T-cell reactivity against HSP60 relates to early but not advanced atherosclerosis

M. Knoflach [a,*], S. Kiechl [a], B. Mayrl [b], M. Kind [b], J.S.H. Gaston [c], R. van der Zee [d], A. Faggionato [e], A. Mayr [f], J. Willeit [a], G. Wick [b]

[a] *Department of Clinical Neurology, Innsbruck Medical University, Anichstr. 35, A-6020 Innsbruck, Austria*
[b] *Division of Experimental Pathophysiology and Immunology, Biocenter, Innsbruck Medical University, Innsbruck, Austria*
[c] *Department of Medicine, University of Cambridge, Cambridge, UK*
[d] *Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands*
[e] *Pneumology Service, Sanitätsbetrieb Bozen, Italy*
[f] *Departments of Laboratory Medicine, Bruneck Hospital, Italy*

Abstract

Background: Anti-heat-shock protein 60 (HSP60) antibody-levels have been linked to carotid atherosclerosis and cardiovascular risk in a variety of studies. The potential role of cellular immune reactions against HSP60 has so far attracted little attention in epidemiological research.
Methods and results: In vitro T-cell reactivity to various HSP60s and tuberculin was assessed in blood samples from a elderly subpopulation of the Bruneck study (100 men, 50–69 years) and the young participants of the ARMY study (141 men, 17–18 years), and analyzed for a potential association with common carotoid artery intima-media thickness (IMT). In vivo skin reaction against tuberculin was recorded in subjects of the Bruneck study and correlated with the *in vitro* proliferative response to tuberculin ($P = 0.004$). T-cells isolated from peripheral blood of all individuals proliferated upon stimulation with HSP60s. In multivariate linear regression analysis adjusted for standard risk factors, T-cell stimulation was significantly related to IMT in the ARMY ($P = 0.005$ for human HSP60 and $P = 0.064$ for mycobacterial HSP60) but not in the Bruneck study.
Conclusions: T-cell reactivity against HSP60s correlated with IMT in male youngsters but not in men aged 50 and over, indicating a more prominent role of specific cellular immunity to HSP60s in the young and very early stages of atherosclerosis.
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Keywords: Atherosclerosis; Inflammation; Lymphocyte; Heat-shock protein 60; HSP60; Antibody; Youth; Ageing; Human; IMT

1. Introduction

More than a century ago Virchow was the first to seriously consider atherosclerosis as an inflammatory condition [1]—nowadays a generally accepted concept [2–4]. Histological studies demonstrated the presence of immune cells in the arterial vessel wall forming an immunological network termed 'vascular associated lymphoid tissue (VALT)' by analogy with the local immune network of the gut ('mucosa associated lymphoid tissue—MALT') [5]. Early atherosclerosis is characterized by a prominent infiltration of mononuclear cells especially monocytes/macrophages and T-lymphocytes [6]. In late lesions the latter cells were shown to be activated and a sizeable proportion react against heat-shock proteins (HSPs) in particular the 60 kDa protein HSP60 [7,8]. These highly conserved molecular chaperones – e.g. HSP60 of *Escherichia coli* and human origin share an over 50% amino acid sequence identity [9] – have been implicated in the pathogenesis of atherosclerosis [3]. Levels of antibodies cross-reactive between human and microbial HSP60 are associated with prevalent and incident human

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* Corresponding author. Tel.: +43 512 504 81697; fax: +43 512 504 23986.
  E-mail address: Michael.Knoflach@uibk.ac.at (M. Knoflach).

atherosclerosis and cardiovascular risk [10]. The role of cellular immune reactivity against HSP60, however, has mainly been studied in animal models and in human late complicated lesions so far. Of note, induction of atherosclerotic lesions by immunisation of rabbits with mycobacterial HSP60 [11] can be blocked by depleting the animals of T-lymphocytes [12] while a transfer of HSP60-reactive T-lymphocytes to LDL-receptor deficient mice is capable of inducing pronounced atherosclerotic vessel wall changes [13]. Moreover, induction of tolerance by mucosal administration of HSP60 decreased atherosclerosis in mice [14]. Interestingly, T-cells reactive against HSP60 accumulate in early atherosclerotic lesions in rabbits [15] as compared to the peripheral blood.

The current study measures reactivity of T-cells from peripheral blood to various HSP60s and other antigens, tests for a potential association of specific T-cell responses with common carotid artery intima-media thickness (IMT) a validated surrogate of atherosclerosis and vascular risk in two independent studies and ties up to our previous investigations in the field of anti-HSP60 immunity and atherosclerosis [3,10–12,15–17]

2. Methods

2.1. Study population

The Bruneck study is a prospective population-based survey of the epidemiology and pathogenesis of atherosclerosis [18,19]. At the 1990 baseline evaluation, the study population was recruited as a sex- and age-stratified random sample of all inhabitants of Bruneck (Bolzano Province, Italy) 40–79 years old (125 women and 125 men in the fifth to eighth decades each; $n = 1000$). A total of 93.6% participated, with the data assessment completed for 919 subjects. Between 1990 and 2000 follow-up for carotid ultrasound among survivors exceeded 90%. For the present investigation as part of the 2000 re-evaluation the subsample of men aged 50–69 years ($n = 100$) was assigned to undergo isolation of peripheral blood mononuclear cells (PBMCs) and tuberculin skin tests. In 90 men the dataset was complete (four did not show up for the blood donation and six refused conduction of a tuberculin skin test). Details of clinical history taking and medical examination are given elsewhere [18,19].

The atherosclerosis risk factors in male youngsters (ARMY) study is a cross-sectional evaluation of young men. 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 six volunteers among those who registered at the recruiting office in Innsbruck on randomly selected 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. Details of clinical history taking and medical examination are given elsewhere [16,20].

In both studies, 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.

2.2. Clinical history and examination

The participants of both studies underwent a thorough clinical examination and completed the same standardized questionnaires on current and past exposure to vascular risk factors [16,21]. Smoking status and alcohol consumption were recorded as detailed previously [16,21,19]. Hypertension was defined as blood pressure $\geq 140/90$ mmHg (mean of three independent measurements obtained with a standard mercury sphygmomanometer after at least 10 min of rest) or the use of anti-hypertensive drugs. Pulmonary function was assessed as maximum expiratory flow (MEF) by a standard spirometer (Flowscreen V2.0, Jaeger, Höchberg, Germany).

2.3. Measurement of the intima-media thickness

All participants underwent high resolution B-mode ultrasonography with a 10 MHz imaging probe (ATL8 in the Bruneck and HDI3000 in the ARMY study, both ATL Ultrasound, Bothell, Washington) of common carotid arteries (CCA). In the Bruneck study, all scans were performed by the same experienced sonographer (J.W.) using different scanning angles (anterior and posterolateral) to identify the greatest wall thickness. The intima-media thickness (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 and averaged for the left and right sides. This method was found to be highly reproducible (intraobserver coefficient of variation [CV] 10%) when applied to two independent assessments of IMT in a group of 100 individuals [18]. In the ARMY study, ultrasound evaluations were performed by a single experienced sonographer, and measurements were made from stored digital images by the same investigator as in the Bruneck study (J.W.) [16]. Source images were assessed using different scanning angles with the ultrasound beam directed through the axis of the vessel. Although differences in the ultrasound methods applied (real-time assessment of IMT in the Bruneck study versus readings from stored images in the ARMY study) represents a limitation we consider it unlikely that this procedure had influenced the results obtained. Further details about the method and variability of the ultrasound methods have been extensively described previously [22,23].

2.4. Tuberculin skin testing

Skin testing was performed by a specialist for pulmonary diseases. In brief, five international units of standardised purified protein derivate of a culture filtrate of tubercle bacilli (PPD, Tuberculin) (Biocine-Test PPD Liofillo, Chiron S.p.A, Siena, Italy) was injected intradermally (Mantoux method) into the volar surface of the forearm. The reaction to the antigen was recorded 72 h later and considered positive if induration was ≥10 mm [24].

2.5. Laboratory methods

Blood samples were drawn after an overnight fast and transported to the lab within 3 h. Standard routine parameters were assessed in our iso-certified lab.

An enzyme-linked immunosorbent assay was used to determine antibody titers to mycobacterial HSP60, as described previously [10]. The use of PBMC proliferation assays to determine T-lymphocyte reactivity to different antigens *in vitro* followed methods detailed before [16,25]. PBMCs were separated by density gradient centrifugation (Ficoll, Pharmacia, Uppsala, Sweden). About 10^5 cells were cultured for 7 days in 200 µl 1640 RPMI (Sigma, Vienna, Austria) supplemented with antibiotics (1% penicillin 10,000 Units/ml and streptomycin 10 mg/ml (both, Invitrogen, Carlsbad, CA)) with 10% autologous plasma, alone or after addition of various antigens: 10 µg/ml recombinant mycobacterial HSP60 (mHSP60) (obtained from the EC-sponsored facility on HSP-reagents [project BMH4-CT98-3935]), 20 µg/ml recombinant human HSP60 (hHSP60) or 10 µg/ml recombinant *Chlamydia trachomatis* HSP60 (cHSP60) (all prepared and standardized in our laboratories [25,26]), 10 µg/ml HSP60 of *E. coli* (GroEL) (Boehringer Mannheim, Germany), 10 µg/ml tuberculin (PPD, Statens Seruminstitut, Copenhagen, Denmark) in the Bruneck study and recombinant mycobacterial and human HSP60 from the same sources [25,26] in the ARMY study. Endotoxin contents were low in all HSP60 preparations (<100 Units/mg hHSP60 and <1500 Units/mg mHSP60, cHSP60 or PPD). Experiments were performed in the same laboratory by the same technician for both studies.

Phytohemagglutinin (PHA, 1 µg/ml) and concanavalin A (ConA, 3 µg/ml), both purchased from Sigma (Vienna, Austria), served as positive controls. Proliferation was assessed by [^{3}H]thymidine incorporation [25]. Results were expressed as stimulation indices: [(counts/min in presence of the antigen) − (counts/min in absence of the antigen)]/(counts/min in absence of the antigen).

2.6. Statistical analysis

Levels of variables were presented as mean values ± S.D. and as absolute numbers and percentages (dichotomous variables). Correlations were estimated by Spearman rank correlation coefficients. Standard linear regression analysis was applied to test the associations of specific T-cell reactivity to HSP60s and other antigens with IMT. Base models were unadjusted. Multivariable analyses were controlled for fixed standard sets of established and potential vascular risk factors as established in previous analyses [16,21]. In the Bruneck study, analyses were adjusted for age, HDL and LDL cholesterol level, hypertension, smoking, ferritin concentration, leukocyte count, alcohol consumption, microalbuminuria, hypothyroidism and diabetes mellitus (ADA criteria); in the ARMY study, vascular risk factors included diastolic blood pressure, smoking, alcohol consumption, HDL cholesterol level, antibody reactivity against HSP60, and pulmonary function (maximum expiratory flow at 50% of vital capacity). Regression coefficients (95% CI) were calculated for a 1-SD unit increase of given stimulation indices. Findings were very similar when stimulation indices were ln-transformed. Thus, for ease of presentation and interpretation only data derived from analyses using untransformed variables are presented. Finally, the findings remained robust when the models were adjusted for all covariates listed in Table 1. Calculations were performed using SPSS version 12.0 (SPSS Inc., Chicago, Ill) and BMDP (SAS Institute Inc., Cary, NC) soft-

Table 1
Characteristics of study participants in the ARMY and the Bruneck study

Variables	ARMY study (N = 141)	Bruneck study (N = 90)
Age (years)	17.8 ± 0.6	60.0 ± 3.3
Body-mass index (kg/m^2)	22.5 ± 3.8	25.2 ± 3.2
Obesity (BMI ≥ 25) no. (%)	24 (17.0)	45 (50.0)
MEF50 (l/sec)	6.0 ± 1.3	–
IMT (µm)	468 ± 66	977 ± 148
Systolic blood pressure (mmHg)	117.3 ± 10.3	133.7 ± 16.0
Diastolic blood pressure (mmHg)	77.5 ± 8.1	83.5 ± 7.4
Hypertension no. (%)	15 (10.6)	22 (24.4)
HDL-cholesterol (mg/dl)	37.6 ± 11.9	54.4 ± 13.9
LDL-cholesterol (mg/dl)	108.0 ± 25.5	144.7 ± 36.6
Smoking no. (%)	77 (54.6)	25 (27.8)
Alcohol consumption		
Mean (g/d)	14.4 ± 14.7	41.4 ± 39.5
No alcohol	19 (13.5)	26 (28.9)
1–50 g/d, no. (%)	118 (83.7)	38 (42.2)
51–99 g/d, no. (%)	4 (2.8)	13 (14.4)
≥100 g/d, no. (%)	0 (0.0)	13 (14.4)
Microalbuminuria (mg/l)	–	24.4 ± 108.3
Feritin concentration (µg/l)	41.0 ± 22.6	171.1 ± 158.9
Hypothyroidism, no. (%)	–	4 (4.4)
Anti-mHSP60 antibody titer steps	1.97 ± 1.0	2.93 ± 1.1
hHSP60 stimulation index	3.22 ± 5.2	2.35 ± 3.6
mHSP60 stimulation index	5.13 ± 7.2	2.75 ± 4.2
cHSP60 stimulation index	NA	4.07 ± 5.8
GroEL stimulation index	NA	2.06 ± 4.0

Plus–minus values are means ± S.D. IMT, intima-media thickness of the common carotid arteries; MEF50, maximum expiratory flow at 50% of the vital capacity during spirometry; HDL, high-density lipoprotein; LDL, low-densitiy lipoproteins; mHSP60, mycobacterial heat-shock protein 60; hHSP60, human heat-shock protein 60; cHSP60, chlamydial heat-shock protein 60 and GroEL the heat-shock protein 60 of *E. coli*. To convert values for cholesterol to millimoles per liter, multiply by 0.02586.

Table 2
Spearman rank correlation between *in vitro* PBMC proliferation to different HSP60s in the Bruneck study ($n = 90$)

	hHSP-SI	mHSP-SI	cHSP-SI
GroEL-SI	0.434[**]	0.553[**]	0.563[**]
cHSP-SI	0.517[**]	0.590[**]	
mHSP-SI	0.530[**]		

hHSP, recombinant *human* HSP60; mHSP, recombinant HSP60 of *mycobacterium tuberculosis*; cHSP, recombinant HSP60 of *C. trachomatis*; GroEL, recombinant HSP60 from *E. coli*; SI, stimulation index as determined by lymphocyte proliferation assay.

[**] $P < 0.001$.

ware packages. A two-sided P-value < 0.05 was considered significant.

3. Results

Characteristics of the study populations are summarized in Table 1. T-cells isolated from peripheral blood of all individuals proliferated upon stimulation with various eu- and prokaryotic HSP60s. In both studies, *in vitro* proliferation expressed as stimulation index of PBMCs upon stimulation by distinct HSP60s yielded high correlations: the correlation coefficient between the responses to mycobacterial and human HSP reached 0.518 ($P < 0.001$) in the ARMY study, and a similar magnitude in the Bruneck study, as summarised in Table 2. The strength of correlation was similar to that observed to exist between cellular reactions to the two mitogens phytohemagglutinin and concanavalin which served as positive controls in each experiment of the Bruneck ($r = 0.510$; $P < 0.001$) and ARMY ($r = 0.623$; $P < 0.001$) study. Levels of antibodies against mycobacterial HSP60 were not correlated to cellular immune reactions against mycobacterial and human HSP60 in both studies (Bruneck study: $r = -0.096$ or 0.020 and ARMY study: $r = -0.069$ or -0.048; $P > 0.05$ each). Proliferation was more pronounced in young individuals of the ARMY study than among elderly men in the Bruneck study.

T-cell reactivity against mycobacterial and human HSP60 were correlated with IMT in young males (ARMY study) [16]. After adjustment for vascular risk factors, the association remained significant for the human HSP60 stimulation index ($P = 0.008$) and approached significance for the mycobacterial HSP60 stimulation index ($P = 0.064$). In contrast, in the Bruneck study no association was observed to exist between T-cell reactivity of PBMCs against various HSP60s (*E. coli*, chlamydial, human, mycobacterial) and IMT in uni- or multivariate analysis (Table 3).

Of the 90 men tested, 20 yielded a skin reaction ≥10 mm in the Mantoux test. Correlation between *in vitro* T-cell reaction to tuberculin (PPD) and size of the *in vivo* skin reaction was significant ($r = 0.311$; $P = 0.004$), yet no correlation was found with *in vitro* T-cell reactivity to mHSP60 ($r = 0.022$; $P = 0.837$). Similar findings have been reported previously [27]. Finally, there was no correlation between *in vitro* or

Table 3
Linear regression of T-cell reactivity to various antigens (per standard deviation unit) or tuberculin skin test (Mantoux test) on CCA IMT

	Regression coefficient	95% CI	*P*-value
ARMY study			
hHSP-SI			
Univariate	0.015	0.004–0.026	0.007
Multivariate[a]	0.016	0.005–0.027	0.005
mHSP-SI			
Univariate	0.013	0.001–0.025	0.029
Multivariate[a]	0.011	−0.001 to 0.023	0.064
Bruneck study			
hHSP-SI			
Univariate	0.022	−0.009 to 0.053	0.17
Multivariate[b]	0.023	−0.007 to 0.054	0.13
mHSP-SI			
Univariate	0.011	−0.021 to 0.042	0.50
Multivariate[b]	0.015	−0.015 to 0.044	0.32
cHSP-SI			
Univariate	−0.021	−0.052 to 0.010	0.19
Multivariate[b]	−0.016	−0.047 to 0.014	0.29
GroEL-SI			
Univariate	−0.005	−0.036 to 0.027	0.76
Multivariate[b]	0.003	−0.028 to 0.034	0.86
PPD-SI			
Univariate	−0.014	−0.049 to 0.020	0.41
Multivariate[b]	−0.008	−0.041 to 0.024	0.62
PPD in vivo[c]			
Univariate	−0.020	−0.096 to 0.055	0.59
Multivariate[b]	−0.025	−0.097 to 0.046	0.49

hHSP, human heat-shock protein 60; mHSP, mycobacterial HSP60; cHSP, chlamydial HSP60; GroEL, HSP60 of *E. coli*; PPD, tuberculin; CCA, common carotid artery; IMT, intima-media thickness.

[a] Adjusted for smoking, diastolic blood pressure, alcohol consumption, high-density lipoprotein cholesterol, maximum expiratory flow at 50% of the vital capacity during spirometry and anti-mycobacterial HSP60 antibody titer.

[b] Adjusted for age, leukocyte count, high-density lipoprotein cholesterol, low-densitiy lipoprotein cholesterol, hypothyroidism, ferritin level, smoking, alcohol consumption, hypertension diabetes mellitus and micoalbuminuria.

[c] Positive Mantaoux test (skin induration in mm).

in vivo reaction to tuberculin and IMT in the Bruneck study (Table 3).

4. Discussion

In animal models of early atherosclerosis, such as rabbits on a high cholesterol diet or LDL-receptor deficient mice, the pathogenic relevance of T-cells has been well documented. Amongst others, it has been shown that T-cell depletion in rabbits confers excellent protection against atherosclerosis [12] and that transfer of HSP60 reactive T-lymphocytes from immunized mice to littermates induces formation of atherosclerotic lesions [13]. These findings in experimental animals derive strong epidemiological support from results

of the current investigation. In the ARMY study involving a cohort of young male subjects, T-cell reactivity against HSP60, as estimated by *in vitro* proliferations tests using lymphocytes from the peripheral blood, showed a significant association with common carotid artery IMT a valid surrogate for atherosclerosis and vascular risk. This is especially true for human and less so for mycobacterial HSP60. Of note, the association emerged as independent of classic vascular risk factors. Both, the animal models and analyses in the ARMY study deal with early stages of atherosclerosis, like fatty streak formation. The situation, however, may substantially change with advancing vessel pathology. Benagiano et al. [8] demonstrated the presence of T-cells highly reactive against HSP60 in late atherosclerotic lesions. T-cell lines from the peripheral blood of the same individuals, in contrast, did not show reactions to HSP60. In close agreement, unpublished data from our own laboratory (Rossmann et al.) indicate that T-cell lines derived form advanced human atherosclerotic lesions yield a considerably higher immune reaction to HSP60s than those from the peripheral blood. With this in mind, lack of an association between IMT and reactivity of peripheral T-cells to various HSP60s in men aged 50–69 from the Bruneck study is not unexpected. Hypothetically, a majority of T-cells specific for HSP60s has already homed from blood to the site of inflammation in atherosclerotic plaques. Lymphocytes isolated from the peripheral blood may no longer represent the specific antigen repertoire of cells in the vessel wall. We assume a situation similar to that in Hashimoto thyroiditis. In obese strain (OS) chickens, an animal model for human autoimmune thyroiditis, disease can be transferred to other animals by PBMCs only in the 1st weeks (early stages of illness). However, lymphocytes (predominantly T-cells) isolated from the site of the highest autoantigen expression, i.e. the infiltrated thyroid itself, are capable of inducing experimental thyroiditis in all stages of disease [28]. In addition, lessons from animal experimental research also indicate a more prominent involvement of T-cells in early atherosclerosis [29] and the palette of antigens that maintain vessel wall inflammation is likely to spread in advanced stages of disease [30]. Finally, part of the T-cell reactivity assessed in the current study may represent a regulatory response elicited to control inflammation, which at older age, when the stress responses weaken, becomes obsolete [31].

By analogy with the *in vitro* results for HSP60s, *in vivo* T-cell reactivity to tuberculin was not related to IMT in the Bruneck study involving elderly subjects. In this context it is worth mentioning that *in vitro* and *in vivo* tests (Mantoux) for T-cell reactivity against tuberculin yielded highly correlated results.

The current demonstration of a relationship between T-cell responses to HSP60 and IMT does not on its own infer causality. Taken together with the many intriguing previous epidemiological and experimental studies [3,10–12,15–17], however, our findings lend strong support to the concept of a pathogenic role of cellular immune reactions to HSP60 in early human atherosclerosis and are in keeping with the autoimmune hypothesis of atherosclerosis [3].

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