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Immunology of Atherosclerosis

Demonstration of Heat Shock Protein 60 Expression and T Lymphocytes Bearing α/β or γ/δ Receptor in Human Atherosclerotic Lesions

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Our previous work revealed the presence of a great number of activated T lymphocytes in early human atherosclerotic lesions, and we were able to induce atherosclerosis in normocholesterolemic rabbits by immunization with* Mycobacterium tuberculosis *heat-shock protein (HSP) 65. We hypothesized this latter phenomenon to arise from cross-reactivity of mycobacterial HSP 65 with the endogenously expressed homologous 60-kd form of this stress protein. To study HSP 60 expression and the phenotype of intima infiltrating T lymphocytes relative to the T cell receptor (TCR) in human atherosclerotic lesions, specimens of aorta, carotid arteries, and internal mammary arteries and veins, as well as saphenous veins and vena cava from 27 subjects, aged 23 to 80 years, were examined using immunohistochemical and immunofluorescence techniques on serial frozen tissue sections. HSP 60 was detected on endothelium, smooth muscle cells, and/or mononuclear cells of all carotid and aortic specimens, whereas vessels of smaller diameter, serving as reference specimens for normal intima without atherosclerotic lesions and mononuclear infiltration, showed no detectable expression of this stress protein. Furthermore, although the majority of $CD3^+$ cells within the mononuclear cell infiltrates of atherosclerotic lesions bear the α/β TCR, a considerable portion also consisted of $\gamma/\delta\ TCR^+$ cells. Thus, 9.7% of T cells in the transition zone between normal intima and fatty streaks carry the γ/δ TCR, a proportion that decreases to 6.6% and 4.3% in fatty streaks and atherosclerotic plaques, respectively. We conclude that the intensity of HSP 60 expression correlates positively with the atherosclerotic severity and that most lymphocytes participating in atherogenesis bear the α/β TCR, although $\gamma/\delta\ TCR^+$ cells are also enriched in atherosclerotic lesions. Expression of HSP 60 by intimal cells, caused, eg, by hemodynamic shear forces, may be responsible for recruitment of HSP-sensitized T cells, thus leading to the induction of an initiating inflammatory process in atherosclerosis. Other risk factors, such as high serum cholesterol levels, contribute to the final outcome of the disease. (Am J Pathol 1993, 142:1927–1937)

Recent immunological investigations of atherosclerotic lesions indicated an involvement of cellular and humoral immune reactions in atherogenesis. Histological evaluation revealed not only the presence of macrophages and smooth muscle cells (SMCs), but also of T lymphocytes in atherosclerotic lesions of humans and experimental animals.[1–8] Furthermore, humoral immunological processes have been implicated in atherogenesis, and the C3 receptor and C5b-9 complement factors that form the cytolytic complex have been observed in human atherosclerotic plaques.[9–11] Chemoattractive complement activation products may stimulate the inflammatory process. Vascular SMCs, as well as endothelial cells,

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infiltrating macrophages, and T cells, are known to release numerous immununological and regulatory mediators, composed of platelet-derived growth factor (PDGF), interleukin (IL)-1, IL-2, IL-6, IL-8, tumor necrosis factor-α and tumor necrosis factor-β, and interferon-γ (for review, see refs. 12 to 15). Although the mediation mechanisms of adhesion and penetration of inflammatory cells at sites of vascular lesions is unclear, a role for certain adhesion molecules is evident. Moreover, SMCs and endothelial cells in atherosclerotic lesions aberrantly express major histocompatibility complex class II antigens, induced by interferon-γ-secreting lymphocytes.[6,16,17] In line with this hypothesis, most infiltrating T cells proved to be activated based on IL-2 receptor expression.[5] Stemme et al recently reported that T lymphocytes in human atherosclerotic plaques are primarily memory cells expressing CD45-RO and the integrin VLA-1[18] and are polyclonal in origin.[19] However, the proportional role α/β T cell receptor-(TCR) and γ/δ TCR-bearing cells, respectively, and their immunological appearance in different stages of atherogenesis remain to be clarified.

In human peripheral blood, γ/δ TCR$^+$ cells amount only to about 3 to 5% of all CD3$^+$ lymphocytes.[20] A γ/δ T cell subtype expressing the Vδ1 receptor segment is considered site-specific, as is the mucosa-associated lymphoid tissue (MALT) of local immunity.[21] γ/δ TCR$^+$ clones, derived from peripheral blood of mice and synovial fluid of rheumatoid arthritis patients, have been demonstrated to recognize *Mycobacterium tuberculosis* HSP 65 as the major antigenic determinant.[22–24] However, the subset of Vγ9/Vδ2 TCR-bearing lymphocytes has been shown to be activated by a lectin-binding, protease-resistant mycobacterial ligand.[25]

Heat-shock proteins (HSPs) or stress proteins comprise about two dozen proteins belonging to several families, and cognates thereof, based on their molecular weight.[26,27] They are among the most highly conserved and abundant proteins in the biosphere[28] and serve vital roles in the cell. HSPs are induced in response to factors threatening the integrity of the cell metabolism, such as heat shock, oxygen radicals, heavy metals, anoxia, infection, high concentration of ethanol, ionophores, some inhibitors of mitochondrial function, thiol reactive agents, etc. Thus, stress comprises all factors leading to induction of protein translation with concomitant induction of HSPs, which may be due to loss or denaturation of proteins or the requirement of newly synthesized peptides to guarantee a steady state of cell metabolism. Expression of *E. coli* HSP 60 is, eg induced by heat shock from a basic value of 1.6 to 15% of total protein.[29] Immune responses to HSP can be highly cross-reactive and even autoreactive due to their extensive interspecies amino acid sequence homology. The monoclonal antibody (MAb) ML30, originally raised against mycobacterial HSP 65, also shows binding to mammalian cells expressing HSP 60 as a homologue.[30] HSPs have been shown to be involved in the development of various diseases such as adjuvant arthritis,[31] diabetes mellitus in the nonobese diabetic mouse,[32] rheumatoid arthritis,[33] and systemic lupus erythematosus.[34] Interestingly, atherosclerotic lesions can be induced in normocholesterolemic rabbits by immunization with HSP 65–containing material or recombinant mycobacterial HSP 65.[8,35] An immune response to endogenously expressed HSP 60 may initiate the development of atherosclerosis in this animal model.[36]

In the present study, we observed HSP 60 expression in different stages of human atherosclerosis and co-localization of HSP 60 with markers for T lymphocytes, endothelial cells, macrophages, and SMCs, using a series of antibodies of monoclonal and polyclonal origin. Simultaneously, we investigated the T cell population in atherosclerotic lesions with regard to α/β and γ/δ TCR positivity on cryocut sections.

Materials and Methods

Patients and Tissues

Twenty-five specimens of carotid bifurcation and ascending aorta were obtained for routine pathohistological diagnosis from nine patients at the time of carotid endarterectomy and 16 patients with aorto-coronary bypass operation. In the latter cases, histological and immunohistological analysis of five internal mammary arteries and veins and of nine saphenous veins were carried out as a reference for normal intima. The group of patients, aged 33 to 80 years with an average of 62.4 years, included 20 male and five female subjects. The tissue was immediately placed into phosphate-buffered saline (PBS, pH 7.4), chilled on ice, and transported to the laboratory. Routinely, less than 1 hour elapsed between surgical removal and analyses. Although most of the specimens represented advanced lesions, usually involving the whole arterial circumference and often complicated with ulceration, calcification, and thrombosis, transition zones from fatty streak to atherosclerotic plaque, fatty streaks, and atherosclerotic plaques could be obtained by excluding complicated areas. Furthermore, we obtained three samples of abdominal vena cava within 8 hours post mortem from routine autopsy. A portion

of the specimens was fixed in 4% buffered (pH 7.4) formaldehyde, embedded in paraffin, and sectioned for routine hematoxylin and eosin staining. Another portion was snap-frozen and stored in liquid nitrogen.

Reagents

Table 1 summarizes specificities and sources of all primary antibodies used in this study. Fluorescein isothiocyanate- (FITC) labeled streptavidin for visualization of biotinylated antibodies, tetramethylrhodamin isothiocyanate- (TRITC) and FITC-labeled rabbit anti-mouse Ig antibodies, rabbit anti-mouse Ig bridging antibody, and alkaline phosphatase anti-alkaline phosphatase (APAAP) complex with mouse Ig were purchased from Dakopatts (Copenhagen, Denmark). Poly-L-lysine, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid), naphthol AS-MX phosphate, dimethylformamide, levamisole, Fast Red TR salt, and bovine serum albumin were purchased from Sigma (Munich, Germany). Recombinant mycobacterial HSP 65 (*Mycobacterium bovis* BCG 65K, batch MA-10B) was a gift from Dr. J.D.A. van Embden (National Institute of Public Health and Environmental Protection, Bilthoven, The Netherlands). Polyclonal antibodies against mycobacterial HSP 65 were obtained by immunizing New Zealand White rabbits (Savo/Charles River Co.; Kisslegg im Allgäu, Germany) with HSP 65 (200 μg/rabbit) three times at 3-week intervals. IgG from rabbit serum was isolated by means of a protein G column (Micon Co., Lexington, MA) and labeled with biotin (Sigma Chemical Co., St. Louis, MO) in our laboratory. Gelvatol was purchased from Monsanto (Duesseldorf, Germany), glycerin-gelatine and Meyer's hemalum from Merck (Darmstadt, Germany).

Immunohistochemistry

The entire procedure was performed at room temperature. Cryostat sections (4 μ), mounted on poly-L-lysine coated slides, were air-dried for 20 minutes, and fixed for 7 minutes with acetone. To obtain optimal binding of the primary antibody we used acetone/chloroform (1:2, v/v) for 7 minutes when using anti-γ/δ TCR antibodies.

The sections were preincubated with 10% normal human serum for 20 minutes to block any unspecific binding, rinsed with 0.005 mol/L Tris-HCl-buffered saline (TBS, pH 7.6), and incubated in a humidified chamber with primary antibody, followed by bridging antibody rabbit anti-mouse and APAAP complex for half an hour each with intermittent washes in Tris-HCl-buffered saline for 5 minutes. Optimal concentrations of antibodies and secondary reagents were determined by chessboard titration on lymphoid tissue. For amplification, application of bridging antibody and APAAP was repeated before Fast Red substrate solution was added. The substrate solution was prepared using 9.8 ml Tris-HCl-buffered saline (0.1 mol/L, pH 8.2), 0.2 ml dimethylformamide, 8 mg naphthol AS-MX phosphate, 3 mg levamisole, and 10 mg Fast Red TR salt. For optimal labeling, staining was observed and controlled under the microscope during the final reaction step. Finally, sections were washed with tap water, counterstained with Meyer's hemalum and mounted with glycerin-gelatine. The negative controls for all staining procedures were performed us-

Table 1. *Antibodies Used*

Code	Ig isotype	Specificity	Cells identified	Source
VIT3b	IgG1	CD3	T lymphocytes	a
VIT8	IgG1	CD8	T suppressor/cytotoxic cells	a
13B8.2	IgG1	CD4	T helper/inducer cells	b
4D7	IgG	Vγ9	γ/δ T cells	c
4G6	IgG	Vδ2(J1–3)	γ/δ T cells	c
TCRδ1	IgG1	Cδ1	pan-γ/δ T cells	d
βF1	IgG1	β chain of TCR	α/β T cells	d
EBM 11	IgG1	CD68	Macrophages	e
F8/86	IgG1	Factor VIII-related antigen	Endothelial cells	e
ASM1	IgG2a	α-actin	Smooth muscle cells	f
ML30	IgG1	HSP 65	Stressed cells (HSP 60)	g
Rabbit Ig	Polyclonal	HSP 65	Stressed cells (HSP 60)	h
Rabbit Ig	Polyclonal	Chicken thyroglobulin	Control antibody	h
53/c2	IgG	Human chorionic gonadotropin	Control antibody	i

a: O. Majdic, Institute of Immunology, University Vienna, (Vienna, Austria). b: Immunotech (Marseille, France). c: K. Pfeffer, Institute for Medical Microbiology and Hygiene, Technical University Munich, (Munich, Germany). d: T-Cell Sciences (Cambridge, MA). e: Dakopatts (Copenhagen, Denmark). f: Boehringer-Mannheim (Vienna, Austria). g: J. Ivanyi, MRC Tuberculosis and Related Infections Unit, Hammersmith Hospital, (London, UK) (ref 30). h: Our laboratory. i: P. Berger, Institute for Biomedical Aging Research, Austrian Academy of Sciences (Innsbruck, Austria).

ing an unrelated primary antibody directed to human choriogonadotropic hormone or biotinylated unrelated rabbit anti-chicken thyroglobulin primary antibody, respectively, and also by omitting the primary antibody before applying the appropriate conjugates.

As detailed previously,[6] we operationally classified the specimens as a) normal intima, showing largely unaffected morphology of tissue without mononuclear infiltrate; b) transition zone between normal intima and the core of fatty streaks with infiltration by macrophages and lymphocytes, but without appearance of foam cells; c) fatty streak with the mentioned cellular infiltrate plus formation of foam cells; or d) atherosclerotic plaque with the characteristic composition by a fibrous cap, shoulder regions, and a necrotic core.

For semi-quantitative determination of T cells in these different stages of atherosclerotic lesions, the complete intimal area of serial sections was examined and all positive cells, ie, CD3, α/β and γ/δ TCR-bearing lymphocytes, were counted and the proportion of α/β and γ/δ TCR^+ cells from total $CD3^+$ cells was calculated.

Enzyme-Linked Immunosorbent Assay (ELISA)

To assess the specificity of polyclonal antibodies against mycobacterial HSP 65, a competitive ELISA with MAb ML30 to mycobacterial HSP 65 was established. Microtiter plates (96 wells, Petra Plastic, Würzburg, Germany) were coated with 1 µg of recombinant mycobacterial HSP 65 per ml and blocked with 1% bovine serum albumin. ML30 (0.1 µg/ml) with increasing concentrations of rabbit IgG against HSP 65 were added in 100 µl PBS per well. After washing with Tween 20-PBS, rabbit anti-mouse Ig-peroxidase was used. After further washing, 100 µl citrate phosphate buffer (0.1 mol/L, pH 4.2) containing 0.35 mg/ml 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) was added. Absorbance was measured at 490 nm after 30 minutes with a Microelisa Autoreader (Dynatech Laboratories Inc., Alexandria, VA). For further specification of this polyclonal antibodies we also performed double staining with MAb ML30, affording in an identical pattern of binding.

Immunofluorescence

The procedure for immunofluorescence studies has been described detailed previously.[6] Briefly, cryostat sections were air-dried for 20 minutes, fixed in acetone for 10 minutes, preincubated with 10% normal human serum for 20 minutes, incubated with the primary antibody under study in a humidified chamber, rinsed with PBS, and finally incubated with FITC- or TRITC-labeled anti-mouse Ig and rinsed again. The latter four steps lasted half an hour each. Double staining was performed by extension of this process, adding biotinylated primary antibodies, detected by FITC-streptavidin. All steps were done at room temperature. Slides were mounted in gelvatol and examined in a fluorescence epi-illumination microscope, equipped with an appropriate filter combination for the two wavelength method (Leitz Ortholux II, Wetzlar, Germany)

Results

T Lymphocytes in Atherosclerotic Lesions

Areas of approximately 30 mm^2 each were studied using serial section staining of 25 atherosclerotic cryocut samples of human aorta and carotid artery. Enumeration of α/β TCR^+ cells among $CD3^+$ T lymphocytes showed that approximately 90% of T cells in early stages of atherosclerosis, and 95% in atherosclerotic plaques bear the α/β TCR (Figure 1, A and B). These cells were interspersed in the thickened intima and predominantly occurred in transition zone and fatty streaks concomitant with infiltrating macrophages. However, it could not be determined from these studies which of these mononuclear cells are the very first infiltrating population. In advanced lesions, a characteristic distribution was again seen, with macrophages predominantly appearing around the necrotic core and α/β TCR^+ lymphocytes in the shoulder regions of the atherosclerotic plaque. In lesion cap regions, α/β TCR^+ cells could also be observed.

To compare the present immunohistochemical observations to those based on previous results by immunofluorescence staining,[6] $CD4^+$ and $CD8^+$ T cells in different stages of atherosclerosis, including transition zone, fatty streaks, and atherosclerotic plaques, were enumerated in 15 arterial specimens. The data revealed a predominance of T helper cells three times higher than $CD8^+$ cells in transition zone (CD4/CD8 ratio = 3.2 ± 1.1) and fatty streak (CD4/CD8 ratio = 3.4 ± 2.2). Conversely, the atherosclerotic plaques contained a greater proportion of T cytotoxic/suppressor cells with a CD4/CD8 ratio of 0.77 ± 0.13, similar to previous observations.[6]

Using monoclonal antibody TCR δ1, which recognizes all T cells bearing γ/δ TCR, 9.7% γ/δ T cells

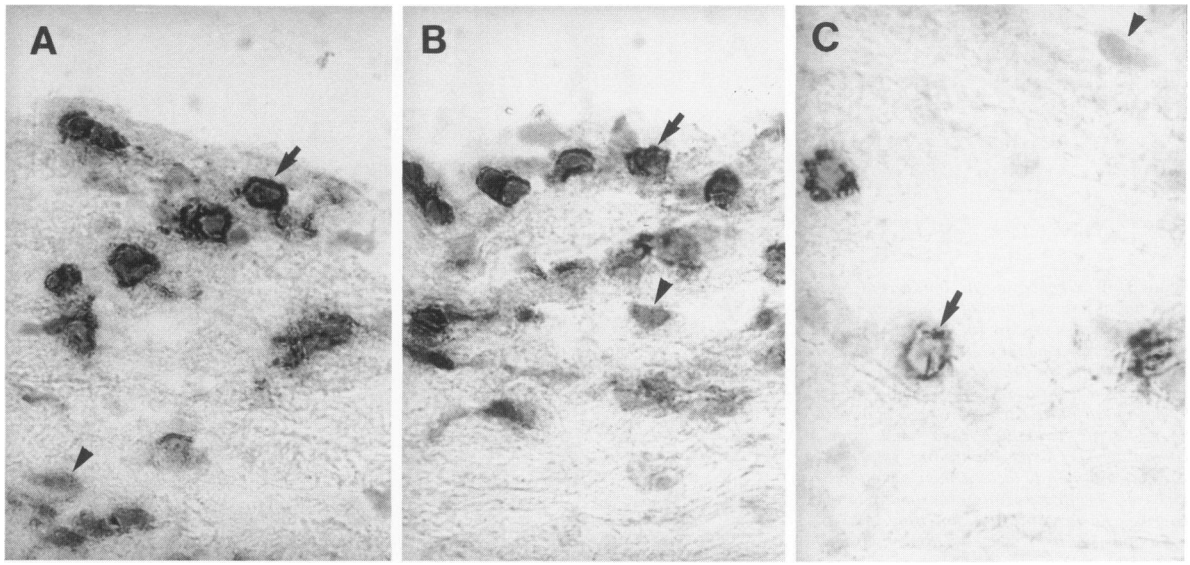

Figure 1. *T lymphocytes in atherosclerotic lesions. Immunohistochemical detection of CD3⁺ cells* **(A)**, *α/β TCR⁺ cells* **(B)** *and γ/δ TCR-bearing cells* **(C)** *in an aortic fatty streak of a man at 42 years of age. Arrows indicate positive cells appearing with a dark reaction product of alkaline phosphatase. Arrowheads show negative cells, counterstained with Mayer's hemalum. Original magnification × 500.*

were observed in transition zone, 6.6% in fatty streak, and 4.3% in atherosclerotic plaque (Figures 2 and 1C). The highest proportion of γ/δ T cells was found in early stage lesions containing relatively few $CD3^+$ cells. Later, however, the recruitment of α/β TCR-bearing cells prevails. α/β as well as γ/δ T cells were found in regions of atherosclerotic lesions with high expression of HSP 60 in mononuclear cells and SMCs, but also in fatty streaks with very low levels of HSP 60 expression detectable by immunofluorescence in endothelial cells.

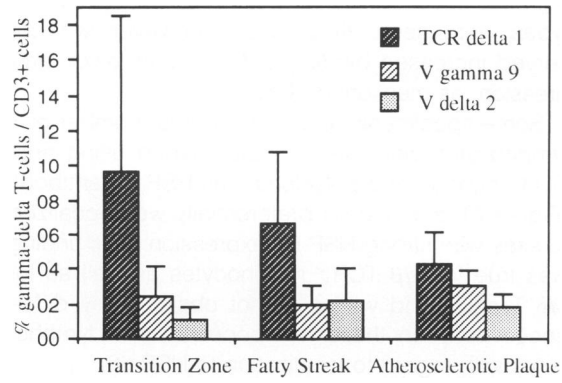

Figure 2. *Relative γ/δ T cell frequency in atherosclerotic lesions. Comparison of transition zone, fatty streak, and atherosclerotic plaque shows a decreasing tendency of relative γ/δ T cell percentage with disease progression. Columns show mean ± SEM cell numbers, counted in 25 human arterial specimens. δ1 TCR antibody was used as pan-γ/δ T cell marker and demonstrated a maximum of 9.7% of these T cells in transition zone. Vγ9 and Vδ2 antibodies detected percentages of γ/δ T cells characteristic for peripheral blood lymphocytes. This γ/δ TCR-bearing T cell subset constitutes a consistent number of all $CD3^+$ cells in all types of lesions, ranging from 1.1 to 3.3%.*

Several subtypes of γ/δ T cells were described recently with varying distributions and specificities.[37] In the present experiments, T lymphocytes identified by monoclonal antibodies directed to Vγ9 and Vδ2 were found in a consistent proportion in all stages of atherosclerotic lesions, ranging from 1.1 to 3.1% of total $CD3^+$ cells, which corresponds to those described for peripheral blood.[20] This subset has been shown to be activated by mycobacterial antigens but not by HSP 65.[25] Thus, these non-HSP 65 reactive T cells are not increased in atherosclerotic lesions.

Our specimens of normal intima of internal mammary vein and long saphenous vein controls showed neither HSP 60 expression nor mononuclear cell infiltration. However, three specimens of vena cava interestingly showed the same alterations observed in aortic and carotid specimens. Endothelial cells expressed HSP 60, and infiltrating T cells were seen in the intima (Figure 3). Phenotypic analysis of the TCR expression revealed a γ/δ T cell frequency corresponding to early atherosclerotic arterial lesions.

Expression of HSP 60 by Intima Cells

To test the specificity of our own rabbit anti-*M. bovis* HSP 65 IgG, a competitive ELISA was performed with ML30, specific for a sequential epitope within residues 311 to 322 of the mycobacterial HSP 65 as well as human HSP 60.[30] ML30 binding to HSP 65

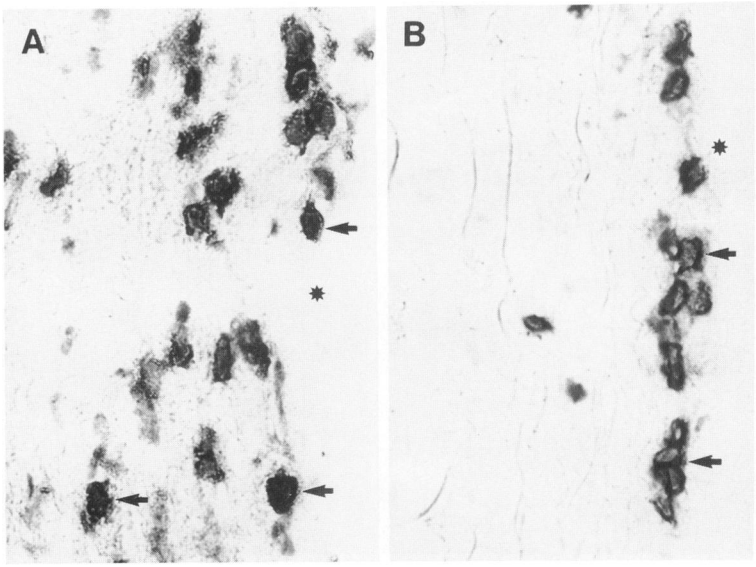

Figure 3. *T lymphocytes in aorta and vena cava. Immunohistochemical demonstration of CD3⁺ T-lymphocytes in an arterial* (A) *and venous* (B) *specimen by the APAAP method. Aorta represents the advanced lesion of an fatty streak, taken from a 42-year-old male patient. T cells appear black (arrows). The venous sample represents abdominal vena cava from a woman aged 23 years. Infiltrating lymphocytes (arrows) are mainly located near the endothelial layer, forming an infiltrate similar to that described for arterial transition zone. Asterisks mark lumen of the vessels. Original magnification × 500.*

was effectively competed by the polyclonal rabbit IgG, suggesting a common HSP epitope binding by both antibodies (Figure 4).

By means of this polyclonal reagent, double labeling immunofluorescence tests were performed to study HSP 60 expression in different intimal cells (Figure 5). Co-localization of factor VIII–related antigen and HSP 60 indicated expression of this stress protein by endothelial cells only in aortic and carotid specimens, not in internal mammary artery/veins or saphenous veins. The first cells to show constant HSP 60 expression were macrophages in the transition zones, fatty streaks, and atherosclerotic plaques. More advanced lesions also revealed HSP 60 positivity of SMCs and lymphocytes, identified by α-actin and CD3-specific antibodies, respectively. The highest levels of HSP 60 expression were found around the necrotic core of the plaques. An association between endothelial immunofluorescence intensity and underlying HSP 60 expression of mononuclear cells and SMCs was not found on individual sections, including transition zone, fatty streak, and plaque. All combinations of low- and high-fluorescence intensity on these different cell types were seen. In principle, however, we observed increased binding of ML30 with lesion progression, as mentioned above.

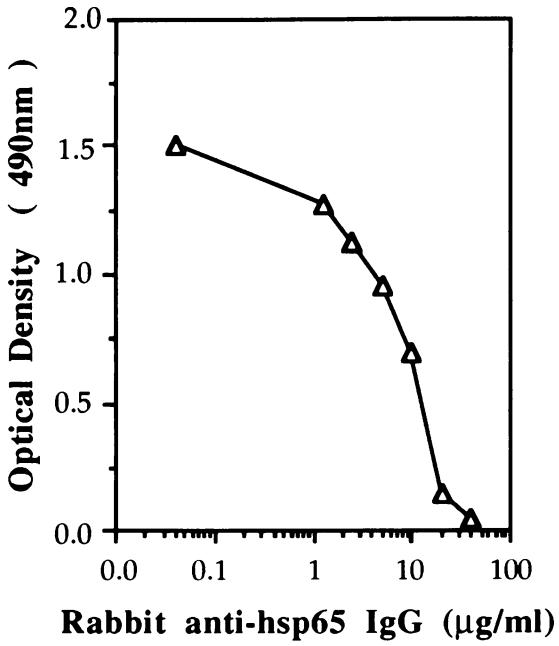

Figure 4. *Antibody competitive ELISA. Polyclonal rabbit anti-HSP 65 antibody was tested for binding specificity by a competition with MAb ML30 antibody to recombinant mycobacterial HSP 65. Bound ML30 was detected by peroxidase conjugated rabbit anti-mouse Ig. A decreasing optical density at a wavelength of 490 nm was observed with increasing concentration of the rabbit IgG, which indicates an effective blocking of the HSP 65 epitope by the polyclonal antibody.*

Some specimens containing a great number of T lymphocytes were also double stained using anti-CD3 antibody and polyclonal anti-HSP 65 antibody (Figure 6), and T cells preferentially were localized to sites with strong HSP 60 expression. This finding was true for α/β TCR⁺ lymphocytes as well as for γ/δ T cells, and we could not observe any differences between these two populations of lymphocytes with regard to localization to HSP 60.

HSP 60 Staining in Aortic and Carotid Atherosclerotic Lesions

HSP 60 in thirty specimens derived from 25 patients, was detected by using MAb ML30 in immu-

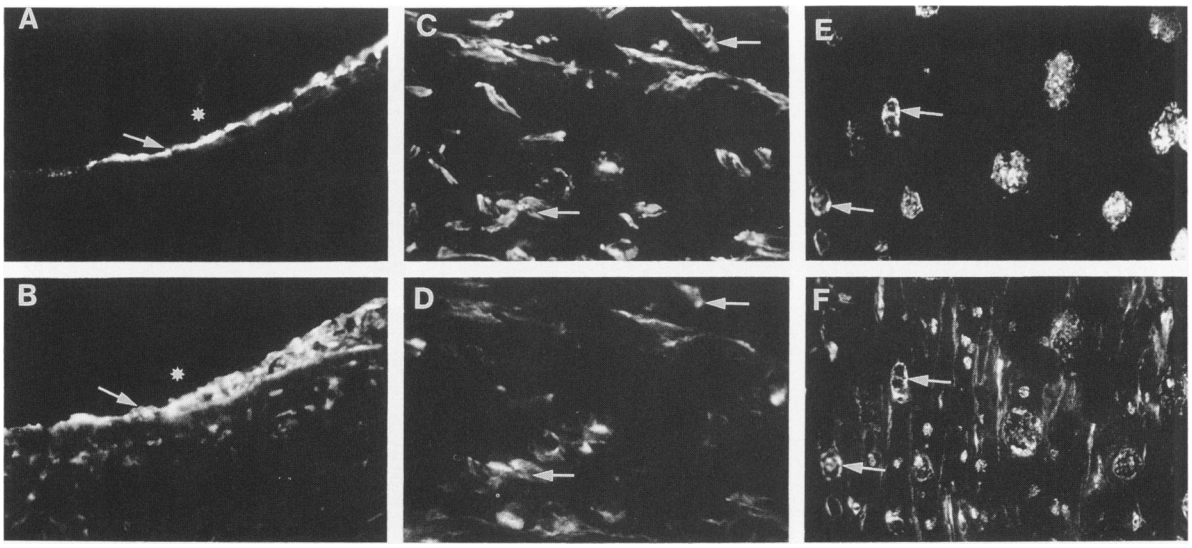

Figure 5. *Expression of HSP 60 in different intima cells. Double labeling immunofluorescence photographs of endothelial cells (factor VIII–related antigen,* **A**), *SMCs (α-actin,* **C**), *and macrophages (CD 68,* **E**), *all detected by TRITC-labeled conjugate, with the corresponding photographs showing FITC-stained HSP 60* (**B, D,** *and* **F**). *Samples were taken from the aorta of a male patient aged 44 years. Asterisks mark lumen of the vessels. Arrows indicate selected double positive cells on corresponding pictures, which demonstrate HSP 60 expression on endothelial cells, SMCs, and macrophages, respectively. Original magnification ×250* (**A, B**) *and ×500* (**C** *to* **F**), *respectively.*

nofluorescence tests. Normal intima, showing diffuse thickening without mononuclear cell infiltration was found in the internal mammary artery, a vessel very rarely afflicted with atherosclerosis,[38] in the internal mammary vein, and the long saphenous vein only. HSP 60 expression was not detectable in either case.

However, with the occurrence of infiltrating macrophages, HSP 60 could be detected in some cells interspersed in the thickened intima (Figure 7). In this type of very early lesion, designated as transition zone,[6] staining of the endothelium also occurred in about half of the aortic and carotid specimens studied. The intensity of endothelial fluorescence was not associated with that of similarly stained mononuclear cells and SMCs. With progression of the atherosclerotic process and formation of fatty streaks and atherosclerotic plaques, the endothelium became constantly positive and the rest of the intimal area showed more extensive HSP 60 expression in heterogenous distribution, mainly mononuclear cells and SMCs. The highest intensity of

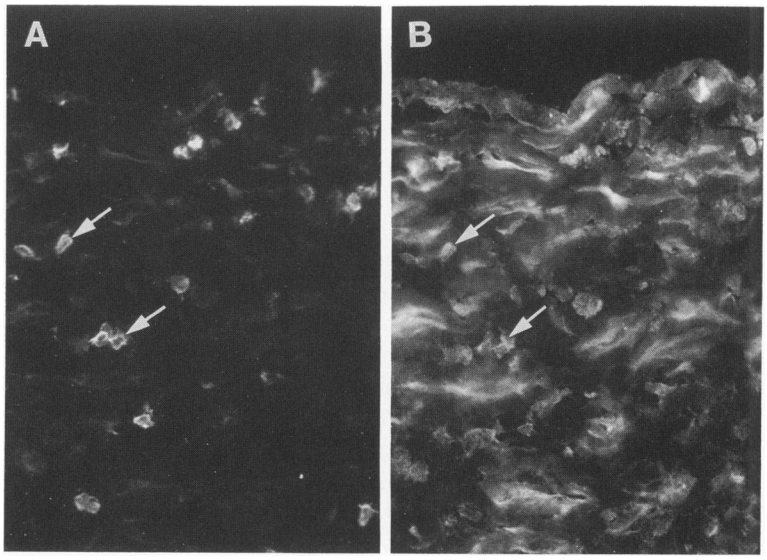

Figure 6. *Co-localization of T lymphocytes with HSP 60 expressing intima cells. Immunofluorescence double staining of CD3⁺ cells* (**A**) *detected by TRITC-labeled conjugated, and HSP 60 demonstration by polyclonal biotinylated primary antibody detected by FITC-streptavidin conjugate* (**B**) *of specimens taken from carotid plaque of a male patient aged 62 years shows that T lymphocytes co-localize to the stress-protein positive areas and also express HSP 60 by themselves too (arrows in* **B**). *Original magnification ×250.*

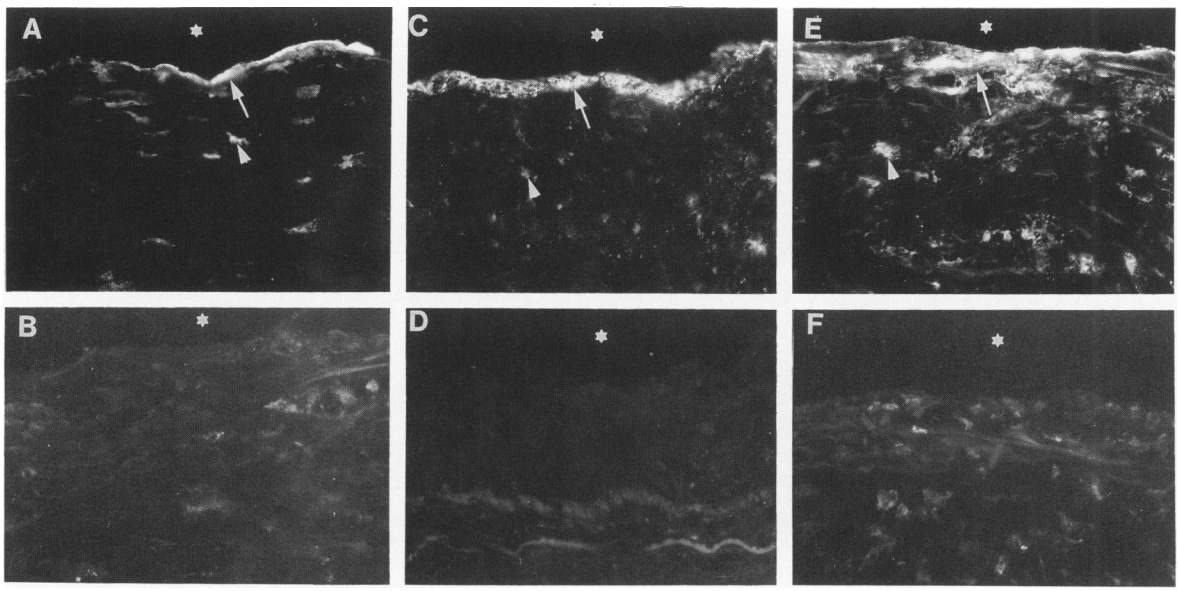

Figure 7. *Distribution of HSP 60 in atherosclerotic lesions. TRITC-immunofluorescence staining by MAb ML30 shows expression of HSP 60 in transition zone* (A), *fatty streak* (C), *and atherosclerotic plaque* (E). B, D, *and* F *show the corresponding negative controls of an adjacent serial section. The representative specimens depicted in the figure were taken from the carotid bifurcation of a male patient, aged 76 years* (A, B, E, F) *and from the carotid bifurcation of another male patient aged 62 years* (C, D). *These types of lesions reveal positivity of endothelial cells (arrows) and different intimal cells (arrowheads). Staining can be observed from transition zone onwards with increasing brightness to the lesions of fatty streak and atherosclerotic plaque. Asterisks mark lumen of the vessels. Original magnification ×250.*

HSP 60 staining was seen in the shoulder regions and around the necrotic core of atherosclerotic plaques.

Discussion

The work described herein has shown that most T lymphocytes involved in atherogenesis bear the α/β TCR. However, in the earliest stage of atherogenesis, we found an average of 9.7% γ/δ T cells, the function of which is still elusive. γ/δ TCR$^+$ cells have been proposed to constitute a first line of defense,[24] and our results also could point to a possible participation of γ/δ T cells in the early stages of atherosclerosis. High numbers of human γ/δ T cells have been found only in lymphoid organs and in small and large intestine, which contain percentages of 12.5 ± 8.1 and 20 ± 10, respectively.[20,39] The Vγ9/Vδ2 subset of TCR segment–bearing lymphocytes, characteristic in peripheral blood and for proliferation to mycobacterial antigens but not to HSP 65, was not increased relative to known numbers in peripheral blood,[20] which argues against a superantigenlike triggered expansion of γ/δ T cells,[40] but seems to support recruitment or expansion of γ/δ T cells expressing Vδ1, which are characteristic for local immunity and may show proliferation to HSP 65, and α/β TCR-bearing lymphocytes during disease progression. Thus, it can be hypothesized that the increase of γ/δ TCR-bearing cells in the very early stages of atherosclerosis is in accordance with their suspected function as a first line of defense by local surveillance, but it seems evident that α/β T cells are the disease-mediating population. Based on TCR V regions analysis, T cells in advanced atherosclerotic lesions have been reported to represent a heterogenous population, thus arguing against local clonal expansion and for recruitment and retention of lymphocytes from peripheral blood.[19] We confirmed our previous finding that the first α/β TCR$^+$ lymphocytes infiltrating the atherosclerotic lesion exhibit the CD4 phenotype. In transition zone and fatty streak, they represent the predominant population of CD3$^+$ cells, but in atherosclerotic plaques, CD4$^+$ to CD8$^+$ cell distribution reverts to a preponderance of the latter. This result suggests that CD4$^+$ cells may initiate an immunological process in the vascular intima. In three samples of vena cava, which served as controls, some α/β and γ/δ TCR$^+$ lymphocytes were also found in the intima, albeit in small numbers. The role of T cells observed in venous intima remains to be elucidated.

HSP 70, also found to be expressed at high levels in atherosclerotic lesions,[41] was demonstrated to increase arterial cell survival, suggesting that these proteins are associated with a protective

mechanism in normal and diseased arteries.[42] Studies revealing HSP 70 messenger RNA expression in selected tissues of surgically stressed rats showed the highest increase of HSP 70 induction in the aorta, exceeding basal expression by more than 20 times.[43] Because the factors inducing HSP expression are similar for different families of these proteins, it is likely that induction of HSP 70 expression is concomitant to HSP 60 expression. A characteristic of stress protein induction of certain families, eg, HSPs 70 and 60, is that in response to various stress factors in some instances even expression on the cell surface can be demonstrated, which makes them accessible to the immune system. However, it remains to be clarified if HSP 70 is involved pathogenically in the development of atherosclerosis, as we postulate for HSP 60, or reflects an increased protein turnover only.

The present study describes HSP 60 expression by endothelial cells of the human vascular system as well as the occurrence of this molecule in cells at some distance from the blood flow, such as resident macrophages, SMCs, and infiltrating lymphocytes. In 30 specimens stained with ML30 for HSP 60, no obvious association between intensity of superficial, ie, endothelial staining, and HSP 60 expression on macrophages, SMCs, and lymphocytes in deeper layers of the intima was observed. From these results, we deduced that HSP 60 expression by endothelial cells depends on hemodynamic factors in large arteries, but expression by SMCs and mononuclear cells seems to result from the inflammatory process itself. As previously pointed out, the factors leading to HSP 60 expression in detail are numerous, but they can be summarized as influences threatening cellular integrity with possible lethal effects.

Cell types performing phagocytosis, such as histiocytes or mesangial cells of the kidney, were shown to express high levels of HSP 60,[30,44] and we found this to also be true for macrophages involved in atherogenesis. That such expression indicates a superficial presence of this stress protein has also been shown.[45] Cytotoxic oxidized low density lipoproteins taken up by scavenger receptors may represent an important factor leading to this phenomenon.[46] It can be assumed that presentation of HSP 60 peptides by major histocompatibility complex class II molecules over time leads to increasing immunity to different epitopes of this autologous stress protein. Infection with several strains of bacteria containing large amounts of HSP 65 in a form very homologous to their mammalian counterpart may amplify the process, or lead to autonomous induction of anti-HSP 65/60 immunity. The significant association of elevated titers of anti-HSP 65 antibodies in humans with the severity of atherosclerosis supports a possible causal relationship between immunity to HSP 60/65 and development of atherosclerosis.[47]

The fatty streak is considered a reversible type of lesion that occurs even in children.[48] Progression to the atherosclerotic plaque in adult humans might be explained by increased cellular and humoral immunity to HSP 65 in combination with the endogenously expressed form of this protein by hemodynamically stressed endothelial cells, thus leading to a self-perpetuating chronic inflammatory process stimulated by HSP 60 in advanced lesions. Additionally, SMCs and endothelial cells, after stimulation with lymphocyte-derived interferon-γ, express major histocompatibility complex class II,[49,50] which, together with HSP 60 expression, may lead to a vicious circle entailing an autoimmune disease.

We have shown that HSP 60 expression occurs in endothelial cells of large human vessels, subject to stronger hemodynamic forces than small vessels, which means that the pulsatile blood flow and pressure as well as turbulent flow and shear stress at branching sites occurs mainly in the aorta and carotid arteries, that therefore are predisposed to development of atherosclerotic lesions.[51] The internal mammary artery, which shows in relation to aorta and carotid arteries a considerable difference in radius and velocity of blood flow and therefore is not subject to the same intensity of shear forces by change from laminar to turbulent flow and is known to be rarely afflicted with atherosclerosis, showed neither mononuclear infiltration nor HSP 60 expression. However, it should be mentioned that endothelial cells of normal intima of rat aorta, despite the absence of mononuclear infiltration expressed this stress protein (Q. Xu, unpublished observations), probably due to hemodynamic stress. The human aortic samples available to us did not include specimens without cellular infiltrate, and thus we cannot define HSP 60 expression in normal human aorta. Psychosocial risk factors leading to increased blood pressure by sympathoadrenal medullary activation,[52] or hypertension for genetic or metabolic reasons, can be integrated into the concept of atherogenesis initiated by increased expression of HSP 60 due to endothelial injury. Thus, our concept of an immunological initiation of atherosclerosis also includes the role of many additional risk factors involved in this disease.

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