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

Stress proteins, or heat-shock proteins (hsps), 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 hsps to protect themselves against these unfavorable conditions.[2–4] Pathologically, hsps may be involved in atherogenesis due to a cross-reaction between the hsps of microorganisms and cellular "self" components giving rise to an autoimmune reaction against such hsps.[5]

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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 al[11] 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 hsps. In fact, increased human hsp60 expression on

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endothelial cells, macrophages, and smooth muscle cells in human atherosclerotic lesions has been observed.[12] Thus, hsps expressed in the vessel wall may serve as (auto)antigens, resulting in immune reactions.[5]

Our previous study[13] demonstrated that serum antibodies to mycobacterial hsp65 were significantly increased in subjects with carotid atherosclerosis, which has subsequently been confirmed by several laboratories.[14–17] 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 hsp60, chlamydial hsp60, and *Escherichia coli* GroEL; correlate with the presence of antibodies to bacterial endotoxins; and mediate vascular cytotoxicity of stressed endothelial cells.[18,19] 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.[13,20] 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.[13,20,21] 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.[13] 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.[13] 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 for antibodies against hsp65 if the optical density at 410 nm exceeded 0.400.

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.[20,21] 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.[20] Various stages in atherogenesis were differentiated[21]: (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.[20] 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).[22] 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.[23]

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 fluorometric 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.[24] Erythrocyte sedimentation rate and blood leukocyte count were expressed as mm/h and cells$\times 10^9$/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.[25] 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.[25] Hazard ratios of 5-year mortality were calculated with Cox models.[26]

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-

TABLE 1. Changes in Anti-Hsp Antibody Titers During Follow-Up (1990 vs 1995)*

	1995								
1990	20	40	80	160	320	640	1280	2560	5120
20	4	11	7	4	...	...	...	...	...
40	...	10	20	12	3	...	...	...	...
80	...	15	35	16	3	...	...	...	...
160	...	...	47	149	81	...	...	...	...
320	...	...	1	43	171	4	...	...	...
640	...	...	...	1	65	18	5	...	...
1280	...	...	...	...	...	7	11	3	...
2560	...	...	...	...	...	...	1	1	1
5120	...	...	...	...	...	...	...	...	...

* $r=0.78$, $P<0.0001$, n=750.

pectedly 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 2. Comparison of Anti-Hsp Antibodies and Other Risk Factors in 4 Categories of Atherosclerotic Lesions

	AS^- 1990 (n=453)		AS^+ 1990 (n=297)	
Variable	AS^- 1995 (n=333)	AS^+ 1995 (n=120)	$Stenosis^-$ 1995 (n=215)	$Stenosis^+$ 1995 (n=82)
Anti-hsp65 Ab, titer	260±276	325±601	360±373	501±711†
Age, y	51.8±8.9	57.7±9.6	65.3±9.1	68.5±7.9
Sex, % male	39.6%	57.5%	53.5%	63.4%
BMI, kg/m^2	24.8±3.7	25.2±3.8	25.3±3.9	24.9±4.0
ApoB, mg/dL	115.4±29.3	134.0±57.4‡	122.7±30.4	132.9±44.6*
ApoA, mg/dL	164.9±28.2	161.8±29.4	161.5±28.3	163.7±39.5
Total cholesterol, mg/dL	214.9±36.4	231.7±40.9‡	226.0±41.5	237.0±48.2*
Triglycerides, mg/dL	106.0	123.5*	108.0	126.0*
Lp(a) >32 mg/dL	11.7%	15.8%*	15.3%	26.8%†
Fibrinogen, mg/dL	245.1±52.9	267.6±48.8*	271.3±58.3	294.4±64.4†
ATIII, %	98.5±12.5	99.1±14.3	95.9±13.1	91.7±16.4
ESR, mm/h	10.9±7.4	11.1±7.1	14.4±10.4	18.1±13.9
Ferritin, $\mu g/L$	107.5±110.9	191.0±195.0‡	164.7±178.8	200.5±173.6
Leukocytes, $10^9/L$	6.3±1.6	6.6±1.6	6.4±1.9	6.7±1.5
Systolic BP, mm Hg	137.8±18.1	144.6±20.4*	152.2±21.9	157.8±23.0*
Smoking, %	23.1%	25.8%*	19.1%	36.6%†
Smoking, y	9.3±13.3	15.5±17.3*	15.8±19.9	23.5±22.3*
IGT, %	4.2	12.5*	7.9	20.7†
Diabetes	3.0%	6.7%	7.9%	22.0%‡

HSP-Ab indicates anti-hsp65 antibody titer; BMI, body mass index; apo, apolipoprotein; Lp, lipoprotein; ATIII, antithrombin III; ESR, erythrocyte sedimentation rate; BP, blood pressure; and IGT, impaired glucose tolerance. The left 2 columns focus on incident atherosclerosis (AS) in subjects without plaques on the 1990 baseline, whereas the right 2 columns focus on incident stenosis in subjects with preexisting AS. Values are mean±SD or proportion (%).

* $P<0.05$, † $P<0.01$, ‡ $P<0.001$ (column 1 vs 2 and 3 vs 4).

TABLE 3. Multiple Linear Regression Analysis of 5-Year Changes of Atherosclerosis Scores (IMT) With Anti-Hsp Antibodies and Other Risk Factors

Variable	Regression Coefficient	Standardized Regression Coefficient	*P*
Anti-hsp65 antibodies	1.6375 (0.0145)	0.2516 (0.0597)	0.0353 (0.0385)
Age	0.0537 (0.0110)	0.2516 (0.501)	<0.0001 (<0.0001)
Apolipoprotein B	0.0086 (0.0006)	0.1368 (0.0922)	<0.0001 (0.0013)
Smoking	0.0217 (0.0023)	0.1611 (0.1625)	<0.0001 (<0.0001)
Lipoprotein(a)	0.0088	0.0686	0.0334
Hypertension	0.3025 (0.0626)	0.1761 (0.1174)	0.0863 (0.0001)
Fibrinogen	0.0047	0.1144	0.0015
Ferritin	0.0179 (0.00014)	0.1203 (0.0904)	0.0004 (0.0023)

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.

antibody titers and this particular stage in atherosclerosis (Table 4). Conversely, a total of 82 subjects of 297 with preexisting atherosclerosis developed stenosis (>40%) or showed progression of stenotic disease. In this advanced stage of atherosclerosis, hsp65 antibodies ranked among the strongest independent risk predictors (Table 5). These findings suggest that higher antibody titers are predictive for severe atherosclerosis. Complete risk profiles of early and advanced stages of atherosclerosis are given in Tables 4 and 5. Furthermore, 53 of the 867 subjects died between 1990 and 1995; advanced lesions and higher antibody titers had been detected in 1990 in most of those who died. Survival analysis showed a significant association of baseline antibody levels and mortality after adjustment for age and sex (hazard ratio, 1.52 per 1-SD unit change in hsp65 antibody titers; 95% CI, 1.14 to 2.03; $P<0.001$).

In addition, the association between atherosclerosis progression and hsp65 antibodies applied equally to men and women, smokers and nonsmokers, and various subpopulations defined by the presence or absence of the risk variables mentioned above. In other words, established risk factors did not modify the association between atherosclerosis and hsp65 antibodies.

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

Variable	Regression Coefficient (SE)	Odds Ratio (95% CI)	*P*
Anti-hsp antibodies	−0.0043 (0.1273)	1.00 (0.78–1.27)	0.4729
Age	0.0498 (0.0130)	1.60 (1.26–2.03)	0.0001
ApoB	0.0072 (0.0034)	1.33 (1.02–1.75)	0.0427
Hypertension	0.9922 (0.2674)	2.70 (1.60–4.55)	0.0002
Lipoprotein(a)	0.0129 (0.0064)	1.26 (1.01–1.57)	0.0426
Smoking	0.0189 (0.0080)	1.32 (1.05–1.66)	0.0187
Ferritin	0.0284 (0.0084)	1.40 (1.19–1.90)	0.0007

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.

Discussion

We have previously shown an association of serum anti-hsp65 antibodies to carotid atherosclerosis,[13] and reports from several groups have demonstrated increased antibody titers in patients with arteriosclerosis or coronary heart diseases,[14,17] which declined after angioplasty or myocardial infarction[15,16] because of immune complex formation, with autologous hsp60 released as a consequence of tissue necrosis.[26a] In the present follow-up study, we provide evidence of a sustained elevation of anti-hsp65 antibody titers in subjects with atherosclerosis and demonstrate a predictive value for the progression of atherosclerotic lesions with high risk of mortality. The findings could be

TABLE 5. Multiple Logistic Regression Analysis of Advanced Atherosclerosis With Anti-Hsp Antibodies and Other Risk Factors

Variable	Regression Coefficient (SE)	Odds Ratio (95% CI)	*P*
Anti-hsp antibodies	0.4227 (0.1619)	1.42 (1.02–1.98)	0.0393
Age	0.0396 (0.0192)	1.55 (1.12–2.16)	0.0091
Fibrinogen	0.0059 (0.0026)	1.43 (1.05–1.95)	0.0226
Antithrombin III	−0.0309 (0.0112)	0.64 (0.47–0.88)	0.0057
Lipoprotein(a)	1.1733 (0.3736)	3.23 (1.55–6.72)	0.0017
Smoking	0.7551 (0.1791)	1.86 (1.39–2.48)	<0.0001
Impaired glucose tolerance	1.2987 (0.4228)	3.66 (1.61–8.35)	0.0021
Diabetes	1.678 (0.4408)	5.36 (2.26–12.69)	<0.0001

Regression coefficient, odds ratio, and 95% CI were derived from forward stepwise logistic regression analysis, which selects variables for inclusion among all these listed in Table 2 in subjects (n=297) with preexisting carotid atherosclerosis at the 1990 baseline.

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 hsp60 proteins could induce an anti-self response through molecular mimicry in susceptible individuals.[27] Second, the protein could become immunogenic because of structural alteration or posttranslational modification resulting from oxidation or metabolic alterations.[28] Third, other foreign or self antigens could interact with hsp60 to form immunogenic complexes in which B cells recognize hsp60 and T cells direct their response at the associated antigen.[29] Therefore, circulating anti-hsp antibody titers could be maintained at higher levels via different mechanisms.

The possible role of circulating hsp65 antibodies in atherogenesis may involve an autoimmune reaction to endothelial cells that express high levels of hsps due to stress, such as local infections[9] and mechanical (eg, hemodynamic) stress.[2] Xu et al[30,31] demonstrated that restraint (ie, psychological stress) or hypertensive agents result in selective hsp70 induction in rat aortas, supporting the role of high blood pressure in stimulation of hsp expression in the arterial wall. Likewise, Frostegård et al[32] provided evidence that serum anti-hsp antibodies correlate positively with hypertension, further supporting the effects of altered hemodynamic stress on hsp and anti-hsp antibody inductions. Oxidized LDL, an established risk factor for atherosclerosis, has been demonstrated to stimulate monocytes/macrophages producing hsp60.[33] Cytokines expressed at high levels in atherosclerotic lesions[7] may also stimulate hsp expression in situ. In general, hsp60 proteins 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. Evidence points to an additional surface location of hsp60 proteins in endothelial cells.[34] Hsps may also be released from dead cells and evoke inflammatory reactions in the vessel wall.[26a,35] 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.[5] Thus, immune reactions mediated by anti-hsp antibodies could play an important role in the pathogenesis of atherosclerosis.

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