Serum Immunoglobulin G Antibodies to Chlamydial Heat Shock Protein 60 but Not to Human and Bacterial Homologs Are Associated With Coronary Artery Disease

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Background—Evidence for an association between Chlamydia pneumoniae infection and coronary artery disease (CAD) has been reported by numerous studies, cross-reactive heat shock protein (Hsp) antibody responses have been causally linked to CAD, and the severity of chlamydial disease pathogenesis correlates with Hsp serology. Our aim was to determine if chlamydial Hsp (cHsp) antibody responses are predictive of CAD.

Methods and Results—Patients were recruited in a case-control study: 250 cases had angiographically significant CAD (stenosis ≥70%), and 250 controls had normal coronary arteries (stenosis <10%). Serum immunoglobulin G reactivity to Hsp10 and Hsp60 antigens (chlamydial, Escherichia coli, and human), and C pneumoniae whole organisms were measured by ELISA. Univariate analysis confirmed that classical CAD risk factors were predictors of CAD. Univariate analysis showed that cHsp60 (P=0.001, OR 3.9), cHsp10 (P=0.045, OR 3.8), E coli Hsp60 (P=0.04, OR 1.5) and C pneumoniae (P=0.03, OR 1.8) ELISA optical density (OD) values were significantly different between cases and controls. Multivariate analysis found that only upper-quintile cHsp60 seroreactivity remained a significant predictor of CAD after controlling for classical CAD risk factors and seroreactivity to the other antigens (cHsp60 OD, P=0.005, OR 3.9 per OD unit; cHsp60 quintile, 5 versus 1 to 4; P=0.01, OR 2.1).

Conclusions—The presence of elevated anti-cHsp60 immunoglobulin G antibodies, but not anti-human or anti-E coli homologs, was independently associated with CAD. This finding argues against previous suggestions that cross-reactive or autoimmune Hsp60 responses may contribute to disease progression. High anti-cHsp60 antibody response appears to identify the subset of patients with chlamydial infection and significant CAD. (Circulation. 2002;106:1659-1663.)

Key Words: coronary disease • antibodies • atherosclerosis • proteins

Atherosclerosis is regarded as a chronic inflammatory disease.1 The initiation and development of atherosclerotic lesions include the development of foam cells from macrophages, leading to the deposition of cholesterol-containing low-density lipoproteins (LDL) and the oxidation of lipoproteins at the site of lesion development, which directly contribute to tissue damage.2 Inflammation causes fragility in atheromatous plaques, which may in turn initiate and perpetuate vascular endothelial damage, thereby contributing to both the development and progression of coronary artery disease (CAD).

Although it is known that local and systemic inflammatory processes are stimulated during infections, the theory proposing that infectious agents contribute to lesion development in atherosclerosis is still controversial. It is, however, supported by reports on associations between atherosclerosis and certain persistent bacterial (Chlamydia pneumoniae) and viral (cytomegalovirus) infections.3

Consistent results linking C pneumoniae infection and different stages of atherosclerosis have been obtained from several kinds of studies. Evidence for the association between C pneumoniae and atherosclerosis includes serological findings of immunoglobulin G (IgG) antibodies against C pneumoniae in CAD patient specimens,4 detection of the organism or its components in atherosclerotic plaques from patients at autopsy,5 and the presence of viable organisms in atheromatous lesions.6 Cell culture systems have been used to study how C pneumoniae infection contributes to the pathogenesis of atherosclerosis. Results from studies showing that C pneumoniae infection induces human macrophage foam cell formation when cultured in the presence of LDL and also that C pneumoniae induces cellular oxidation of LDL provide evidence that directly links the organism to events thought to contribute to atherogenesis.7,8 However, because no causal relationship has been conclusively established, it is still not clear whether the organism initiates
Heat shock proteins (Hsps) belong to a family of approximately two dozen proteins whose amino acid sequences are highly homologous between widely divergent species, from bacteria to humans, and function to protect other proteins from denaturation. Because of this high degree of homology, there is a risk of immunological cross-reactions between microorganisms and vascular autoantigens.9 Hsps are usually produced in response to stress, including elevated temperatures, infection, oxygen free radicals, nutrient deprivation, and inflammatory reactions.10 They are also known as molecular chaperones, because their functions include stabilizing and protecting newly synthesized proteins during folding, translocating proteins across membranes, and removal of denatured proteins. Hsps are subdivided into multimeric families on the basis of the molecular weights of the proteins encoded (eg, Hsp10, Hsp60, and Hsp70).

There is some evidence that Hsps may play a role in the pathogenesis of chlamydial infections.11 It has been suggested that the severe sequelae in chlamydial infections may be attributable to an immunopathological response to the chlamydial Hsp60 (cHsp60),12 and Hsp60 can serve as a proinflammatory modulator in a manner similar to that described for LPS.13,14

It is possible that autoimmune and cross-reactive immune responses to host and pathogen Hsps may be induced because of molecular mimicry between chlamydial, human, and other bacterial Hsp epitopes.15,16 We specifically addressed this question by analyzing the immune response to whole C pneumoniae organisms and Hsp from Chlamydia, human, and Escherichia coli in patients with CAD and matched controls.

Data indicate that in multivariate analysis, only the presence of upper-quintile cHsp60 IgG Abs was significantly associated with CAD. Immune responses to the human homolog were found to be unrelated to the subject’s disease status, and immune responses to the E coli homolog were found to be dependent on cHsp60. These findings argue against a cross-reactive or an autoimmune role for Hsp60 and CAD but support the hypothesis that C pneumoniae infection may contribute to disease progression in a subset (~20%) of CAD patients.

Methods

Patient Population

Patients were selected from among those recruited for participation in the catheterization registry of the Intermountain Heart Collaborative Study carried out at the LDS Hospital, Salt Lake City, Utah. The study design was case-control and included subjects of either sex who were older than 18 years of age and had available risk factor information. The case group was composed of 250 patients with significant, angiographically documented CAD (stenosis ≥70%) who became candidates for bypass surgery, percutaneous coronary intervention, or postangiography cardiovascular medical therapy. The control group was composed of 250 subjects with angiographically normal coronaries (stenosis <10%) who received no form of intervention therapy for CAD except for any secondary prevention for known risk factors. Both groups of patients were evaluated by angiography during the same time period. Blood samples were collected in EDTA from each individual and plasma obtained. Samples were stored at -80°C until analysis.

Written consent for a blood draw for use in confidential research studies was obtained from each patient in the study. The study was approved by the Research and Human Rights Committee, LDS Hospital, Salt Lake City, Utah, and the Health Sciences Human Subjects Committee, University of Wisconsin, Madison.

Assessment of Demographic and Cardiovascular Risk Factors

Demographic and cardiovascular risk factor assessments were included in the study to control for their possible confounding influences on each other, as well as their influence on immune responses to Hsps or whole chlamydial organisms. Factors assessed were age, sex, diabetes, family history of early CAD, history of hypertension, history of smoking, and presence of a C-reactive protein (CRP) concentration. Age was analyzed as a continuous variable. Diabetes was defined as a fasting blood sugar >126 mg/dL, glycosylated hemoglobin >7.5%, or patient undergoing anti diabetic therapy. Family history was positive if a first-order relative had suffered cardiovascular death, myocardial infarction (MI), or coronary revascularization before age 65 years. Hyperlipidemia was defined as a history of total cholesterol >180 mg/dL, LDL >130 mg/dL, or use of lipid-lowering therapy. Hypertension was defined as a history of systolic blood pressure >160 mm Hg, diastolic blood pressure >90 mm Hg, or use of antihypertensive therapy. Tobacco use was considered present for subjects who were present smokers or had a past smoking history of >10 pack-years. Testing for CRP was performed using a regular-sensitivity fluorescence polarization immunoassay (Abbott Diagnostics), and CRP concentration was analyzed as a continuous variable.

Assessment of Risk Factors Related to Infection

Antigens

A panel of antigens was tested. E coli Hsp10 and Hsp60 (GroES and GroEL) and human Hsp10 and Hsp60 (recombinant human chaperonin10 [Cpn10] and recombinant human Hsp60) were obtained from Stressgen Biotechnologies Corp (Victoria, BC, Canada). C trachomatis Hsp10 and Hsp60 were purified as previously described,17,18 C pneumoniae whole organisms were from isolate TW183. They were grown in HeLa cells, and the infectious stage of the organism known as elementary bodies (EBs) was harvested as described elsewhere19 and stored at -80°C until use.

ELISA

The ELISA method used was a modification of that previously reported.20 Briefly, Immunolon 2 plates (Dynex Technologies) were coated with 0.5 µg of each antigen in PBS for 48 hours at 4°C. After this period, plates were washed 3 times (with wash buffer containing PBS/0.1% Tween 20) using a Labsystems Wellwash 4 Mk 2 plate washer and then blocked for 90 minutes at 37°C with PBS/3% ovalbumin (grade II)/0.1% Tween 20. Plates were then washed 3 times and incubated for 1 hour at 37°C with a 1:250 dilution of patient sera in PBS/0.1% ovalbumin (grade V)/0.05% Tween 20. After this step, plates were washed 3 times, followed by incubation with alkaline phosphatase–conjugated goat anti-human IgG (Jackson Immunoresearch Laboratories, West Grove, Pa) for 30 minutes at 37°C. Finally, plates were washed 3 times, followed by a rinse with Tris-buffered saline. The substrate, p-nitrophenylphosphate (SigmaFAST tablets; Sigma Chemical Co), was added and incubated for 30 minutes at 37°C. Absorbance was read as optical density (OD) at 405 nm on a Perkin Elmer HTS 7000 Bio Assay Reader. For each serum, the OD value of a PBS-coated well that had no antigen (antigen-blank) was subtracted from the values for all test wells for that antigen. Triplicate blanked test OD values for each antigen were averaged and reported for each patient. Laboratory personnel performing the ELISA test were blinded to clinical information on the patients.

Statistical Analysis

ELISA results of seroreactivity to each antigen were reported as OD from the assay measurements. In instances where the OD values
were below zero in the raw data, the positive value of the lowest result for each antigen was added to every patient result for that antigen to provide an absolute baseline value. Statistical results were equivalent regardless of this minor adjustment. Evaluation of the association of seroreactivity to each antigen to the presence of significant CAD was performed by ANOVA. In cases where the variables were not normally distributed, a natural logarithm transformation was performed before analysis. Multiple variable logistic regression analysis was performed to determine adjusted estimates of risk associated with the infection-related factors using SPSS, v10.0 software. Models were built to include significant (P<0.05), near significant (P<0.10), and confounding variables. ORs and 95% CIs are presented with 2-tailed probability values, designating 0.05 as the critical level of statistical significance.

Results

General Population Characteristics

Overall characteristics of the study population for the classical demographic and cardiovascular risk factors were evaluated for prediction of CAD. Univariate analysis confirmed that known risk factors assessed in the study were significant predictors of CAD (Table 1); these were age (P<0.001), male sex (P<0.001), diabetes (P=0.006), family history of early CAD (P<0.001), hyperlipidemia (P<0.001), hypertension (P<0.001), smoking (P=0.003), and CRP concentration (P=0.045).

Antibody Responses and Risk of CAD

ELISA was used to measure IgG Ab responses (ODs) to chlamydial, human, and E coli antigens. The mean OD values from patients in the case and control groups were compared for each antigen and are reported in Table 2. Univariate analysis of OD values (with ORs per OD unit) showed that cHsp60 (P=0.001), cHsp10 (P=0.045), C pneumoniae EBs (P=0.03), and E coli Hsp60 (P=0.04) ODs were significantly different between cases and controls, but that E coli Hsp10 (P=0.72), hHsp60 (P=0.48), and hHsp10 (P=0.16) were not significant.

Multivariate Analysis for CAD Prediction

It was not possible using univariate analyses to determine if antibody responses associated with CAD were confounding because of cross-reactive responses; therefore, a multivariate analysis was done to adjust the predictive effect of each antigen on CAD diagnosis. We found that in bivariate and multivariate analyses including all antigens tested, cHsp60 retained its statistical significance and predictive power (all P<0.05, all OR>3.1), whereas ODs for cHsp10 (P=0.38, OR 1.9), C pneumoniae EBs (P=0.35, OR 1.4), and E coli Hsp60 (P=0.88, OR 1.04) lost statistical significance and predictive power because of the effect of cHsp60 (Table 3). Additionally, the predictive power of E coli Hsp10 (P=0.21, OR 0.65), hHsp60 (P=0.50, OR 1.7), and hHsp10 (P=0.92, OR 1.2) were also diminished by cHsp60.

In additional multivariate analyses, classical risk factors shown to confound the association of antibody response to CAD were as follows: cHsp10 (age, smoking, and diabetes), C pneumoniae EBs (age), and E coli Hsp60 (age). No confounder of cHsp60 was identified, and when modeled as a continuous variable with adjustments for other classical risk factors, it remained a significant, independent predictor of CAD (P=0.006).

Because cHsp60 was the only antigen that remained associated with disease in a multivariate analysis, we examined cHsp60 responses in more detail. The cHsp60 OD values

<table>
<thead>
<tr>
<th>TABLE 2. Univariate Analysis of IgG Seroreactivity to Hsp Antigens and C pneumoniae in Cases and Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Antigen</td>
</tr>
<tr>
<td>-------------------</td>
</tr>
<tr>
<td>Chlamydia Hsp60</td>
</tr>
<tr>
<td>Chlamydia Hsp10</td>
</tr>
<tr>
<td>C pneumoniae EBs</td>
</tr>
<tr>
<td>E coli Hsp60</td>
</tr>
<tr>
<td>E coli Hsp10</td>
</tr>
<tr>
<td>Human Hsp60</td>
</tr>
<tr>
<td>Human Hsp10</td>
</tr>
</tbody>
</table>

Values are mean OD±SD.
TABLE 3. Multivariate Analysis for the Effect of the Antigens on CAD Prediction

<table>
<thead>
<tr>
<th>Antigen</th>
<th>OR</th>
<th>95% CI</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chlamydial Hsp60</td>
<td>all &gt;3.1</td>
<td>...</td>
<td>all &lt;0.05</td>
</tr>
<tr>
<td>Chlamydial Hsp10</td>
<td>1.9</td>
<td>0.46–7.9</td>
<td>0.38</td>
</tr>
<tr>
<td>C pneumoniae EBs</td>
<td>1.4</td>
<td>0.71–2.6</td>
<td>0.35</td>
</tr>
<tr>
<td>E coli Hsp60</td>
<td>1.04</td>
<td>0.64–1.7</td>
<td>0.88</td>
</tr>
<tr>
<td>E coli Hsp10</td>
<td>0.65</td>
<td>0.33–1.29</td>
<td>0.21</td>
</tr>
<tr>
<td>Human Hsp60</td>
<td>1.7</td>
<td>0.35–8.8</td>
<td>0.5</td>
</tr>
<tr>
<td>Human Hsp10</td>
<td>1.2</td>
<td>0.07–18.9</td>
<td>0.92</td>
</tr>
</tbody>
</table>

TABLE 4. Proportions of CAD Cases and Controls in Each of the Chlamydial Hsp60 OD Quintiles

<table>
<thead>
<tr>
<th>Quintile</th>
<th>OD Values</th>
<th>% Cases</th>
<th>% Controls</th>
<th>OR</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>&lt;0.01266</td>
<td>51.1</td>
<td>48.9</td>
<td>...</td>
<td>...</td>
</tr>
<tr>
<td>2</td>
<td>0.01266–0.07309</td>
<td>50.0</td>
<td>50.0</td>
<td>1.0</td>
<td>0.5–1.7</td>
</tr>
<tr>
<td>3</td>
<td>0.07310–0.1608</td>
<td>59.6</td>
<td>40.4</td>
<td>1.4</td>
<td>0.8–2.5</td>
</tr>
<tr>
<td>4</td>
<td>0.1609–0.2891</td>
<td>55.7</td>
<td>44.3</td>
<td>1.2</td>
<td>0.7–2.1</td>
</tr>
<tr>
<td>5</td>
<td>&gt;0.2891</td>
<td>69.4</td>
<td>30.6</td>
<td>2.2</td>
<td>1.2–3.9</td>
</tr>
</tbody>
</table>

Discussion

Evidence for an association between *C pneumoniae* infection and atherosclerosis has been obtained from numerous studies in which different methods were used for detecting the organism.4–6 Chlamydial Hsp60 seroreactivity has also been previously implicated in the pathogenesis of chlamydial infections and in the development of severe sequelae after infections.11,12,21–25 It has also been directly linked to the pathogenesis of atherosclerosis,14,26 and quite recently a strong correlation between anti-Hsp60 IgG levels and coronary heart disease was reported.27 It has therefore been suggested that Hsps may be the link between *C pneumoniae* infections and atherogenesis. However, because of the high degree of homology between the amino acid sequences of different Hsp60 orthologs, it is possible that antibody response to these molecules could lead to immunological cross-reactivity and autoimmune-mediated damage.28

*C trachomatis*, not *C pneumoniae* Hsp60 antigen, was used for this study. Amino acid sequence comparisons of these molecules are 92% identical, whereas 61% similarity is found between *C pneumoniae* and *E coli* and 50% between *C pneumoniae* and human Hsp60s.

We found that antibodies against chHsp60, chHsp10, *C pneumoniae* EBs, and *E coli* Hsp60 were associated with disease in univariate analysis. However, chHsp10, *C pneumoniae* EBs, and *E coli* Hsp10 antibodies lost statistical significance and predictive power in multivariate analysis between antigens tested and after adjustment for other classical risk factors. Several studies have shown a correlation between *C pneumoniae* antibodies and CAD.4,29 However, the lack of independent serological association of *C pneumoniae* to atherosclerotic risk observed in our study has also been reported in other studies in which different criteria were used for seropositivity.30,31 These results highlight the importance of distinguishing between dependent and independent risk factors by multivariate testing.

Seroreactivity to chHsp60 remained a significant predictor of CAD after correction for possible confounding factors. Other studies have found serum antibodies to Hsp60 from *Chlamydia*, human, and various microorganisms to also be associated with atherosclerosis.27,32–34 Therefore, it has been suggested that immune reactions to bacterial Hsp60 evoke anti-self immune response because of its high sequence homology with human Hsp60. We did observe some correlation between OD values for Hsp antigens from chlamydial, human, and *E coli* homologs. However, because of the independent association of chHsp60 antibodies with CAD found in our study, the results do not support the idea that cross-reactive or autoimmune responses are responsible for the contribution of Hsp to the progression of disease. If the amino acid sequences for chHsp60 epitopes recognized by CAD sera were identical to those from the *E coli* and human Hsp60s, then seroreactivity to those antigens would also be independently associated with CAD.

A recent study looking at antibodies to chHsp60 and the risk of CAD found no association of serum antibody responses with the severity of CAD.35 In that study, the criteria for defining cases and controls was different from our study,
because cases were defined as having ≥50% vessel stenosis and controls included patients with no angiographic signs of CAD as well as those having lesion occupying <50% of the vessel lumen. In our study, the criteria for differentiating cases and controls was stricter and therefore more likely to significantly distinguish the groups.

Our study does not address the question about whether the cHsp60 OD values reflect an immunological response contributing to pathology or whether it represents a secondary phenomenon in the disease process. However, the high predictive power obtained from seroreactivity to cHsp60 we observed suggests that it could be used as a marker for CAD. This will be especially true at high OD values where anti-cHsp60 serology may be used to determine if chlamydial infection contributes to the lesion process. It is possible that patients with elevated cHsp60 ODs (5th quintile) are the ones who would benefit from antibiotic treatment trials and therefore should be the subset considered when evaluating efficacy.

Acknowledgments

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References
