

# Isolation and Characterization of Lymphatic Microvascular Endothelial Cells From Human Tonsils

EMIRENA GARRAFA,<sup>1\*</sup> GIULIO ALESSANDRI,<sup>2</sup> ANNA BENETTI,<sup>3</sup> DANIELA TURETTA,<sup>4</sup>  
ATTILIO CORRADI,<sup>5</sup> ANNA MARIA CANTONI,<sup>5</sup> EDOARDO CERVI,<sup>6</sup> STEFANO BONARDELLI,<sup>6</sup>  
EUGENIO PARATI,<sup>2</sup> STEFANO MARIA GIULINI,<sup>6</sup> BARBARA ENSOLI,<sup>7</sup> AND ARNALDO CARUSO<sup>1\*</sup>

<sup>1</sup>Department of Microbiology, University of Brescia, Brescia, Italy

<sup>2</sup>Carlo Besta Neurological Institute, Milan, Italy

<sup>3</sup>Department of Pathological Anatomy, University of Brescia, Brescia, Italy

<sup>4</sup>ENT Clinic, Ospedale Sacro Cuore, Negrar, Verona, Italy

<sup>5</sup>Department of Animal Health, University of Parma, Parma, Italy

<sup>6</sup>Department of Medical and Surgical Science, Surgical Section,  
University of Brescia, Brescia, Italy

<sup>7</sup>Laboratory of Virology, ISS, Rome, Italy

Human lymphatic endothelial cells (LECs) have isolated prevalently from human derma and tumors. As specialized lymphatic organs within the oropharynx, palatine tonsils are easily obtained and rich in lymphatic venules. Using a two-step purification method based on the sorting of endothelial cells with Ulex Europaeus Agglutinin 1 (UEA-1)-coated beads, followed by purification with monoclonal antibody D2–40, we successfully purified LECs from human palatine tonsils. The LECs were expanded on flasks coated with collagen type 1 and fibronectin for up to 8–10 passages and then analyzed for phenotypic and functional properties. Cultured cells retained the phenotypic pattern of the lymphatic endothelium of palatine tonsils and expressed functional VEGFR-3 molecules. In fact, stimulation with VEGFR-3 ligand, the vascular endothelium growth factor C, induced a marked increase in cell proliferation. Similarly to blood endothelial cells (BECs), LECs were able to form tube-like structure when seeded in Cultrex basement membrane extract. Comparative studies performed on LECs derived from palatine tonsils and iliac lymphatic vessels (ILVs), obtained with the same procedures, showed substantial discrepancies in the expression of various lymphatic markers. This points to the existence of micro- and macrovessel-derived LECs with different phenotypes, possibly involving different biological activities and functions. Palatine tonsil- and ILV-derived LECs may, therefore, represent new models for investigating function and biochemical properties of these lymphatic endothelia. *J. Cell. Physiol.* 207: 107–113, 2006. © 2005 Wiley-Liss, Inc.

The lymphatic system is made up of an extensive network of capillaries, collecting the vessels and ducts that permeate most organs (Ryan and Curri, 1989). This system collects the extravasated protein-rich fluid and lymphocytes from the tissue and reintroduce them into circulation. Lymphatic vessels are therefore essential for the continuous removal of interstitial fluid and proteins, as well as being an important entry point for leukocytes and tumor cells (Ryan et al., 1986; Ryan and Curri, 1989). Although lymphatic vessels were discovered about 300 years ago, at the same time as blood vessels, the lymphatic system was investigated less extensively than the vascular system; as a result, the molecular mechanisms regulating human lymphatic endothelial cell (LEC) functions have remained largely elusive, partly due to the lack of specific lymphatic markers. This circumstance may also account for misconceptions regarding the gene expression and function of microvascular blood endothelial cells (BECs), as BEC cultures can often be “contaminated” by LECs. Lymphatic vessels were initially identified by the absence of erythrocytes in their lumen and by the presence of less elaborated cell junctions, as compared with blood vessels (Leak, 1970; Gerli et al., 1990; Erhard et al., 1996). LECs also have lower levels of CD34 and von Willebrand factor (vWf) expression than BECs. Finally, LEC growth is selectively regulated by VEGF-C, a member of the vascular endothelial growth factor (VEGF) family (Ferrara and Davis-Smyth, 1997; Veikkola et al., 2003), via VEGF receptor-3 (VEGFR-3) (Clauss, 2000; Ferrara, 2002).

Very few attempts have been made in the past to isolate LECs but this has recently become possible

thanks to the identification of such new lymphatic markers as transmembrane mucoprotein Podoplanin (Matsui et al., 1999), hyaluronidase receptor Lyve-1, (Banerji et al., 1999), VEGFR-3 (Jussila and Alitalo, 2002), transcription factor Prox-1 (Petrova et al., 2002), and the D2-40 monoclonal antibody (mAb), which recognizes O-linked glycoprotein (Kahn et al., 2002).

**Abbreviations:** BME, basal membrane extract; EC, endothelial cell; HUVEC, human umbilical vein endothelial cell; ILV-LEC, Iliac lymphatic vessel derived LEC; LEC, lymphatic endothelial cell; mAb, monoclonal antibody; PT-LEC, palatine tonsil derived LEC; UEA-1, Ulex Europaeus Agglutinin 1; VEGF-C, vascular endothelial growth factor-C; VEGFR-3, vascular endothelial growth factor receptor-3.

To the memory of Prof. Adolfo Turano “Accidit quod patrem plus etiam quam non modo tu sed quam ipse scit amo” M.T. Cicerone.

Emirena Garrafa and Giulio Alessandri equally contributed to this work.

Contract grant sponsor: ISS AIDS; Contract grant numbers: 30D.15, PRIN 2002 prot 2002074251.

\*Correspondence to: Emirena Garrafa and Arnaldo Caruso, Department of Applied and Experimental Medicine, Institute of Microbiology, Piazzale Spedali Civili, 1, 25123 Brescia, Italy. E-mail: emirenagarrafa@libero.it; caruso@med.unibs.it

Received 30 August 2005; Accepted 9 September 2005

DOI: 10.1002/jcp.20537

LEC isolation was successfully performed from human derma (Kriehuber et al., 2001) and lymphatic vascular tumors (Mancardi et al., 1999; Weninger et al., 1999), using mAb to VEGFR-3 or polyclonal antibodies (Abs) to Podoplanin (Kriehuber et al., 2001). This allowed preliminary LEC characterization in terms of phenotype and biological functions. As BEC biological functions are related to the size of vessels and to the organs they originate from (Turner et al., 1987; Page et al., 1992; Thorin and Shreeve, 1998), we suggested that purification of LECs from vessels of various size and from different tissues may give rise to phenotypically different cells and improve our understanding of their functional activities. The present study describes a new method for the purification and in vitro expansion of human micro- and macrovessel-derived LECs from palatin tonsils (PTs) and from iliac lymphatic vessel (ILVs). Furthermore PTs are easily obtained by surgery and their weight and dimension insure a large number of LECs.

## MATERIALS AND METHODS

### Processing of PT and ILV tissues

Human PT and ILV specimens were obtained from patients undergoing therapeutic surgery, according with the principles listed in the Helsinki declaration. We avoid using PT fragments from patient with chronic and recurrent tonsillitis but obtained PT specimens from patients with obstructive sleep apnea. After surgical removal, tissue samples were immediately transferred to the laboratory in cold RPMI 1640 supplemented with fungizone (10 µg/ml), penicillin (400 U/ml), streptomycin (200 µg/ml), vancomycin (200 µg/ml), and gentamycin (200 µg/ml), and 20% fetal bovine serum (FBS) (Wetherby, West Yorkshire, UK). Tissues were washed several times with PBS supplemented with the antimicrobial agents as above, and PT specimens were squeezed over a 100 µm nylon mesh screen to remove the connective capsula, which mostly contain high venule blood capillaries (Baekkevold et al., 1999). PT and ILV fragments were then finely minced with scissors and subjected to enzymatic digestion for 3 h at 37°C with 0.25% (w/v) collagenase/dispase solution (Boehringer Mannheim, Mannheim, Germany). The resulting digestion product was filtered through a 30 µm pore size filter and the cells washed and cultured in T25 flask coated with collagen type I (5 µg/cm<sup>2</sup>; Boehringer Mannheim) and fibronectin (1 µg/cm<sup>2</sup>; Sigma-Aldrich, St. Louis, MO), in the presence of endothelial growth medium (EGM, BioWhittaker, Walkersville, MD). Six hours later, non-adherent cells were removed and discarded, whereas the adherent ones were washed two times with PBS added with antibiotics, and incubated with EGM until confluence (usually reached in 4–6 days).

### LEC isolation and culture

Primary cultures were harvested by trypsinization, centrifuged, resuspended at a concentration of 10<sup>6</sup>/ml and incubated with magnetic tosyl-activated beads (Dynal, Oslo, Norway) coated with lectin UEA-1 (Sigma-Aldrich; cell:bead ratio 1:1) as reported by Jackson et al. (1990), or with magnetic beads coated with anti-CD31 mAb, purchased from Dako (Carpinteria, CA), as previously described (Alessandri et al., 1999, 2001). All endothelial cells (ECs) positive to UEA-1 or CD31 selection recovered by magnetic particle concentrator were cultured on collagen type I and human fibronectin-coated wells in the presence of EGM. They were cultured for 4–5 days and harvested by trypsinization; LECs were positively purified from ECs with magnetic beads (ratio cells:beads 1:5) coated with D2–40 mAb (Signet Laboratories, Dedham, MD) or VEGFR-3 mAb (kindly provided by Kari Alitalo, University of Helsinki, Finland) at a mAb concentration of 5 µg for 4 × 10<sup>7</sup> goat anti-mouse IgG beads (Dynal). LECs were then seeded onto collagen type I and fibronectin-coated wells and cultured in EGM added with VEGF-C (50 mg/ml) (R&D System, Inc., Minneapolis, MN). This was assumed as the first in vitro

passage. LEC cultures, routinely examined by light microscopy, were serially subcultured at a split ratio of 1:3 and grown for 8–10 passages. All the experiments described here were performed between the 3rd and 5th in vitro passage. HUVECs were isolated as described by Jaffe et al. (1973), cultured in EGM, and expanded every 3–4 days at a split ratio 1:3. These cells were not used for 5 in vitro passages. All cell types were also grown on collagen type I and fibronectin-coated glass slides.

### Immunohistochemistry

Four-micrometer serial sections of formalin fixed and paraffin-embedded PT were transferred to glass slides coated with poly-lysine and rehydrated by immersion in 100% xylene, followed by graded ethanol (100%, 95%, 90%, 80%, and 70%). To enhance antigenicity and allow epitope unmasking, the sections used for UEA-1 staining were heat-treated three times in a microwave in the presence of citrate buffer (pH 6.0), while those used for D2–40 staining underwent same treatment but in the presence of EDTA (pH 8.0). Endogenous peroxidase was inhibited by incubation of tissue sections with 3% hydrogen peroxide for 15 min at room temperature (RT), while aspecific epitope binding was avoided by incubation for 20 min with 20% human serum. Biotin-conjugated UEA-1 (dilution 1:20) (Sigma), D2–40 mAb (dilution 1:40) and diluent buffer alone were added to serial sections for 30 min at RT. After washings, the sections treated with D2–40 mAb and with diluent buffer were incubated for 30 min with biotin-conjugated anti-mouse. All samples were then processed according to the avidin/biotin peroxidase complex method along with the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Peroxidase activity was detected with 3,3'-diaminobenzidine (Menarini-Biogenex, San Ramon, CA) in PBS.

### Immunocytochemistry

Immunocytochemical studies were performed on LECs and HUVECs seeded on glass slides coated with collagen and fibronectin, fixed in cold 4% paraformaldehyde in PBS, pH 7.4, for 10 min at RT. Cells were then washed twice with PBS, incubated with 10% goat serum (Gibco, Grand Island, NY) to block aspecific binding, then incubated for 90 min at 37°C with mAb to CD31, Ki-67 (dilution 1:100; Dako), KDR (dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA), and D2-40 (dilution 1:160), or with rabbit antisera to vWf (dilution 1:80; Sigma), Lyve-1, Podoplanin and Prox-1 (dilution 1:160; Reliatech, Braunschweig, Germany). After two washings with PBS, cells were incubated for 45 min at RT with 1:300 diluted cyanine dye-labeled goat anti-mouse or goat anti-rabbit IgG (Jackson Immunoresearch, West Grove, PA). For UEA-1 staining, cells were incubated with biotin-conjugated UEA-1 and then with a rabbit anti-UEA-1 antibody (Sigma). The complex was revealed using cyanine dye-labeled anti-rabbit IgG. Air-dried cells were then mounted with Fluorosave (Calbiochem, La Jolla, CA) and photographed using a Zeiss Axiophot-2-microscope (Oberkochen, Germany). To identify the presence of false positives, due to non-specific binding of the secondary antibody, all the cells were treated in the same way, with buffer replacing the primary antibodies.

### Cord formation on culture basement membrane extract (BME)

Two hundred microliters of Cultrex BME (10 mg/ml) (Bioscience International, Saco, MA) at 4°C were transferred to prechilled 24-well culture plates using sterile tips that had been cooled to –20°C before use. After gentle agitation to insure coating, the plates were incubated for 1 h at 37°C to allow Cultrex BME to solidify. The LECs were then seeded at a concentration of 6 × 10<sup>4</sup>/well in EGM containing VEGF-C. Cord formation was obtained after 24 h of incubation.

### Proliferation assay

In three independent experiments, LECs and HUVECs were detached from culture flasks using a trypsin solution. One milliliter of EGM containing 2 × 10<sup>9</sup> cells was seeded into 24-multiwell plates previously coated with collagen type I and

fibronectin. After 4 h, the medium was removed and replaced with fresh EGM supplemented or not with VEGF-C (50 ng/ml). At day 5 and 10, the cells were trypsinized, washed, stained with Trypan blue solution and counted.

#### Immunofluorescence

Cells cultured with EGM medium supplemented or not with VEGF-C (50 ng/ml) in 24-well plates containing fibronectin and collagen type I coverslips, were fixed for 20 min at RT using 100% ice-cold methanol. Monolayers were incubated for 90 min at 37°C with mouse mAb to CD105 (Sigma-Aldrich) (dilution 1:20). After two washings with PBS, the cells were incubated with FITC-conjugated secondary antibodies for 45 min at room temperature. Air-dried cells were then mounted with Fluorsave and photographed using a Zeiss Axiophot-2-microscope.

### RESULTS

#### Identification of specific lymphatic markers for PT

PT tissue which appeared normal on histologic examination, showed lymphatic capillaries distinguishable from blood capillaries for their very thin vessel wall and the presence of lymphocytes in the lumen. Blood vessels usually had a thicker wall and contain red blood cells. PT specimens were evaluated for reactivity to EC-specific reagents, namely UEA-1 and mAb to CD31, and for the presence of LEC-specific markers such as VEGFR-3, D2-40, Lyve-1, Prox-1, and Podoplanin. PT-derived tissue showed that LECs were markedly stained by UEA-1 (Fig. 1a), while BECs were principally stained by anti CD31 mAb (data not shown). Furthermore, D2-40 mAb strongly stained lymphatic vessels but not blood vessels (Fig. 1b). Other lymphatic markers, such as Podoplanin, VEGFR-3, Lyve 1, and Prox-1, were expressed on LECs to a lesser degree (data not shown).

#### Isolation and culture of PT-LECs

Specimen obtained from PT were processed and subjected to enzymatic digestion. Bacteria and fungi adhering to their surface were removed by several washings in a solution containing high concentrations of vancomycin, penicillin, streptomycin, fungizone, and gentamicin. The resulting cultures produced a heterogeneous adherent cell population consisting mainly of elements with an endothelial morphology. Because of the strong specificity of UEA-1 for PT-EC surface, this lectin was used to coat tosyl-activated magnetic beads and allow purification of all ECs from processed tissue (Jackson et al., 1990). At confluence, 40%–60% of UEA-1<sup>+</sup> selected cells were recognized by D2-40 mAb (Fig. 1c). At confluence, about 5% of the D2-40 negative selection was stained by D2-40 mAb (Fig. 1d), arguably because these cells were not selected during positive sorting. We therefore employed HUVEC as control, because being derived from the umbilical vein lumen, they contain no ECs of lymphatic origin. When we avoided pre-selection with UEA-1 and tried to purify PT-LECs by a single purification with D2-40 mAb-coated beads, only a very low recovery of LECs was obtained. Similarly, the use of VEGFR-3 mAb, which interacts with a receptor highly expressed on LECs and almost absent on BECs (Jussila and Alitalo, 2002), always produced a LEC recovery lower than that obtained, give raise to a LECs recovery lower than that obtained, under the same experimental condition, with D2-40 mAb, in both unselected and UEA-1-enriched preparation. Using a UEA-1 and D2-40 mAb-based method, we were also able to successfully purify and expand in vitro macrovessel-derived LECs from ILV (ILV-LECs). Under

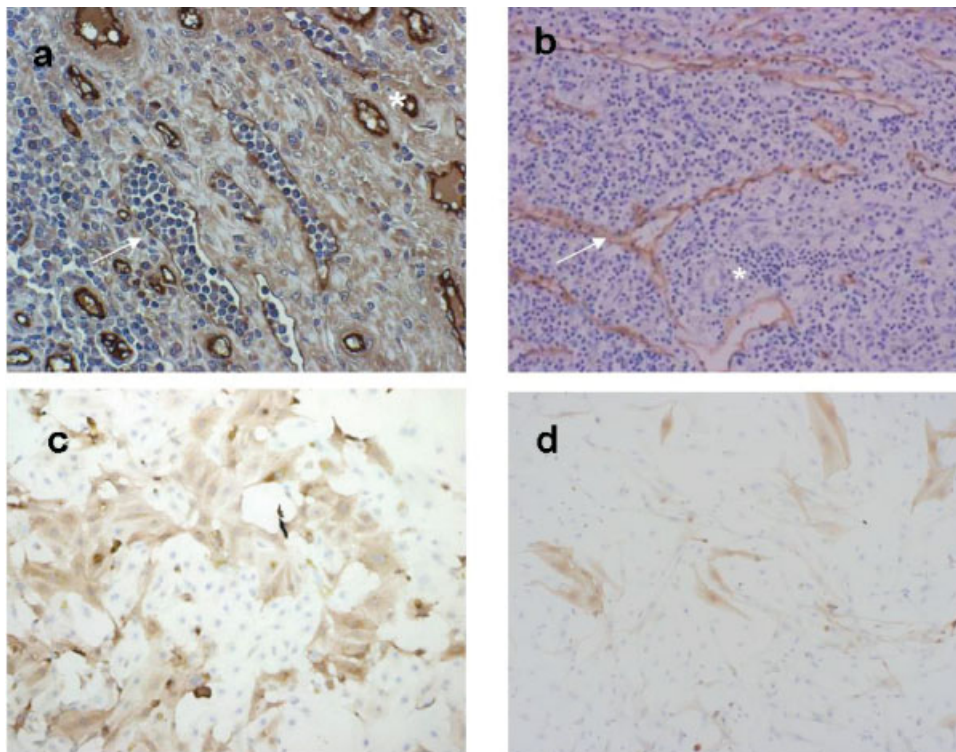


Fig. 1. Immunohistochemical examination of tonsil sections for blood and lymphatic vascular markers. Lymphatic vessels are indicated by arrows, while blood vessels are marked by an asterisk. Both blood and lymphatic vessels strongly reacted to UEA-1 (a), while D2-40 mAb specifically stained the endothelium of lymphatic vessels (b). Expression of D2-40 on UEA-1<sup>+</sup> and UEA-1<sup>+</sup>/D2-40<sup>-</sup> ECs selection. c: In

UEA-1<sup>+</sup> EC culture, immunolabeling with anti-D2-40 revealed multicellular islands of D2-40<sup>+</sup> LECs surrounded by D2-40<sup>-</sup> ECs. d: The D2-40 negative selection of UEA-1<sup>+</sup> ECs is not homogeneously negative for D2-40 as shown here. Original magnification in (a) and (b): 10×; in (c) and (d): 20×.

microscopy, the morphology of ILV-LECs was similar to that of PT-LECs; both LEC types reflected the typical morphology of ECs, with a prominent nucleus. Furthermore, they were both propagated in the presence of EGM containing VEGF-C on collagen and fibronectin-coated flasks. The use of fibronectin led to improved attachment and subsequent spreading of LECs, as compared to collagen alone (data not shown). However, PT-LECs had a lower growth rate than ILV-LECs, with doubling times of 6 and 4 days, respectively.

#### Comparative phenotype analysis of lymphatic and blood vascular markers on PT-LECs, ILV-LECs, and HUVECs

PT-LECs were strongly stained by D2-40 mAb and Prox-1 antisera (Fig. 2a,d). Antibodies to other lymphatic markers such as VEGFR-3 (data not shown) and podoplanin (Fig. 2c) stained PT-LECs but at lower intensity than D2-40 mAb. Since Schacht et al. (2005) recently demonstrated that D2-40 mAb recognizes an epitope on the podoplanin molecule, the weakness of podoplanin expression as compared to D2-40 mAb is probably due to the polyclonal origin of the anti-podoplanin antibody used. Interestingly, only few elements of PT-LECs were positive for Lyve-1 staining (Fig. 2b). All these markers were expressed on ILV-LECs (Fig. 2e-h) and absent on HUVECs (Fig. 2i-l). As expected, PT-LECs and ILV-LECs were CD31<sup>+</sup> (Fig. 3a,e), von vWf<sup>+</sup> (Fig. 3b,f) and UEA-1<sup>+</sup> (Fig. 3c,g), but the expression levels of these vascular markers were lower than on HUVECs (Fig. 3i-l). KDR, the main human receptor responsible for the VEGF activity in both physiological and pathological vascular develop-

ment (Duval et al., 2003), was well expressed on HUVECs (Fig. 3l) and almost absent on PT-LECs and ILV-LECs (Fig. 3d,h). The expression level of these different markers on PT-LECs, ILV-LECs and HUVECs is summarized in Table 1. With the only exception of low Lyve-1 expression, the phenotypic pattern of cultured PT-LECs closely resemble that observed in the tissue sections of PT lymphatic vasculature (data not shown); this proves that cultures maintain in vitro the original expression level of specific markers. When subjected to tube-forming assay on Cultrex BME, PT-LECs were able to form tube-like structures in the presence of EGM plus VEGF-C, after 24 h of incubation (Fig. 4).

#### Proliferative response of PT-LECs and ILV-LECs to VEGF-C

The growth response of PT-LECs and ILV-LECs to VEGF-C was assessed on pooled cultures obtained from tissue of different donors in order to minimized individual variability. Cells were seeded on collagen type I and fibronectin-coated flasks and incubated with different concentrations of VEGF-C (ranging from 5 to 100 ng/ml). After 5 and 10 days, PT-LECs and ILV-LECs cultured in presence of 50 ng/ml of VEGF-C, which is the best concentration in promoting LEC growth (data not shown), showed a proliferation rate twice that of cultures growth in the absence of growth factor (Fig. 5a). Increased proliferative activity was evident at all the time point analyzed. On the contrary, VEGF-C did not increase HUVEC proliferation even at the highest concentrations. The effect of growth factor on PT-LEC proliferation was confirmed by the strong reactivity of

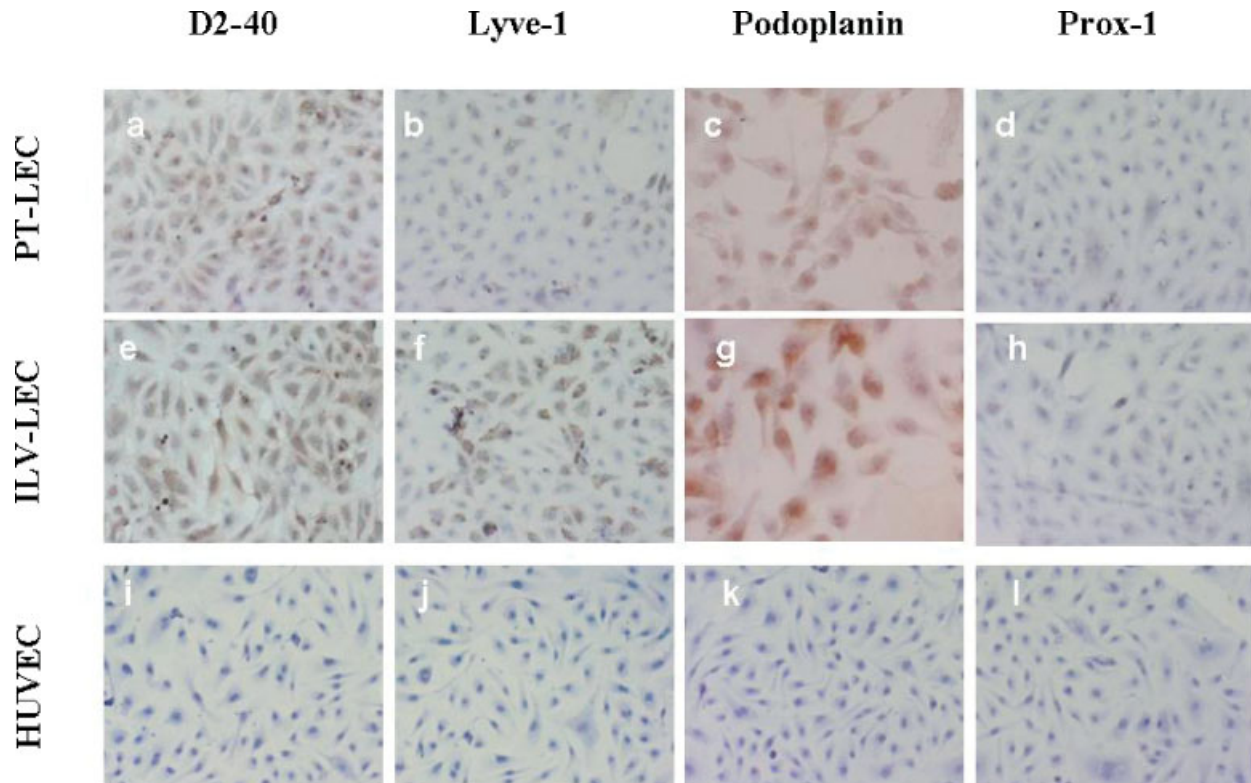


Fig. 2. Immunostaining of PT-LECs, ILV-LECs and HUVECs for lymphatic markers (magnification 20 $\times$ ). All elements of cultured PT-LECs express D2-40 (a), and Prox-1 (d), while they express podoplanin (c) weakly and only a few element express the hyaluronan receptor Lyve-1 (b). ILV-LECs are positive to D2-40 (e), Podoplanin (g), and Prox-1 (h) staining, and almost all elements express Lyve-1 (f), while all the lymphatic markers tested are absent on HUVECs (i, j, k, l).

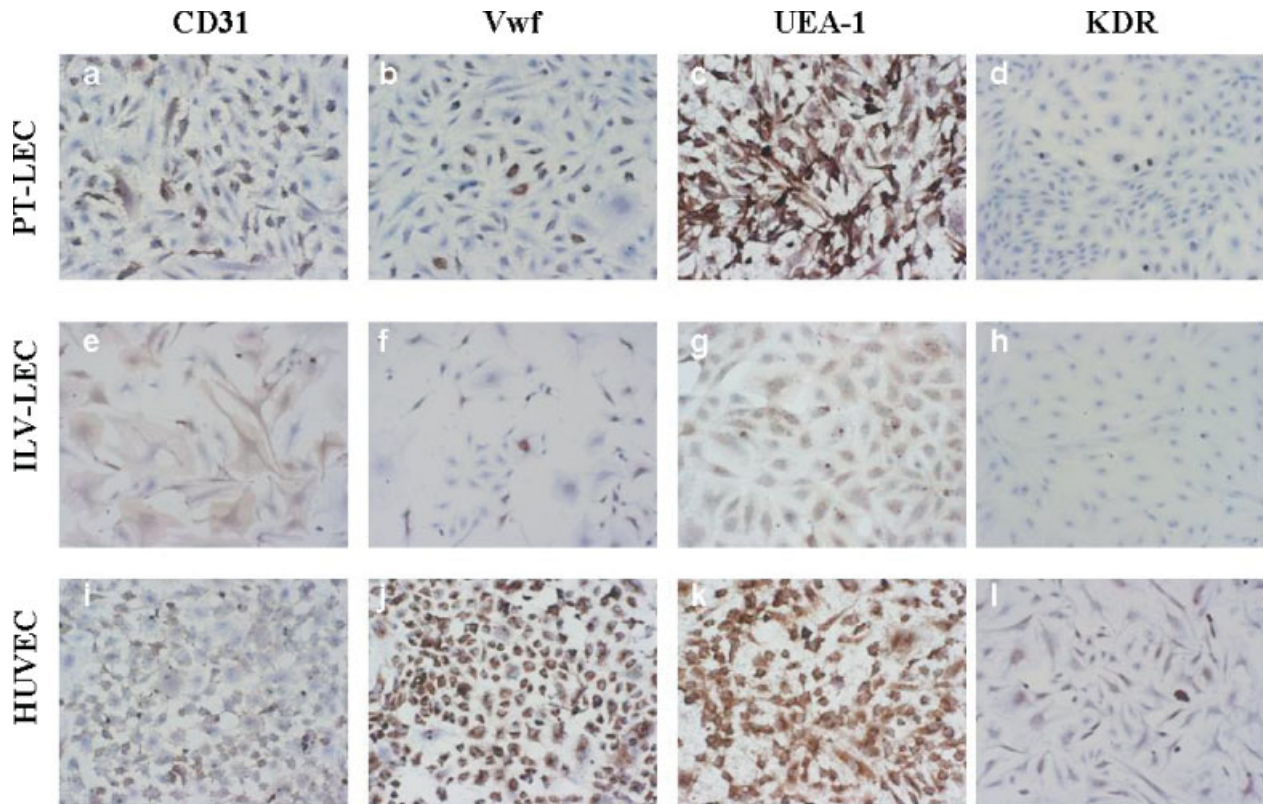


Fig. 3. Immunostaining of PT-LECs, ILV-LECs and HUVECs for blood vascular markers. PT-LECs and ILV-LECs immunostained for CD31 (a, e), and for vWf (b, f) but at lower intensity than HUVECs (i, j), which strongly expressed these markers; positive staining with UEA-1 is evident on all the ECs type used in our experiments (c, g, k) while KDR was almost absent on PT-LECs (d) and ILV-LECs (h) and well expressed by HUVECs (l). Magnification 20 $\times$ .

cells incubated in the presence of VEGF-C with mAb to Ki-67 (Fig. 5b), a nuclear marker linked to cell proliferation (Brown and Gatter, 2002), as compared to cells cultured in its absence (Fig. 5c). Immunofluorescence studies with CD105 mAb, which recognizes another marker of highly proliferating ECs (Ferrara, 2002; Duff et al., 2003; Fonsatti et al., 2003) produced similar result (data not shown). Despite the greater proliferative activity observed with both PT- and ILV-LECs cultured in the presence of VEGF-C, the number of serial culture propagations did not increase and never exceeded 8–10 passages.

**DISCUSSION**

Several attempts were made in the past to obtain a pure LEC population, mainly employing lymphatic cells from vascular tumors (Mancardi et al., 1999; Weninger et al., 1999). The lack of specific markers, however made it impossible to define a precise phenotype of cultured

cells or the histogenetic origin of the increased population. LEC purification and growth have become a reality only recently, since the discovery of specific lymphatic markers and the identification of specific lymphatic growth factors and substrates (Kriehuber et al., 2001; Makinen et al., 2001). This study is the first to report the isolation of LECs from PT and ILV, using a mAb named D2–40 recognizing the O-linked ptyaloglycoprotein, specifically expressed on LECs. PTs are indeed an

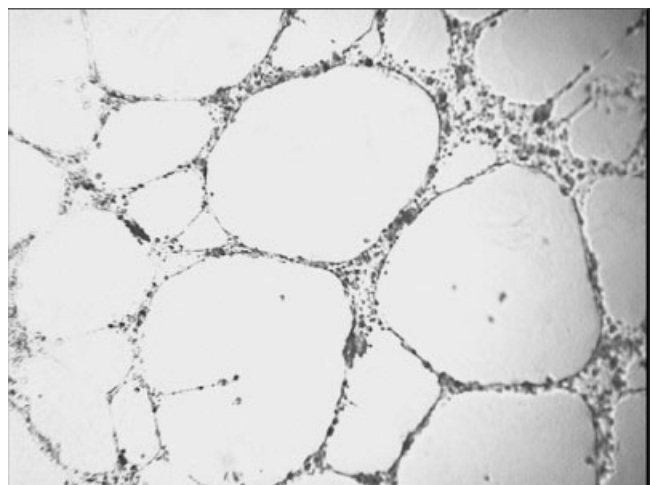


Fig. 4. Tube forming assays. PT-LECs are able to form capillary-like structures when seeded on Cultrex BME. The UEA1<sup>+</sup>/D2–40<sup>+</sup> cells form long, thick tube-like structures interconnecting occasional clumps of cells. Magnification 10 $\times$ .

TABLE 1. Summary of comparative results of phenotypical characterization between PT-LECs, ILV-LECs and HUVECs by immunocytochemistry

Marker	PT-LECs	ILV-LECs	HUVECs
D2–40	+++	+++	–
Lyve-1	+/-	+	–
Podoplanin	++	++	–
Prox-1	+++	+++	–
CD 31	++	++	+++
vWf	+	+	+++
UEA 1	+++	+++	+++
KDR	+/-	+/-	++

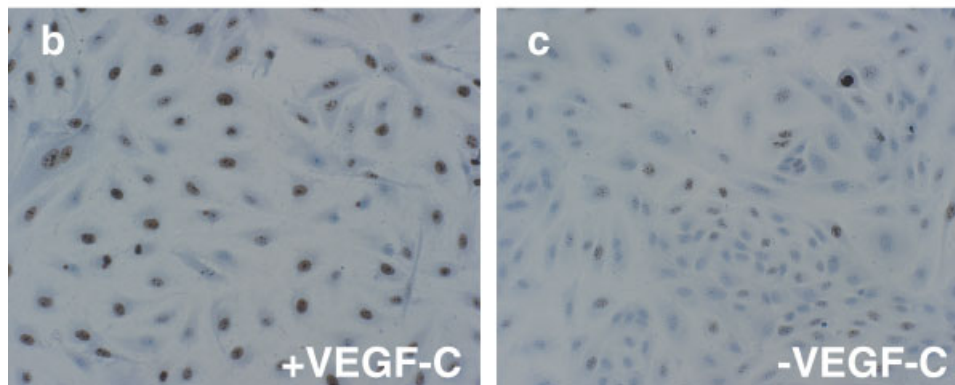
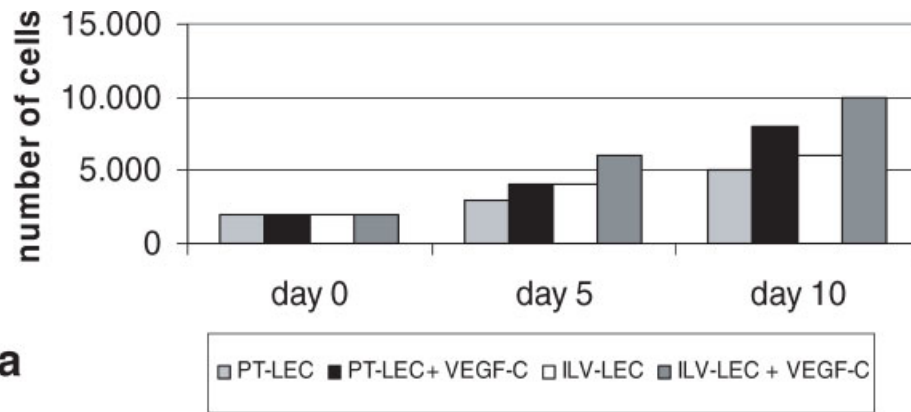


Fig. 5. Effect of VEGF-C on LECs. (a) Cell count at day 5 and 10: growth in the presence or absence of VEGF-C demonstrates that VEGFR-3 signaling is sufficient to promote LEC proliferation, as shown by the ability of its specific ligand VEGF-C (50 ng/ml) to increase both PT- and on ILV-LEC proliferative activity. Data presented are means of three replicates per assay (standard deviation

<10%) and are representative of three independent experiments with similar results. (b): Proliferation markers Ki67 is expressed more strongly on PT-LECs, when cultured in the presence of VEGF-C (50 ng/ml), as compared to PT-LECs cultured in the absence of growth factor (c). Magnification in b and c: 20 $\times$ .

extremely useful as source of LECs because they are easy to obtain from material discarded after therapeutic surgery and because of their dimension, weight and abundance of lymphatic capillaries. Generating LEC cultures required tissue dispersion, removal of non-adherent cells after 12–18 h of plating, immune preselection with UEA-1-coated magnetic beads and selection with magnetic beads coated with D2–40 mAb. Anti-CD31 mAb was less efficient than UEA-1-coated beads in purifying ECs from dispersed PT tissue: a finding that appears to correlate with the differential expression of these two markers on PT microvessels. Similarly, D2–40 mAb-coated beads allowed better separation of UEA-1 selected cells, as compared with anti-VEGFR-3-coated beads. In both cases, however, we obtained a morphologically and phenotypically similar LEC population, though the percentage of LECs obtained with anti-VEGFR-3 mAb was always much lower than that obtained with D2–40 mAb-coated beads. This might be due to the down modulation of VEGFR-3 in primary cultures, to presence of FCS in the culture medium, or even to the use of extracellular matrix components (Makinen et al., 2001). PT-LEC purification with polyclonal antibodies against other specific lymphatic membrane markers, such as Podoplanin or Lyve-1, was ineffective in terms of satisfactory LEC recovery. A reasonable explanation might be the inefficient binding of polyclonal antibodies to Podoplanin and Lyve-1 on magnetic beads (Gaudernack et al., 1986). LECs are known to interact directly in vivo with

the extracellular matrix (Gerli et al., 1990, 2000) and, indeed, both PT-LECs and ILV-LECs were successfully propagated on collagen and fibronectin-coated flasks in the presence of EGM and VEGF-C. However, LECs cultured on collagen and fibronectin adhered better and proliferated more efficiently than those grown on culture dishes coated with collagen alone, in line with data suggesting that fibronectin provides additional signals for LEC adhesion, survival and proliferation (Grinnel, 1980; Kleinman et al., 1981). Furthermore, when seeded on Cultrex BME, PT-LECs formed long, thick capillary-like structures, thus proving their capability to form lymphatic vessels in vitro. PT-LECs and ILV-LECs were morphologically similar to BECs, although their shape appeared more elongated. Most of the known blood vascular markers, such as CD31 and vWf, were present at low levels on the LEC surface but KDR was almost absent, in agreement with previous studies of derma-derived LECs (Kriehuber et al., 2001). Lymphatic markers were differentially represented on PT-LECs because strongly stained by D2–40 mAb; they almost entirely expressed Prox-1 and Podoplanin, and only a few were LYVE-1<sup>+</sup>. Conversely, nearly all the cultured ILV-LECs equally expressed all the lymphatic markers tested. Stimulation of PT- and ILV-LECs cultured with the specific lymphatic endothelial growth factors VEGF-C induced proliferation on both PT- and ILV-LEC cultures but not on HUVECs; this indicates the presence of functional VEGFR-3 molecules on the LEC surface throughout the period of culture. As

suggested by Podgrabinska et al. (2002), different markers expressions reflect the presence of LECs with different activities and functions. This hypothesis is supported by studies performed on BECs, which exhibit phenotypic and functional differences according to their origin (adult vs. fetal), anatomical position and vessels size (large vs. capillary vessel) (Turner et al., 1987; Page et al., 1992).

The main indication yielded by our results, there, is that palatine tonsils are a valuable and easily available source for isolating human lymphatic microvessel ECs. We were able to culture of PT- and ILV-LECs in the presence of specific growth factors and extracellular matrixes without losing any of their different properties and functional activity. PT-LECs and ILV-LECs may provide new tools for investigation of genetic, phenotypic and functional divergences between macro- and microvessel-derived LECs isolated from different organs and tissues. Their use could improve our understanding of specialized lymphatic functions, of their role in tumor lymphatic tumor metastatic dissemination and their capability to support virus replication, as already demonstrated for BECs (Lathey et al., 1990; Ricotta et al., 2001; Caruso et al., 2003).

#### ACKNOWLEDGMENTS

We thank Dr. L. Imberti and Dr. P.G. Natali for helpful discussion and Giuseppe Crea for technical assistance.

#### LITERATURE CITED

- Alessandri G, Chirivi RG, Fiorentini S, Dossi R, Bonardelli S, Giulini SM, Zanetta G, Landoni F, Graziotti PP, Turano A, Caruso A, Zardi L, Giavazzi R, Bani MR. 1999. Phenotypic and functional characteristics of tumour-derived microvascular endothelial cells. *Clin Exp Metastasis* 17:655–662.
- Alessandri G, Girelli M, Taccagni G, Colombo A, Nicosia R, Caruso A, Baronio M, Pagano S, Cova L, Parati E. 2001. Human vasculogenesis ex vivo: Embryonal aorta as a tool for isolation of endothelial cell progenitors. *Lab Invest* 81:875–885.
- Baekkevold ES, Jahnsen FL, Johansen FE, Bakke O, Gaudernack G, Brandtzaeg P, Haraldsen G. 1999. Culture characterization of differentiated high endothelial venule cells from human tonsils. *Lab Invest* 79:327–336.
- Banerji S, Ni J, Wang SX, Clasper S, Su J, Tammi R, Jones M, Jackson DG. 1999. LYVE-1, a new homologue of the CD44 glycoprotein, is a lymph-specific receptor for hyaluronan. *J Cell Biol* 144:789–801.
- Brown DC, Gatter KC. 2002. Ki67 protein: The immaculate deception? *Histopathology* 40:2–11.
- Caruso A, Favilli F, Rotola A, Comar M, Horejsh D, Alessandri G, Grassi M, Di Luca D, Fiorentini S. 2003. Human herpesvirus-6 modulates RANTES production in primary human endothelial cell cultures. *J Med Virol* 70:451–458.
- Clauss M. 2000. Molecular biology of the VEGF and the VEGF receptor family. *Semin Thromb Hemost* 26:561–569.
- Duff SE, Li C, Garland JM, Kumar S. 2003. CD105 is important for angiogenesis: Evidence and potential applications. *FASEB J* 17:984–992.
- Duval M, Bedard-Goulet S, Delisle C, Gratton JP. 2003. Vascular endothelial growth factor-dependent down-regulation of Flk-1/KDR involves Cbl-mediated ubiquitination: Consequences on nitric oxide production from endothelial cells. *J Biol Chem* 278:20091–20097.
- Erhard H, Rietveld FJ, Brocker EB, de Waal RM, Ruiters DJ. 1996. Phenotype of normal cutaneous microvasculature. Immunoelectron microscopic observations with emphasis on the differences between blood vessels and lymphatics. *J Invest Dermatol* 106:135–140.
- Ferrara N. 2002. Role of vascular endothelial growth factor in physiologic and pathologic angiogenesis: Therapeutic implications. *Semin Oncol* 29:10–14.
- Ferrara N, Davis-Smyth T. 1997. The biology of vascular endothelial growth factor. *Endocr Rev* 18:4–25.
- Fonsatti E, Sigalotti L, Arslan P, Altomonte M, Maio M. 2003. Emerging role of endoglin (CD105) as a marker of angiogenesis with clinical potential in human malignancies. *Curr Cancer Drug Targets* 3:427–432.
- Gaudernack G, Leivestad T, Ugelstad J, Thorsby E. 1986. Isolation of pure functionally active CD8+ T cells. Positive selection with monoclonal antibodies directly conjugated to monosized magnetic microspheres. *J Immunol Methods* 90:179–187.
- Gerli R, Iba L, Fruschelli C. 1990. A fibrillar elastic apparatus around human lymph capillaries. *Anat Embryol (Berl)* 181:281–286.
- Gerli R, Solito R, Weber E, Agliano M. 2000. Specific adhesion molecules bind anchoring filaments and endothelial cells in human skin initial lymphatics. *Lymphology* 33:148–157.
- Grinnel F. 1980. Fibroblast cell-substratum interactions: Role of cold insoluble globulin (plasma fibronectin). *Experientia* 36:505–507.
- Jackson CJ, Garbett PK, Nissen B, Schriber L. 1990. Binding of human endothelium to Ulex europaeus 1-coated Dynabeads: Application to the isolation of microvascular endothelium. *J Cell Sci* 96:257–262.
- Jaffe EA, Nachman RL, Bejcek CG, Minick CR. 1973. Culture of human endothelial cells derived from human umbilical veins: Identification of morphologic and immunologic criteria. *J Clin Invest* 32:2745–2756.
- Jussila L, Alitalo K. 2002. Vascular growth factors and lymphangiogenesis. *Physiol Rev* 82:673–700.
- Kahn HJ, Bailey D, Marks A. 2002. Monoclonal antibody D2-40, a new marker of lymphatic endothelium, reacts with Kaposi's sarcoma and a subset of angiosarcomas. *Mod Pathol* 15:434–440.
- Kleinman HK, Wilkes CM, Martin GR. 1981. Interaction of fibronectin with collagen fibrils. *Biochemistry* 20:2325–2330.
- Kriehuber E, Breiteneder-Geleff S, Groeger M, Soleiman A, Schoppmann SF, Stingl G, Kerjaschki D, Maurer D. 2001. Isolation and characterization of dermal lymphatic and blood endothelial cells reveal stable and functionally specialized cell lineages. *J Exp Med* 194:797–808.
- Lathey JL, Wiley CA, Verity MA, Nelson JA. 1990. Cultured human brain capillary endothelial cells are permissive for infection by human cytomegalovirus. *Virology* 176:266–273.
- Leak LV. 1970. Electron microscopic observations on lymphatic capillaries and the structural components of the connective tissue-lymph interface. *Microvasc Res* 2:361–391.
- Makinen T, Veikkola T, Mustjoki S, Karpanen T, Catimel B, Nice EC, Wise L, Mercer A, Kowalski H, Kerjaschki D, Stacker SA, Achen MG, Alitalo K. 2001. Isolated lymphatic endothelial cells transduce growth, survival and migratory signals via the VEGF-C/D receptor VEGFR-3. *EMBO J* 20:4762–4773.
- Mancardi S, Stanta G, Dusetti N, Bestagno M, Jussila L, Zweyer M, Lunazzi G, Dumont D, Alitalo K, Burrone OR. 1999. Lymphatic endothelial tumors induced by intraperitoneal injection of incomplete Freund's adjuvant. *Exp Cell Res* 246:368–375.
- Matsui K, Breiteneder-Geleff S, Soleiman A, Kowalski H, Kerjaschki D. 1999. Podoplanin, a novel 43-kDa membrane protein, controls the shape of podocytes. *Nephrol Dial Transplant* 14(Suppl 1):9–11.
- Page C, Rose M, Yacoub M, Pigott R. 1992. Antigenic heterogeneity of vascular endothelium. *Am J Pathol* 141:673–683.
- Petrova TV, Makinen T, Makela TP, Saarela J, Virtanen I, Ferrell RE, Finegold DN, Kerjaschki D, Yla-Herttuala S, Alitalo K. 2002. Lymphatic endothelial reprogramming of vascular endothelial cells by the Prox-1 homeobox transcription factor. *EMBO J* 21:4593–4599.
- Podgrabinska S, Braun P, Velasco P, Kloos B, Pepper MS, Jackson DG, Skobe M. 2002. Molecular characterization of lymphatic endothelial cells. *Proc Natl Acad Sci USA* 99:16069–16074.
- Ricotta D, Alessandri G, Pollara C, Fiorentini S, Favilli F, Tosetti M, Mantovani A, Grassi M, Garrafa E, Dei Cas L, Muneretto C, Caruso A. 2001. Adult human heart microvascular endothelial cells are permissive for non-lytic infection by human cytomegalovirus. *Cardiovasc Res* 49:440–448.
- Ryan TJ, Curri SB. 1989. Blood vessels and lymphatics. *Clin Dermatol* 7:25–36.
- Ryan TJ, Mortimer PS, Jones RL. 1986. Lymphatics of the skin. Neglected but important. *Int J Dermatol* 25:411–419.
- Schacht V, Dadras SS, Johnson LA, Jackson DG, Hong YK, Detmar M. 2005. Up-regulation of the lymphatic marker podoplanin, a mucin-type transmembrane glycoprotein, in human squamous cell carcinomas and germ cell tumors. *Am J Path* 166:913–921.
- Thorin E, Shreeve SM. 1998. Heterogeneity of vascular endothelial cells in normal and disease states. *Pharmacol Ther* 78:155–166.
- Turner RR, Beckstead JH, Warnke RA, Wood GS. 1987. Endothelial cell phenotypic diversity. In situ demonstration of immunologic and enzymatic heterogeneity that correlates with specific morphologic subtypes. *Am J Clin Pathol* 87:569–575.
- Veikkola T, Lohela M, Ikenberg K, Makinen T, Korff T, Saaristo A, Petrova T, Jeltsch M, Augustin HG, Alitalo K. 2003. Intrinsic versus microenvironmental regulation of lymphatic endothelial cell phenotype and function. *FASEB J* 17:2006–2013.
- Weninger W, Partanen TA, Breiteneder-Geleff S, Mayer C, Kowalski H, Mildner M, Pammer J, Sturzl M, Kerjaschki D, Alitalo K, Tschachler E. 1999. Expression of vascular endothelial growth factor receptor-3 and podoplanin suggests a lymphatic endothelial cell origin of Kaposi's sarcoma tumor cells. *Lab Invest* 79:243–251.