Phenotype of Normal Cutaneous Microvasculature

Immunoelectron microscopic observations with emphasis on the differences between blood vessels and lymphatics

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The lymphatic system has been poorly characterized in comparison to the blood vessels. We investigated the expression of microvasculature markers in cutaneous lymphatics and blood microvessels in normal skin. Scrotal skin was chosen because of its high density of both types of microvessels. A pre-embedding peroxidase-conjugated immunoelectron microscopy technique was used, allowing both the visualization of the lymph and blood vessels and their immunohistochemical staining. The markers studied included endothelial antigens (recognized by PAL-E, EN-4, and von Willebrand factor/factor VIII-related antigen), structural molecules of the vascular wall (α-smooth muscle actin, heparan sulfate proteoglycan, collagen type IV), and adhesion molecules (endothelial leukocyte adhesion molecule-1 [E-selectin], intercellular adhesion molecule-1 [ICAM-1], platelet endothelial adhesion molecule-1 [PECAM-1], vascular cell adhesion molecule-1 [VCAM-1]). It is shown that lymphatics of normal skin are phenotypically different from blood microvasculature, only weakly expressing endothelial markers (EN-4+, von Willebrand factor/factor VIII-related antigen, PAL-E+), mural markers (α-smooth muscle actin−, heparan sulfate proteoglycan−, collagen type IV−) and do not express the studied adhesion molecules except PECAM-1 (E-selectin−, ICAM-1−, PECAM-1+, VCAM-1−). The results were substantiated by a double-labeling immunoelectron microscopic technique, which facilitates detection and assessment of microvascular segments. By this technique, collagen type IV, recognized by a peroxidase-labeled 2nd antibody, stains the basal lamina by a linear pattern, whereas a second optional epitope is visualized as grains by a silver-enhanced ultra-small gold-conjugated antibody. Our study shows that not only morphology but also antigenic phenotype of lymphatics differs significantly from blood vessels.

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The microvasculature of the skin has been characterized by early morphological studies in the 1950s and 1960s. Conventional electronmicroscopic investigations have revealed distinctive microvascular segments based on ultrastructural features [1,2]. The later introduction of vascular markers, initially those of endothelial binding lectins with varying specificity (Ulex europaeus agglutinin), and later on those of more specific polyclonal antibodies (e.g., against von Willebrand factor/factor VIII-related antigen [vWF/FAVIII-rA] [3] and angiotensin-converting enzyme) and monoclonal antibodies (MoAb) against endothelial-specific antigens (e.g., EN-4 [4] and PAL-E [5]), initiated extensive immunohistochemical studies. This has facilitated study of the histogenesis and classification of wound healing, angiogenesis associated with melanoma [6], and Kaposi sarcoma [7].

Remarkably, the cutaneous lymphatic network has been rather neglected, probably due to the lack of specific markers. A limited number of ultrastructural studies [8,9] showed the structure of initial cutaneous lymphatics to be markedly different from that of vascular capillaries. Lymphatics are flattened tubes lined by an extremely attenuated endothelium encompassed only by subendothelial “basal lamina-like” material, whereas surrounding pericytes are lacking [10]. The lymphatic endothelium contains very few pinocytotic vesicles and lacks Weibel-Palade bodies and fenestration [10].

As no comparative studies of the microvascular marker phenotypes of vascular capillaries and lymphatics in normal skin have been performed, we were interested in whether differences could be detected in the expression of common endothelial antigens, structural microvascular molecules, and certain functional antigens such as adhesion molecules.

MATERIAL AND METHODS

Tissue Specimens Fresh surgical biopsies of normal scrotal skin were obtained from four middle-aged patients undergoing a vasectomy at the Department of Urology, University Hospital, Nijmegen, The Netherlands. The samples were minced by a razor blade into smaller pieces of a maximum size of 5 × 5 × 2 mm and fixed for 4 h at room temperature, rotating in freshly prepared periodate-lysine-2% paraformaldehyde fixative, cryopero-
Antibodies

As endothelial markers the mouse anti-human MoAbs PAL-E and EN-4 (both from Sanbio, Uden, The Netherlands) were used. As structural markers the mouse anti-human MoAbs anti-vWF/FVIII-rA (F8/86; Dakopatts, Copenhagen, Denmark), anti-α-smooth muscle actin (1A4; Sigma Chemical Co., St. Louis, MO), anti-heparan sulfate proteoglycan (anti-HSPG) (JM-72; gift of Dr. L.W.P.J. vd Heuvel, Department of Paediatrics, University Hospital, Nijmegen), and the mouse anti-rabbit polyclonal antibody anti-collagen type IV (Organon Teknika, Tumhout, B) were used.

For detection of adhesion molecules, the following monoclonal anti-human MoAbs were purchased from British Biotechnology Ltd. (Abingdon, U.K.): anti-endothelial leukocyte adhesion molecule-1 (anti-E-selectin) (BBIG-E6), anti-intercellular adhesion molecule-1 (anti-ICAM-1) (CD54) (BBIG-11), anti-platelet endothelial adhesion molecule-1 (anti-PECAM-1) (CD31) (BBIG-P1), and anti-vascular cell adhesion molecule-1 (anti-VCAM-1) (BBIG-V1).

Indirect Immunoelectron Microscopy

Sections of 30 μm were cut at −20°C and washed in phosphate-buffered saline with 0.1 M glycine at room temperature for 1 h to quench free aldehyde groups. The sections were incubated at 4°C overnight with the first antibody at an appropriate dilution. Polymeric peroxidase was diluted in phosphate-buffered saline (pH 7.4), containing 1% bovine serum albumin (BSA). Washing procedures were performed with phosphate-buffered saline. After washing for 1.5 h, the sections pretreated with a MoAb were incubated for 1.5 h at room temperature with peroxidase-labeled goat anti- mouse immunoglobulin (RAML-Po-Ig; dilution 1/40; Dakopatts) the sections with a polyclonal first antibody were incubated with peroxidase-labeled swine anti-rabbit Ig (SwAR-Po-Ig; dilution 1/100; Dakopatts). For incubation and washing steps a rotary shaker was used. After washing for 1.5 h at room temperature, demonstration of the peroxidase product, dehydration, and embedding was carried out as described previously [11]. The representativity of the results found for scrotal skin was assured by control biopsies from distinct skin areas. Appropriate irrelevant antibodies were used as controls to assure the specificity of the used antisera.

Double Staining Immunoelectron Microscopy

Thirty-nanometer-thick cryomicrotome sections of peridote-lysine-2% paraformaldehyde-fixed normal scrotal skin were washed three times and incubated with the first MoAb (i.e., EN-4 or anti-PECAM-1) in an appropriate dilution overnight at 4°C. After three washing steps followed by incubation in ultra-small gold-conjugated Fab2 goat anti-mouse IgG (Fab2) GaM-IgG; dilution 1/40 in BSA-C; Aurion, Wageningen, The Netherlands) for 5 h at 37°C, pools of PECAM-1 and anti-collagen type IV at 2.5% glutaraldehyde for 5 min and silver-enhanced over 45 min according to Danscher’s method [12]. After intense washing, the sections were incubated with polyclonal antibody collagen type IV at an appropriate dilution in phosphate-buffered saline overnight at 4°C, washed again for 1.5 h and incubated with SwAR-Po-Ig (dilution 1/100, Dakopatts). After washing for 1.5 h at room temperature, demonstration of the peroxidase product, dehydration, and embedding was carried out as described previously [11]. Controls for cross-reactivity of the two antibodies used in the dual-labeling experiment included: omission of (i) the primary antisera, (ii) the ultra-small gold incubation step, or (iii) the peroxidase immunolabeling technique. In addition, the distribution of the antigens was compared with the patterns found with singly labeled antisera.

Light microscopic Immunohistochemistry

As a control for peridote-lysine-2% paraformaldehyde-fixation, we performed immunohistochemistry for anti-E-selectin and anti-VCAM-1 antibodies on acetone-fixed scrotal skin samples using a three-step ABC-method.

RESULTS

Indirect Immunoelectron Microscopy

Representative immunoelectron micrographs with cross-sections of blood vessels and lymphatics demonstrating the phenotype of normal cutaneous microvasculature are shown in Figs 1 through 5.

Phenotype of Blood Vessels

Blood vessels were identified and classified in distinct segments of cutaneous microvasculature by morphologic criteria as reported previously [1]. A set of specific markers distinguishing the distinct components of the vessel wall (endothelium, basal lamina, perivascular cells) was used.

PAL-E stained the endothelium of venous and arterial capillaries with moderate intensity (Fig 1a). Micropinocytotic vesicles, mainly at the luminal side of the endothelial cells, were clearly labeled, accompanied by a delicate linear staining at the luminal endothelial membrane (Fig 1a). Focal staining was observed at endothelial interdigitations. Arterioles, characterized by an endothelial lining with numerous basal protrusions to a complete encompassing layer of smooth muscle cells, were unlabeled.

EN-4 stained all segments of the dermal microvasculature. The vascular endothelium showed an intense discrete luminal staining and a diffuse, focally intensified staining at the abluminal side (Fig 1c). Part of the micropinocytotic vesicles were also labeled.

In the cytoplasma of endothelial cells lining arterioles and venules, a marked staining of organelles, compatible with Weibel-Palade bodies, was found to be vWF/FVIII-rA positive (not shown). The reaction product was also present in the subendothelial space. The luminal endothelial membrane stained weakly.

The basal lamina, which forms the inner elastic vascular sheet, revealed an homogeneous intense staining with polyclonal antibody anti-collagen type IV. In arterioles, the basal lamina adjacent to endothelial cells and smooth muscle cells was single layered, while in postcapillary venules the basal lamina was multilaminated (Fig 1f). Heparan sulfate proteoglycan presented an almost identical staining pattern as collagen type IV. In the cytoplasm of smooth muscle cells and pericytes of the vascular lamina media α-smooth muscle actin was expressed homogeneously sparing cytoplasmic organelles (Fig 2).

In a high proportion of the microvascular endothelium ICAM-1 positivity (estimated more than 75% of the vascular cross-section) could be detected. Venous and arterial capillaries, especially when located in the upper papillary dermis, were frequently unstained. ICAM-1 positive vessels showed a marked linear labeling restricted to the luminal plasma membrane of the endothelial cells (Fig 3). Sparse staining of micropinocytotic vesicles was intensified in areas of interendothelial contact. No other part of the microvascular wall revealed ICAM-1 staining.

Anti-PECAM-1 marked the cutaneous microvasculature including lymphatics intensively (Fig 4a,b). The majority of blood microvessels showed an intense linear labeling at the luminal aspect of the endothelium. In areas of intercellular contact between endothelial cells, focal positivity with linear to diffuse staining pattern was seen. Part of the micropinocytotic vesicles, mainly at the luminal side, was stained. Few capillaries (Estimated <10%) had a strikingly different staining pattern. They were stained exclusively either at the luminal or at the abluminal side of the endothelial cell. Occasionally, capillaries showed no staining for PECAM-1.

No staining could be detected for E-selectin and VCAM-1 in normal cutaneous microvasculature. This observation was light-microscopically confirmed by a standard indirect immunoperoxidase procedure on acetone-fixed scrotal skin.

Phenotype of Lymphatics Significantly Differs from Blood Vessels

Dermal lymphatics were identified by morphologic criteria: an attenuated endothelium with areas of endothelial overlaps and loose openings, an indistinct and discontinuous basal lamina, and anchoring filaments linking the endothelium to the surrounding connective tissue.

Lymphatics did not stain with MoAbs against the PAL-E antigen (Fig 1b). HSPG or the adhesion molecules E-selectin, ICAM-1, and VCAM-1. Lymphatic capillaries, in contrast to collecting lymphatics, lacked an encompassing sheet of lamina media and did not stain for α-smooth muscle actin (not shown). Endothelial marker EN-4 and anti-PECAM-1 revealed strong reactivity with lymphatic capillaries and collecting lymphatics (Fig 4b,d) and presented a distinct, almost complete lining at the luminal endothelial aspect with pronounced staining at endothelial interdigitations, interendothelial overlaps, and abluminal paracellular clefts. In these areas, staining at the abluminal side could also be detected. In areas of extremely attenuated endothelium, diffuse cytoplasmic staining was
Figure 1. Vascular markers PAL-E, EN-4, and anti-collagen type IV identify distinct components of lymph and blood vessels in normal human skin. a) MoAb PAL-E stains blood vessel with linear staining of the luminal endothelial membrane and focally of the abluminal membrane. Labeling of the micropinocytotic vesicles is not identifiable in this magnification. b) Lymphatic endothelium (right side) is clearly not stained with PAL-E. Note the capillary (left side) with staining of the luminal endothelial membrane. MoAb EN-4 stains blood vessel c) and lymphatics d) with intense linear labeling of the luminal and abluminal surface of endothelial cells. Areas of interendothelial junction show pronounced staining (arrow). e) Polyclonal antibody anti-collagen type IV marks the single-layer basal lamina adjacent to the pericytes of a capillary (upper vessel) and the multilaminated basal lamina of a postcapillary venule (lower vessel) with homogeneous and intense staining. Left upper corner; a peripheral nerve (*) with stained outer layer of the epineurium enveloping axons (arrow). f) Cross-section of a lymph vessel with stained intermittent “basal lamina-like material” reflecting its permeable nature. Arrows, gaps. E, endothelial cell; L, lumen; P, pericyte. Scale bars: a) 2 μm; b) 4 μm; c) 5 μm; d) 4 μm; e) 5 μm; f) 7 μm.

Whereas Heparan sulfate proteoglycan presented a staining pattern in blood vessels almost identical to that of collagen type IV, the subendothelial “basal lamina-like” material in lymph vessels did not stain at all (not shown). The subendothelium of lymphatics sporadically showed a weak diffuse staining with antibody to vWF/FAVIII-rA. In the endothelial cytoplasm, however, Weibel-Palade bodies were not detected.
Figure 2. α-Smooth muscle actin is present in the cytoplasm of pericytes and pericytic dendrites of an arteriole. Cell organelles are spared. Encompassed endothelial cells are also unlabeled. Right upper corner, a tangentially sectioned pericyte. E, endothelial cell; L, lumen; P, pericyte. Scale bar, 4 μm.

The most striking differences in phenotype between cutaneous blood microvessels and lymphatics are listed in Table I.

Double-Labeling Immunoelectron Microscopy

Double-labeling immunoelectron microscopy visualizes the linear peroxidase signal of basal lamina produced by antibody to collagen type IV as described above and results in a highly selective localization of silver-enhanced gold particles at sites with a staining pattern typical for EN-4 or PECAM-1, respectively (Fig 5).

DISCUSSION

In this study, we have demonstrated by immunoelectron microscopy that consistent differences exist in the expression of microvascular markers in normal cutaneous lymphatics compared with blood vessels. The immunoelectron microscopic approach allows identification of the different levels and components of the microvasculature and optimal discrimination between lymph vessels and blood vessels. The results obtained by a pre-embedding indirect immunoelectron microscopic technique were substantiated by a pre-embedding double-labeling immunoelectron microscopy method. The reliability of the results was assured by a panel of appropriate external and internal controls and by examination of a large number of ultrathin sections.

Findings in lymphatic endothelium indicated in Table I are consistent with earlier immunohistochemical reports by light microscopy with various tissues [4,6,13]. In human skin, the absence of PAL-E staining in a microvessel of capillary size appears to indicate that it is a lymphatic. Extension of this feature to neoplastic microvasculature supports the conclusion of previous studies [7,14] that Kaposi sarcoma may have a lymphatic origin. The EN-4 staining pattern of lymphatics, which is different at the ultrastructural level from that of blood vessels, is strikingly similar to that found with anti-PECAM-1. This raises the question whether EN-4 and anti-PECAM-1 do recognize different epitopes of the same antigen.

Regarding the mural microvascular antigens, lymphatics lack the expression of α-smooth muscle actin and of HSPG, whereas the collagen type IV staining is intermittent. This expression pattern is compatible with the permeable nature of their vascular wall [10] and reflects the absence of pericytes. In blood vessels, both pericytes and endothelial cells contribute to the formation of the vascular basal lamina [15], which is therefore more pronounced and continuous. It has been assumed that the different basal lamina composition in lymph capillaries (e.g., the lack of HSPG) may be essential for a free influx and/or efflux of fluids and proteins [16]. It may also facilitate the intravasation of tumor cells. Since pericytes were found to be involved in the earliest phase of angiogenesis appropriate external and internal controls and by examination of a large number of ultrathin sections.

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Figure 4. MoAb anti-ICAM-1 stains a blood vessel with marked linear labeling restricted to the luminal side of endothelial cells. E, endothelial cell; L, lumen; P, pericyte. Scale bar, 5 μm.

Figure 3. MoAb anti-ICAM-1 stains a blood vessel with marked linear labelling restricted to the luminal side of endothelial cells. E, endothelial cell; L, lumen; P, pericyte. Scale bar, 5 μm.
Table I. Summary of the Expression of Microvascular Markers in Cutaneous Blood and Lymph Vessels

<table>
<thead>
<tr>
<th>Antibody/Marker</th>
<th>Blood Vessels</th>
<th>Lymph Vessels</th>
</tr>
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<tbody>
<tr>
<td>PAL-E</td>
<td>Intense staining of the microvascular vessels and focal linear positivity of the luminal endothelial surface</td>
<td>No staining</td>
</tr>
<tr>
<td>EN-4</td>
<td>Linear staining of the luminal endothelial surface, microvascular vesicles stained</td>
<td>Focal staining at endothelial interdigitations or overlaps, microvascular vesicles partly stained</td>
</tr>
<tr>
<td>vWF/FAVIII-rA</td>
<td>Marked cytoplasmic staining of organelles, compatible with Weibel-Palade bodies, and of the subendothelial space; weak staining of the luminal endothelial surface</td>
<td>Sporadically weak staining of the subendothelium</td>
</tr>
<tr>
<td>Collagen type IV</td>
<td>Intense linear staining of basal lamina enclosing endothelium and smooth muscle cells at arterial side, multilaminated linear staining of the basal lamina at venous side</td>
<td>Linear staining of subendothelial matrix encompassing the endothelium, interrupted at interendothelial gaps</td>
</tr>
<tr>
<td>α-Smooth muscle actin</td>
<td>Cytoplasmic staining of surrounding smooth muscle cells/pericytes</td>
<td>No staining of capillaries</td>
</tr>
<tr>
<td>HSPG</td>
<td>Staining pattern like collagen type IV, but less intense</td>
<td>No staining</td>
</tr>
<tr>
<td>E-selectin</td>
<td>Linear staining at the luminal endothelial surface</td>
<td>No staining</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>Linear staining at the luminal endothelial surface, microvascular vesicles stained</td>
<td>Focal staining at interendothelial interdigitations or overlaps, microvascular vesicles partly stained</td>
</tr>
<tr>
<td>PECAM-1</td>
<td>No staining</td>
<td>No staining</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>No staining</td>
<td>No staining</td>
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[p617], their absence may be one reason for the apparent lack of lymphangiogenesis in tumors.

ICAM-1, which is constitutively expressed on endothelial cells of normal skin [18,19], is upregulated by various cytokines such as interleukin-1, tumor necrosis factor, and interferon-γ [20]. The present study confirms the ICAM-1 expression of normal cutaneous microvasculature. We demonstrate that ICAM-1 is exclusively present at the luminal endothelial cell-surface of the blood vessels, whereas lymphatics of comparable size do not stain for ICAM-1. This may indicate that trafficking of immune response-related cells (i.e., of lymphocytes and Langerhans cells) through blood microvessels is a selective process based on cell-cell adherence, whereas in lymphatics either it is of a more passive nature, other adhesion receptors are involved or it is absent. The major role of ICAM-1 in lymphocyte recruitment by promoting lymphocyte-endothelial cell-contact is in agreement with the luminal localisation. Furthermore, it indicates that establishing and maintaining of cell-surface polarity [21] on the endothelial cell-surface is essential for the function of adhesion molecules. The frequent staining of microvascular vesicles may reflect transportation and delivery of synthesized molecules to the endothelial cell-membrane and endocytosis of surface molecules.

Another adhesion molecule, PECAM-1, a member of the immunoglobulin superfamily is present on leukocytes, platelets, and endothelium. Recent studies [22-24] postulate a role in lymphocyte recruitment and transmigration, platelet-leukocyte-endothelial interaction during thrombosis and intercellular adhesion of endothelial cells. We demonstrate its expression both on blood vessels and lymphatics, but in different expression patterns. PECAM-1 may function in lymphatics predominantly as an interendothelial adhesion molecule or in recirculation of cells from the tissue into the lymph vessel, whereas in blood vessels, its distribution suggests a "lumen-directed" function. Since PECAM-1 is constitutively expressed on the surface of endothelial cells, which are not involved in spontaneous aggregation or coagulation, PECAM-1-induced cell-cell adhesion may require an additional signal, as has been demonstrated for the LFA-1/ICAM-1 interaction, or may depend on the concentration of expressed molecules. Our observations provide further evidence that PECAM-1, which has been shown to influence the aggregation of transfected cells in vitro [25], plays a role in establishing or maintaining cell-cell interactions both in blood and lymph vessels.

The lack of immunoreactivity for E-selectin and VCAM-1 in normal cutaneous microvasculature is consistent with recent studies [19,26]. These adhesion molecules are upregulated under pathologic conditions in cutaneous inflammatory and neoplastic diseases [19]. Future studies may also reveal whether other adhesion molecules may be expressed in cutaneous lymphatics during pathologic conditions.

Figure 5. Double-labeling with MoAb anti-PECAM-1 (ultra-small gold conjugated) and anti-collagen type IV (peroxidase conjugated) in two postcapillary venules. Note the linear peroxidase staining of the multilaminated basal lamina (anti-collagen type IV) and a highly selective localization of silver-enhanced gold particles at the luminal and abluminal side of the endothium typical for MoAb anti-PECAM-1. E, endothelial cell; L, lumen. Scale bar, 7 μm.
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