Serum Soluble Heat Shock Protein 60 Is Elevated in Subjects With Atherosclerosis in a General Population

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Background—Work from our laboratory has proven that increased titers of anti-heat shock protein 60 (HSP60) antibodies are associated with atherosclerosis and that HSP60-reactive T-cells are present in atherosclerotic lesions. Recent studies from others demonstrated that HSP60 directly activates endothelial cells and macrophages.

Methods and Results—To explore the possibility that HSP60 exists in the circulation, where it could exert its functions, we performed a population-based study with 826 subjects aged 40 to 79 years. The following items were measured in all participants: serum soluble HSP60 (sHSP60); anti-*Escherichia coli* lipopolysaccharide; anti-HSP65, anti-*Chlamydia*, and anti-*Helicobacter pylori* antibodies; and a variety of acute phase reactants (C-reactive protein, α_1-antitrypsin, and ceruloplasmin) and markers of systemic inflammation. Carotid atherosclerosis was assessed twice (1990 and 1995), and 15 other risk factors were evaluated. Our data show that levels of sHSP60 were significantly elevated in subjects with prevalent/incident carotid atherosclerosis and that these levels were correlated with common carotid artery intima/media thickness. Multiple logistic regression analysis documented these associations as independent of age, sex, and other risk factors. Interestingly, sHSP60 was also correlated with anti-lipopolysaccharide, anti-*Chlamydia* and anti-HSP60 antibodies, various markers of inflammation, and the presence of chronic infections. The risk of atherosclerosis associated with high sHSP60 levels was amplified when subjects had clinical and/or laboratory evidence of chronic infections.

Conclusions—Our data provide the first evidence of a strong correlation between sHSP60 and atherosclerosis, suggesting that sHSP60 may play important roles in activating vascular cells and the immune system during the development of atherosclerosis. **(*Circulation*. 2000;102:14-20.)**

Key Words: heat shock proteins ■ chaperonin 60 ■ aging ■ infection ■ inflammation ■ atherosclerosis

In response to stress stimuli, including high temperature, mechanical stress, infections, surgical stress, and oxidant and cytokine stimulation, cells produce high levels of heat shock protein (HSP) to protect themselves against these unfavorable conditions.[1,2] HSPs belong to a group of ≈2 dozen proteins and cognates that show highly homologous sequences between different species, from bacteria to humans. They are highly expressed in cardiovascular tissues.[2–4]

Recently, reports from 2 independent groups have demonstrated that both chlamydial and human HSP60 have a cytokine-like activity and induce tumor necrosis factor-α and matrix metalloproteinase production in human and mouse macrophages.[5,6] Interestingly, both chlamydial and human HSP60 induced E-selectin, intercellular adhesion molecule–1 (ICAM-1), and vascular cell adhesion molecule-1 expression and interleukin-6 production in endothelial cells.[7] These findings suggest that HSP60 directly stimulates vascular endothelial cells, leading to an inflammatory response, which contributes to the pathophysiology of atherosclerosis.

Data from our laboratory have shown that atherosclerotic lesions can be induced in normocholesterolemic rabbits by immunization with recombinant mycobacterial HSP65.[8] Levels of serum antibodies to HSP65 were significantly increased in clinically healthy human subjects with carotid atherosclerosis compared with those without lesions. The elevation of antibody levels was independent of age, sex, and other classic risk factors.[9] Because HSPs show a high degree of amino acid homology between different species from prokaryotes to humans,[1–4] an autoimmune reaction may be evoked because of the immunological cross-reaction between microbial and human HSP60.[10]

The question arose regarding whether HSP60, which is normally an intracellular protein, exists in the circulation of healthy individuals, where it could directly contact the arterial

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wall and immune cells. The present study was designed to assess the serum levels of soluble HSP60 (sHSP60) in the general community (Bruneck study) and to investigate its association with the development of carotid atherosclerosis. We had a special focus on potential interactions with chronic infections or inflammation.

Methods

Subjects

Population recruitment was performed as part of the Bruneck Study.[11–14] The survey area is located in the north of Italy (Bolzano province). Special features of the study design and protocol have been described in detail previously.[11–14] In brief, in 1990, the study population was recruited as a sex- and age-stratified random sample of all inhabitants of Bruneck aged 40 to 79 years, such that 125 women and 125 men in each decade, from the 5th to 8th decades of age, were selected (n=1000). A total of 93.6% of these subjects finally participated in the study. Among these subjects, 58 died between the summer of 1990 and 1995. At the first re-evaluation of the study cohort in 1995, the follow-up rate among survivors was high (96.5%; n=826).[15] These 826 subjects were the basis for the current study. All participants gave their informed consent before entering the study.

sHSP60 Assays

sHSP60 was determined as part of the 1995 evaluation.[11] Blood was obtained after subjects had fasted and abstained from smoking overnight.[11] A sandwich enzyme-linked immunosorbent assay (ELISA) was performed using 2 types of monoclonal antibodies against different epitopes of HSP60 (II-13[16] and ML-30[17]). In short, 96-well microtiter plates were coated with 1 μg/mL PBS of the antibody II-13 overnight, washed with PBS, and incubated with 100 μL of human serum diluted in PBS (1:10). After washing, biotin-labeled IgG ML-30 (1 μg/mL) was added to the plates and incubated at room temperature for 1 hour. Streptavidin conjugated with horseradish peroxidase was added and developed with the substrate. Absorbance at 410 nm was measured after 30 minutes. A serum concentration (in ng/mL) of sHSP60 was calculated according to the standard curves obtained by using recombinant human HSP60 (StressGen Biotechnology Co) in the sandwich ELISA.

Chromatography and Western Blot Analysis

Monoclonal antibody II-13 was coupled to supports using the Affi-Gel HZ Immunoaffinity kit (Bio-Rad) according to the manufacturer's instructions. sHSP60 was separately isolated from sera with higher sHSP60 levels from several subjects. Isolated proteins were electrophoresed, transferred to the membrane, probed with the antibody, and visualized with an enhanced chemiluminescent kit (Amersham Co).[18]

Assays of Anti-Lipopolysaccharide, Anti-HSP65, Anti-*Chlamydia,* and Anti-*Helicobacter pylori* Antibodies

The procedure used for determining anti-lipopolysaccharide (LPS) antibodies was similar to that described elsewhere.[19] In short, microtiter plates were coated with 1 μg/mL polylysine in PBS (Sigma) at 37°C for 1 hour; they were then washed and incubated with *Escherichia coli* LPS (Sigma) overnight. After washing, the plates were incubated with 100 μL of human serum diluted in PBS (1:10 to 1:5120). A serum dilution was considered positive for antibodies against LPS if the optical density absorbance at 410 exceeded 0.200. Serum anti-HSP65 antibodies were measured using the ELISA, as described previously.[9] Anti-*Chlamydia* and anti-*H. pylori* antibodies were determined using commercial kits (Medac) according to the manufacturer's instructions.[18]

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.[11–14] Atherosclerotic lesions were defined by the following 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 the addition of all plaque diameters. The accuracy of this procedure was established previously.[11] Incident atherosclerosis was defined as the occurrence of new plaques in previously normal segments (1990 to 1995). Intima/media thickness was also documented, and it was correlated with both the atherosclerosis scores (r=0.64) and the 5-year changes in the scores (r=0.48).

Clinical History and Examination

The average number of cigarettes smoked per day and the pack-years smoked (used as a measure of cumulative exposure) were noted for each smoker and ex-smoker. Regular alcohol consumption was assessed in terms of grams/day. 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 current 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 of 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 >11.1 mmol/L (200 mg/dL) (World Health Organization criteria). The body mass index and waist-to-hip ratio were used as obesity indices. Subjects with chronic infections (n=268) were identified by extensive clinical and laboratory screening, as described elsewhere.[20]

Other Laboratory Assays

Total and HDL cholesterol were determined enzymatically (CHOD-PAP and GOD-PAP method, Merck; coefficient variation [CV], 2.2% to 2.4%), lipoprotein(a) concentrations were determined with ELISA (Immuno; CV, 3.5% to 6.3%), and apolipoproteins were determined with a nephelometric fixed-time method (apolipoprotein AI: CV, 5.7%; apolipoprotein B: CV, 2.4%).[21,22] LDL cholesterol was calculated with the Friedewald formula and corrected for lipoprotein(a) cholesterol.[21] Fibrinogen was assayed according to the method of Clauss.[23] Blood leukocyte counts were expressed as cells$\times 10^9$/L. Endotoxin levels were measured with commercial ELISA kits.[20] Serum-soluble ICAM-1 was determined with commercial ELISA kits (Boehringer Ingelhein Co). All other parameters, including microalbuminuria, α_1-antitrypsin, ceruloplasmin, C-reactive proteins, and antithrombin III, were assessed by standard assays.[20,24]

Statistical Analysis

Strength and type of association between sHSP60 concentration and 5-year progression of carotid atherosclerosis (changes in the atherosclerosis score, size of lesions, or intima/media thickness) were assessed using standard linear regression analysis. Linear regression models were supplemented by logistic regression analyses that used prevalent or incident atherosclerosis as dichotomized outcome variables. The test procedure was based on maximum likelihood estimators, and the accuracy of fit of each model was assessed by the test of Hosmer and Lemeshow.[25] For ease of presentation, all multivariate regression models were adjusted for the same set of established and putative vascular risk factors.

Results

In ≈33% of study subjects (n=281), serum sHSP60 was not detectable; in others, serum levels ranged up to 11 000 ng/mL. Five percent of subjects had concentrations >1000

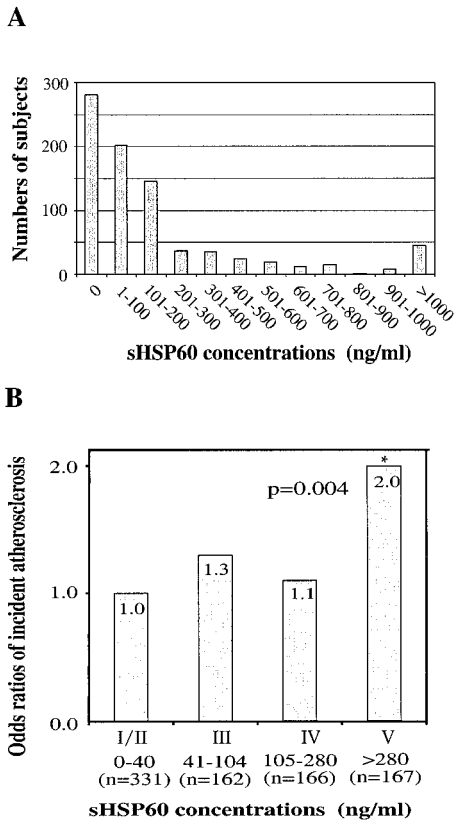

Figure 1. Distribution of sHSP60 in the general community (A) and association with incident carotid atherosclerosis (B). ORs were derived from logistic regression analysis of incident carotid atherosclerosis on quintiles of sHSP60 concentrations, age, sex, and baseline atherosclerosis (reference category, sHSP≤104 ng/mL).

ng/mL; these high concentrations produce a maximal activation of macrophages and endothelial cells in vitro.[5–7] The distribution of sHSP60 levels in the general community of Bruneck is shown in Figure 1A. Western blot analysis revealed that sHSP60 was present in the circulation as 60-kDa proteins (Figure 2).

Table 1 depicts means and SDs of selected demographic characteristics and risk factors according to levels of sHSP60. The probability value for differences in risk factor levels between low sHSP60 (first to fourth quintile) and high sHSP60 (fifth quintile; levels >280 ng/mL) were derived from the *t* test (unadjusted) or from ANOVA (adjusted for age and sex). Data indicate that high sHSP60 was significantly correlated with markers of infection and inflammation, including anti-LPS antibodies, anti-HSP65 and anti-*Chlamydia* antibodies, concentrations of circulating endotoxin, α_1-antitrypsin, C-reactive protein, ceruloplasmin, and the presence of chronic infections, as ascertained by clinical criteria ($P<0.05$ to $P<0.001$); however, high sHSP60 levels were not correlated with classic risk factors for atherosclerosis, except LDL cholesterol (apolipoprotein B) (Table 1).

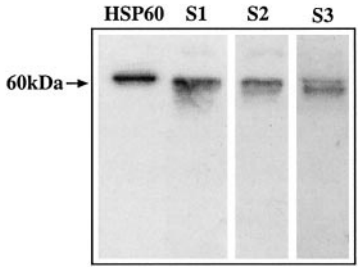

Figure 2. Chromatography and Western blot analysis. Monoclonal antibody II-13 was coupled to supports using an immunoglobulin-coupling kit. sHSP60 was isolated separately from 3 sera with higher sHSP60 levels, as determined by ELISA. Isolated proteins were electrophoresed, transferred to the membrane, probed with the antibody, and visualized with the enhanced chemiluminescent kit. S indicates subject.

Log_e-transformed serum concentrations of sHSP60 showed a significant association with prevalent carotid atherosclerosis (odds ratios [ORs], 1.28 and 1.24 per 1-SD unit increase in sHSP60; $P<0.05$) and the common carotid artery intima/media thickness ($P<0.05$). When sHSP60 levels were subdivided into 5 equally-spaced categories (quintiles), the increase in atherosclerosis risk was mainly confined to the top category (fifth quintile, >280 ng/mL) (Figure 1B). Table 2 depicts the associations between high sHSP60 and the various ultrasound measures of atherosclerosis extent and progression.

Analyses of incident atherosclerosis and changes in the atherosclerosis score over time were performed in light of a long-term intraindividual consistency of sHSP60 levels (n=100; correlation coefficient, $r=0.42$; $P<0.0001$) between sHSP60 levels from 2 independent samplings at a 5-year interval; the coefficient $\kappa=0.44$ indicates substantial accordance in the classification of subjects with respect to their sHSP60 level (quintiles 1 through 4 versus quintile 5). To exclude the possible confounding effects of vascular risk factors, multiple logistic regression models were adjusted for the potential effects of age, sex, smoking, alcohol consumption, hypertension, LDL and HDL cholesterol, ferritin concentrations, microalbuminuria, and hypothyreosis (Table 2). The significant association between sHSP60 and atherosclerosis applied equally to men and women, smokers and nonsmokers, and various age groups. Finally, analyses were virtually unchanged when systolic or diastolic blood pressure was substituted for hypertension (yes versus no) or apolipoproteins (AI and B) for cholesterol fractions (LDL and HDL).

A large number of epidemiological studies reported an association between atherosclerosis and certain persistent bacterial and viral infections, including *Chlamydia pneumoniae* and herpesviruses.[26–28] Surprisingly, we found that anti-*E. coli* LPS antibody titers were negatively correlated with incident carotid atherosclerosis (OR, 0.85 for a 1-titer increase in anti-LPS antibodies; $P=0.03$) (Figure 3), intima/media thickness (regression coefficient, −0.017; $P=0.011$), and 5-year changes in the atherosclerosis score (regression coefficient, −0.076; $P=0.02$) and that these associations were independent of age, sex, and other vascular risk predictors. Notably, low titers of anti-LPS antibodies and high levels of sHSP60 seemed to synergistically affect atherosclerosis risk (Figure 4). ORs of incident atherosclerosis were 5.1 (model adjusted for age, sex, and baseline atherosclerosis) or

TABLE 1. Comparison of Infectious and Inflammatory Markers and Risk Factors for Atherosclerosis Between Low and High Levels of sHSP60

	sHSP60 Level			
Variable	<280 ng/mL	≥280 ng/mL	*P*	Adjusted* *P*
Age	57.8±11.1	58.3±10.9	0.604	NS
Systolic BP, mm Hg	148.1±20.3	149.3±22.3	0.506	NS
Diastolic BP, mm Hg	87.1±9.1	87±9.7	0.924	NS
Hypertension, %	38.1	33.3	0.262	NS
Anti-*E. coli* LPS antibodies	302±261	637±607	<0.001	<0.001
Anti-HSP60 antibodies, titer	254±256	375±312	<0.001	<0.001
Anti-*Chlamydia* IgG, titer	169±277	219±221	0.007	0.004
Anti-*H. pylori* antibodies, titer	63.9±51.2	67.9±49.2	0.356	NS
Microalbuminuria, mg/L	39.7±203.9	23.9±54.5	0.329	NS
Leucocyte count, 10^9 cells/L	6.53±1.7	6.37±1.7	0.285	NS
α_1-Antitrypsin, mg/dL	197.4±36.7	205.3±37.9	0.015	0.038
Ceruloplasmin, mg/dL	26.7±4.9	28.6±6.1	<0.001	0.002
Soluble ICAM-1, ng/mL	326.4±87.6	338.5±112.1	0.156	0.025
High C-reactive proteins, %	6.5	8.6	0.342	NS
Chronic infection, %	30.7	38.9	0.044	0.019
LDL, mg/dL	146.6±36.4	140.1±42.4	0.050	0.013
HDL, mg/dL	58.5±16.4	57.7±15.3	0.543	NS
ApoA, mg/dL	165.6±27.6	163.9±27.3	0.500	NS
ApoB, mg/dL	117.5±31.3	110.3±30.9	0.009	0.024
Lp(a), mg/dL	15.9±18.6	15.4±16.2	0.756	NS
Body mass index, kg/m^2	25.1±3.6	24.8±4.2	0.486	NS
Waist-to-hip ratio	0.93±0.1	0.92±0.1	0.019	NS
Physical activity, score	4.39±1.5	4.25±1.6	0.261	NS
Antithrombin III, %	99.2±11.2	99.4±12.7	0.824	NS
Fibrinogen, mg/dL	289.1±76.3	291.6±75.4	0.697	NS
Fast glucose, mg/dL	102.9±26.4	99.7±14.2	0.136	NS
Diabetes mellitus, %	10.6	7.4	0.221	NS
Endotoxin, pg/mL	22.4±29.0	34.5±51.0	0.003	0.010
Smoking, %	20.6	14.8	0.094	NS
Smoking, pack-years	13.4±27.5	12.9±29.9	0.860	NS
Alcohol consumption, %	54.2%	41.4%	0.009	NS

Values are mean±SD unless otherwise indicated. BP indicates blood pressure; Apo, apolipoprotein; Lp(a), lipoprotein(a); and NS, no significant differences.

*Adjusted for age and sex.

6.6 (multivariate adjustment) when both conditions coexisted in a single individual.

Accumulating evidence supports the view that atherosclerosis is a chronic inflammatory disease and that infection plays an important role in this scenario.[29] In this population-based prospective study, we demonstrated that markers of inflammation (C-reactive protein or soluble ICAM-1) and the presence of chronic infections (as ascertained by clinical criteria) are strongly associated with the development of atherosclerosis in the carotid arteries. The data shown in Figure 5 indicate that the predictive significance of sHSP60 for atherosclerosis progression grows with laboratory and/or clinical evidence of chronic infection/inflammation.

Discussion

Data from our laboratories showed an association between serum anti-HSP65 antibodies and carotid atherosclerosis[9] and the presence of T-lymphocytes specifically responding to HSP60/65 in atherosclerotic lesions.[30] Other groups reported increased antibody titers in patients with atherosclerosis or coronary heart diseases,[31,32] which declined after angioplasty or myocardial infarction.[33,34] Recently, novel findings demonstrated that HSP60, like cytokines, stimulates the release of tumor necrosis factor-α from macrophages and the expression of E-selectin, ICAM-1, and vascular cell adhesion molecule-1 by endothelial cells; this could lead to inflammatory responses in the arterial wall and eventually to the development of atherosclerosis.[5–7] In the present population-

TABLE 2. Association of sHSP60 with Carotid Atherosclerosis

	OR (95% CI)	*P*
Incidence of atherosclerosis (1990 to 1995)		
sHSP60*	1.74 (1.15–2.62)	0.009
sHSP60†	1.92 (1.24–2.98)	0.004
Prevalent atherosclerosis		
sHSP60*	1.58 (1.01–2.50)	0.047
sHSP60†	1.64 (1.04–2.60)	0.034
	Regression Coefficient (95% CI)	
Intima/media thickness		
sHSP60*	0.0034 (0.0000–0.0068)	0.050
sHSP60†	0.0040 (0.0003–0.0077)	0.041
5-Year changes in carotid atherosclerosis		
sHSP60*	0.0249 (0.0033–0.0465)	0.033
sHSP60†	0.0297 (0.0013–0.0581)	0.049

ORs were derived from logistic regression analysis of incident/prevalent carotid atherosclerosis on sHSP60 levels and age/sex (*adjusted for age and sex); in the multivariate† approach, they were adjusted for age/sex, cigarette smoking, hyperthyreosis, hypertension, HDL and LDL cholesterol, microalbuminuria, ferritin, and alcohol consumption. Regression coefficients were derived from corresponding linear regression models.

based study, we provide the first evidence of an elevation of a soluble form of circulating HSP60 in subjects with atherosclerosis. The findings provide solid evidence to explain how HSP60 contacts vascular and immune cells and, thus, could significantly enhance our understanding of the role of HSP60 in the pathobiology of atherosclerosis.

Proteins of the HSP60 family were previously considered to be located intracellularly in the mitochondria, where they facilitate protein translocation and act as chaperones protecting proteins from harmful enzymatic attacks during folding.[1] Accumulating evidence now points to an additional surface location of HSP60 proteins on eukaryotic[35,36] and even prokaryotic cells, such as *H. pylori*.[37]

In the present study, we found that sHSP60 was present in the human circulation, which tempts us to hypothesize about

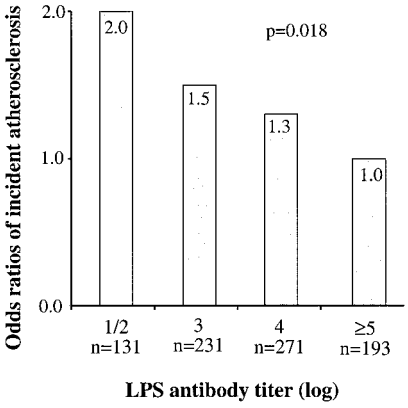

Figure 3. Association of anti-LPS antibody titers with incident carotid atherosclerosis. ORs were derived from logistic regression analysis of incident carotid atherosclerosis on LPS antibody titer (log), age, sex, and baseline atherosclerosis (reference category, titer 5).

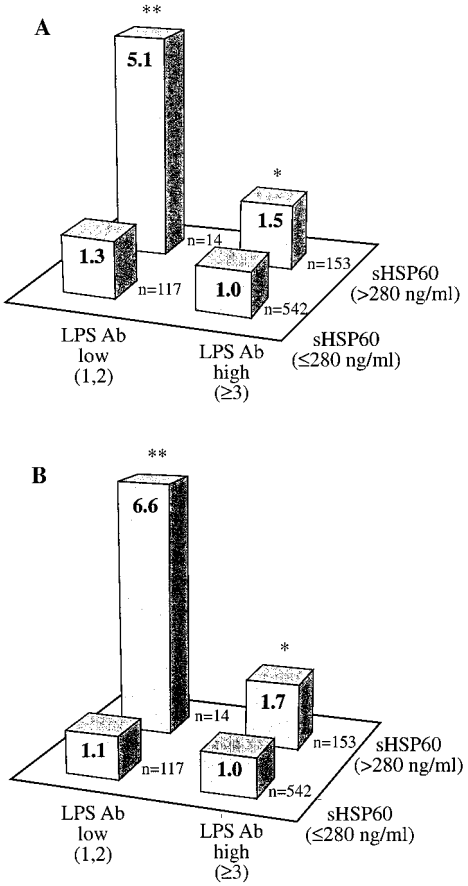

Figure 4. Association of sHSP60 and anti-LPS antibodies with the risk of carotid atherosclerosis. ORs were derived from logistic regression analysis of incident carotid atherosclerosis on LPS antibodies/sHSP60 categories, age, sex, and baseline atherosclerosis (A) plus cigarette smoking, hypothyreosis, hypertension, HDL and LDL cholesterol, microalbuminuria, ferritin, and alcohol consumption (B). *$P<0.05$; **$P<0.01$ (reference category, high anti-LPS antibodies [Ab]/low sHSP60).

various possibilities resulting in HSP60 releases into the blood. First, infectious agents may be the major cause of sHSP60 release from themselves and human cells. For example, during their life cycle, chlamydiae undergo both phases of chronic, persistent, nonlytic infection, in which they remain viable but do not replicate, and phases of lytic

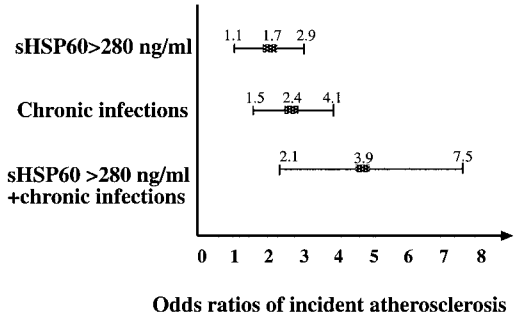

Figure 5. Risk of carotid atherosclerosis according to baseline serum concentrations of sHSP60, chronic infection, and the combination of both parameters. ORs were derived from logistic regression analysis of incident carotid atherosclerosis on sHSP60 categories and chronic infection.

infection.[38] During these lytic phases, the host cells release both their own HSP60, which is produced during the previous chronic phase of infection, and also the chlamydial HSP60, which has been produced in the host cell in response to the infection and to previous noninfectious stimuli.[39] Support for this notion is the fact that sHSP60 levels are significantly correlated with anti-*Chlamydia* antibodies and that both chlamydial and human HSP60s exist at high levels in human atherosclerotic lesions.[5,40] Second, sHSP60 release could be from the dead cells of tissues suffering from chronic inflammation and atheroma, because several observations support the occurrence of cell death within atheroma.[41,42] Our data demonstrate the correlation of sHSP60 concentrations with chronic infection and markers of inflammation. Therefore, HSP60 release into the circulation is an important event because it can then activate vascular cells and the immune system.

The second novel finding of the present study is that anti-LPS antibody titers are negatively correlated with atherosclerosis, which further supports the impact of infections. *E. coli* infections may result in the release of endotoxin-LPS into the circulation. LPS may stimulate the body to produce specific antibodies that will neutralize and remove LPS from the circulation. In fact, Wiedermann et al[20] demonstrated that circulating endotoxin is positively correlated with atherosclerosis in this population. Thus, anti-LPS antibodies may have a protective role for the arterial wall.

In summary, we demonstrated an association between sHSP60 and atherosclerosis in the general population; this association relates to infection and inflammation. The functional role of circulating sHSP60 may involve the direct stimulation of vascular cells and the immune system, thus promoting the progression of atherosclerosis. In addition, sHSP60 could serve as a diagnostic marker for atherosclerosis risk, especially when combined with other infectious and inflammatory markers. Thus, this new approach to arterial risk assessment may be useful in the clinic.

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