Association of Serum Antibodies to Heat-Shock Protein 65 With Carotid Atherosclerosis
Clinical Significance Determined in a Follow-Up Study

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Background—Previous work has proved that increased titers of antibodies against heat-shock protein (hsp) 65 are associated with atherosclerotic lesions independently of other established risk factors. The present follow-up study was designed to further scrutinize the association of hsp antibodies and atherosclerosis and evaluate the possible predictive value of these antibodies for the development and/or progression of lesions in the same population.

Methods and Results—A total of 750 subjects 45 to 74 years old were recruited, and the rate of participation was 93.6%; 58 subjects died between 1990 and 1995. All participants were subjected to determination of serum antibodies against hsp65 and sonography to assess carotid atherosclerotic lesions and evaluate other risk factors, ie, age, sex, body mass index, blood cholesterol, apolipoprotein B, apolipoprotein A, triglycerides, lipoprotein(a), fibrinogen, leukocyte number, antithrombin III, ESR, ferritin, hypertension, smoking, and diabetes mellitus. Our data show that hsp65 antibody titers in the population emerged as highly consistent over a 5-year observation period ($r=0.78$, $P<0.0001$). Titers were significantly elevated in subjects with progressive carotid atherosclerosis and correlated with intima/media thickness. Multiple linear regression analysis documented these associations to be independent of age, sex, and other risk factors. Subanalyses revealed a preferential association of hsp65 antibody titers with advanced lesions (odds ratio, 1.42; 95% CI, 1.02 to 1.98; $P=0.039$). Other risk factors neither confounded nor modified this association. Finally, hsp65 antibody titers significantly predicted the 5-year mortality (hazard ratio, 1.52; 95% CI, 1.14 to 2.03; $P<0.001$).

Conclusions—These findings indicate a sustained existence of anti-hsp65 antibodies in subjects with severe atherosclerosis, which is predictive for mortality. (Circulation. 1999;100:1169-1174.)

Key Words: atherosclerosis ■ antibodies ■ immunology ■ follow-up studies

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stress proteins, or heat-shock proteins (hsp65), belong to a group of ~2 dozen proteins and cognates showing highly homologous sequences between different species, from bacteria to humans. Because of their function, eg, involvement in protein folding and transport crossing intracellular membranes, they have also been named chaperonins.1 In response to stress or injury, including infections, mechanical stress, oxidants, and cytokine stimulation, cells of the arterial wall produce high levels of hsp65 to protect themselves against these unfavorable conditions.2–4 Pathologically, hsp65 may be involved in atherogenesis due to a cross-reaction between the hsp65 of microorganisms and cellular “self” components giving rise to an autoimmune reaction against such hsp65.

See p 1148

Atherosclerosis is largely viewed as a chronic inflammatory disease,6 and cellular and humoral immune reactions are involved in the development of lesions.5,7 The discovery of activated T lymphocytes, dendritic cells, mast cells, and macrophages in atherosclerotic lesions, the detection of HLA class II antigen expression, and the finding of lytic complement complexes support the concept that immune and inflammatory responses play an important role in the pathogenesis of atherosclerosis.5–7 What are the pathogens or (auto)antigens that evoke such responses? A large number of studies have reported on the association of atherosclerosis and certain persistent bacterial and viral infections, including Chlamydia pneumoniae and herpesviruses.8–10 Interestingly, a recent report from Kol et al11 demonstrated that chlamydial hsp60 is present in macrophages of atherosclerotic lesions, because chlamydiae can produce large amounts of hsp60 during chronic, persistent infections and stimulate host cells to induce hsp60. In fact, increased human hsp60 expression on
endothelial cells, macrophages, and smooth muscle cells in human atherosclerotic lesions has been observed. Thus, hsps expressed in the vessel wall may serve as (auto)antigens, resulting in immune reactions. Our previous study demonstrated that serum antibodies to mycobacterial hsp65 were significantly increased in subjects with carotid atherosclerosis, which has subsequently been confirmed by several laboratories. This increased antibody level was independent of other established risk factors, such as hyperlipidemia, smoking, hypertension, diabetes mellitus, and obesity. These serum antibodies cross-react with human hsps60, chlamydial hsp60, and Escherichia coli GroEL; correlate with the presence of antibodies to bacterial endotoxins; and mediate vascular cytotoxicity of stressed endothelial cells. However, previous studies were cross-sectional in design and thus did not permit demonstration of the temporal sequence of high baseline antibody titers and subsequent progression of atherosclerosis. Our follow-up study in this population demonstrated a sustained correlation between serum anti-hsp65 antibodies and carotid atherosclerosis and indicated a predictive value for lesion advancement and mortality, respectively.

Methods

Subjects
Population recruitment was performed as part of the Bruneck Study. The survey area was located in the north of Italy (Bolzano Province). Special features of the study design and protocol have been described in detail previously. In brief, at the 1990 baseline, the study population was recruited as a sex- and age-stratified random sample of all inhabitants of Bruneck 40 to 79 years old such that 125 women and 125 men each from the fifth to eighth decades of age were selected (n=1000). A total of 93.6% of these subjects finally participated, and frozen serum samples for the measurement of hsp65 antibodies (see below) were available from 867. Among these subjects, 58 died between summer 1990 and 1995. At the first reevaluation of the study cohort in 1995, the follow-up rate among survivors was high, at 93% (n=750). All participants gave their informed consent before entering the study.

Assays of Antibodies to hsp65
Blood was obtained between 7 and 10 AM. All subjects were required not to eat breakfast that day. The procedure used for the ELISA of anti-hsp65 antibodies was similar to that described previously. In short, microtiter plates were coated with 1 µg/mL PBS of recombinant mycobacterial hsp65 (StressGen Biotechnologies Co) overnight, incubated with 100 µL human serum diluted in PBS 1 in 10 to 5120. A serum dilution was considered positive if the optical density at 410 nm in PBS 1 in 10 to 5120. A total of 125 women and 125 men each from the fifth to eighth decades of age were selected (n=1000). A total of 93.6% of these subjects finally participated, and frozen serum samples for the measurement of hsp65 antibodies (see below) were available from 867. Among these subjects, 58 died between summer 1990 and 1995. At the first reevaluation of the study cohort in 1995, the follow-up rate among survivors was high, at 93% (n=750). All participants gave their informed consent before entering the study.

Determination of Carotid Atherosclerosis
The ultrasound protocol involves scanning the internal (bulbous and distal segments) and common carotid arteries (proximal and distal segments) on both sides with a 10-MHz imaging probe and a 5-MHz Doppler scan. Atherosclerotic lesions were defined by 2 ultrasound criteria: (1) wall surface (protrusions into the lumen or roughness of the arterial boundary) and (2) wall texture (echogenicity). A sensitive and reproducible atherosclerosis score was calculated by addition of all plaque diameters. The accuracy of this procedure was established previously. Various stages in atherogenesis were differentiated: (1) small atherosclerotic lesions were defined by the occurrence of new (incident) plaques in previously normal vessel segments and (2) advanced atherosclerosis by the progression of preexisting small to medium-sized lesions to vessel stenosis. The latter process was assumed when the relative increase in the plaque diameter exceeded the double measurement error of the method (distal internal carotid artery, 35%; bulbous, 30%; common carotid artery, 20%) and the lumen was obstructed by >40%. Intima/media thickness (IMT) was also documented and was found to be correlated with the atherosclerosis scores (r=0.64) and with 5-year changes in the scores (r=0.48).

Clinical History and Examination
The study protocol included a complete clinical examination with cardiological and neurological priorities. The average number of cigarettes smoked per day and pack-years as a measure of cumulative exposure were noted for each smoker and ex-smoker. Systolic and diastolic blood pressures were taken with a standard mercury sphygmomanometer after ≥10 minutes of rest while the subject was in a sitting position. The values used in the present analysis were means of 3 measurements taken by the same investigator at ~1-hour intervals. Hypertension was defined by a blood pressure ≥160/95 or the current use of antihypertensive drugs. A standardized oral glucose tolerance test (75 g glucose in 10% solution) was performed in all subjects except those with well-established diabetes mellitus. Diabetes mellitus was diagnosed when fasting glucose levels exceeded 7.8 mmol/L (140 mg/dL) and/or a 2-hour value was higher than 11.1 mmol/L (200 mg/dL) (WHO criteria). Body mass index was used as an obesity index. Subjects with inflammatory, neoplastic, and autoimmune diseases (n=85) were identified by an extensive clinical and laboratory screening as described elsewhere.

Other Laboratory Assays
Triglycerides (interassay coefficient of variation [CV], 4.3% to 5.4% for different standards) and total and HDL cholesterol were determined enzymatically (CHOD-PAP and GOD-PAP methods, Merck; CV, 2.2% to 2.4%). Lipoprotein(a) concentrations with ELISA (Immuno; CV, 3.5% to 6.3%), apolipoproteins by a nephelometric fixed-time method (apolipoprotein AI: CV, 5.7%; apolipoprotein B: CV, 2.4%), and serum ferritin with a fluoro- metric assay (CV, 3.9% to 4.9%). LDL cholesterol was calculated with the Friedewald formula and corrected for lipoprotein(a) cholesterol. Fibrinogen was assayed according to the method of Clauss. Erythrocyte sedimentation rate and blood leukocyte count were expressed as mm/h and cells/10/L, respectively.

Statistical Analysis
Strength and type of association between baseline hsp65 antibody titers and 5-year progression of carotid atherosclerosis (changes in the atherosclerosis score, size of lesions, or IMT) were assessed by multivariate linear regression analysis. Antibody titers were normalized by logarithmic transformation. Linear regression models were supplemented by logistic regression analyses that used incident nonstenotic atherosclerosis (early atherosclerosis) or incident stenosis (advanced atherosclerosis) as dichotomized outcome variables. The test procedure based on maximum-likelihood estimators and the accuracy of fit of each model was assessed by the test of Hosmer and Lemeshow. Multivariate logistic regression models were again built with a forward stepwise selection procedure (P values for entry and removal, 0.05 and 0.10). For comparability, ORs given in the tables were calculated for a 1-SD unit of given variables. The test procedure was done with maximum-likelihood estimation. Hazard ratios of 5-year mortality were calculated with Cox models.

Results
More than 85% of the population had antibody titers between 80 and 320, but a few subjects exceeded 1280. Changes of antibody titers during follow-up were unex-
expectedly low (Table 1; r=0.78, P<0.0001). These results suggest that anti-hsp65 antibody titers are a consistent characteristic of given study subjects during a 5-year period.

Table 2 depicts means and proportions of selected demographic characteristics and risk factors according to categories of atherosclerosis progression. The 2 left columns address incident atherosclerosis in subjects without detectable atherosclerosis in 1990, and the 2 right columns focus on incident carotid stenosis in subjects with prevalent atherosclerosis at the 1990 baseline (advanced lesions). P values for differences of risk factor levels across atherosclerosis categories (column 1 versus 2 and 3 versus 4) were adjusted for age and sex (logistic regression analysis) (Table 2). Marked elevation of baseline antibody titers in subjects with incident carotid stenosis, ie, advanced lesions, was found (P<0.01).

To exclude possible effects of other established risk factors on the association of baseline hsp65 antibodies with 5-year progression of atherosclerosis (change in the lesion summing score and IMT), multiple linear regression analyses were fitted with a forward stepwise selection procedure. These models allowed for all variables listed in Table 2, and the findings indicate that increased hsp65 antibody titers are associated with atherosclerosis independently of other risk factors (Table 3). Analyses were virtually unchanged when systolic or diastolic blood pressure was substituted for hypertension (yes versus no). We next attempted to clarify whether anti-hsp65 antibodies preferentially correlated with the development of small or advanced stenotic lesions in the carotid arteries. In the 5-year follow-up, 120 of 453 subjects with no detectable atherosclerosis in 1990 developed atherosclerotic lesions in their carotid arteries. Multiple logistic regression analyses failed to obtain a significant relation between anti-hsp...
TABLE 3. Multiple Linear Regression Analysis of 5-Year Changes of Atherosclerosis Scores (IMT) With Anti-Hsp Antibodies and Other Risk Factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Coefficient (SE)</th>
<th>Standardized Regression Coefficient</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-hsp65 antibodies</td>
<td>−0.0043 (0.1273)</td>
<td>1.00 (0.78–1.27)</td>
<td>0.4729</td>
</tr>
<tr>
<td>Age</td>
<td>0.0498 (0.0130)</td>
<td>1.60 (1.26–2.03)</td>
<td>0.0001</td>
</tr>
<tr>
<td>ApoB</td>
<td>0.0072 (0.0034)</td>
<td>1.33 (1.02–1.75)</td>
<td>0.0427</td>
</tr>
<tr>
<td>Hypertension</td>
<td>0.9922 (0.2674)</td>
<td>2.70 (1.60–4.55)</td>
<td>0.0002</td>
</tr>
<tr>
<td>Lipoprotein(a)</td>
<td>0.0129 (0.0064)</td>
<td>1.26 (1.01–1.57)</td>
<td>0.0426</td>
</tr>
<tr>
<td>Smoking</td>
<td>0.0189 (0.0080)</td>
<td>1.32 (1.05–1.66)</td>
<td>0.0187</td>
</tr>
<tr>
<td>Ferritin</td>
<td>0.0284 (0.0084)</td>
<td>1.40 (1.19–1.90)</td>
<td>0.0007</td>
</tr>
</tbody>
</table>

Regression coefficients and P values were derived from linear regression analyses of changes in the atherosclerosis score (IMT) between 1990 and 1995 on hsp antibodies and other risk factors. The model was fitted with a forward stepwise selection procedure (P values for entry and removal, 0.05 and 0.10) that allowed for all variables given in Table 2, n=750. When subjects with neoplasms, infections, and autoimmune diseases or liver diseases (n=85) were excluded, the regression coefficient (0.1684) for hsp antibodies (P=0.0314) was virtually unchanged.

TABLE 4. Multiple Logistic Regression Analysis of Small Atherosclerotic Lesions With Anti-Hsp Antibodies and Other Risk Factors

<table>
<thead>
<tr>
<th>Variable</th>
<th>Regression Coefficient (SE)</th>
<th>Odds Ratio (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anti-hsp antibodies</td>
<td>−0.0031 (0.1273)</td>
<td>1.00 (0.78–1.27)</td>
<td>0.4729</td>
</tr>
<tr>
<td>Age</td>
<td>0.0498 (0.0130)</td>
<td>1.60 (1.26–2.03)</td>
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<tr>
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<tr>
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Regression coefficient, odds ratio (OR), and 95% CI were derived from forward stepwise logistic regression analysis, which selects variables for inclusion among all those listed in Table 2 in subjects (n=453) without detectable carotid atherosclerosis at the 1990 baseline. ORs were calculated for a 1-SD unit change of given variables.
significant for understanding the possible role of antibodies in the pathogenesis of atherosclerosis and for prognosis of lesion advancement.

A striking finding of the present study is that anti-hsp65 antibody titers were relatively stable over a 5-year period. Circulating antibodies to hsp65 might be induced or maintained by several different mechanisms. First, infection with agents that contain homologous hsps may induce an anti-self response through molecular mimicry in susceptible individuals. Second, the protein could be maintained at higher levels via different mechanisms.

The possible role of circulating hsps antibodies in atherosclerosis may involve an autoimmune reaction to endothelial cells that express high levels of hsps due to stress, such as local infections and mechanical (e.g., hemodynamic) stress. Xu et al. demonstrated that restraint (i.e., psychological stress) or hypertensive agents result in selective hsps induction in rat aortas, supporting the role of high blood pressure in stimulation of hsp expression in the arterial wall. Likewise, Frostegård et al. provided evidence that serum anti-hsp antibodies correlate positively with hypertension, further supporting the effects of altered hemodynamic stress on hsps and anti-hsp antibody inductions. Oxidized LDL, an established risk factor for atherosclerosis, has been demonstrated to stimulate monocytes/macrophages producing hsp60. Cytokines expressed at high levels in atherosclerotic lesions may also stimulate hsp expression in situ. In general, hsps are 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. Evidence points to an additional surface location of hsp60 proteins in endothelial cells. Hsps may also be released from dead cells and evoke inflammatory reactions in the vessel wall.

Preexisting antibodies could react with these surface-exposed or released hsp60 components, causing further endothelial and macrophage injury and perpetuating the progress of atherosclerotic lesions. Thus, immune reactions mediated by anti-hsp antibodies could play an important role in the pathogenesis of atherosclerosis.
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