-react with human HSP60 in a biologically important manner. If biologically important cross-reactivity exists, then anti–mycobacterial HSP65 antibodies would recognize and interact with human HSP proteins that are postulated to be overexpressed in stressed vascular wall cells, leading to atherogenic changes induced by immunopathological mechanisms.

Recent studies, however, have demonstrated that although some cross-reactivity exists, it is at most only partial. Mayr and coworkers examined cross-reactivity of anti–HSP antibodies against *Escherichia coli* HSP and mycobacterial HSP65. On Western blot, each recognized its human and bacterial HSP, mycobacterial HSP65, and *Chlamydia* HSP60 demonstrated only partial inhibition and therefore only partial cross-reactivity.

Importantly, Prohaszka and coworkers tested the potential for cross-reactivity using a biologically relevant functional assay. They noted that complement activation contributes to the development of atherosclerosis by several mechanisms and examined whether complement can be activated by HSP60 antibodies. They also determined whether such an effect, if it occurred, required the formation of immune complexes with anti-HSP60 antibodies. They found both of these to be the case. Complement was activated by recombinant human HSP60 in a dose-response manner, but not in the absence of antibodies, indicating that activation necessitated an immune complex formation between HSP and its relevant antibodies. Of note, they found that antibodies against human HSP60 and mycobacterial HSP65 differed from each other in their antigen recognition and complement-activating capacity. Whereas homologous HSPs competed strongly for binding of the relevant antibody with its homologous protein, HSP60 only minimally inhibited binding of anti–mycobacterial HSP65 antibody with mycobacterial HSP65, and vice versa. Moreover, whereas there was a highly positive correlation between HSP60-induced complement activation and anti–human HSP60 antibodies, the extent of activation did not correlate with the level of anti–mycobacterial HSP65 antibodies. Similar results regarding the lack of strong cross-reactivity between antibodies recognizing human HSP60 versus mycobacterial HSP65 were reported by Handley et al.

The impetus for the present investigation derived from these considerations. Thus, the correlation between atherosclerosis and anti–mycobacterial HSP65 antibodies may derive from the association of these antibodies with infection, which could be the primary determinant of the correlation. This is suggested by the finding that the correlation of anti–mycobacterial HSP65 antibodies with ultrasound-defined carotid thickening was observed to occur only in older age groups, in which antibodies to various infectious diseases are known to be detected with greater frequency.

The results of our investigation demonstrate that there is a strong correlation between the presence of CAD and anti–human HSP60 antibodies. The mean titers of HSP60 antibodies were significantly higher in CAD patients than in non-CAD patients. A highly significant correlation between severity of CAD and increasing prevalence of HSP60 antibodies was found. The relationship persisted after adjustment for traditional CAD risk factors (age, race, sex, smoking, diabetes, hypercholesterolemia, hypertension, and CRP levels).

No association was found between HSP60 antibodies and antibodies against 6 different infectious agents previously linked to atherosclerosis (cytomegalovirus, *C pneumoniae*, *H pylori*, hepatitis A virus, and herpes simplex virus types 1 and 2). This finding can be interpreted as indicating that although cross-reacting anti-pathogen antibodies may be contributing to an immunopathological component of atherogenesis, an independent association exists between disease and antibodies to human HSP60. The lack of an association between infection and antibodies to human HSP60 suggests that if an association between anti–pathogen and anti–host HSP antibodies does in fact exist, it is masked by other noninfectious inducers of anti–human HSP60 antibodies. The data also are compatible with the concept that bona fide autoreactivity may arise against biochemically altered autologous HSP60.
thereby contributing to a true autoimmune atherogenesis mechanism.

Several caveats should be considered relating to our conclusions. First, the study design of this investigation is cross-sectional and therefore cannot establish causality. It can only establish an association. Hence, any conclusions derived from such a study must be considered preliminary and hypothesis-generating rather than hypothesis-proving. Second, it is possible that our conclusions may be limited to the particular population we studied. Third, our non-CAD control group consisted of individuals who, on clinical evaluation, had some suspicion of CAD. These individuals may not be representative of other individuals without CAD who lack clinical features triggering the decision to perform coronary angiography.

In summary, this is the first study demonstrating a strong association between anti–human HSP60 antibodies and both the presence and severity of CAD. Although not conclusive, these findings are compatible with the concept that autoimmunity plays a role in atherogenesis and that HSP is one of the autoantigenic determinants.

References
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