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Antigenic Heterogeneity of Vascular Endothelium

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The antigenic status of vascular endothelium from different sites of the normal adult and fetal human cardiovascular system was investigated. Tissues included aorta (n = 9), pulmonary artery (n = 8), coronary artery (n = 6), ventricle/atrium (n = >10), lymph node (n = 2), fetal whole heart (n = 3), and umbilical cord (n = 7). Frozen sections were studied using monoclonal antibodies recognizing endothelial markers (EN4, vWf, Pal-E, and 44G4), vascular adhesion molecules (ICAM-1, ELAM, VCAM, and PECAM), the monocyte/endothelial marker (OKM5), and major histocompatibility complex (MHC) molecules (class I and class II). Results demonstrate that capillary endothelium is phenotypically different from endothelial cells (EC) lining large vessels. Capillary EC strongly express MHC classes I and II, ICAM, and OKM5, which are variably weak to undetectable on large vessels. In contrast, the large vessels strongly express vWf and appear to constitutively express ELAM-1. This suggests that the capillary EC may be more efficient at antigen presentation or more susceptible to immune attack in vivo. ***Interestingly, normal coronary arteries, unlike all other large vessels, express MHC class II and VCAM molecules. Future studies should concentrate on comparative functional studies between capillary, coronary, and large vessel EC. (Am J Pathol 1992, 141:673–683)***

It is now well appreciated that endothelial cells (EC) contribute actively to the development of local vascular immune and inflammatory responses. Specific aspects of the role of vascular endothelium in inflammation include the initiation of coagulation by von Willebrand factor (vWf), modulation of leukocyte–vessel wall adhesion, and participation as antigen-presenting cells. Modulation of leukocyte–vessel wall adhesion is thought to be mediated by constitutive or cytokine–upregulated expression of some or all of the following vascular endothelial molecules: endothelial leukocyte adhesion molecule-1 (ELAM-1),[1] vascular adhesion molecule (VCAM),[2] intercellular adhesion molecule (ICAM-1),[3] and platelet EC adhesion molecule (PECAM), a molecule (CD31) reported to be found on all endothelial cells[4] and platelets. Endothelial cells have been shown to share various phenotypic and functional properties with antigen-presenting cells (APC) of the macrophage/monocyte lineage, including expression and induction of HLA-DR,[5–7] induction of Fc and complement receptors,[8] expression of monocyte cell antigens,[9,10] antigen-induced T-cell proliferation,[11,12] and presentation of peptides to primed T cells.[13] Endothelial cells can be stimulated to secrete immunoregulatory factors such as interleukin-1[14] and interleukin-6.[15]

Recent studies have shown that vascular EC in different anatomic compartments of the liver,[16,17] lung,[18] and kidney[19,20] expressed different patterns of surface antigens. In the current study, we have used monoclonal antibodies and immunocytochemical methods to investigate the expression of endothelial antigens functionally involved in different processes, including coagulation, adhesion, and antigen presentation in vascular endothelium from different regions of the human cardiovascular system (adult and fetal), and have also included umbilical vessels and human lymph nodes.

Materials and Methods

Tissue Specimens

Histologically normal tissue from different anatomic compartments of adult human cardiovascular system were obtained from the donor (aorta, coronary artery, pulmonary artery, right ventricular biopsy, or pieces of atrium and lymph node) or explanted recipient heart (coronary artery) at the time of cardiac transplantation (Table 1). Fetal tissue included whole heart, (from fetuses at 16 to

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Table 1. *Human Tissue Specimens*

Adult	Number
Donor aorta	9
Donor pulmonary artery	8
Donor coronary artery	3
Recip. coronary artery	2
Donor ventricle/atrium (capillaries/endocardium)	>10
Lymph node	2
Fetal	
Heart (capillaries/endocardium)	3
Umbilical cord	7

18 weeks gestation), obtained from the Medical Research Council Tissue Bank (Royal Marsden Hospital, London, UK) and umbilical cords (taken at full term), obtained from the maternity unit at Hillingdon Hospital, Middlesex, United Kingdom. Local ethical permission for use of fetal hearts was obtained. Specimens were snap frozen and stored in liquid nitrogen until required. Frozen cryostat sections (6 μ) were cut, air dried, and fixed in acetone. For each block of frozen tissue, a section was cut and stained with hematoxylin and eosin (H&E), and sequential sections were stained with a panel of mouse anti-human monoclonal antibodies (Table 2).

Immunocytochemistry

Immunocytochemical investigation was carried out using a standard APAAP technique.[21] Briefly, the primary mouse monoclonal antibody was followed by rabbit anti-mouse IgG, then by mouse monoclonal anti-alkaline phosphatase and alkaline phosphatase complexes. Sites of alkaline phosphatase fixation were visualized by incubation with substrate (fast red TR salt (4 mmol/l), dissolved in TRIS buffer, pH 8.2 (100 mmol/l) containing naphthol as MX phosphate (0.5 mmol/l) and levamisole (0.5 mmol/l) added to inhibit nonspecific staining); prepared immediately before use. The final antibody concentration (Table 2) had been determined by serial dilutions on sections of human heart. A control section with the primary antibody omitted was included for each tissue specimen. Sections were counterstained with Harris' hematoxylin and mounted in Apathy's medium. The reactivity of the various monoclonals was graded, taking account of both the intensity and the extent (ie, whether patchy or uniformly distributed) of staining, using the following scale (vw = very weak, w = weak) listed in Table 3.

Table 3. *Scoring System*

Score	Intensity	Extent
7	++	Uniform
6	+	Uniform
5	+	Patchy
4	w+	Uniform
3	w+	Patchy
2	vw+	Uniform
1	vw+	Patchy
0	−	(comparable with control section)

Each tissue specimen was scored individually by two observers, unaware of the origin of the tissue and the arithmetic mean value for the group tabulated (Tables 4, 5). Although all results were taken from sections stained using APAAP, in some instances, for the purpose of illustration, sections were restained with immunoperoxidase for better black and white contrast.

Results

Endothelial Markers EN4, Pal-E, 44G4 vWf

In adult tissue (Table 3), EN4 stained all endothelial cells, within all vessels, regardless of size (Figure 1A, B), including individual capillary cells within the myocardium. Immunohistology shows that virtually all the interstitial cells within the adult heart are EN4 positive (Figure 1A). Platelet EC adhesion molecule exhibited a pan-endothelial staining pattern, very similar to EN4 reactivity,

Table 2. *Mouse Anti-human Monoclonal Antibodies*

MAb	Specificity	Dilution	Source	Ref.
EN4	EC	1/40	Becton Dickinson	(22)
Pal-E	Restricted EC	1/40	Bradshaw Biologicals	(35)
44G4	EC	1/10	A. Gougos	(36)
vWf	Restricted EC	1/50	Dakopatts	(31)
OKM5	Monocytes	1/25	Orthodiagnostics	(29)
OKM1	Monocytes	1/25	Orthodiagnostics	(29)
W6/32	HLA-A,B,C	1/100	Dakopatts	(37)
L243	HLA-DR	1/40	Becton Dickinson	
B721	HLA-DP	1/40	Becton Dickinson	
Leu10	HLA-DQ	1/40	Becton Dickinson	
BB19-I1	ICAM-1 (CD54)	1/200	British Biotech. Ltd.	(38)
BB19-P1	PECAM (CD31)	1/200	British Biotech. Ltd.	(39)
BB19-V1	VCAM-1	1/200	British Biotech. Ltd.	(40)
BB19-E6	ELAM-1	1/200	British Biotech. Ltd.	(1)

Table 4. *Adult Endothelium*

MAb	Capillary	Coronary artery	Aorta	Pulmonary artery	Endocardium	Lymph node (HEV)
EN4	6	6	6	6	6	6
vWf	3	7	7	6	6	6
Pal-E	3	1	3	3	4	6
44G4	6	5	4/5	3	6	ND
MHC-I	6	4/5	4	3/5	3	1/2
MHC-II -DR	6	4/5	0	0	0	0/1
-DP	4	0	0	0	0	ND
-DQ	2	0	0	0	0	ND
ICAM-1	6	5/4	4	3/4	4	4
PECAM	6	6	6	6	6	6
VCAM-1	0	4	0	0	2/3	0
ELAM-1	0	2/3	2	2/3	2/3	4
OKM5	6	0	0	0	0	6
OKM1	0	0	0	0	0	0

The level of staining was graded on an increasing rate of 1 (low) to 7 (high); taking account of both the intensity and extent of binding. 0 represents negative staining comparable to control sections.

in all tissues included here (Figure 2A, B). The reactivity of Pal-E antibody with the various vascular endothelium was commonly reduced in comparison to the intensity or the frequency of EN4 binding. This antibody preferentially bound venules and not arterioles in the heart. In the lymph node, HEV were clearly positive. The 44G4 monoclonal antibody (MAb) reacted with all endothelium, with variation existing between, rather than within, tissues. The pattern of staining was frequently diffuse in the larger vessels and appeared to be more intense on the fetal EC. Antibody against vWf intensely stained the endothelium lining the large vessels and endocardium of adult heart, umbilical cord (Figure 3) vessels, and (HEV) within lymph nodes strongly expressed vWf. In contrast, vWf was comparatively weaker and less frequent on capillary EC of adult heart.

In fetal tissues (Table 4), EN4 and 44G4 showed intense staining of capillaries of the heart and umbilical vessels. Von Willebrand's factor was weakly expressed on the capillaries of the heart. Pal-E was strongly expressed on capillaries within the heart but weakly on the vessels.

MHC Antigens and OKM5

The different endothelia examined showed diverse expression of MHC products. Class I (HLA-A, -B, -C) and class II (HLA-DR) antigens were expressed strongly on the capillary EC in adult heart. The immunohistology was similar to that obtained with EN4 (Figure 4A, B). Expression of class II molecules on these cells was such that HLA-DR > DP > DQ. The endocardium and larger vessels often exhibited a patchy distribution of MHC I, varying in intensity. Class II MHC antigens were absent from all large vessels, including endocardium (Figure 5A), except for coronary artery. All five specimens of coronary artery showed medium to strong expression of MHC

Table 5. *Fetal Endothelium*

	Heart		Umbilical cord	
MAb	Capillary	Endocardium	Vein	Artery
EN4	6	6	6	6
vWf	3	2	7	7
Pal-E	6	6	4	4
44G4	6	6	6	6
MHC-I	6	ND	4/5	4/5
MHC-II -DR	0	0	0	0
-DP	0	0	0	0
-DQ	0	0	0	0
ICAM-1	3	0	4/5	5/6
PECAM	6	6	6	6
VCAM-1	0	0	<1	1/2
ELAM-1	0	0	3/4	1
OKM5	6	0	0	0
OKM1	0	0	0	0

The level of staining was graded on an increasing scale of 1 (low) to 7 (high); taking account of both the intensity and extent of binding. 0 represents negative staining comparable to control sections.

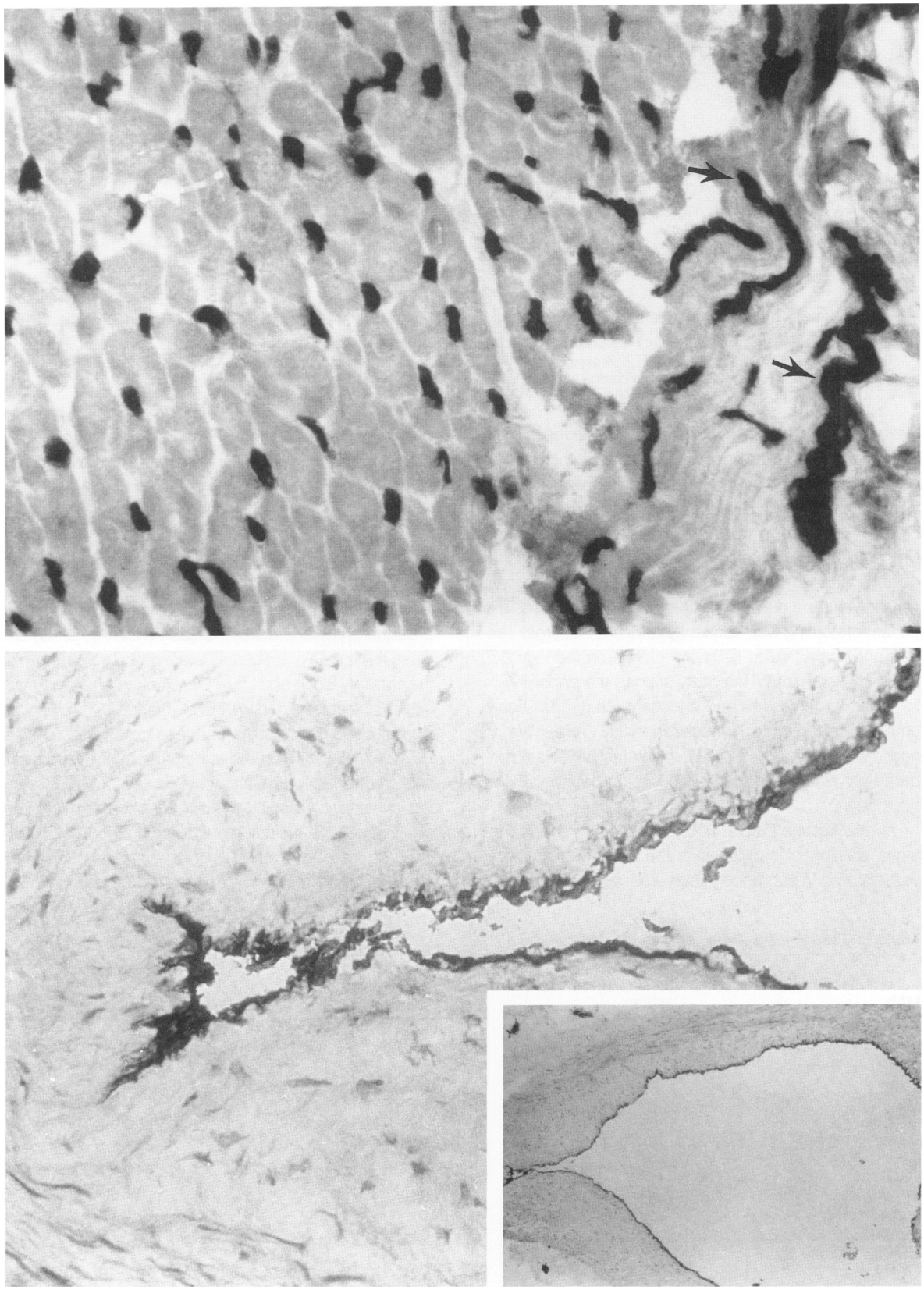


Figure 1. *Photomicrograph (immunoperoxidase) of 6-μm cryostat sections of* **(A)** *normal donor left atrium (×400) and* **(B)** *donor coronary artery (×400, inset ×100) stained with EN4 against endothelial cells. Immunoperoxidase. Arrows indicate longitudinal sections of microvessels which are EN4 positive.*

class II (Figure 5B) as well as class I antigens. Lymphoid HEV exhibited weak or no reactivity for HLA-A, -B, -C, and HLA-DR.

In fetal tissue, capillary cells within the heart strongly expressed class I and OKM5 but were devoid of staining for class II determinants. The umbilical vein and artery were moderately positive for class I but negative for class II and OKM5. Monoclonal antibody OKM1 did not react with any of the endothelium studied. Expression was extremely minimal on interstitial cells within the adult myocardium.

Adhesion Molecules

Intercellular adhesion molecule ICAM-1 was detected to varying degrees on all endothelium examined. It was most strongly and consistently expressed on capillary endothelium in adult heart. The expression of ICAM-1 on EC lining the endocardium, the larger vessels, arterioles and venules, including the HEV, varied from weak to moderate (both within and between specimens) (Figure 6A). Endothelial leukocyte adhesion molecule-1 and VCAM were not detected on capillary EC. However, ELAM staining was found on larger-vessel endothelium (Figure 6B), albeit patchy and to a lesser degree than ICAM-1. The endocardium exhibited some patchy ELAM and VCAM staining. Vascular adhesion molecule was not found on capillary endothelium (Figure 7A) or in any of the large vessels except the coronary arteries (Figure 7B). In the five pieces examined (from three donors, two recipients), there was patchy expression on the endothelial cells. In the lymph node, some HEV would appear quite strongly ELAM positive; others were clearly negative. Vascular adhesion molecule was strongly expressed within the secondary follicular zones of the lymph node, but not detected on HEV.

In fetal heart, neither ELAM nor VCAM were detected and ICAM was found infrequently.

Discussion

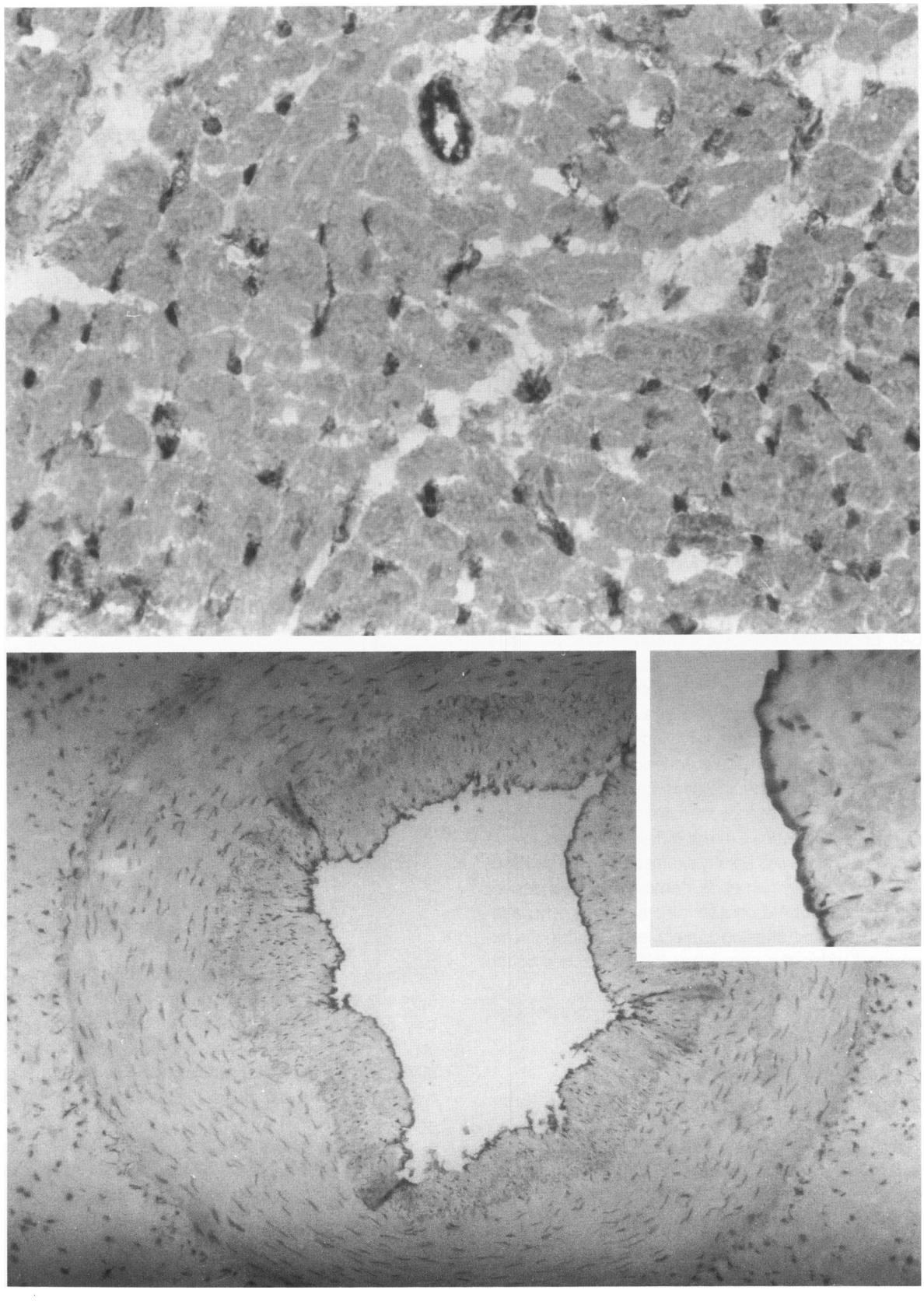
The only two antibodies found in this study to bind to all endothelial cells were EN4 and PECAM. EN4 was one of the earliest anti-endothelial monoclonal antibodies reported as staining all endothelial cells,[22] and our previous studies showed that it stained all capillaries in the human heart.[6] The antigen to which EN4 binds has not been identified. Platelet EC adhesion molecule or CD31 has been shown to be present on endothelial cells from a wide variety of tissues.[4] Both EN4 and MAb against PECAM do not stain infiltrating cells within human cardiac allografts. EN4 binds large foamy macrophages within the lung,[23] as does PECAM (Taylor, in preparation).

The presence of antigens within fetal hearts of 16 to 18 weeks' gestation is an indication of constitutive expression and probably not dependent on immunologic upregulation. EN4, Pal-E, 44G4, MHC class I, PECAM, and OKM5 were all found on the endothelium in the fetal heart, suggesting that expression of the other antigens, vwF, MHC class II antigens DR, DP, and DQ, ICAM-1, VCAM, and ELAM-1 may be inducible.

The important implications of this study are that the vascular endothelium from vessels of different sizes and from different anatomical compartments can express different phenotypic properties and as such may play different roles in normal and diseased states. Endothelial cells have been shown to share many phenotypic and functional properties with antigen-presenting cells,[5,8,11,24,25] suggesting that they may be important effector cells for immune responses. Our results show these similarities are confined *in vivo* to capillary EC and not to EC of large vessels. Previous studies of the antigen-presenting ability of EC have been derived from cultures of large vessels (umbilical vein or aorta) after treatment with cytokines. These studies suggested that the ability of EC to contribute to antigen presentation may occur as an epiphenomenon during inflammatory reactions. Our *in situ* study, however, shows the immunogenic nature of "resting" capillary endothelium in adult human heart, which expresses proteins that are essential for lymphocyte interaction (HLA-A, -B, -C, HLA-DR, ICAM-1, OKM5). It has been suggested that cardiac capillary EC have the capacity to evoke an immune response and play a major role in the initiation of cardiac allograft rejection.[6] These immunohistochemical characteristics of cardiac capillaries are similar to those of sinusoidal EC in the liver,[17] to those of the alveolar septa of the lung[18] and renal capillaries.[19]

A surprising finding of this study, and one of possible significance to the cause of coronary artery disease, was that the EC lining the coronary vessels expressed MHC class II molecules, unlike all the other large vessels studied. Salomon et al[26] found expression of MHC class II on

Figure 2. *Photomicrographs of 6-μm sections of* **(A)** *normal donor left ventricle (immunoperoxidase, ×400) and* **(B)** *coronary artery (APAAP, ×100, inset ×400) stained with α-PECAM MAb. Illustrates the ubiqitous expression of PECAM on endothelium.*



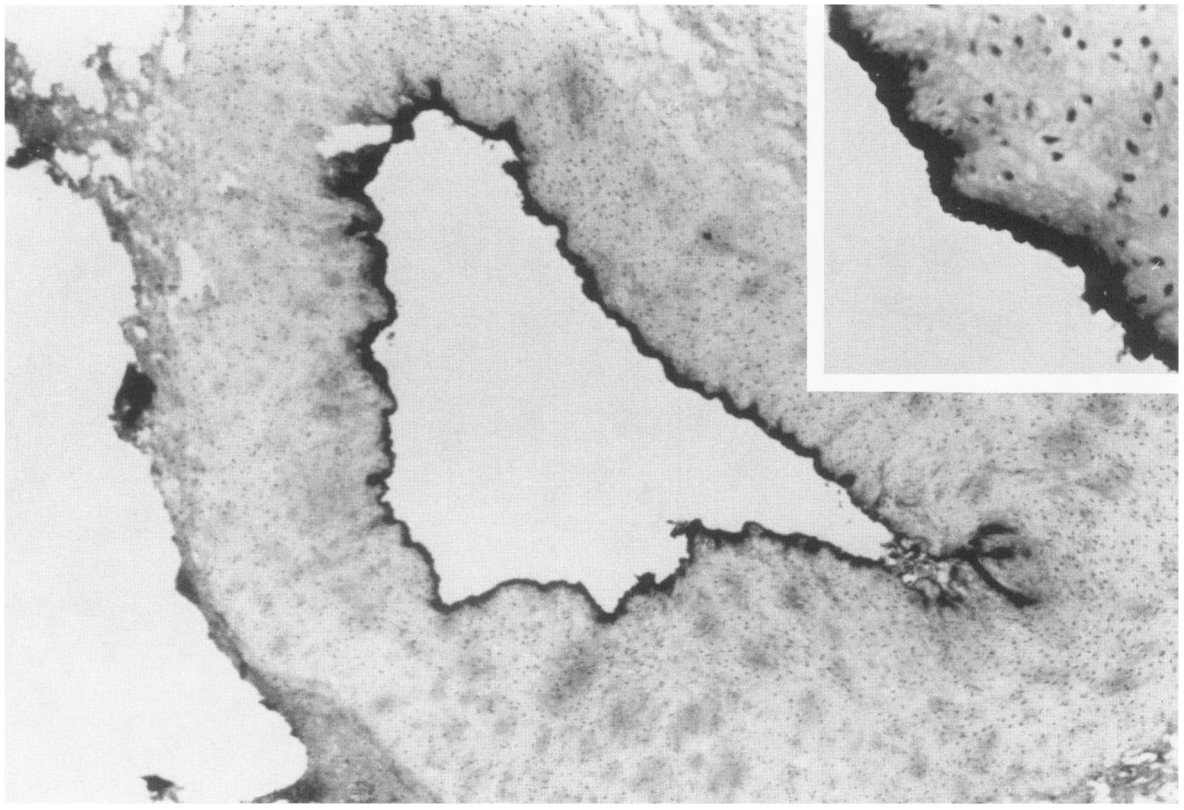


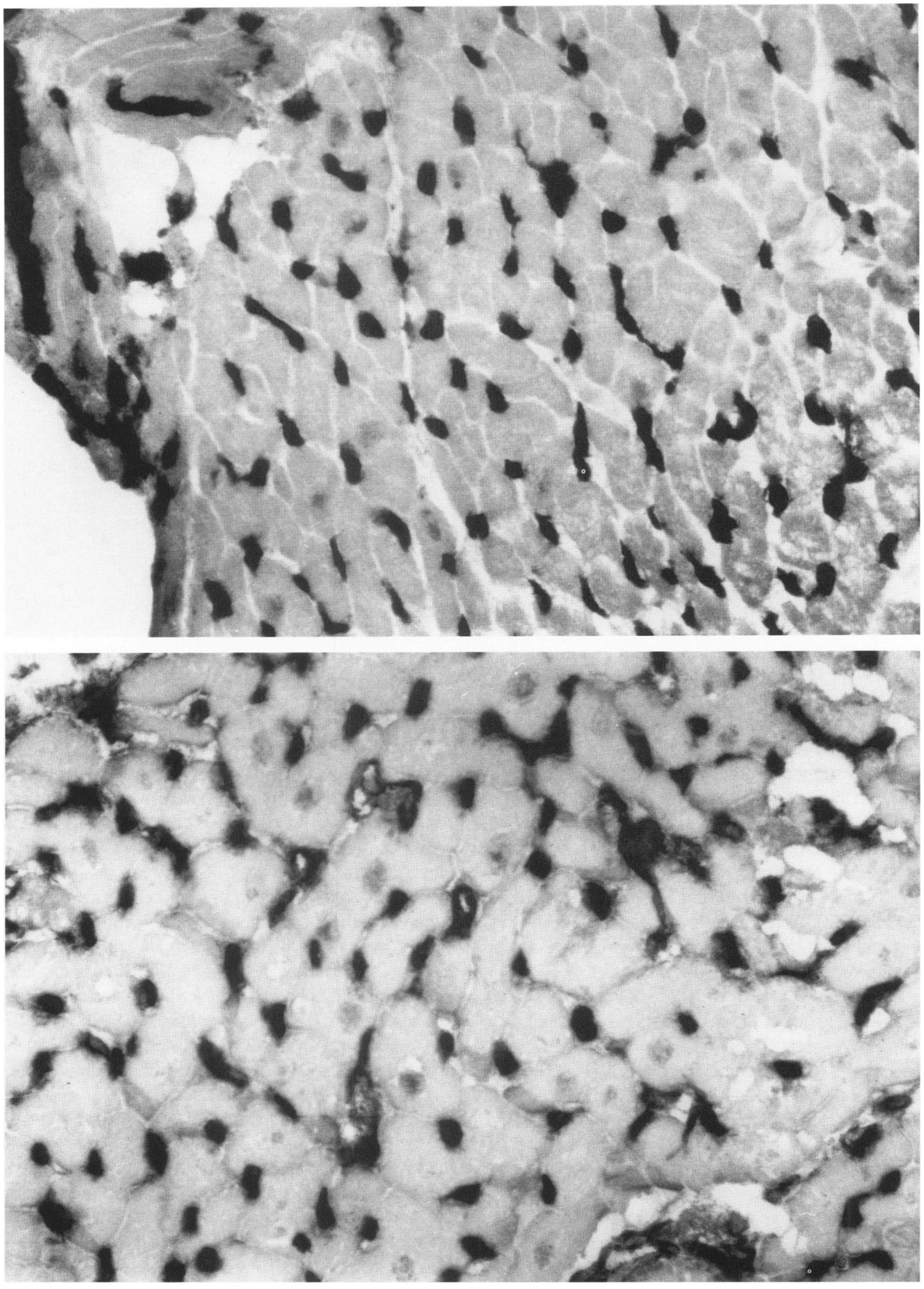
Figure 3. *Photomicrograph (immunoperoxidase, ×50, inset ×400) of 6-μm cryostat section of normal human umbilical vein stained for vWf. Illustrates the strong expression of vWf by the luminal endothelium.*

EC lining the coronary vessels of patients with accelerated coronary artery disease after cardiac transplantation. They suggested that such expression was associated with the pathogenesis of this disease, because no class II was found on EC from mammary vessels, and EC from typical coronary artery plaques were rarely class II positive. The absence of class II from mammary vessels would be consistent with the current study, which finds no class II on EC lining the large vessels: aorta, pulmonary artery, and the umbilical artery and vein. We have not investigated EC in coronary artery disease.

Like Salomon et al,[26] we used the monoclonal antibody L-243 to detect HLA -DR determinants on coronary vessels. We found unequivocal staining of the EC lining the lumen. It will be extremely difficult to obtain fresh normal coronary artery vessels. The five coronary vessels used in this study all appeared histologically normal. Two were from recipients and three from donors not used for transplantation. The recipients had cardiomyopathy, and there is no published evidence for inflammatory involvement of coronary vessels in this disease. The donors were deemed unsuitable for transplantation because of lack of information regarding cardiovascular dynamics. All five vessels examined here also expressed VCAM, which was not found on any other vessel, including capillaries. Vascular adhesion molecule is inducible; like other workers[27] we have found induction of VCAM on capillaries of the heart during transplant rejection.[28] It cannot be excluded that the coronary vessels used in this study have been activated by cytokines, released during the various traumatic procedures employed to prolong life (in the case of the donor heart), and bypass surgery (in the case of the explanted hearts). Clearly a large study of "normal" coronary endothelium, from different sources, is warranted to clarify this important issue.

Monoclonal antibodies OKM1 and OKM5 detect antigenic determinants distributed on functionally distinct human peripheral blood APC subsets.[29] Our study has demonstrated that capillary EC, (in both adult and fetal human heart) clearly reacted with OKM5. Such reactivity was not found on EC of larger vessels. Evidence suggests that expression of monocyte-related antigens by endothelial cells is important in kidney transplant rejection.[30]

Figure 4. *Photomicrograph (imunoperoxidase, ×400) of 6-μm cryostat sections of normal donor left ventricle (of same origin as Figure 1A) stained with* **(A)** *L243 mAb against HLA-DR and* **(B)** *W6/32 MAb against monomorphic MHC class I* **(B).**



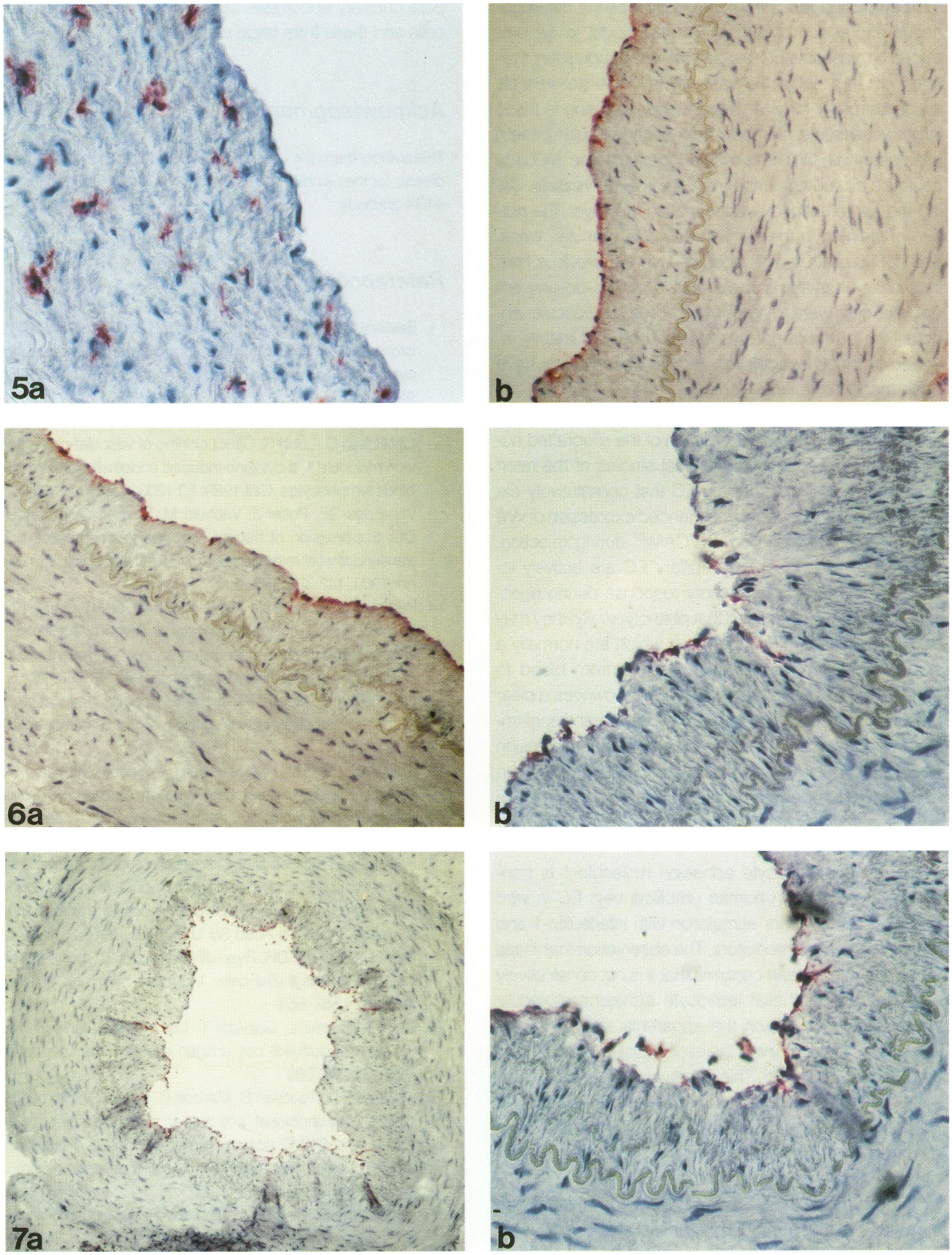


Figure 5. *Color photomicrograph (APAAP, ×400) of 6-μm cryostat sections of* **(a)** *donor left atrium and* **(b)** *coronary artery. Stained with L243 MAb against HLA-DR. Illustrates the absence of DR expression by endocardial EC, such is found on the luminal EC of coronary artery. Note positive cells are present beneath the endothelial monolayer on both sections.*

Figure 6. *Color photomicrographs (APAAP, ×400) of 6-μm cryostat sections of coronary artery. Stained with* **(a)** *α-ICAM-1 and* **(b)** *α-ELAM-1. Illustrates the expression of these adhesion molecules on the luminal EC.*

Figure 7. *Color photomicrograph (APAAP) of 6-μm cryostat sections of coronary artery stained with α-VCAM-1 MAb.* **(a)** *×100,* **(b)** *×400.*

A surprising result from this study was the heterogeneous expression of vWf, which is thought to be produced in vascular EC as part of the coagulation system.[31] In this study, the vascular EC of larger vessels stained strongly for vWf. The pattern of staining in these larger vessels did not show the typical granular pattern often seen with vWf. This may suggest that the antibody could be diluted out further for use on large vessels. Using the same antibody concentration, however, the pattern of staining in the capillary EC was granular, weak, and not ubiquitous. This accords with our previous findings in the heart that only about 30% of EN4-positive endothelial cells were vWf positive.[32] Thus, the vascular endothelium of the larger vessels may be that mainly involved in blood coagulation. Similar observations have been made in the lung.[18]

A perivascular infiltrate of mononuclear cells is characteristic of cell-mediated rejection of the allografted human heart.[33] Immunocytochemical studies of the heart have shown that the capillary EC that constitutively express class II antigens show enhanced expression of vWf and Pal-E[32] and induction of VCAM[28] during rejection. These results suggest the capillary EC are actively involved in the local inflammatory response during rejection. We therefore reasoned that phenotypically, they may be similar to HEV in lymph nodes, which are normally a site of rapid lymphocyte extravasation from blood to node. In the normal human lymph node, however, a clear pattern of vWf staining of the HEV was apparent, confirming previous reports,[34] but there was minimal expression of class II. This is in contrast to previous studies[34] in which lymph nodes from patients with various reactive and neoplastic conditions demonstrated HEV strongly reactive for HLA-DR.

Endothelial leukocyte adhesion molecule-1 is transiently expressed on human umbilical vein EC *in vitro* only after 2 to 8 hours' stimulation with interleukin-1 and other inflammatory mediators. The observation that it was not found in fetal heart confirms that it is not constitutively expressed. Endothelial leukocyte adhesion molecule was found, however, on the apparently normal, noninflamed endothelium lining the large vessels of adult heart and the umbilical cord. This observation plus the finding that ELAM was also found on a population of HEV within lymph node (where neutrophils rarely extravasate) suggests that ELAM is not solely involved in allowing neutrophils to migrate across the endothelium.

In conclusion, these studies describe a marked heterogeneity of expression of endothelial antigens from different vessels. These differences could reflect responses to different microenvironments but are more likely to reflect specialization of EC to perform different functions. It is clearly important to perform functional studies to compare capillary endothelial cells with coronary endothelial cells and those from large vessels.

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