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Adhesion Molecules on the Endothelium and Mononuclear Cells in Human Atherosclerotic Lesions

Allard C. van der Wal, Pranab K. Das, Albert J. Tigges, and Anton E. Becker

From the Department of Cardiovascular Pathology, University of Amsterdam, Academic Medical Center, Amsterdam, The Netherlands

Atherosclerotic lesions show features of a cell-mediated immune inflammatory process. From this viewpoint, the potential role of arterial endothelium in the recruitment of mononuclear cells (T lymphocytes and macrophages) was studied. The endothelium of diffuse intimal thickening (DIT) and atheromatous plaques (AP) in human coronary arteries and abdominal aortas was characterized for the expression of adhesion molecules ELAM-1, ICAM-1, and the major histocompatibility complex (MHC) class II antigens HLA-DR/DP. A marked increase in expression of ICAM-1 and ELAM-1, and to a lesser extent HLA-DR/DP was observed on endothelial cells that were adjacent to subendothelial infiltrates of T lymphocytes ($CD3^+$, $CD11a^+$, HLA-DR/DP^+) and macrophages ($CD14^+$, $CD11a^+$, $CD11c^+$, HLA-DR/DP^+). This contrasted with a lower or absent expression of these activation markers at sites without prominent inflammatory cell infiltrates. These findings could be demonstrated in DIT as well as in AP. The observations suggest that cytokines produced by the subintimal infiltrates may activate the endothelium in a similar way as is observed in the microvasculature at sites of immune inflammation. The expression of these activation markers in the microvasculature is associated with enhanced leukocyte adhesion, permeability for macromolecules, and procoagulant activity, features known to occur also in early experimental atherosclerosis. The findings therefore support the concept that arterial endothelium plays an active role in the recruitment of mononuclear cells in atherosclerotic lesions. (Am J Pathol 1992, 141: 1427–1433)

It is currently acknowledged that atherosclerotic lesions contain an immune-mediated inflammatory reaction.[1–5] To this extent, the involvement of endothelial cells is considered to be important. This is based particularly on previous observations that cultured endothelium as well as endothelial cells of the microvasculature are known to become activated under the influence of the cytokine network.[6–13] The activated endothelial cells gain new functional status in relation to leukocyte adherence, as well as to changes in permeability of macromolecules and in coagulation activation. These alterations have been observed also in cases of experimental atherosclerosis.[14] These altered functions at the level of the microvasculature are known to be associated with the expression of newly defined adhesion molecules.[10,12,13] Adhesion molecules can be identified with the use of immunohistochemical techniques.

For these reasons, the study of the activation state of endothelial cells, particularly with regard to the expression of adhesion molecules, in relation to the inflammatory cellular infiltrates in atherosclerotic lesions, has been undertaken.

Materials and Methods

Tissue Sampling

Arterial tissues were obtained from autopsies of 14 patients who died of cardiovascular diseases (n = 10) or neurologic diseases (n = 4). Exclusion criteria were immunologic diseases, sepsis, and pulmonary hypertension.

The ages of the patients ranged from 30 to 64 years (mean age, 58), and the material was sampled within 2 to 9 hours after death (mean time span, 5 hours). Selected tissues included segments of the left coronary artery (ring-shaped) and of the abdominal aorta (1 cm length). These were taken from sites that were macroscopically normal and from small elevated, but otherwise uncomplicated, plaques. In addition, five segments of pulmonary

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Address reprint requests to Dr. A. E. Becker, Department of Cardiovascular Pathology, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam-Zuidoost, The Netherlands.

arteries (1 cm length) and aortic tissue from five infant autopsies (0 to 4 years) was sampled. Immediately after sampling, the segments were snap-frozen in liquid nitrogen and stored at −70°C. Cryostat sections, stained with hematoxylin and eosin, were used to classify the lesions.

Immunohistochemical Techniques

Immuno-single staining was performed in a three-step indirect immunoperoxidase assay.[15] Briefly, monoclonal antibodies (MAbs) were applied to 6-μ frozen sections, followed by a second-step incubation with peroxidase-labeled rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark) and a third-step horseradish peroxidase-labeled swine anti-rabbit immunoglobulin (Dakopatts).

Peroxidase activity was visualized with 3-amino-9-ethylcarbazole (AEC), and the sections were faintly counterstained with hematoxylin.

Immuno-double staining was performed combining the detection of an unlabeled MAb and a fluorescein isothiocyanate (FITC)-labeled antibody, in which FITC served as a hapten.[15] Briefly, the first unlabeled MAb was applied to the sections, followed by alkaline phosphatase-labeled goat anti-mouse immunoglobulin (Dakopatts). This reaction was terminated using normal mouse serum (blocking step). The FITC-labeled second MAb was applied, followed by incubation with rabbit anti-FITC immunoglobulin (Dakopatts) and subsequently coupled with peroxidase-conjugated swine-anti-rabbit immunoglobulin (Dakopatts). Finally, alkaline phosphatase was visualized with fast blue BB and the peroxidase with AEC.

The MAbs used are listed in Table 1, including their specificities relevant to the current study, appropriate dilution, sources, and selected references.[13,16–19] For double-staining method, the following combinations were used: FVIII-RA/Leu-4, FVIII-RA/Leu-M3, FVIII-RA/HLA-DR, ICAM-1/Leu-4, ELAM-1/Leu-4, ICAM-1/Leu-M3, LFA-1/Leu-4, HLA-DR/Leu-4, HLA-DR/Leu-M3.

Control tissues for testing the specificity of the reactivity of the antibodies included the microvasculature of human noninflamed skin and human psoriatic skin, in accordance with a previous communication in this context.[20]

Sections of arterial tissue, treated in identical fashion but with an irrelevant MAb of the same isotype, were used as negative controls.

Results

The aortic and coronary artery segments were classified either as diffuse intimal thickening (DIT, flat fibrocellular thickening of the intima) or as atheromatous plaques (AP, raised lesions with a central core of necrotic lipid rich debris surrounded by a fibrocellular cap). The pulmonary artery and infant aortas showed only minimal intimal thickening. The number of specimens is shown in Table 2.

Subendothelial infiltrates (Figure 1A, B) were defined

Table 1. *List of Antibodies Used for Immunohistochemistry*

Antibody	CD-code	Specificity relevant to present study	Dilution		Source
			Single staining	Double staining	
FVIII-RA	—	Endothelial cells	1:800	1:400	a
Anti-HLA-DR	—	MHC class II molecules (HLA-DR)	1:100	1:50	b
Anti-HLA-DP	—	MHC class II molecules (HLA-DP)	1:20	—	b
Leu 4	3	Pan T cell	1:20	1:10	b
Leu M3	14	Monocytes, macrophages	1:10	1:10	b
Anti-ELAM-1 (MAb 1.2 B6)	n.d.	Adhesion molecule[16] on activated endothelium	1:10	1:10	c
Anti-ICAM-1 (MAb 6.5 B5)	54	Adhesion molecule[13,16] on endothelium, macrophages, lymphocytes	1:20	1:10	c
Anti-LFA-1	11a	Complementary ligand[17,18] to ICAM-1 expressed on macrophages, lymphocytes	1:10	1:5	d
P150,95	11c	Complementary ligand (?)[17,19] expressed mainly on macrophages	1:10	—	a

a, Dakopatts (Glostrup, Denmark); b, Becton and Dickinson (Mountain View, California, USA); c, Dr. D. O. Haskard Royal Postgraduate Medical School (London, UK); d, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (Amsterdam, The Netherlands).

Table 2. *The Number of Tissue Segments Studied and the Reactivity of the Endothelium with Anti-adhesion Molecules and Anti-MHC Class II Antibodies*

Anatomic site	Histology	Total	Expression of activation markers on endothelium					Clusters of mononuclear cells
			ICAM-1	ELAM-1	HLA-DR	HLA-DP	F-VIII	
Coronary artery	DIT	13	11	5	5	1	13	9/13
	AP	9	8	5	4	3	9	8/9
Abdominal aorta	DIT	10	7	4	3	0	10	5/10
	AP	17	12	10	9	7	17	15/17
Pulmonary artery		5	2	0	1	0	5	—
Infant aortas		5	2	0	1	0	5	—

as focal accumulations of T-lymphocytes (Leu 4^+) and macrophages (Leu 3^+).

Immunophenotype of Arterial Endothelium

The reactivity pattern of the endothelium, taken from different anatomic sites, using anti-adhesion molecules, is summarized in Table 2. Anti–FVIII-RA staining in most cases showed a continuous lining of endothelial cells in, but in a small number there were interruptions of staining. All the lesions showed anti–FVIII-RA reactivity.

With anti-adhesion molecules, a patchy staining was found, which ranged from a few scattered cells to areas of continuous lining. Anti–ELAM-1 binding was found in coronary arteries and aortas associated with DIT and AP, but more often in larger areas in AP.

Of all the tested activation markers, anti–ICAM-1 binding was observed most frequently and most abundantly in DIT and AP, in which the shoulders of the plaques were preferentially involved. Moreover, two of five infant aortas and two pulmonary arteries showed a faint anti–ICAM-1 staining. Anti-MHC class II reactivity appeared to be the most variable marker in DIT as well as in AP, of which anti–HLA-DR staining was present more frequently than anti–HLA-DP staining. Using these anti-MHC class II antibodies, the staining intensity of endothelial cells was much lower as compared with the strong reactivity of subendothelial mononuclear cells. In contrast, the staining intensity of anti–ICAM-1 was about the same or even stronger in endothelial cells. Reactivity with the different activation markers occurred at about the same localization in one and the same lesion, as demonstrated by serial sections (Figure 1C, D). Expression of activation markers related to subendothelial infiltrates.

Foci of activated endothelium, as identified by the above-mentioned MAbs, were screened for the presence of subendothelial clusters of T lymphocytes (Leu-4^+) and macrophages (Leu-M3^+). Only scattered lymphocytes and macrophages could be detected in the thin intimal layer of pulmonary arteries and infant aortas.

Segments of adult coronary arteries and abdominal aortas showed prominent subendothelial clusters of mononuclear inflammatory cells in sections with DIT (14 of 23 cases) and in AP (23 of 26 cases). In AP, these infiltrates were located subendothelially in the fibrous cap and, most abundantly, in the shoulder region of the lesions. Here a low number was noticed scattered through the intima or deep in the plaques around the atheroma as foamy macrophages. The expression of activation markers on endothelium overlying subendothelial inflammatory infiltrates is shown in Figure 2.

In sections containing a subendothelial cellular infiltrate, an almost uniform, albeit patchy expression of ICAM-1 was found in DIT (92% of sections) and AP (86% of sections). Conversely, ICAM-1 expression was also found in lesions devoid of inflammatory infiltrates, but in a much lower number (41%). The expression of ELAM-1, HLA-DR, and HLA-DP molecules on endothelial cells, adjacent to infiltrates, was less common than ICAM-1 expression. The incidence in DIT was 64%, 50%, and 7%, respectively, and in AP, 65%, 56%, and 43%, respectively. Inflammatory infiltrates were absent and the expression of ELAM-1 and HLA-DP dropped to zero; the expression of HLA-DR dropped to 16%. In a selected number of cases the immuno-double staining (ICAM-1/Leu 4, ELAM-1/Leu 4, ICAM-1/Leu M3) clearly visualized adherence and margination of T-cells and monocytes/macrophages at sites of activated endothelial cells (Figure 1E through G).

Immunophenotype of the Subendothelial Infiltrates

The T lymphocytes and macrophages were characterized further with antibodies directed to the aforementioned adhesion molecules and activation markers (Table 1). There was a strong anti–HLA-DR expression of T cells and macrophages. Part of the overall staining positivity with anti–HLA-DR was accounted for by smooth muscle cells. The HLA-DR^+ macrophages were identified by their coexpression of Leu-M3. In addition, most of these cells expressed HLA-DP molecules and CD-11a, CD-

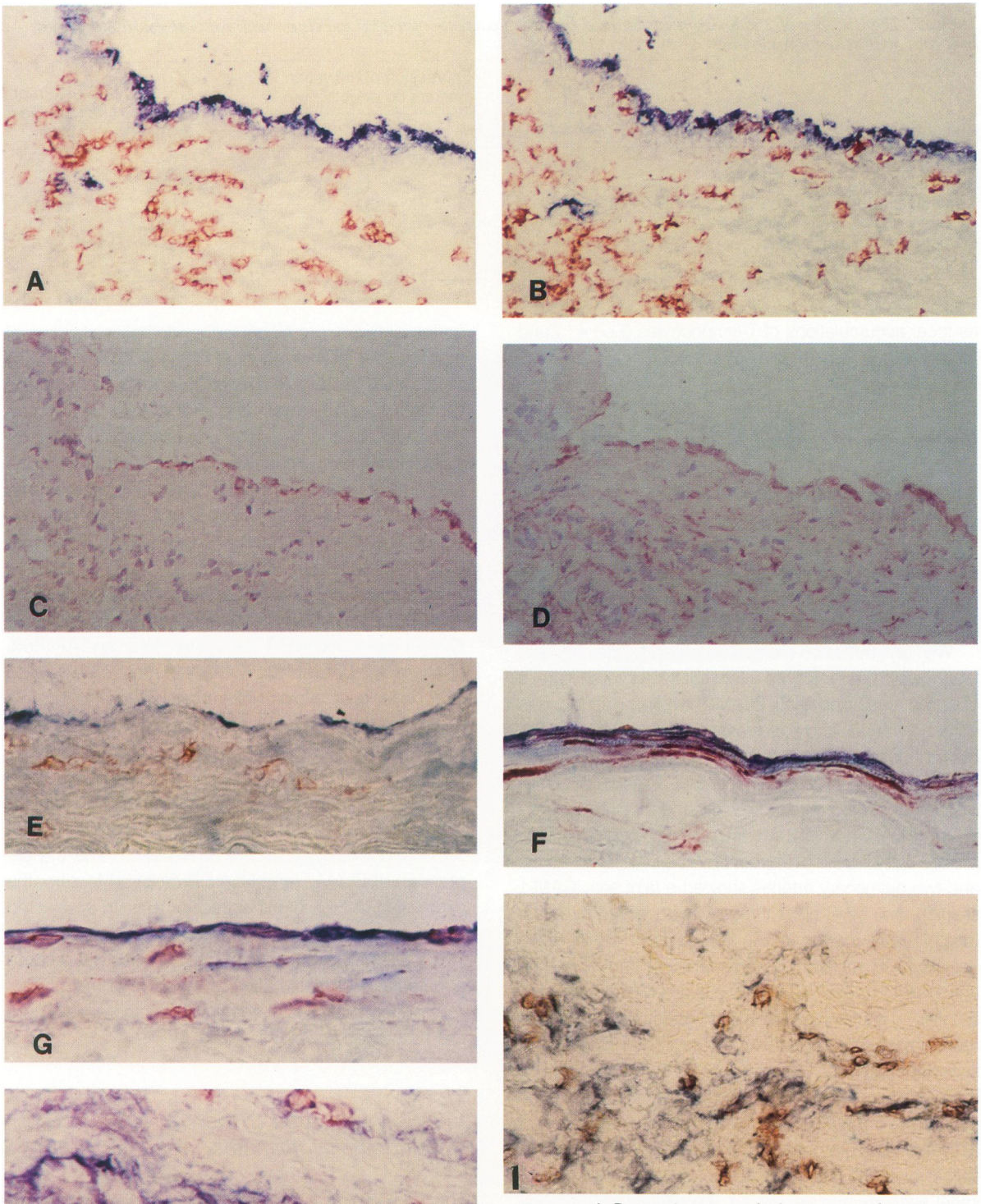

Figure 1. A–D: *Serial sections of atheromatous plaque in coronary artery.* A: *Factor-VIII-RA (blue) and Leu 4 (red) double stain showing subendothelial of T lymphocytes.* B: *FVIII-RA (blue) and Leu M3 (red) showing subendothelial macrophages.* C: *anti-ELAM-1 staining of endothelium.* D: *anti-ICAM-1 staining of endothelium and subendothelial mononuclear cells.* (A–D, ×140). E–G: *Diffuse intimal thickening in abdominal aorta.* E: *Anti-ELAM-1 (blue) and Leu 4 (red) double stain showing adhesion and margination of T lymphocytes.* F: *Factor-VIII-RA (blue) and anti-HLA-DR (red) show weak reactivity of the endothelium (double stained) and strong reactivity of the inflammatory cells with anti-HLA-DR.* G: *Anti-ICAM-1 (blue) and Leu M3 (red) show margination of macrophages.* (E–G, ×350). H,I: *Subendothelial infiltrate in atheromatous plaque, aorta.* H: *anti-ICAM-1 (blue) and Leu4 (red)* (×540). I: *LFA-1 (blue) and Leu 4 (red) double stain* (×350).

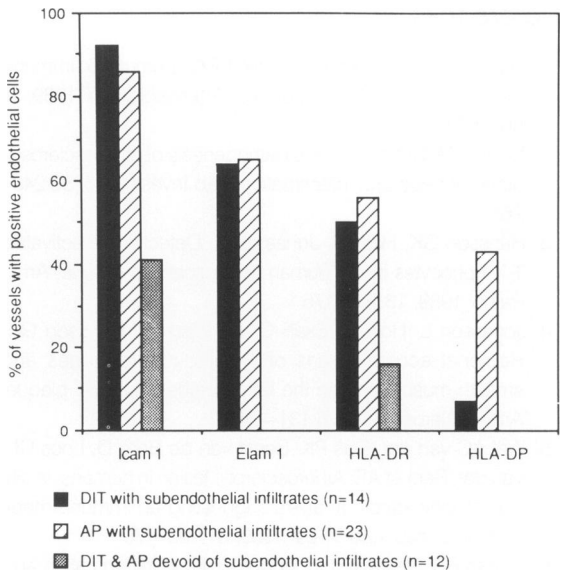

Figure 2. *The expression of activation markers on endothelium in relation to the presence of subendothelial inflammatory infiltrates.*

11c, and ICAM-1, which resulted in a membrane-bound staining pattern of variable intensity. T lymphocytes showed a ring-shaped CD-11a (LFA-1) reactivity. Once related to the pan T-cell reactivity (Leu-4) of the infiltrates, approximately 50% of the T cells was considered to express LFA-1 positivity. Intimate contacts between lymphocytes and macrophages, expressing adhesion molecules, was apparent with the double-staining experiments (Fig. 1H, I; LfA-1/Leu-4, ICAM/Leu-4).

Discussion

In atherosclerotic lesions, inflammatory infiltrates, composed of activated T cells and macrophages, occur.[3–5]

The results of the current study indicate that arterial endothelium in the proximity of these infiltrates shows features of an activated state, as based on the expression of adhesion molecules (ELAM-1, ICAM-1) and to a lesser extent MHC-class II molecules (HLA-DR/DP). Thus far, the expression of adhesion molecules ELAM-1 and ICAM-1 on endothelial cells related to atherosclerotic lesions has not yet been reported in humans. Recently, Cybulsky and Gimbrone[21] detected a newly defined inducible adhesion molecule, expressed by arterial endothelial cells overlying foam cell lesions in rats, which they have called "athero-ELAM." Cytokines produced by the subendothelial inflammatory cells are a likely source for activation of these endothelial cells. Indeed, the cytokines tumor necrosis factor α (TNF-α) and γ-interferon (γ-IFN) have recently been identified in atherosclerotic lesions by means of immunocytochemical techniques.[3,22] In those reports, macrophages, lymphocytes, and smooth muscle cells were considered to produce these cytokines. The current study seems to support this concept because an almost uniform expression of ICAM-1 was found in the endothelium overlying clustered inflammatory cells in DIT and AP.

The fact that ICAM-1 staining occurred also in sections without prominent inflammatory infiltrates, as well as in sections of some normal pulmonary arteries and infant aortas, may lead to a conclusion that the staining reflects the baseline expression of ICAM-1 in these instances.[6,8] Nevertheless, ICAM-1 expression is markedly enhanced in cases of DIT and AP with clustered inflammatory infiltrates.

Intercellular adhesion molecule ICAM-1 uses LFA-1 (CD11a) as counterstructure. ICAM-1 on endothelium mediates the adhesion of LFA-1 positive mononuclear cells.[19] LFA-1 is expressed by T lymphocytes in the lesions, and their presence therefore may point toward the involvement of the LFA-1/ICAM-1 receptor ligand pathway in the recruitment of T lymphocytes in these lesions. The CD11a and CD11c (P150, 95) receptors on monocytes/macrophages mediate the adhesion of these cells to endothelium. Both epitopes were found to be expressed on the monocytes/macrophages in the lesions. Although the ligand for the CD11c receptor on endothelium is still unknown,[23] we observed the adhesion and margination of monocytes preferentially at places of activated endothelium (ELAM-1$^+$, ICAM-1$^+$).

Not only endothelium, but also subendothelial macrophages, expressed ICAM-1. Because the receptor molecules LFA-1 and P150, 95, as well as an abundance of MHC class II antigens, are expressed together in the clusters of T cells and macrophages, this finding emphasizes cellular immune reactions at the subendothelial level.

ELAM-1 is an adhesion molecule unique for endothelium and becomes up-regulated during activation. We found ELAM-1 expression in lesions with prominent infiltrates, but not in the arterial segments devoid of infiltrates. *In vitro* ELAM-1 mediates adhesion of polymorphonuclear cells,[12] and *in vivo* expression is generally associated with an active (granulomatous) inflammation, and delayed hypersensitivity and certain cytokine reactions in the skin.[8,10] In our experiments we found only occasionally polymorphonuclear cells as identified with anti-elastase, which is in accordance with the observations of Jonasson et al,[4] who demonstrated that among all cells involved in atherosclerotic plaques only 0.1% could be identified as polymorphonuclear. Recently, the ELAM-1 has been reported to be involved in cytokine-mediated adhesion of T cells,[24] identified as memory T cells.[25] *In vivo* immunocytochemical experiments have demonstrated ELAM-1 expression in rheumatoid arthritis, a chronic inflammatory process.[26]

The expression of MHC class II antigens of endothe-

lium HLA-DR and HLA-DP was less pronounced than that of the anti-adhesion molecules, and in contrast to the strong HLA-DR and HLA-DP reactivity of subendothelial mononuclear cells. Because MHC class II antigens on endothelium generally are considered to bind processed antigens for T cell recognition,[7] it seems unlikely that antigen presentation is a major function of the arterial endothelium in atherosclerosis. Nevertheless, the presence of activated inflammatory cells indicates an ongoing inflammatory reaction, with an almost certain interaction with the overlying endothelium.

Focal absence of endothelium, as judged by absence of anti–factor-VIII-RA staining was found in a limited number of atherosclerotic plaques with dense mononuclear infiltrates. This loss of endothelium has been observed also at the ultrastructural level[27] and in other immunocytochemical studies.[28] The spotty loss of endothelial identification could, of course, be due to an artifact secondary to autolysis or histologic processing. Conversely, one may speculate also, purely on theoretical grounds, that such endothelial cell loss could be due also to cytotoxic effects of locally produced cytokines (mainly TNF).[29]

The results of this study indicate that the endothelium synthesizes adhesion molecules at sites of atherosclerotic lesions associated with subendothelial inflammatory infiltrates. The analogy with the endothelium of the microvasculature,[6–8] and with observations in experimental atherosclerosis,[14,21] suggests that the arterial endothelium is actively involved in the recruitment of mononuclear cells. *In vivo* studies of endothelial cell adhesion molecules are needed to verify whether this phenomenon contributes to progression or regression of lesions.

Note Added in Proof

After submitting the manuscript, at the request of one reviewer, we have studied the reactivity of endothelium with a second anti-ELAM antibody. Segments of coronary and carotid arteries obtained at autopsy that contained atherosclerotic plaques were used. Single staining experiments were carried out with a commercially available anti–ELAM-1 antibody, clone ENA-1 (Sanbio BV, Uden, The Netherlands). This antibody has been demonstrated to react with human endothelial cells activated with TNF and IL-1.[30,31] Positive staining of endothelial cells with this antibody was obtained in a similar manner as described with the anti-ELAM-1 antibody 1.2B6.

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