Cardiovascular Risk Factors and Atherosclerosis in Young Males

ARMY Study (Atherosclerosis Risk-Factors in Male Youngsters)

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Background—Necropsy studies suggest that atherosclerosis begins in childhood, but in vivo confirmation of this concept is sparse and limited to selected population samples. Furthermore, new risk concepts of atherosclerosis focusing on inflammation, infections, and immunity have not yet been evaluated in this age group.

Methods and Results—This study was conducted in a sample of 141 17- to 18-year-old white males homogenous in age and sex. In addition to classic risk factors, C-reactive protein and the humoral and cellular immune reactivity to heat-shock proteins (HSPs) were assessed. Intima-media thickness (IMT) was quantified at 4 vessel segments of the carotid and femoral arteries. High IMT was considered to be present if the IMT of at least 1 vessel segment exceeded the 90th percentile. In a multivariate logistic regression analysis, cigarette smoking, high diastolic blood pressure, prominent immune reactivity to human and/or mycobacterial HSP60s, alcohol consumption (inverse), and low HDL cholesterol levels were all associated with high IMT. The prevalence of high IMT substantially increased from 0 to 60% when the number of risk conditions in a single individual increased from 0 to 4 (P < 0.001 for linear trend).

Conclusions—Our study supports the concept that atherosclerosis begins in the first decades of life and suggests a role of the immune system, especially immunoreactivity against HSP60s, in atherosclerosis of young individuals. (Circulation. 2003;108:1064-1069.)

Key Words: aging | atherosclerosis | immune system | lymphocytes

In recent years, it has become apparent that atherosclerosis is not a paradigmatic disease of the elderly, but starts early in life, presumably during childhood. Two necropsy evaluations, the PDAY study and the Bogulosa Heart Study, demonstrated a high prevalence of atherosclerotic lesions in individuals from 2 to 39 years old and provided evidence that classic vascular risk factors are relevant to these initial stages of vessel pathology.1-3

Advances in high-resolution B-mode ultrasound techniques have allowed noninvasive assessment of structural atherosclerotic lesions in a young population. The intima-media thickness (IMT) is the most commonly used and best validated ultrasound measure of early and intermediate stages of atherosclerosis.4 It is a powerful predictor of vascular diseases5 and permits comprehensive estimation of systemic vessel pathology if obtained in various vascular beds.6 Thus far, IMT measurements have been extensively used in studies of atherosclerosis risk factors in adults, but similar evaluations in young individuals are sparse and limited to selected populations, such as offspring of patients with premature myocardial infarction,7 heart transplant recipients,8,9 or young adults with familial hypercholesterolemia.10

Atherosclerosis research has recently been enriched by new pathogenetic concepts targeting inflammatory and infectious risk conditions, and examining the potential role of the immune system in atherogenesis. We have proposed that (chronic) infections induce antibodies to microbial heat-shock protein (HSP), which cross-react with human HSP60 (molecular mimicry), expressed on stressed endothelial cells, as well as biochemically altered HSP60 (bona fide autoimmunity). This process triggers vascular inflammation and endothelial damage, implicit in the development of atherosclerotic lesions.11,12

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The present study was designed to evaluate the relation of traditional vascular risk factors, markers of inflammation, and levels of humoral and cellular immune reactivity to HSP60s with carotid and femoral artery IMT in a population of male youngsters.

Methods

Subjects
In Austria, every male citizen undergoes a thorough physical examination by experienced medical personnel to assess physical fitness for recruitment into the Austrian army in the year he turns 18, except for those suffering from chronic diseases (e.g., diabetes) or permanent disabilities (<1.5%). In the study period between January and June 2001, the first 6 volunteers among those who registered at the recruiting office in Innsbruck on randomly selected on Mondays or Tuesdays were included in our study. A total of 159 individuals agreed to participate and were subjected to a B-mode ultrasound study of the carotid and femoral arteries and a variety of additional examinations on the day they were dismissed from the recruiting office. Data assessment was incomplete in 18 participants, which left 141 subjects for the current analysis. All participants were informed about the purposes and scope of the study, which was approved by the local Ethics Committee, and signed appropriate consent forms.

Assessment of Vascular Risk Factors
Assessment of candidate risk factors was performed according to standardized protocols as validated and used previously in the Bruneck Study. It consisted of a clinical history, an additional physical examination, and detailed questionnaires on risk behaviors.

The average number of cigarettes smoked per day was noted for each smoker and assessed by a standardized interview. Subjects were categorized as "smokers" if they reported regular consumption of at least 1 cigarette per week and a lifetime consumption ≥40 cigarettes. Subjects were instructed to indicate their customary alcohol consumption (frequency, amount, and type of alcohol). Average alcohol consumption was quantified in terms of grams per day.

Blood pressure was calculated as a mean of 3 independent measurements taken on the seated subject after at least 5 minutes rest. Hypertension was defined as a systolic blood pressure ≥140 and/or diastolic blood pressure ≥90 mm Hg. No regular use of antihypertensive or other types of drugs was reported. Social status was deduced from the occupation of the parents, ie, rated 1 for an unskilled worker, 2 for a skilled or supervisory worker, or 3 for academics. The family history was considered positive if at least 1 first- or second-degree relative had suffered a stroke or myocardial infarction before the age of 60 years. The gingival sulci of 6 index teeth were gently probed to provide the papillary bleeding index for gingivitis, as described.

Height, weight, and lung function (Flowscan V2.0 spirometer, Jaeger) were assessed as part of military medical examination.

Laboratory Methods
Blood samples were drawn during the recruiting procedure after an overnight fast and triglycerides, glucose, total, and HDL cholesterol were determined by standard colorimetric assays and high-sensitivity C-reactive protein with a latex-enhanced immunologic assay (Roche). An enzyme-linked immunosorbent assay was used to determine antibody titers to mycobacterial HSP60, as described previously.

The use of peripheral blood mononuclear cell (PBMC) proliferation assays to determine T-lymphocyte reactivity to different antigens in vitro has been detailed before. A total of 10^9 PBMCs were separated by density gradient centrifugation and cultured for 7 days in 1640 RPMI (Sigma) with 10% autologous plasma, alone or after addition of 10 µg/mL recombinant mycobacterial HSP60 (1500 Endotoxin Units/mg; obtained from the EC-sponsored facility on HSP-reagents [project BMWH-CT98-3935]) or 20 µg/mL recombinant human HSP60 (100 Endotoxin Units/mg), both prepared and standardized in our laboratories.

Phytohemagglutinin (1 µg/mL) and concanavalin A (3 µg/mL), both purchased from Sigma, served as positive controls. Proliferation was assessed by [3H]thymidine incorporation. Results were expressed as stimulation indices: [(counts per minute in presence of the antigen) – (counts per minute in absence of the antigen)]/(counts per minute in absence of the antigen).

Because 2 preparations of human HSP60 with slightly different capacities to stimulate lymphocyte proliferation were used in the course of the study, standardized human HSP60 stimulation indices (computed by subtraction of the mean and division by the SD in both groups) were applied in all calculations.

High-Resolution Ultrasound Studies
The ultrasound protocol involved scanning of the internal carotid artery, carotid bulb, common carotid artery, and superficial femoral artery on both sides with a 5- to 8-MHz broadband linear transducer on a HDI 3000 (ATL, Bothell). All scans were performed by the same sonographer using different scanning angles (anterior and posterolateral) to identify the greatest wall thickness. Longitudinal images directed through the center of the artery were taken at each vessel site. Measurements were made from stored digital images by an experienced reader. The IMT was assessed at the far wall as the distance between the interface of the lumen and intima and the interface between the media and adventitia. The maximal IMT was recorded at each of the 4 vessel segments and averaged for the left and right sides. High IMT was considered to be present when at least 1 segment-specific IMT exceeded the 90th percentile (Figure 1). This classification was found to be highly reproducible (Kappa coefficient 0.87) when applied to 2 independent assessments of IMT in a group of 100 individuals. This calculation was performed using data from a study of similar design, the Bruneck Study.

Statistical Analysis
Differences in the means of anthropometric variables, vascular risk attributes, and markers of inflammation and immunity in subjects with and without high IMT were analyzed with the Student t test (χ² or Fisher exact test for proportions). Triglyceride and C-reactive protein levels were converted to logarithmic values to approximate a gaussian distribution, and comparisons were made between sets of log-transformed data. The association of candidate risk factors with high IMT was examined by logistic regression analysis, with the test procedure determined by the maximum likelihood estimators. The model was selected by a stepwise strategy applied to all variables given in Table 1 with a P<0.10 (entry criterion) and all variables that are well-established risk factors in adults irrespective of the probability value. To further evaluate the type of association, continuous variables were subdivided into categories (usually quartiles) and modeled with indicator variables in separate analyses. Trends were estimated by visual inspection of plots of the logit against midpoints of variable categories and by the application of orthogonal polynomials.

Results
Results of the ultrasound studies, including segment-specific IMT means and distributions, are depicted in Figure 1. A total of 41 subjects met the criteria of high IMT. Clinical characteristics of the study population and comparison of variable levels between subjects with and without high IMT are shown in Table 1. Significant or near significant differences were observed for HDL-cholesterol levels, cigarette smoking, maximum expiratory flow at 50% of the vital capacity (MEF50), anti-mycobacterial HSP60 antibody titers, and human and mycobacterial HSP60 stimulation index.

Measurements of humoral and cellular immunity to HSP60s were not correlated with each other (Spearman rank correlation coefficient −0.01 [anti-mycobacterial HSP60 antibody and human HSP stimulation index] and −0.02 [anti-
Mycobacterial HSP60 antibody and mycobacterial HSP60 stimulation index).

Results of the stepwise logistic regression analysis are summarized in Table 2. The multivariate risk profile was composed of traditional risk conditions, such as cigarette smoking, low HDL-cholesterol levels, and high diastolic blood pressure and of enhanced immune reactivity to HSP60. In supplementary logistic regression analyses applying categorized variables, the risk of high IMT increased across variable quartiles, indicating a dose-response type of relation, which was confirmed by the use of orthogonal polynomials (linear component $P<0.05$ for each variable). For example, the hHSP-SI odds ratios (95% CI) for high IMT in quartiles 1 to 4 amounted to 1.0, 1.4 (0.4 to 5.2), 2.6 (0.8 to 8.1), and 5.1 (1.4 to 18.2) (for linear trend $P<0.01$). Corresponding data for cigarette smoking were as follows: nonsmokers ($n=64$) 1.0; 1 to 9 cigarettes per day ($n=29$) 2.7 (0.8 to 9.0); 10 to 19 cigarettes ($n=27$) 3.6 (1.1 to 12.4); and $\geq$20 cigarettes ($n=21$) 5.0 (1.4 to 17.0) (for linear trend $P<0.01$).

Next, we defined a risk profile with 4 individual risk components, as follows: smoking, low to medium HDL cholesterol levels (quartiles 1 to 3), moderate to high diastolic blood pressure (quartiles 2 to 4), and high anti-mycobacterial HSP60 antibody titers and/or high human HSP60 stimulation index (higher half each). In this risk model, we did not include MEF50 because of the unclear nature of this association, nor alcohol consumption because of the associated health hazards. As shown in Figure 2, there was a consistent trend toward a higher prevalence of high IMT with an increasing number of risk conditions clustering in a single individual ($\chi^2$ test for linear trend $P<0.001$).

Discussion

The PDAY study and the Bogulosa Heart Study, 2 large necropsy investigations, provided the first convincing evidence that atherosclerotic transformation of arteries begins in childhood and that the presence of incipient plaques is associated with classic vascular risk factors as ascertained postmortem or antemortem in an ongoing (since 1970) survey of children and young adults, respectively. Our study confirms and extends these fundamental observations in several respects, as follows.

(1) It provides in vivo confirmation of necropsy data. High IMT, an accepted surrogate marker of atherosclerosis, was associated with traditional vascular risk factors such as low HDL-cholesterol levels, high diastolic blood pressure, and cigarette smoking (Table 2). Diastolic blood pressure emerged as a significant risk predictor only after multivariate adjustment because of its correlation with hHSP-SI and alcohol consumption.

These findings correspond well with the results of previous ultrasound evaluations and also with investigations on arterial distensibility, an indicator of disturbed arterial physiology with an assumed relevance to atherosclerosis. In most evaluations, at least 1 component of blood pressure and lipid status was significantly associated with the presence of premature atherosclerosis. Low LDL-cholesterol levels were correlated with increased IMT in a sample of high school students aged 13 to 18 years old and in PDAY subjects aged 25 to 34 years, whereas in other studies, LDL, non-HDL, and/or total cholesterol qualified as significant risk predictors.
Lack of an association of between LDL cholesterol and IMT in our study may be explained by the fact that in a majority of subjects LDL levels were low and beneath the limits predictive of atherosclerosis in adults. 

HDL cholesterol values in our study group are clearly lower than those usually measured in children. This may be explained by observations from the Bogalusa Heart Study demonstrating a marked drop in HDL cholesterol at puberty with lowest levels reached around an age of 20. 

In addition to classic risk conditions, low MEF50, a parameter of lung capacity and pulmonary obstructive disease, was related to high IMT in our study, although the reason for this association is not immediately apparent. It is possible that low MEF50 serves as a marker of poor physical fitness, chronic respiratory infections, or increased susceptibility to the adverse effects of cigarette smoke. The consumption of low amounts of alcohol was inversely related to high IMT, as is well established in adults. Given the overrepresentation of smokers among those reporting alcohol consumption, this latter association gained significance only after adjustment for smoking.

(2) Unlike the necropsy and few previous ultrasound evaluations in young individuals, most of which were conducted in highly selected populations, our study population was clinically healthy and homogenous in age (17 to 18 years) as well as sex, which rules confounding by these variables as relevant to other studies.

(3) The present study focused on new pathogenetic concepts that suggested a role of inflammation and the immune system in atherogenesis. We previously described the possible significance of HSPs as an autoantigen in vascular pathology. HSP60 is expressed on the surface of stressed, but not normal, endothelial cells, and may act as a target epitope for the attack of anti-HSP antibodies stimulated by infections and other stress conditions. There is a prominent crossreactivity between microbial and human HSPs.
TABLE 2. Stepwise Logistic Regression Analysis of High IMT on Potential Vascular Risk Factors

<table>
<thead>
<tr>
<th>Variables</th>
<th>OR (95% CI)</th>
<th>P</th>
<th>Step of Entry</th>
</tr>
</thead>
<tbody>
<tr>
<td>hHSP60 stimulation index*</td>
<td>2.18 (1.32–3.60)</td>
<td>0.0023</td>
<td>1</td>
</tr>
<tr>
<td>MEF50</td>
<td>0.52 (0.33–0.82)</td>
<td>0.0047</td>
<td>2</td>
</tr>
<tr>
<td>Smoking</td>
<td>3.58 (1.34–9.54)</td>
<td>0.0108</td>
<td>3</td>
</tr>
<tr>
<td>HDL cholesterol</td>
<td>0.56 (0.36–0.89)</td>
<td>0.0144</td>
<td>4</td>
</tr>
<tr>
<td>anti-mHSP60 antibody titer</td>
<td>1.52 (1.00–2.31)</td>
<td>0.0514</td>
<td>5</td>
</tr>
<tr>
<td>Alcohol consumption</td>
<td>0.51 (0.30–0.87)</td>
<td>0.0133</td>
<td>6</td>
</tr>
<tr>
<td>Diastolic blood pressure</td>
<td>1.61 (1.03–2.52)</td>
<td>0.0374</td>
<td>7</td>
</tr>
</tbody>
</table>

ORs and 95% CIs were derived from stepwise logistic regression analysis applied to all variables listed in Table 1. ORs were calculated for a 1-SD unit change in given variables. Pooled SDs were used (n=141, Table 1).

*Of both cellular stimulation indices, only the human one was chosen because it was more strongly related to high IMT, and the human and mycobacterial HSP60 stimulation indices were highly correlated (Spearman rank correlation coefficient = 0.31). If the human HSP60 stimulation index was not allowed to enter the model, the OR (95% CI) for the mycobacterial HSP60 stimulation index amounted to 1.54 (0.99–2.41) and the set of variables selected was essentially the same.

(Spearman rank correlation coefficient = 0.31) due to high structural homology (molecular mimicry). Previous evaluations documented a significant relation between antimycobacterial HSP60 antibody titers and atherosclerosis in adults.26,27 and the present study suggests that these findings may extend to the initiating stages of atherosclerosis in young males (P = 0.0514). In animal experiments, circulating T cells specific for HSP60 have been shown to play a role in the formation of fatty streaks.28,29 Accordingly, T cells are frequent constituents of atherosclerotic lesions in young individuals.2 The present study demonstrates for the first time that individual T-cell reactivity to human and mycobacterial HSP60s is significantly associated with high IMT. Taken together, our findings advocate a role of the cellular and potentially humoral immune reaction to HSP60 in atherosclerosis of male adolescents.

Compelling evidence has been accumulating recently in favor of inflammatory risk conditions in atherogenesis. In adults, C-reactive protein, a marker of systemic inflammation, was shown to be an excellent predictor of atherosclerosis risk.30,31 In our study, C-reactive protein levels in subjects with high IMT surpassed those seen in the low IMT group, but the difference was not significant. Interpretation of this finding should take into account the substantially lower C-reactive protein levels of adolescents compared with adults. For example, the geometric mean C-reactive protein in our survey (0.49 mg/L) was beneath the upper limit of the lowest C-reactive protein quartile in the Physician Health Study (0.55 mg/L).30,31

The Issue of Multiple Risk Conditions

Our study yields clear evidence that the prevalence of high IMT increases along with the number of risk conditions clustering in a single individual (Figure 2). Notably, all subjects with no or a single risk condition were free of high wall thickening. This group, however, accounted for <10% of the overall population, highlighting the need for prevention.

Conclusion

In summary, our study supports the concept that atherosclerosis begins in the first decades of life, demonstrates a significant association of various risk factors with atherosclerosis in a population of healthy male adolescents, and suggests a role of the immune system, particularly the specific immune reactivity to HSP60, in atherogenesis. We believe that initiating prevention programs in childhood holds great promise for delaying atherogenesis and lowering the risk of its clinical sequelae with aging. It may be important to focus on multiple rather than specific risk factors. Interventions based on lifestyle modifications are appropriate in this age group.

Acknowledgments

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References

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