Endothelial Cytotoxicity Mediated by Serum Antibodies to Heat Shock Proteins of *Escherichia coli* and *Chlamydia pneumoniae*

**Immune Reactions to Heat Shock Proteins as a Possible Link Between Infection and Atherosclerosis**

Manuel Mayr, MB; Bernhard Metzler, MD; Stefan Kiechl, MD; Johann Willeit, MD; Georg Schett, MD; Qingbo Xu, MD, PhD; Georg Wick, MD

**Background**—Growing evidence suggests that an immunological reaction against heat shock proteins (HSPs) may be involved in atherogenesis. Because HSPs show a high degree of amino acid sequence homology between different species, from prokaryotes to humans, we investigated the possibility of “antigenic mimicry” caused by an immunological cross-reaction between microorganisms and autoantigens.

**Methods and Results**—Serum antibodies against the *Escherichia coli* HSP (GroEL) and the 60-kDa chlamydial HSP (cHSP60) from subjects with atherosclerosis were purified by use of affinity chromatography. Western blot analyses and competitive ELISAs confirmed the cross-reaction of the eluted antibodies with human HSP60 and the bacterial counterparts. The cytotoxicity of anti-GroEL and anti-cHSP60 antibodies was determined on human endothelial cells labeled with ⁵¹Cr. A significant difference (40% versus 8%) was observed in the specific ⁵¹Cr release of heat-treated (42°C for 30 minutes) and untreated cells, respectively, in the presence of these anti-HSP antibodies and complement. This effect was blocked by addition of 100 μg/mL recombinant GroEL. In addition, seropositivity against specific non-HSP60 *Chlamydia pneumoniae* antigens is more prominent among high–anti-HSP titer sera than low-titer sera.

**Conclusions**—Serum antibodies against HSP65/60 cross-react with human HSP60, cHSP60, and GroEL; correlate with the presence of antibodies to *C pneumoniae* and endotoxin; and mediate endothelial cytotoxicity. These findings suggest that humoral immune reactions to bacterial HSPs, such as cHSP60 and GroEL, may play an important role in the process of vascular endothelial injury, which is believed to be a key event in the pathogenesis of atherosclerosis. *(Circulation. 1999;99:1560-1566.)*

**Key Words:** stress ■ immunology ■ antibodies ■ *C pneumoniae* ■ atherosclerosis

Recent evidence indicates that the first steps of atherosclerosis are inflammatory in nature.¹ The discovery of macrophages, T lymphocytes, dendritic cells, and mast cells in atherosclerotic lesions; the detection of HLA class II antigen expression; and the finding of secretion of several cytokines point to the involvement of immune inflammatory mechanisms in the pathogenesis of atherosclerosis.¹² Furthermore, atherosclerotic lesions contain immunoglobulin deposits and complement, including the lytic “membrane attack complex” C5b-C9, strongly suggesting the involvement of complement activation in atherogenesis.³ Bacterial and viral infections have been implicated as potential initiating factors. Infections are known to increase blood viscosity, cause hypercoagulability, and influence the serum lipid profile. Infections by Gram-negative bacteria, endotoxin may also contribute to endothelial cell production of free radicals, which may oxidize LDL.⁴ With respect to potential infectious causes involved in atherosclerosis development, attention has focused on *Helicobacter pylori*, *Chlamydia pneumoniae*, and cytomegalovirus.⁵

A putative antigen maintaining this inflammatory process in the arterial wall must be ubiquitous and present at young age, explaining the overall prevalence of this disease even in young people.⁶ Heat shock proteins (HSPs) serve as a promising target for inducing an immunological attack on endothelial cells and may constitute the missing link between the incriminated microorganisms and autoimmunity. HSP65/60 is a major antigen recognized during various...
bacterial infections, but stress protein induction also occurs in eukaryotic cells after viral infections. Data from our laboratory have shown that atherosclerotic lesions can be induced in normocholesterolemic rabbits by immunization with recombinant mycobacterial HSP65 (mHSP65) and inhibited by simultaneous immunosuppressive treatment (Dr Metzler, unpublished data, 1998). Interestingly, mHSP65-reactive T cells were enriched in atherosclerotic lesions compared with peripheral blood even in animals that had received a high cholesterol diet without mHSP65 immunization. Furthermore, levels of serum antibodies to mHSP65 were significantly increased in clinically healthy human subjects with carotid atherosclerosis compared with those without lesions, and these increased antibody levels were independent of common risk factors.

Methods

Definition of Clinical and Laboratory Methods

Human sera were derived from the Bruneck Study, a large, population-based study on the epidemiology and origin of atherosclerosis and arterial disease. The Bruneck Study population comprises an age- and sex-stratified random sample of men and women who are 40 to 79 years old (baseline evaluation in 1990). Carotid atherosclerosis was quantified by means of high-resolution duplex ultrasound as extensively described previously. Sera for the current evaluation were drawn as part of the first follow-up study in 1995.

Blood samples were taken from the antecubital vein after subjects had fasted and abstained from smoking for ≥12 hours. Clinic and laboratory parameters were examined by standard methods as described previously. Antibodies against mHSP65 and Escherichia coli lipopolysaccharides (L 2880, Sigma Chemical Co) were determined by ELISAs following an established protocol. Purification of serum anti-HSP antibodies was performed following a standard (NH4)2SO4 procedure and incubated by simultaneous immunosuppressive treatment (Dr Metzler, unpublished data, 1998). Interestingly, mHSP65-reactive T cells were enriched in atherosclerotic lesions compared with peripheral blood even in animals that had received a high cholesterol diet without mHSP65 immunization. Furthermore, levels of serum antibodies to mHSP65 were significantly increased in clinically healthy human subjects with carotid atherosclerosis compared with those without lesions, and these increased antibody levels were independent of common risk factors.

Affinity Chromatography of Anti-HSP Antibodies

Purification of serum anti-HSP antibodies was performed following an established method. Briefly, immunoglobulins of pooled HTS were precipitated by a standard (NH4)2SO4 procedure and incubated in a chromatography column with 2 mL agarose gel beads (Affi-Gel Kit, Biorad) coupled with 3 mg recombinant mHSP65, GroEL, or chHSP60. Specific immunoglobulins were recovered by 20 mmol/L HCl acid elution, pooled, and equilibrated with PBS, pH 7.2. Anti-HSP antibody titers of purified immunoglobulins were similar to original HTS (1:1280), whereas unbound immunoglobulin had no measurable HSP antibody titer (<1:20).

Western Blotting

This procedure was also described previously. Briefly, samples were diluted 1:20 (vol/vol) in sample buffer containing 5% 2-mercaptoethanol, 15% glycerol, 3% SDS, and 0.1 mol/L Tris, pH 6.8, and separated on a 12% polyacrylamide gel under reducing conditions. Blots were probed with anti-HSP antibodies (5 μg/mL), including purified anti-HSP antibodies, HTS, and the monoclonal antibody II-13 (a gift from Dr R.S. Gupta, Hamilton, Canada). Reactions were visualized by an enhanced chemiluminescence detection kit (Amer sham) after incubation with a peroxidase rabbit anti-human or anti-mouse immunoglobulin conjugate.

Endothelial Cytotoxicity Assays

Human umbilical vein endothelial cells (HUVECs) were cultured and identified as described previously. Human aortic endothelial cells were purchased from Cascade Biologics. For antibody-mediated cytotoxicity, endothelial cells were heat stressed at 42°C for 30 minutes to induce HSP60 expression, whereas control cells remained at 37°C. The assay was performed following an established protocol and is described in brief in the legend of Figure 5. Supernatant was analyzed for 51Cr-radioactivity (Amer sham) in a gamma counter (Wallac-Wizzard Automatic Gamma Counter). Both human, including LTS and unbound immunoglobulin fraction, and monoclonal mouse antibodies, including mAb anti-a-actin (catalog No. 1148818, Boehringer, Mannheim, FRG), mAb anti-CD3 (catalog No. M756, Dakopatts), and mAb anti-bHCG 58 prepared in our laboratory, were used as controls in the cytotoxicity tests. Blocking experiments were performed by addition of anti-HSP antibody preincubated with 100 μg/mL soluble recombinant GroEL for 1 hour at room temperature. Specifically released radioactivity was determined by calculation according to the following formula: %Cr release in the presence of antibodies minus spontaneous release divided by maximal release minus spontaneous release. Maximal release was obtained by adding 5% Triton X-100 to the cultures. Spontaneous release was determined in the presence of complement without antibodies and did not exceed 15% of maximal release.

Statistical Analysis

The association between anti-HSP antibody titers (category ≤160 versus ≥1280) and atherosclerosis/cardiovascular disease was assessed by means of unconditional logistic regression analysis. ANOVA was performed when ≥2 groups were compared. Paired Student’s t test was used to assess differences between 2 groups. A value of P<0.05 was considered significant.

Specific Tests for C pneumoniae

IgG antibodies to C pneumoniae were measured by 3 different test systems: SeroCP-IgG (Savoy Diagnostics Ltd), microimmunofluorescence (MIF) antibody kit (Labsystems Oy), and chlamydial IgG recombinant lipopolysaccharide (LPS) ELISA (Medac). The analyses were performed and calculated according to manufacturers’ instructions. In contrast to the former test systems that used elementary bodies, a recombinant chlamydial LPS antigen was used for precoating in the last ELISA kit.

Results

Clinical Characterization of Serum Samples

A previous analysis in the Bruneck Study population has shown that increased antibody titers to HSPs were independent of classic risk factors for atherosclerosis (n=867). On comparison of subjects with antibody titers ≥160 and those with titers ≥1280, the latter group faced a 2- to 3-fold increased risk of prevalent carotid atherosclerosis (unadjusted OR, 2.44; 95% CI, 1.56 to 3.80; P<0.0001; adjusted OR, 2.48; 95% CI, 1.58 to 3.89; P<0.0001). Similar results were obtained when prevalent cardiovascular disease was used as the outcome variable (unadjusted OR, 3.63; 95% CI, 1.66 to 7.94; P=0.0013; adjusted OR, 4.35; 95% CI, 1.93 to 9.84; P=0.0004). In this analysis, cardiovascular disease encompasses myocardial infarction, ischemic stroke, transient ischemic attack, and symptomatic peripheral artery disease.

Forty sera with known high or low anti-mHSP65 titers (HTS ≥1280/LTS ≥160) were selected from this population for the present study. No significant difference in classic risk factors for atherosclerosis was observed between these 2 groups (Table 1). In addition, HSP antibody titers did not show a significant correlation with C-reactive protein and blood granulocyte count.
Cross-Reactivity of Serum Antibodies Against HSPs

The specimens (HTS/LTS) were tested for their reactivity against the E coli HSP60 GroEL and the HSP60 of C trachomatis, which is nearly identical to the HSP of C pneumoniae (amino acid sequence homology, >97%). Comparison of these antibody titers to the anti-mHSP65 titer revealed an overall correlation of >90% for GroEL and 75% for cHSP60. The correlation coefficient between the titers of anti-GroEL and anti-cHSP60 antibodies was 0.7 (Figure 1).

We then purified these polyclonal anti-HSP antibodies from pooled HTS by affinity chromatography. The eluted antibodies were capable of recognizing their human and bacterial counterparts, as demonstrated by Western blot analyses (Figure 2). However, the strongest reaction was consistently found with the HSP of E coli, GroEL. Ovalbumin, free of HSP but with an identical molecular weight of 60 kDa, served as negative control and did not interact with the purified antibodies, although it was still recognized by HTS because of anti-ovalbumin antibodies, as confirmed by ELISA experiments. As positive control, a monoclonal antibody to HSP60 (II-13) stained all types of HSPs but not ovalbumin. The cross-reactivity and specificity of the various purified antibodies were confirmed by competitive ELISA experiments (Figure 3). Complete inhibition was achieved only with the HSP used for antibody purification. For anti-GroEL and anti-mHSP65 antibodies, inhibition with cHSP60 was significantly less effective compared with the bacterial counterparts (P<0.05).

Reactivity of Serum Antibodies to Non-HSP60 Bacterial Antigens

Serum samples were assayed for C pneumoniae IgG antibodies by use of an MIF test and 2 commercial ELISA kits. The purified anti-HSP antibodies did not appear to cross-react

Table 1: Clinical Characterization of Subjects With Low and High Anti-HSP Antibody Titers

<table>
<thead>
<tr>
<th></th>
<th>LTS</th>
<th>HTS</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>65.5±10.7</td>
<td>63.1±10.9</td>
<td>0.58</td>
</tr>
<tr>
<td>Male sex, %</td>
<td>70</td>
<td>70</td>
<td>1.00</td>
</tr>
<tr>
<td>Total cholesterol, mmol/L</td>
<td>6.24±1.16</td>
<td>5.55±0.87</td>
<td>0.11</td>
</tr>
<tr>
<td>LDL cholesterol, mmol/L</td>
<td>4.03±1.00</td>
<td>3.49±0.86</td>
<td>0.15</td>
</tr>
<tr>
<td>HDL cholesterol, mmol/L</td>
<td>1.43±0.32</td>
<td>1.49±0.35</td>
<td>0.68</td>
</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.68±0.19</td>
<td>1.69±0.18</td>
<td>0.89</td>
</tr>
<tr>
<td>Body mass index, kg/m²</td>
<td>24.8±2.7</td>
<td>25.0±2.3</td>
<td>0.88</td>
</tr>
<tr>
<td>Hypertension, %</td>
<td>38</td>
<td>23</td>
<td>0.22</td>
</tr>
<tr>
<td>Smoking, %</td>
<td>30</td>
<td>8</td>
<td>0.32</td>
</tr>
<tr>
<td>Alcohol, g/d</td>
<td>44.0±52.1</td>
<td>55.1±43.4</td>
<td>0.56</td>
</tr>
<tr>
<td>Fasting glucose, mmol/L</td>
<td>5.49±0.60</td>
<td>5.54±0.48</td>
<td>0.85</td>
</tr>
<tr>
<td>Leukocyte count, 10⁹/L</td>
<td>5.9±5.2</td>
<td>5.9±1.7</td>
<td>0.92</td>
</tr>
<tr>
<td>Granulocytes, 10⁹/L</td>
<td>3.3±1.9</td>
<td>3.1±0.8</td>
<td>0.64</td>
</tr>
<tr>
<td>CRP, mg/L</td>
<td>2.6±7.1</td>
<td>2.5±3.1</td>
<td>0.84</td>
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<tr>
<td>ESR, mm/h</td>
<td>6.9±4.5</td>
<td>5.2±2.9</td>
<td>0.27</td>
</tr>
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</table>

Hypertension indicates blood pressure ≥160/95 mm Hg; CRP, C-reactive protein; and ESR, erythrocyte sedimentation rate. Data are mean±SD (n=40) or percentages.

*Statistical analyses were performed using Student’s t test and χ² test. When Bonferroni adjustments were performed to control type I error for multiple comparisons (n=15), the value to be achieved for statistical significance decreased to P<0.0033.
with these specific *C. pneumoniae* antigens. Data from all tests revealed that seropositivity against non-HSP60 *C. pneumoniae* antigens was more prominent among HTS than LTS (Table 2). The seroprevalence of IgG antibodies among LTS was 77% all together, but only 31% within this group were considered highly positive in the SeroCP ELISA system. In contrast, all HTS had detectable IgG antibodies; surprisingly, 77% were also highly reactive against *C. pneumoniae* elementary bodies. The mean optical density for LTS was calculated as 2.07, interpreted as positive. The mean optical density for HTS reached 2.88, considered highly positive. MIF data afforded a quantitative determination of IgG antibody responses in both groups. Sera with undetectable IgG titers (<1:32) occurred more often among LTS, whereas medium- (1:32) and high- (1:128) titer sera were more frequent among HTS. Acute, primary infections with *C. pneumoniae* could be ruled out, because none of the serum samples showed a positive reaction for IgM antibodies after absorption with anti-human IgG. Data of the recombinant chlamydial LPS ELISA correlated well with the MIF test and provided clear-cut results: 54% of LTS were negative but about 55% of HTS reached titers of ≥1:200. In parallel to elevated anti-GroEL antibodies, we also detected a higher antibody prevalence against *E. coli* LPS in HTS (Figure 4). Statistical analysis revealed a significant correlation between anti-LPS antibodies and anti-HSP titers (*r* = 0.59, *P* < 0.001).

### Endothelial Cytotoxicity

All purified anti-HSP antibodies exerted effective complement-mediated endothelial cytotoxicity on stressed HUVECs. A specific dose-dependent release of ⁵¹Cr from heat-treated cells was observed in the presence of anti-GroEL and anti-cHSP60 antibodies, with a dramatic increase after antibody concentrations rose >25 μg/mL (Figure 5). Lysis reached ~70% in stressed endothelial cells, whereas an average of only 10% of total radioactivity was released from unstressed cells in the presence of anti-HSP antibodies. This striking effect on stressed endothelial cells could be blocked by the addition of 100 μg/mL recombinant GroEL (Figure 6). Even the anti-cHSP60 antibody lost its cytotoxic potential after preincubation with GroEL. Unbound immunoglobulins from the affinity chromatographic procedure, LTS, and unrelated monoclonal antibodies evoked no significant cyto-toxic effect. In preliminary experiments, anti-GroEL and

### TABLE 2. Determination of Anti-*C. pneumoniae* IgG Antibodies

<table>
<thead>
<tr>
<th></th>
<th>LTS</th>
<th>HTS</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sero-CP ELISA, %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>Borderline</td>
<td>8</td>
<td>0</td>
</tr>
<tr>
<td>Positive</td>
<td>46</td>
<td>23</td>
</tr>
<tr>
<td>Highly positive</td>
<td>31</td>
<td>77</td>
</tr>
<tr>
<td><strong>MIF Test, %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
<td>8</td>
</tr>
<tr>
<td>1:32</td>
<td>38</td>
<td>69</td>
</tr>
<tr>
<td>1:128</td>
<td>8</td>
<td>23</td>
</tr>
<tr>
<td><strong>rcLPS ELISA, %</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Negative</td>
<td>54</td>
<td>15</td>
</tr>
<tr>
<td>Borderline</td>
<td>15</td>
<td>15</td>
</tr>
<tr>
<td>1:100</td>
<td>8</td>
<td>15</td>
</tr>
<tr>
<td>≥1:200</td>
<td>23</td>
<td>55</td>
</tr>
</tbody>
</table>

Sero-CP indicates serology for *C. pneumoniae*; rcLPS, recombinant LPS. IgG antibodies to *C. pneumoniae* antigens were measured by 3 different test systems. Results show that immune reactions against *C. pneumoniae* were more frequent among HTS than LTS.
anti-cHSP60 antibodies were also able to lyse stressed endothelial cells in the presence of high concentrations of normal human peripheral blood mononuclear cells at an effector-to-target ratio of 1:50 via antibody-dependent cellular cytotoxicity (data not shown). To determine whether such an antibody-mediated cytotoxicity also occurs in arterial endothelial cells, human aortic cells and HUVECs were cultivated and treated in parallel. Although no significant difference in the cytotoxic lysis between both types of endothelial cells was found, anti-mHSP65 antibodies markedly induced 51Cr release from heat-stressed cells (Figure 7). In addition, heat stress treatment at 42°C for 30 minutes did not result in endothelial cell necrosis or apoptosis (data not shown).

Discussion

We previously demonstrated a strong association between sonographically detectable carotid atherosclerosis and high levels of serum antibodies to mHSP65 in the Bruneck Study population (n=867).11 Bacterial and viral infections can induce immune reactions against HSPs, which may serve as targets for autoimmune reactions.15 The anti-mHSP65 antibodies react not only with mHSP65 but also with human HSP60 and the bacterial counterparts of E coli and C pneumoniae. Herein, we demonstrate that several epitopes of bacterial HSPs seem to exhibit cross-reactive antibody binding. These findings could signify a possible involvement of infections in atherogenesis, because the cross-reactive epitopes may contribute to the elevated anti-mHSP65 antibodies in subjects with atherosclerosis. The natural microbial flora may also influence such immune reactions to various degrees, eg, GroEL of E coli. However, competitive ELISAs indicate that some additional specific epitopes must exist.

Antibodies to mHSP65 can be induced by several different mechanisms in vivo. First, infectious agents containing homologous HSP60 proteins could induce an anti–self-immune response through molecular mimicry in susceptible individuals.16 Additionally, vaccination (particularly with heat-inactivated bacteria) may contribute to the development of anti-HSP antibodies. Second, viral infection might result in incorporation of HSP60, or at least of HSP-derived peptides, into the envelope of the budding virus.17 Arising from viral infections, HSP60
could also become immunogenic as a result of structural alteration or posttranslational modification. Third, exposure to endogenous HSPs or mimicry proteins might induce immune reactions against HSP60, or endogenous HSPs might interact with other immunogenic proteins.

Proteins of the HSP60 family were considered to be located intracellularly in mitochondria only, where they facilitate protein translocation and act as chaperones protecting proteins from harmful enzymatic attacks during folding. Accumulating evidence now points to an additional surface location of HSP60 proteins on not only eukaryotic but also prokaryotic cells, such as H. pylori. Immune reactions against HSPs could therefore provide a general basic level of protection and limit the spread of infections by acting as an initial defense mechanism that immediately interacts with microbial invaders. In contrast to this benefit, the risk of autoimmunity must be taken into consideration, because HSPs are also induced, eg, on endothelial cells by other exogenous and endogenous stimuli. The endothelium, as a barrier between blood and underlying cells, is subjected to continuous mechanical stress from blood pressure and acute or chronic injury from toxins, such as LPS and importantly oxidized LDL, a known risk factor in atherosclerosis. These stressors and many others, such as cytokines and fever, all induce or augment HSP production by the arterial endothelium to prevent cell damage and maintain homeostasis in the vessel wall. Temperatures of 42°C, as used in our experiments, are indeed encountered in a setting of infection. They are rarely achieved at the body core but might be reached in an inflamed atherosclerotic lesion. Thus, preexisting antibodies and HSP60-specific T cells could react with overexpressed HSP60 components in atherosclerotic lesions, causing endothelial and macrophage injury and subsequently contributing to the initiation and perpetuation of inflammation.

The in vitro phenomenon of anti-HSP serum antibodies also exerting a weak cytotoxic effect on unstressed endothelial cells may be explained by a basal level of HSP60 expression, particularly because in vitro culture is itself stressful to cells compared with in vivo conditions.

Because persistent chlamydial infections are associated with decreased expression of protective antigens, such as the major outer membrane protein and LPS, but an abundance of the immunopathogenic HSP60, they may contribute to the elevated serum antibody levels against mHSP65 found in patients with clinically asymptomatic carotid atherosclerosis. In fact, our data provide evidence that sera with high titers to mHSP65 are highly reactive against C. pneumoniae. Additionally, cHSP60 synthesis in infected cells is likely to be accompanied by an increase in human HSP60 production, because chlamydial infection extensively stresses host cells. cHSP60 appears to be the key antigen in eliciting a delayed hypersensitivity reaction of the host in blinding trachoma or fallopian tube obstruction, resulting in severe tissue damage from C. trachomatis infections with interesting similarities to atherosclerosis. Immunopathology plays a decisive role in chlamydial disease, but anti-HSP60 antibody-mediated immunity may be a possible general link between infections and atherogenesis.

Acknowledgment

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References


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