Expression of heat shock protein–70 by dendritic cells in the arterial intima and its potential significance in atherogenesis

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Objective: **Overexpression of heat shock proteins (HSPs) is an important means of cell protection during physiologic stress such as occurs during atherogenesis. Immune responses are early events in atherosclerosis, with recent studies indicating that both humoral and cellular autoimmune processes in atherogenesis are directed toward HSPs. Dendritic cells are the key cells in the initiation and regulation of immune responses. This study examined whether HSP70 is overexpressed by dendritic cells in atherosclerotic lesions.**

Methods: **Twenty-six carotid artery and 16 aortic specimens obtained at endarterectomy and aortic reconstruction were examined with immunohistochemical techniques. The nature of cells that overexpressed HSP70 was studied in consecutive sections that were double stained with antibodies to HSP70 and cell type–specific markers, including CD1a and fascin (to identify dendritic cells), CD14 (monocytes), CD68 (macrophages), CD3 (T cells), CD15 (mast cells), von Willibrand factor (endothelial cells), and α–smooth muscle actin (smooth muscle cells). Staining with HLA-DR and CD1d was used to identify cells involved in antigen presentation.**

Results: **In advanced atherosclerotic lesions, several cell types, including monocytes, macrophages, dendritic cells, and smooth muscle cells, overexpressed HSP70. In contrast, in early atherosclerotic lesions, only dendritic cells overexpressed HSP70. Dendritic cells that overexpressed HSP70 frequently contacted T cells and also expressed HLA-DR. Furthermore, dendritic cells that clustered with T cells expressed CD1d, a unique molecule responsible for presenting lipid antigens.**

Conclusion: **The results suggest that direct contacts between activated dendritic cells that overexpress HSP70 and T cells might be responsible for T cell activation and might facilitate the presentation of lipid antigens to T cells directly within the arterial wall. In early intimal lesions, HSP70 is overexpressed exclusively by dendritic cells, which suggests that dendritic cells might be involved in the early phases of atherogenesis. (J Vasc Surg 2002;35:368-75.)**

Hypoxia and oxidative stress contribute in atherogenesis[1,2] and, like heat shock, increase the expression of genes that encode stress, the so-called heat shock proteins (HSPs).[1,3,4] HSPs are responsible for the repair or degradation of denatured proteins and, by the maintenance of protein conformation, HSPs enhance the cell's ability to survive metabolic or oxidative stress.[1,3,4] In the absence of stress, many HSPs act as molecular chaperones that play a vital role in healthy cellular processes that facilitate the folding, assembly, and disassembly, and the translocation of other proteins.[3,4] The overexpression of HSPs is considered an important means of cell protection during physiologic stress.[3,4] The different families of HSPs are classified by their molecular masses in kilodaltons.[3,5]

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The association of several HSPs, including HSP27,[5] HSP40 (HDJ-2),[6] HSP47,[7] HSP60,[8,9] and HSP70,[10,11] with vascular cells was identified either in the arterial wall in situ or in vitro experiments. The level of expression of both HSP40 messenger RNA and HSP70 messenger RNA has been shown to be significantly increased in carotid endarterectomy specimens in comparison with the healthy arteries, with the level of HSP70 expression being increased more than 2.5 times.[6] With an immunohistochemical approach, Berberian et al[11] found a homogeneous distribution of HSP70 throughout the intima and media in healthy aortas, and a strong increase in its immunostaining intensity was observed in aortic atherosclerotic plaques. HSP70 was expressed most intensely around areas of necrosis and lipid accumulation in the central portion of atheroma.[11]

Immune responses are an important component of atherosclerosis,[1215] with recent study results indicating that in atherogenesis both humoral and cellular autoimmune processes are directed toward HSPs.[8-10] Chan et al[10] have provided evidence of a correlation between anti-HSP70 antibodies and different types of vascular disease, including atherosclerosis, which suggests that HSP70 might be involved in their pathogenesis.

Dendritic cells (DCs) constitute a family of cells able to induce primary immune responses.[16-18] DCs are the key cells in the initiation and regulation of immune

responses.[16-18] DCs are thought to arise from a common $CD34^+$ progenitor in the bone marrow from where they migrate via the blood stream to settle in different peripheral tissues.[16-18] After engulfing antigen, DCs migrate via afferent lymph to activate T cells.[16-18] DCs express high levels of both class I and class II major histocompatibility complex molecules and costimulatory molecules, which explains their unique ability to activate naive T cells.[16-18]

In previous studies, we found that cells from the family of DCs reside in the intima of large arteries and that these vascular DCs are common in atherosclerotic lesions.[19-22] Vascular DCs exhibit ultrastructural characteristics typical of other DCs[19] and, like Langerhans cells and interdigitating cells,[18] vascular DCs express S-100 protein, actin-bundling protein p55 (fascin), and CD1a, which are markers for their immunohistochemical identification.[20-22] Like other DCs,[18] vascular DCs express HLA-DR[21] and display intercellular adhesion molecule-1 (ICAM-1) and vascular adhesion molecule-1 (VCAM-1).[21]

In the nondiseased arterial wall, DCs are regularly located along the subendothelial layer in numbers similar to those of Langerhans cells in the skin, namely, 2% to 5%.[19,21] In atherosclerotic lesions, the numbers of DCs markedly increase, with more than 90% of DCs colocalized with T cells in neovascularization areas associated with inflammatory infiltrates.[21] Inflammatory infiltrates in atherosclerotic plaques contain activated T cells.[12,15] Antigen-specific T cell activation depends on the interactions of T cell receptors with antigens presented by MHC molecules,[12,15] and we earlier suggested that vascular DCs might be the cells principally responsible for T cell activation in atherosclerosis.[21] ICAM-1/leukocyte function–associated antigen-1 and VCAM-1/very late antigen-4 interactions are critical in T cell activation,[23] and the observations that vascular DCs display HLA-DR, ICAM-1, and VCAM-1 and locate amongst and contact T cells in atherosclerotic lesions[21] imply that DCs might activate T cells directly in the injured arterial wall. Although some DCs cluster with T cells within atherosclerotic lesions, others seem to migrate to regional lymph nodes to activate T cells.[21]

To be capable of activating T cells, DCs need to undergo transition from the so-called DC immature forms into the DC mature forms.[16-18] Ultrastructural study results showed that, in the healthy arterial wall, DCs reside in their immature forms.[19] The microenvironmental signals responsible for DC activation and maturation in atherogenesis are unknown.

The results of several recent studies suggest that HSPs are associated with DC function and possibly trigger DC activation and maturation.[24-29] Complexes of peptides with different HSPs, including gp96, HSP90, and HSP70, have been shown to be taken up by DCs and represented by MHC class I molecules.[29] The importance of HSPs in the activation of DCs is of special interest in atherosclerosis research as evidence accumulates that both humoral and cellular autoimmune processes directed toward HSPs contribute in atherogenesis.[10,13] This study was carried out to determine whether HSP70 is associated with DCs in atherosclerotic lesions.

METHODS

Tissue specimens. Arterial wall segments from 26 carotid arteries and 16 aortas were obtained from patients whose ages ranged from 38 to 73 years. The carotid specimens were obtained at endarterectomy, and the aortic specimens (entire wall segments) were collected during aortic reconstructions at St Vincent's Hospital, Sydney, Australia. The material was collected in accordance with the principles outlined in the Declaration of Helsinki,[30] and the study was approved by the institutional review board of St Vincent's Hospital, Sydney, Australia. Because the specimens were relatively large (as much as 5 cm), it was possible to find different atherosclerotic lesions, including both early and advanced lesions, in the same specimen. Some sites of the arterial wall not affected by atherosclerosis were also identified. From every specimen, small tissue pieces (about 1 mm^3) were cut off and were fixed in 2.5% glutaraldehyde in phosphate-buffered saline solution (PBS; pH, 7.2). The arterial specimens then were divided into two parts. One part from each specimen was immediately embedded in OCT compound (Pelco International, Redding, Calif), rapidly frozen in liquid nitrogen, and stored at –70°C until cryostat sectioning, while the other part of the specimen was kept in ice-cold PBS and used for the preparation of intimal sheets (so called "Hautchen" specimens) for en face immunostaining according to the technique described by Rekhter et al.[31]

Frozen arterial tissue blocks were cut at 5 to 7 μm thickness sections. The sections stained with Mayer's hematoxylin and oil red O were examined to determine the degrees of atherosclerotic alteration of the arterial wall. Microscopic examination showed that every arterial specimen contained sites displaying different stages of atherosclerotic transformation, with 10 carotid artery specimens containing small intimal sites without any signs of atherosclerosis. All the aortic specimens contained extensive areas of nonatherosclerotic arterial wall. Intimal lesions containing some extracellular and intracellular lipids (oil red O staining) and a few foam cells intermingled with inflammatory cells irregularly scattered in the superficial portion of the intima (mainly in the swollen subendothelial layer) were classified as "early" atherosclerotic lesions. These early atherosclerotic lesions corresponded to the initial stage of forming fatty streaks (type I and the initial stage of type II lesions of the classification of Stary et al[32,33]). Thirty-six early intimal lesions were identified. Noncomplicated atherosclerotic plaques with the lipid core still forming did not contain fibrous caps and corresponded to intermediate lesions (type III of the classification of Stary et al[32,33]). "Advanced" atherosclerotic plaques contained lipid (necrotic) cores, fibrous caps, and well-developed neovascularization and were complicated by excessive calcification and in some cases intramural hemorrhages. These plaques mostly corresponded to Va, Vb, and VI

type atherosclerotic lesions of the classification of Stary et al.[33]

Antibodies. Monoclonal antibody to HSP70 (B-6; sc-7298) was purchased from Santa Cruz Biotechnology (Santa Cruz, Calif). Expression of HSP70 was examined in frozen sections in various concentrations (1:50; 1:100; 1:150; 1:200; 1:300) to distinguish between intimal cells expressing and overexpressing HSP70. DCs were identified with both anti-CD1a (Dako, Carpinteria, Calif; NA/34; 1:50) antibody and with antifascin antibody (Dako; 55K-2; 1:50). Macrophages were identified with anti-CD68 antibody (Dako; EBM11; 1:50). T lymphocytes in the arterial intima were identified with anti-CD3 (Dako; CD3; 1:50). Smooth muscle cells were identified with antibody to α–smooth muscle actin (Dako; 1A4; 1:400). Endothelial cells were identified with von Willebrand factor antibody (Dako; F8/86; 1:50). Mast cells were stained with anti-CD15 (Dako; MMA; 1:50). Anti–HLA-DR (Dako; CR/43; 1:50) and anti-CD1d (Research Diagnostic, Inc, Flanders, NJ; NOR3.2; 1:25) were used to identify cells involved in antigen presentation.

Single immunohistochemical staining. Single immunohistochemical staining was used to visualize the expression of HSP70 and the distribution of different cell types in the arterial specimens. An analysis was performed with sets of consecutive parallel sections immunostained with different antibodies. After the elimination of endogenous peroxidase activity with 0.3% H_2O_2 for 5 minutes, the sections were preincubated with nonimmune serum and then tested with avidin-biotin complex (ABC) using the ABC immunoperoxidase method[34] as previously described.[20,21] In brief, after washing in tris-PBS (TPBS), pH 7.6, the sections were incubated with the appropriate secondary antibody. The sections then were washed in TPBS for 5 minutes and treated with avidin-biotin complex (Elite -ABC, Vector, Burlingame, Calif). After washing for 10 minutes in TPBS, a brown staining was produced with the treatment of the sections with 3,3'-diaminobenzidine as used previously. Alternatively, a red staining was produced with treatment with the 3-amino, 9-ethylcarbazole (AEC) substrate kit or ABC substrate kit as previously described.[35] All the incubations were performed at room temperature. For negative control, the primary antibody was omitted or the sections were treated with an immunoglobulin fraction of nonimmune serum as a substitute for the primary antibody. None of the negative control sections showed positive immune staining. Counterstaining was performed with Mayer's hematoxylin, and sections were examined with an Olympus microscope at 10×10 and 10×40 magnifications.

To distinguish between expression and overexpression of HSP70 in the arterial specimens, we stained parallel consecutive sections with HSP70 antibodies diluted in different concentrations. (The term "expression" is used in this article in a wide sense. This term indicates the presence of HSP70 within cells and does not imply the synthesis of this protein only.) Examination of nondiseased portions of the arterial wall (in both aortic and carotid specimens) stained with the antibody diluted as 1:50 showed a low but specific and homogenous expression of HSP70 in all intimal and medial cells, and no positive immunostaining was observed in control sections. In contrast, a very strong intensity of immunostaining in some areas of atherosclerotic plaques was obvious when the antibody was used in the same concentration. When the antibody was gradually diluted, no immunostaining was observed in nondiseased portions of the arterial wall at a 1:300 dilution, and some cells in atherosclerotic lesions still displayed a strong immunopositivity, which suggested that these cells overexpressed HSP70. Therefore, we concluded that a dilution of the antibody to 1:300 is suitable to examine the distribution and the nature of cells that overexpress HSP70 in the arterial wall. All single and double immunostaining in this study were performed with the antibody diluted to 1:300.

Double immunohistochemical staining in sections. For double immunostaining, combinations of the antibody to HSP70 with various cell type-specific antibodies were used. This technique allows simultaneous staining for detection of two different antigens in the same cell. Double immunostaining procedures were performed as previously described.[35,36] In brief, after visualization of the first antigen with the ABC substrate kit or with AEC substrate kit, the tissue sections were washed several times during 60 minutes with 0.1 mol/L glycine-hydrochloric buffer (pH, 2.2) at 4°C. The sections were further incubated overnight at 4°C with the secondary antibody. After rinsing in TPBS, the sections were incubated with a biotinylated antibody and then were incubated with alkaline phosphatase-conjugated streptoavidin (Nichirei, Chiba, Japan) or with avidin-biotin complex (Dako). Alternatively, double immunostaining used a combination of the peroxidase-antiperoxidase and alkaline phosphatase-antialkaline phosphatase techniques as used previously.[21] Negative controls were performed as in single immunostaining. Despite that double immunostaining clearly shows the colocalization of two different antigens in the same cells, the formation of immune reaction product with the first primary antibody might preclude access of the secondary primary antibody to the target antigen. To avoid partial visualization of colocalized antigens, the sequence of the application of the primary antibodies was changed and, in addition, analysis of single staining in parallel consecutive sections was used.

En face double immunohistochemical staining in intimal sheets. Intimal sheets were prepared according to Rekhter et al.[31] En face stained intimal sheets are transparent for light microscopic examination and allow the scanning of the intima at different depths.[31] For en face double immunostaining, acetone-fixed intimal sheets were stained with the primary antibody with the same protocol as described previously for the single immunostaining procedure with the AEC substrate kit in sections. After visualization of the first antigen, the intimal sheets were washed several times during 60 minutes with 0.1 mol/L glycine-hydrochloric buffer (pH, 2.2) at 4°C. The intimal

sheets were further incubated overnight at 4°C with the second primary antibody. After rinsing in PBS, the intimal sheets were incubated with an appropriate biotinylated antibody (Dako) for 12 hours at 4°C. After washing in PBS, the intimal sheets were incubated at room temperature with alkaline phosphatase-conjugated streptoavidin (Nichirei) for 30 minutes and the reaction was then visualized with a fast blue substrate kit (Nichirei) as previously described.[35,36] Because the order of staining might affect the results, the sequence of the primary antibody application was reversed. Negative controls were performed as described previously with the omission of the primary antibodies. None of the negative control sections showed positive immune staining.

Electron microscopic analysis. Electron microscopic examination was performed to analyze the cell composition of the arterial wall, with special attention to the structural characteristics of DCs and their contacts with other intimal cells. After fixation of arterial samples in 2.5% glutaraldehyde in PBS (pH, 7.2), specimens were postfixed in 1% osmium tetroxide, dehydrated in graded ethanol and propylene oxide, and then embedded in Araldite resin as used previously.[19] Serial ultrathin sections were cut on an LKB-III ultratome (Stockholm, Sweden). Ultrathin sections were stained with uranyl acetate and lead citrate and examined with the aid of a Hitachi H7000 electron microscope (Hitachi, Japan) at an accelerating voltage of 100 kV.

RESULTS

Distribution of heat shock protein–70 overexpression in atherosclerotic plaques. Cells that overexpressed HSP70 were detected in all atherosclerotic lesions in both carotid and aortic specimens, and no HSP70 overexpression was identified in all the nondiseased portions of the arterial wall (the approach to distinguish between expression and overexpression of HSP70 in the arterial specimens is described previously, in the Methods section). In some segments of atherosclerotic arterial wall adjacent to plaques, HSP70-positive cells were also present in the media.

The patterns of HSP70 distribution in carotid artery specimens were similar to those in aortic specimens. In different atherosclerotic lesions in the same vessel, numerical differences in HSP70 overexpression were apparent between different layers of the intima and in different zones of the same layers in all types of atherosclerotic lesions (III, V, and VI types of atherosclerotic lesions[32,33]). Within these atherosclerotic plaques, the areas containing HSP70 immunopositive cells bordered with the areas that were free from HSP70 immunopositivity. The variations in the extent of HSP70 expression were obviously not related to the age of the patient or the duration of disease because within the same arterial segments there were lesions with different extents of HSP70 overexpression.

The most intensely immunostained cells were nonfoam oval or elongated cells, which, typically, were longitudinally orientated along the arterial lumen (Fig 1*A*). Some HSP70 immunopositive cells were represented by round or irregularly shaped cells containing a large number of lipid vacuoles (Fig 1*A*), and the intensity of HSP70 immunostaining in these foam cells was typically low. In advanced atheromatous plaques (V and VI types of atherosclerotic lesions[32,33]), the external parts of the lipid (necrotic) core were typically rich in HSP70 immunopositive cells, but the fibrous caps showed a low proportion of HSP70 overexpressing cells.

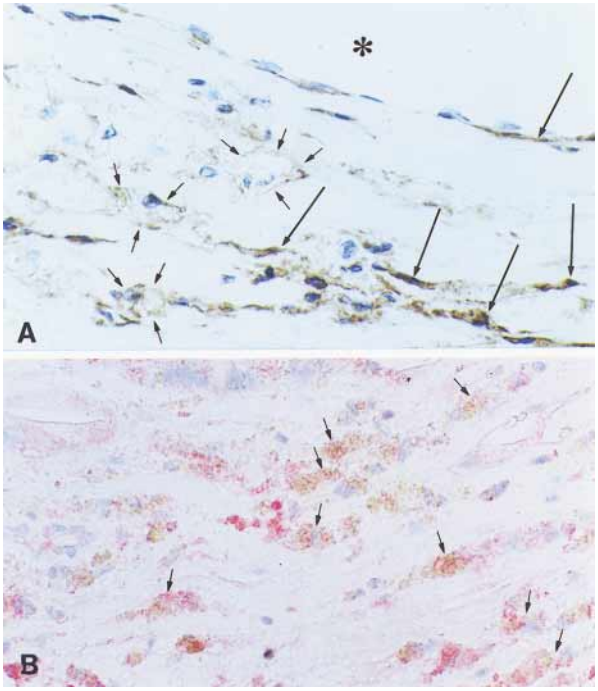

Fig 1. Heat shock protein–70 (HSP70) distribution in atherosclerotic plaques in carotid artery. **A,** Expression of HSP70 with non-foam cells *(large arrows)* and foam cells *(small arrows)* in superficial portion of atheromatous plaque. Lumen of artery is marked with *asterisk*. ABC immunoperoxidase technique. Visualization of HSP70 was produced with diaminobenzidine *(brown-colored product)*. Counterstaining with Mayer's hematoxylin. **B,** Association of HSP70 *(brown)* with CD68 antigen *(rose)* in shoulder area of atheromatous plaque (double immunostained cells are shown with *arrows*). Combination of peroxidase-antiperoxidase and alkaline phosphatase-antialkaline phosphatase immunotechniques using AEC substrate and diaminobenzidine. Counterstaining with Mayer's hematoxylin. Magnification: ×400.

Nature of cells overexpressing heat shock protein–70 in atherosclerotic plaques. Comparison of serial sections stained with cell type-specific antibodies showed that most cells that overexpressed HSP70 were macrophages. Double immunostaining confirmed that HSP70 overexpressing cells also contained CD68 antigen (Fig 1*B*). Macrophages and some macrophage foam cells surrounding the lipid core showed the strongest HSP70 immunopositivity. Analysis of serial sections and double immunostaining also showed that a small proportion of smooth muscle cells (α–smooth muscle actin$^+$) displayed HSP70 overexpression. Comparison of consecutive sec-

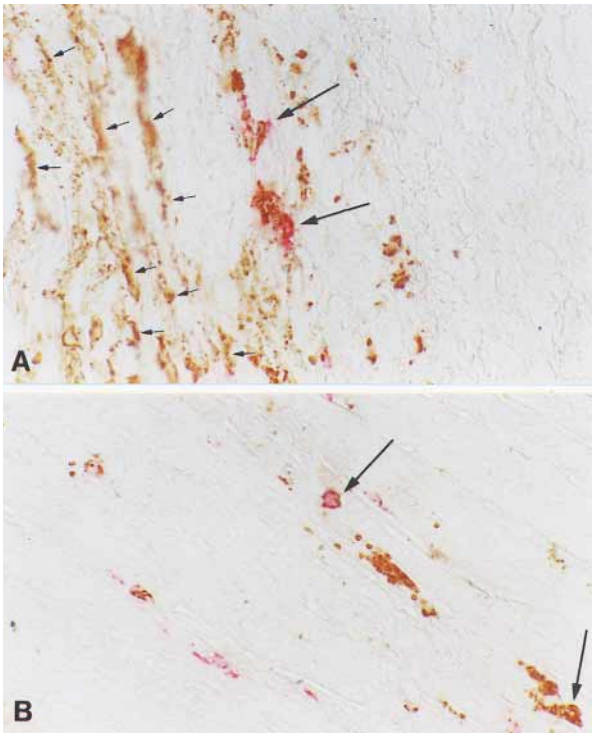

Fig 2. Identification of heat shock protein–70 (HSP70) in dendritic cells in atherosclerotic plaque in carotid artery. **A,** *Arrows* show CD1a+ dendritic cells *(rose)* located on border of necrotic core in complicated atherosclerotic plaque and contained HSP70 *(brown)*. Note intensive expression of HSP70 by foam cells surrounding necrotic (lipid) core (shown with *arrows*). **B,** *Arrows* show CD1a+ dendritic cells *(rose)* located in deep portion of atherosclerotic intima. **A,B,** Combination of peroxidase-antiperoxidase and alkaline phosphatase-antialkaline phosphatase immunotechniques using AEC substrate and diaminobenzidine. Counterstaining with Mayer's hematoxylin. Magnification: ×400.

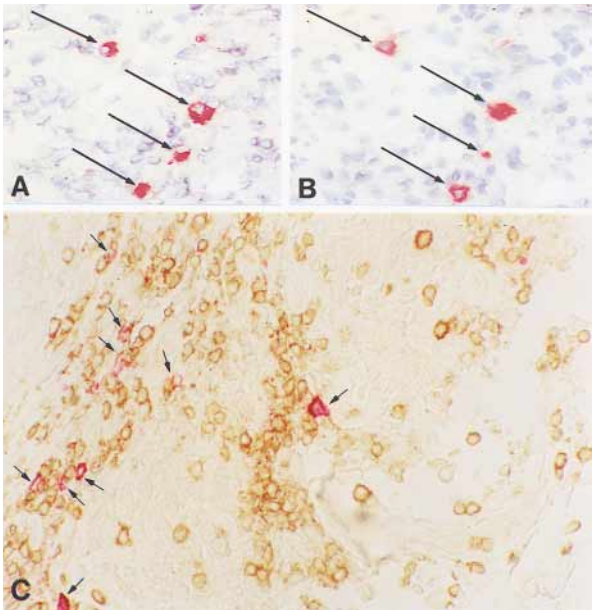

Fig 3. Expression of CD1d by dendritic cells in cell-rich areas in atherosclerotic plaque in carotid artery. **A** and **B** represent consecutive sections that show coexpression of CD1a *(rose;* **A**) and CD1d *(rose;* **B**) by dendritic cells *(arrows)*. Immunoperoxidase technique using AEC substrate; counterstaining with Mayer's hematoxylin. **C,** Colocalization of CD1d+ dendritic cells *(rose; arrows)* with CD3+ T cells *(brown)*. Combination of peroxidase-antiperoxidase and alkaline phosphatase-antialkaline phosphatase immunotechniques using AEC substrate and diaminobenzidine. Magnification: ×400.

tions stained for CD3 and HSP70 immunopositivity indicated that T cells (CD3+) did not overexpress HSP70. When double immunostaining was applied, the colocalization of HSP70 with CD3 antigen was not detected.

In this work, we were particularly interested to see whether DCs overexpress HSP70 in atherosclerotic lesions. Similar to the results of our previous studies,[21,22] this study showed that although CD1a+ DCs were distributed irregularly throughout all the lesions, they were more frequent near the neovasculature or within inflammatory infiltrates. With careful visual scanning, sites where DCs were located separately were found and the patterns of their distribution were compared with the patterns of HSP70 overexpression visualized in parallel consecutive sections. This comparison suggested that the localization of DCs corresponded to the areas that displayed an overexpression of HSP70. Double immunostaining confirmed colocalization of overexpression of HSP70 with CD1a antigen (Fig 2). Practically all DCs located in cell-rich areas and on the border of the necrotic core displayed HSP70 immunopositivity (Fig 2*A*). A large proportion of DCs located in the deep portion of atherosclerotic intima also showed HSP70 immunopositivity (Fig 2*B*). Analysis of parallel consecutive sections showed that in these areas, DCs expressed HLA-DR. HLA-DR was expressed by variety cell types in these areas. Analysis of parallel sections also showed that, in cell-rich areas, DCs coexpressing both CD1a and CD1d were colocalizing with T cells (Fig 3). No other cell types expressed CD1d. Electron microscopic examination confirmed the existence of direct contacts between DC processes and lymphocytes and showed that DCs contained hypertrophied cisterns of the tubulovesicular system, which suggests their activation (Fig 4*A*). Through their processes, DCs also contacted other cell types (Fig 4*B*).

Distribution and nature of cells overexpressing heat shock protein–70 in early intimal lesions. In early intimal lesions (corresponding to type I and the initial stage of type II of atherosclerotic lesions of the classification of Stary et al[32,33]), only some cells located directly under the endothelial monolayer displayed HSP70 overexpression. Analysis of parallel consecutive sections stained with a set of cell type-specific antibodies suggested that this HSP70 overexpression is most likely to be associated with CD1a+ DCs. However, this was not clearly apparent

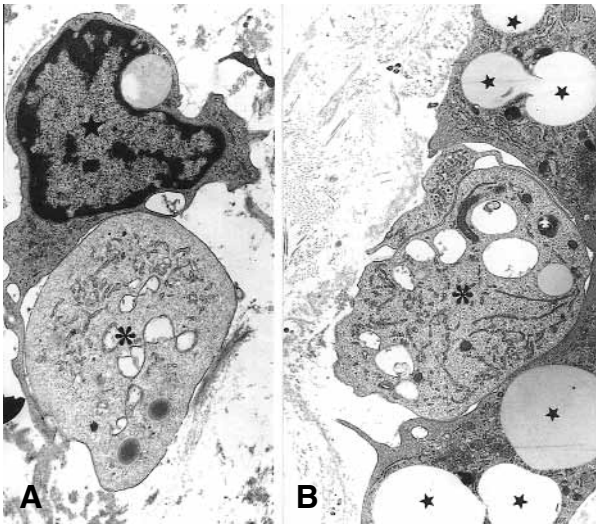

Fig 4. Contacts of dendritic cell processes *(asterisks)*, containing characteristic cisterns of tubulovesicular system, with lymphocyte (*star;* **A**) and foam cell (**B**). **B,** "Lipid droplets" are marked by *stars.* Electron microscopy. Magnification: ×14,000.

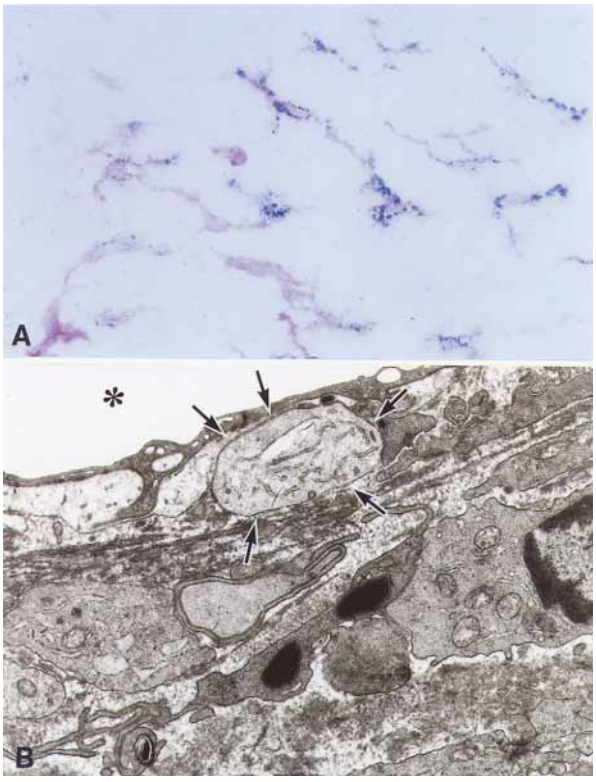

Fig 5. Association of heat shock protein–70 (HSP70) with activated dendritic cells in early intimal lesions of aorta. **A,** Identification of HSP70 *(blue)* within CD1a+ dendritic cells *(rose)* in intimal sheet using en face immunostaining technique. CD1a antigen was visualized with AEC substrate, and visualization of HSP70 was produced with Fast Blue substrate. Magnification: ×400. **B,** *Arrows* show cross-section of dendritic cell process, containing characteristic cisterns of tubulovesicular system, located directly under endothelium. Lumen of aorta is indicated with *asterisk.* Electron microscopy. Magnification: ×19,000.

because it was practically impossible to identify the same cells in parallel sections. In the subendothelial space, DCs were intermingled with both macrophages and T cells, and even though the patterns of HSP70 overexpression were not similar with the patterns of distribution of both macrophages and T cells, unambiguous identification of the nature of overexpressing cells was difficult. For the identification of the nature of cells overexpressing HSP70 in the subendothelial layer, en face staining of intimal sheets was used. Double immunostaining showed that only CD1a+ cells displayed HSP70 immunopositivity (Fig 5*A*). When microscopic fields of the intimal sheets were examined at a magnification of ×400, DCs overexpressing HSP70 were found to constitute as much as 80% of the total number of DCs. Double immunostaining results showed no association between HSP70 and macrophages (CD68+) and between HSP70 and T cells (CD3+).

Electron microscopy showed the presence of cells with characteristics typical of activated DCs. The distinctive feature of these cells was the presence of a tubulovesicular system, which was most developed in the cellular processes (Fig 5*B*). These DC processes were long and twisted, and continuity between the cell body and the cellular processes was established with the analysis of sets of ultrathin consecutive sections.

DISCUSSION

In an earlier immunohistochemical study, Berberian et al[11] found a low homogenous expression of HSP70 throughout the aortic intima and media and a strong irregular HSP70 expression in aortic atherosclerotic plaques. These study results agree with the findings of Berberian at al[11] in relation to aortic specimens, and we also observed a similar pattern of HSP70 expression in carotid specimens. Berberian et al[11] reported that strong HSP70 expression was associated with macrophages and macrophage foam cells. Our observations agree.

We also found that in atherosclerotic plaques, DCs overexpress HSP70, with practically all DCs located on the border of the necrotic core and in cell-rich areas of atherosclerotic lesions that display HSP70 overexpression. In these areas, DCs coexpressed both CD1a and CD1d and colocalized with T cells. CD1 proteins have been recently recognized to be capable of binding and presenting exogenous and endogenous cellular lipids to T cells.[37,38] Lipid antigens are abundant in atherosclerotic lesions, and our observations suggest that lipid antigen presentation by CD1 proteins can contribute to the activation of T cells within plaques and thus initiate the cascade of inflammatory processes, influencing the progression of atherosclerosis.

Evidence of the involvement of immunologic and inflammatory mechanisms in atherogenesis has been accu-

mulating during the past two decades.[12-14] Recently, Wick and coworkers[13] proposed a new autoimmune hypothesis for atherogenesis that postulated: 1, in the healthy arterial intima, disseminated subendothelial accumulations of immunocompetent cells and antigen-presenting DCs form so-called vascular-associated lymphoid tissue, which screens the microenvironment for potentially harmful antigens; and 2, destabilization of vascular-associated lymphoid tissue by autoantigens is responsible for the initiation of immune responses, leading to the development of atherosclerotic lesions. DCs are central to this concept.[13,39] Understanding how DCs are activated could lead to the development of a new approach for therapeutic prophylaxis against atherosclerosis.

We earlier showed that atheroprone aortic areas contain clusters of DCs[19,40] and that some DCs are well-differentiated in early intimal lesions.[41] These observations were further validated by the present electron microscopic examination that showed that the tubulovesicular system in DCs and their cellular processes are hypertrophied. The peculiar network of tubules of the tubulovesicular system, which is a unique feature of DCs, has been suggested to facilitate the internalization and rapid intracellular transport of antigens within the cytoplasm.[18] The observation that the tubulovesicular system is hypertrophied in DCs residing in early intimal lesions implies that these DCs have the capability to engulf and process antigen.

No previous studies have investigated the expression of HSP70 in early atherosclerotic lesions. This work shows that overexpression of HSP70 occurs from the very early stages of atherosclerotic alteration of the arterial intima and that the intimal cells overexpressing HSP70 are DCs. From these findings, we hypothesize that overexpression of HSP70, and possibly some other HSPs, might be important triggers for DC activation. The ability of HSPs to chaperone peptides, including antigenic peptides,[24,26,27] to interact with antigen-presenting cells through a receptor,[28] to stimulate antigen-presenting cells to secrete inflammatory cytokines,[24-26] and to mediate maturation of DCs[29] makes them a unique starting point for the generation of immune responses.

Our work shows that in both early and advanced atherosclerotic lesions, DCs are associated with HSP70. This work used immunohistochemical techniques that have limitations in establishing the origin of HSP70 within HSP70 overexpressing cells. HSP70 might be synthesized directly by DCs. Alternatively, HSP70 might be abundantly accumulated from the extracellular space or large amounts of HSP70 within DCs might be a result of both these events combined. The origin of HSP70 in DCs requires further clarification. However, that HSP70 is associated with DCs in early atherosclerotic lesions suggests that vascular-associated DCs are activated from a very early phase of atherogenesis.

CONCLUSION

In atherosclerotic plaques, various cell types, including macrophages and DCs, overexpress HSP70. DCs that overexpress HSP70 were found frequently in contact with T cells. DCs that cluster with T cells also express CD1d, a unique molecule responsible for presenting lipid antigens. Direct contacts between activated DCs that overexpress HSP70 and T cells might be important in T cell activation because they might facilitate the presentation of lipid antigens to T cells directly within the arterial wall. In early intimal lesions, only DCs overexpress HSP70, which suggests that DCs are activated at a very early stage of atherogenesis.

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