Intrinsic versus microenvironmental regulation of lymphatic endothelial cell phenotype and function

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ABSTRACT **Vascular endothelial cells are characterized by a high degree of functional and phenotypic plasticity, which is controlled both by their pericellular microenvironment and their intracellular gene expression programs. To gain further insight into the mechanisms regulating the endothelial cell phenotype, we have compared the responses of lymphatic endothelial cells (LECs) and blood vascular endothelial cells (BECs) to vascular endothelial growth factors (VEGFs). VEGFR-3-specific signals are sufficient for LEC but not BEC proliferation, as shown by the ability of the specific ligand VEGF-C156S to stimulate cell cycle entry only in LECs. On the other hand, we found that VEGFR-3 stimulation did not induce LEC cell shape changes typical of VEGFR-2-stimulated LECs, indicating receptor-specific differences in the cytoskeletal responses. Genes induced via VEGFR-2 also differed between BECs and LECs: angiopoietin-2 (Ang-2) was induced via VEGFR-2 in BECs and LECs, but the smooth muscle cell (SMC) chemoattractant BMP-2 was induced only in BECs. Both BECs and LECs were able to promote SMC chemotaxis, but contact with SMCs led to down-regulation of VEGFR-3 expression in BECs in a 3-dimensional coculture system. This was consistent with the finding that VEGFR-3 is down-regulated in vivo at sites of endothelial cell–pericyte/smooth muscle cell contacts. Collectively, these data show intrinsic cell-specific differences of BEC and LEC responses to VEGFs and identify a pericellular regulatory mechanism for VEGFR-3 down-regulation in endothelial cells.—Veikkola, T., Lohela, M., Ikenberg, K., Mäkinen, T., Korff, T., Saaristo, A., Jeltsch, M., Augustin, H. G., Alitalo, K. Intrinsic versus microenvironmental regulation of lymphatic endothelial cell phenotype and function. *FASEB J.* 17, 2006–2013 (2003)**

Key Words: LEC · VEGFR-3 · VEGFR-2 · pericyte · SMC

THE GENERATION of new blood vessels requires the coordinated interplay of several effector molecules (1, 2). One of the most potent inducers of blood vessel growth or angiogenesis is vascular endothelial growth factor (VEGF) (3). By binding to its endothelial cell surface receptors, VEGF receptor 1 (VEGFR-1) and VEGFR-2, VEGF mediates vascular leakage, endothelial cell proliferation, and directed migration (4). The VEGF family of growth factors also includes the placenta growth factor (PlGF), VEGF-B, VEGF-C, and VEGF-D. Of these, VEGF-C and VEGF-D have been shown to induce new lymphatic vessel growth via VEGFR-3, which is expressed predominantly in the lymphatic endothelium in adults (5–7). However, the exact molecular mechanisms for the establishment and maintenance of the lymphatic vasculature are incompletely understood.

Newly generated blood vessels must acquire a perivascular cell coating in order to be structurally stabilized (8). However, during adult angiogenesis, periendothelial cells must detach to allow endothelial cell proliferation and new vessel growth. Perivascular smooth muscle cells (SMCs) and pericytes (PCs) communicate in a paracrine manner with the endothelial cells and induce endothelial cell differentiation, quiescence, and survival (9). A lack of periendothelial cells leads to vascular malformations and blood vessel fragility. Several effector molecules that regulate endothelial–periendothelial cell interactions in the blood vascular system have been described. Angiopoietins (Ang) -1 and Ang-2 are secreted factors that mediate their effects by binding to the endothelial-specific Tie-2 receptor tyrosine kinase (10). Ang-1 activates Tie-2, while Ang-2 can behave as a context-dependent antagonist or agonist of Ang-1 (11, 12). In vivo analyses have revealed that Ang-1 recruits and sustains periendothelial cells, thereby stabilizing blood vessels, whereas Ang-2 is presumed to destabilize blood vessels by interfering with constitutive Ang-1/Tie-2 signals in the vessel wall, leading to detachment of the perivascular cells and allowing the vessel endothelium to revert to a more plastic state for angiogenesis (11, 13). Ang-2 is known to be expressed at sites of blood vessel remodeling and invasion (11, 13), and factors that induce angiogenesis in vivo, such as hyp-

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doi: 10.1096/fj.03-0179com

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oxia and VEGF, have been shown to up-regulate Ang-2 in endothelial cell cultures (14–16). Other growth factors that mediate perivascular cell recruitment include platelet derived growth factor (PDGF) -B and bone morphogenic protein (BMP) -2. These factors are expressed in endothelial cells and stimulate the migration of SMCs and PCs to the growing blood vessels (17, 18).

While several factors and mechanisms involved in blood vessel endothelial cell–periendothelial cell interactions have been characterized, such mechanisms are poorly understood in the lymphatic vasculature. We wanted to know whether the effectors involved in the generation of stabilized and functional blood vessels also operate in the lymphatic vascular system. To study the mechanisms of regulation in blood vascular endothelial cells (BECs) and lymphatic endothelial cells (LECs) in parallel, we used human arterial and venous BECs as well as human dermal microvascular endothelial cells (HDMVECs), which were sorted into pure populations of BECs and LECs on the basis of podoplanin expression as previously reported (19). Our results indicate significant cell type-specific differences in the interpretation of VEGFR-specific stimuli in the LECs and BECs. The results also suggest that endothelial–periendothelial cell interactions can regulate gene expression in the LECs, and therefore SMC interactions are likely to modulate the LEC responses to lymphangiogenic stimuli.

MATERIALS AND METHODS

Cell culture and isolation of HUVECs, BECs and LECs

Human umbilical vein endothelial cells (HUVECs) were isolated from human umbilical veins of newborn babies by collagenase digestion and cultured in endothelial cell growth medium from PromoCell (Heidelberg, Germany). The HDMVECs, human umbilical artery endothelial cells (HUAECs), human saphenous vein endothelial cells (HSaVECs), human aortic endothelial cells (HaoECs), human carotid artery SMCs (HCASMCs), and human umbilical artery SMCs (HUASMCs) were purchased from PromoCell and cultured in growth media provided by the supplier. Podoplanin antibodies (20), a kind gift from Dr. Dontscho Kerjascki (Vienna, Austria), MACS colloidal super-paramagnetic MicroBeads conjugated to goat anti-rabbit IgG antibodies, LD and MS separation columns, and Midi/MiniMACS separators (Miltenyi Biotec, Sunnyvale, CA, USA) were used for cell sorting according to the manufacturer's instructions. BECs (CD31+/podoplanin-) were isolated from HDMVECs using LD negative selection columns and a pure population of LECs was subsequently obtained using MS positive selection columns. Both populations were maintained on dishes coated with 1 μg/mL human fibronectin (Sigma, St. Louis, MO, USA), and LECs were propagated in the presence of 100 ng/mL VEGF-C added to the growth medium.

Generation of endothelial cell, SMC, and coculture spheroids

Spheroids of defined cell number were generated as described previously (21). In brief, HUASM or HUVE cells were suspended in corresponding culture media containing 0.25% (w/v) methylcellulose and seeded in nonadherent round bottom 96-well plates (Greiner, Frickenhausen, Germany). Under these conditions all suspended cells contribute to the formation of a single spheroid of defined size and cell number (3000 cells/spheroid) per well. To generate coculture spheroids, equal amounts of suspended SMC, HUVEC, and fibroblasts (1500 SMC/fibroblasts and 1500 HUVEC per spheroid) were mixed and seeded in nonadherent round bottom 96-well plates as described above. Spheroids were cultured for at least 24 h and used for the corresponding experiments. For experimental details, see www.spherogenex.de.

Growth factor stimulation and Northern blot analysis

The endothelial cells were serum starved overnight in microvascular endothelial cell basal medium (PromoCell, Turku, Finland) supplemented with 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, and 0.2% bovine serum albumin. Recombinant human VEGF165 (R&D Systems, Abingdon, Oxon, UK; 10 ng/mL), mature human VEGF-C (Thr103-Leu215, 100 ng/mL), VEGF-C156S (22) (Thr103-Ile225, 500 ng/mL), or VEGF-E NZ2 (23) (50 ng/mL) were added to the cells for defined periods in this medium. Total RNA was extracted with the RNeasy kit (Qiagen, Chatsworth, CA, USA), electrophoresed, transferred to nylon filters, and hybridized with ^{32}P-labeled cDNA probes for human Ang-2 (bp 413-1837, NM_001147), BMP-2A (bp 776-1273, NM_001200), VEGF (bp 57-639, NM_003376.2), VEGF-C (bp 80-2076, NM_005429.2), Tie-2 (bp 149-2374, NM_000459), VEGFR-2 (bp 397-1500, NM_002253.1), or VEGFR-3 (bp 20-1005, NM_002020). Glyceraldehyde-3-phosphate (GAPDH) probe was used as an internal control for equal loading.

3-[4,5-Dimethylthiazol-2-y]-2,5-diphenyltetrazolium bromide (MTT) assay

LECs cultured in full endothelial cell growth medium were allowed to adhere overnight, then stimulated with VEGF, VEGF-C, VEGF-C156S, or VEGF-E NZ2 for 48 h in starvation medium as above. Cells were incubated with the MTT substrate (5 mg/mL) for 4 h at 37°C, lysed in DMSO, and the optical density at 540 nm was measured.

Western blot analysis

Cells cultured as spheroids and monolayers were lysed using sample buffer containing 1% Triton X-100 detergent. Samples were resolved on a 10% SDS PAGE gel and blotted. The blots were probed with anti-human VEGFR-3 antibody (rabbit polyclonal, sc-321, Santa Cruz Biotechnology, Santa Cruz, CA, USA) and reprobed for CD31 (goat polyclonal, sc-1506, Santa Cruz) and actin (goat polyclonal, sc-1616, Santa Cruz) after stripping.

Cell migration assays

Cell migration assays were performed in a 48-well chemotaxis Boyden chamber (Neuro Probe Inc., Gaithersburg, MD, USA) as described previously (19). To produce conditioned media for the SMC migration assays, equal numbers of HCASMCs, BECs, and LECs were maintained for 24 h in starvation medium (as above). After removal of cell debris by centrifugation, the medium was used as chemoattractant. The cells used for the migration assay were starved overnight in the same medium. 25,000 cells/well were allowed to migrate for 4 h at +37°C through a micropore filter coated with 6.7

μg/mL of fibronectin (Sigma). The filter was fixed with cold methanol and stained with hematoxylin (Meyer). Nonmigrated cells on the upper surface of the filter were removed with a cotton swab and the number of migrated cells was counted. The assay was run in quadruplicate and repeated with two independent cell batches.

Morphological and immunostaining analysis

For immunohistochemical staining, tissues, spheroids, and monolayer cultures were fixed in 4% paraformaldehyde (PFA), embedded in paraffin, and sectioned (4 μm). The sections were deparaffinized, rehydrated, and blocked with 3% H_20_2. After washings in PBS, they were incubated for 30 min with blocking solution (10% normal goat serum) followed by incubation with the corresponding primary antibody (mouse monoclonal anti-human VEGFR-3 9D9F9 (24), podoplanin clone 18H5 [kindly provided by Dr. Gerd Zimmer], CD31 clone JC70A [Dako, Carpenteria, CA, USA], CD34 clone QBEND/10 [Novocastra, Newcastle upon Tyne, UK]) in a humid chamber at 4°C overnight. For VEGFR-3 detection in the spheroids and monolayers, sections were heated in a microwave oven in 10 mmol citrate buffer, pH 6.0, before the staining. The tissue sections were then incubated with secondary antibody (biotinylated goat anti-mouse immunoglobulin antibody, Zymed, San Francisco, CA, USA), exposed to streptavidin peroxidase, developed with diaminobenzidine substrate, and weakly counterstained with hematoxylin.

LECs cultured on glass coverslips were fixed in 4% PFA for 10 min, permeabilized with 0.1% Triton-X in PBS for 5 min, and blocked in 5% goat serum followed by staining using rabbit antisera against human LYVE-1 (kindly provided by Dr. David G. Jackson) and monoclonal antibodies against proliferating cell nuclear antigen (clone PC10, Santa Cruz Biotechnology) for 30 min at room temperature, followed by incubation with TRITC-conjugated goat anti-rabbit IgG and FITC-conjugated anti-mouse IgG (Jackson Immunoresearch, West Grove, PA, USA) for 30 min.

For the visualization of functional collecting lymphatic vessels, a small volume of FITC-labeled dextran (MW 464,000, Sigma) was injected intradermally to the periphery of the mouse ear. The ears were dissected and fixed in 4% PFA. Tissue was permeabilized in 0.3% Triton-X in PBS and blocked with 5% normal horse serum (Vector, Burlingame, CA, USA) in the permeabilization buffer for 2 h at room temperature. Samples were incubated with Cy3-conjugated monoclonal antibodies against α-SMC actin (Sigma) and goat polyclonal antisera against mouse VEGFR-3 (R&D Systems) in the blocking buffer at +4°C overnight. After washing, the samples were incubated with FITC-conjugated anti-goat IgG antibodies (Jackson Immunoresearch) and mounted in antifading polyvinyl alcohol mounting medium (Fluka BioChemica, Basel, Switzerland).

RESULTS

In LECs, VEGFR-3 signals are sufficient for proliferation and VEGFR-2 signals are necessary for morphological changes and motility

Striking differences were observed in the LECs when responses to treatment with VEGFR-2 or VEGFR-3 agonists were compared. In the absence of added growth factors, LECs grew in well-demarcated islands consisting of flattened polygonal cells (**Fig. 1*A***), which were not proliferating when analyzed by staining for the proliferating cell nuclear antigen (19; Fig. 1*B*). Treatment of LECs with the VEGFR-3-specific mutant factor VEGF-C156S (22) resulted in cell proliferation as determined by staining for PCNA and by MTT assay, whereas the typical morphological changes, including an elongated cell shape and increased cell motility

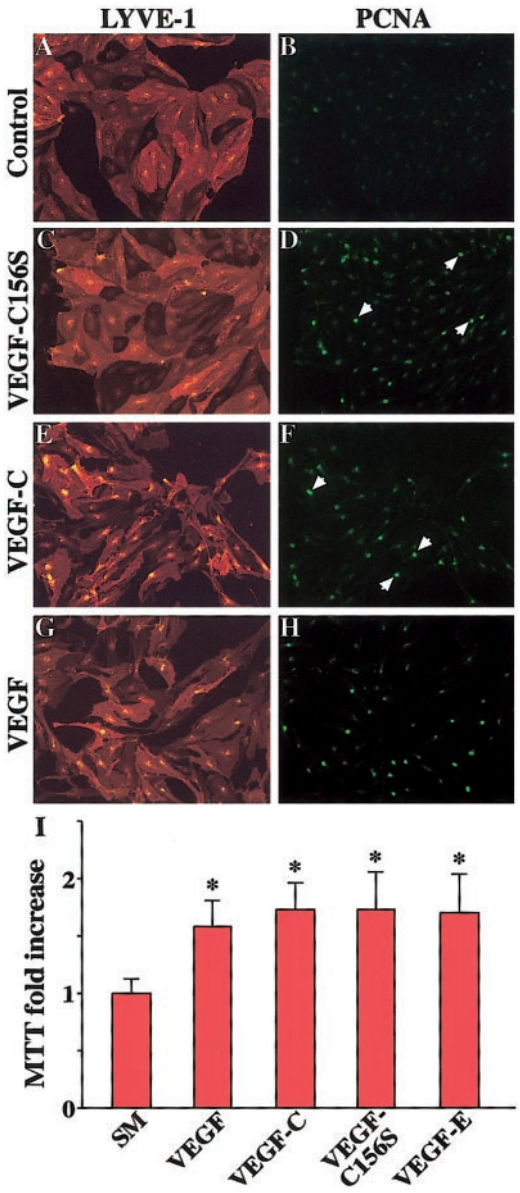

Figure 1. Proliferation and shape change of LECs in response to VEGF, VEGF-C, and VEGF-C156S. *A)* LECs were stimulated with VEGF, VEGF-C, or VEGF-C156S for 48 h and double-stained for the lymphatic marker LYVE-1 (orange, left panel) and for the proliferating cell nuclear antigen (PCNA; green, right panel). Proliferating cells (arrows) are seen in the growth factor-stimulated cultures while very few PCNA-positive nuclei are detected in the unstimulated control culture. *B)* MTT assay for LECs stimulated with VEGF, VEGF-C, VEGF-C156S, or VEGF-E for 48 h. Increase in proliferation is given as fold increase in absorbance ±standard deviation compared with the starvation medium (SM) only. Asterisk indicates significant difference to SM control ($P<0.01$, two-tailed t test).

observed after VEGF-C or VEGF stimulation, did not take place (Fig. 1; see also ref 19). This finding is in line with our observation that compared with VEGF-C and VEGF, VEGF-C156S is a very weak migration-inducing factor for the LECs in a modified Boyden chamber assay (ref 19 and data not shown). In contrast, BECs proliferated in full-growth medium without added growth factors and very small or no changes in their shape occurred upon stimulation with VEGF, VEGF-C, or VEGF-C156S (data not shown).

Ang-2 is up-regulated via VEGFR-2 in both LECs and BECs

Both Ang-2 and BMP-2 have been implicated in blood vessel stabilization by PC/SMC recruitment (11, 17, 25) and VEGF has been reported to stimulate Ang-2 mRNA expression in microvascular endothelial cells (14, 15). The isolated BECs and LECs were stimulated with VEGF, VEGF-C, or VEGF-C156S and total RNA was extracted. VEGF and VEGF-C were found to up-regulate Ang-2 mRNA levels in both BECs and LECs (**Fig. 2*A***). Treatment with VEGF-C156S did not affect Ang-2 mRNA, suggesting that the up-regulation was mediated via VEGFR-2, which is expressed on both types of cells (19). This was further confirmed by the marked up-regulation of Ang-2 in BECs stimulated with VEGF-E, which only binds VEGFR-2 (Fig. 2*C*). Expression levels of Tie-2, VEGFR-2, VEGFR-3 (or endogenous VEGF or VEGF-C) did not change substantially in either cell population in response to stimulation with any of the factors tested (not shown).

VEGFR-2 activation up-regulates BMP-2 in BECs but not in LECs

BMP-2 induces SMC chemotaxis and VEGF is known to up-regulate BMP-2 in endothelial cells (17, 26). To discover if VEGF-C affects BMP-2 expression in the LECs, we probed Northern blots containing RNA from VEGF-C stimulated BECs and LECs. BMP-2 was strongly expressed in the BECs while only very weak expression was detected in the LECs (Fig. 2*B*). Strikingly, an 8 h stimulation with VEGF-C as well as VEGF strongly up-regulated BMP-2 in the BECs, while little if any induction was seen in the LECs (Fig. 2*B*). This suggested that the stimulation was mediated by VEGFR-2, the only receptor shared by VEGF and VEGF-C. We examined this further by stimulating BECs for 24 h, including VEGF-C156S and VEGF-E as controls for VEGFR-3- and VEGFR-2-specific effects, respectively. As expected, VEGF-C156S had no effect on BMP-2, while VEGF-E clearly up-regulated BMP-2 mRNA expression. Induction of BMP-2 in the BECs but not LECs indicates that differential transcriptional programs responsible for the modulation of endothelial–periendothelial cell interactions exist downstream of VEGFR-2 in the two types of endothelial cells.

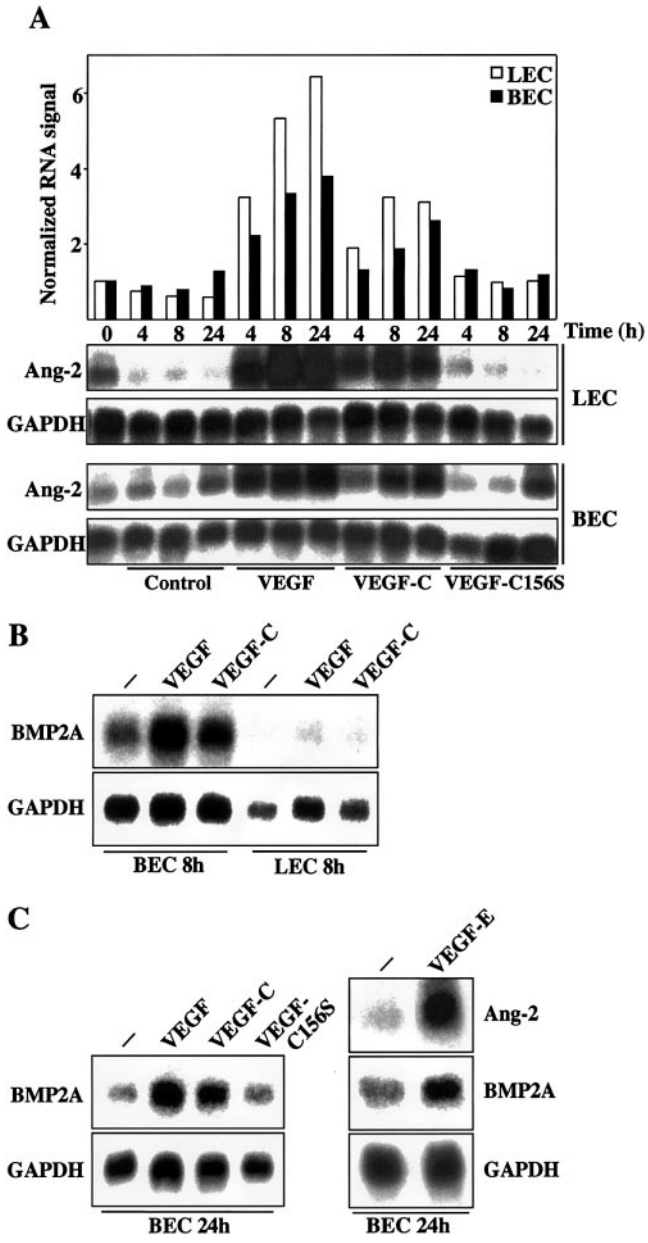

Figure 2. Ang-2 and BMP-2 induction by VEGFR-2 in BECs vs. LECs. *A)* Northern blot analysis of Ang-2 mRNA expression in BECs and LECs stimulated with VEGF, VEGF-C, or VEGF-C156S for the periods indicated. The bar graph represents optical densities of the signals that were quantified and normalized to the GAPDH loading control signals. The experiment was repeated with two independent cell batches. *B)* Northern blot analysis of BMP2A mRNA expression in BECs and LECs stimulated with VEGF or VEGF-C for 8 h. *C)* Northern blot analysis of Ang-2 and BMP2A mRNA expression in BECs stimulated for 24 h.

LECs recruit SMCs in vitro and in vivo

The angiogenic blood vascular endothelium is known to secrete factors such as PDGF-BB and HB-EGF, which stimulate the migration and recruitment of perivascular cells (18, 27). To determine whether LECs are able to recruit SMCs, we tested conditioned medium from LECs in a SMC migration assay. HCASMCs readily

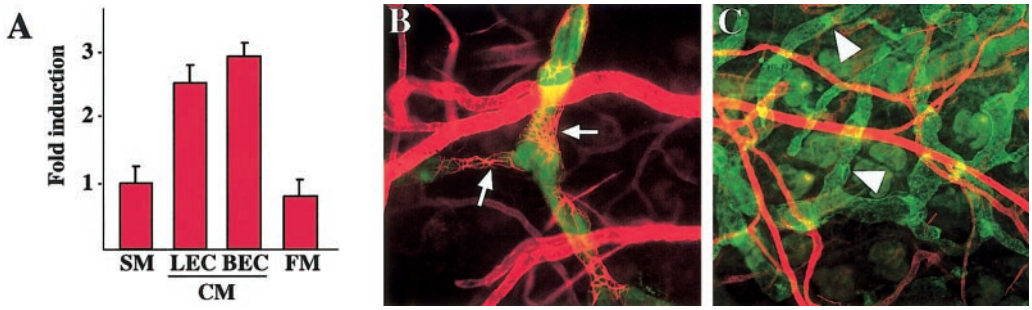

Figure 3. Both BECs and LECs stimulate SMC chemotaxis, but only blood vessels and collecting lymphatic vessels are stained for smooth muscle actin in vivo. *A)* SMC migration toward starvation medium (SM). Full medium (FM), or BEC and LEC conditioned media (CM) was tested in a modified Boyden chamber assay. The assay was run in quadruplicate and repeated with two independent cell batches. *B)* Functional collecting lymphatic vessels in mouse ear were visualized with intradermal injection of FITC-conjugated dextran (*A*, green) and whole mount staining for VEGFR-3 was used to visualize the lymphatic capillaries (*B*, green). Simultaneous whole mount staining for smooth muscle actin (red) was carried out. Blood vessels with SMC coverage are seen in red. The collecting lymph vessel in panel *A* has sparser pericyte/SMC coverage (arrows), whereas the lymphatic capillaries in panel *B* are devoid of PCs/SMCs (arrowheads).

migrated toward conditioned media from both BECs and LECs, whereas in the inverse experiment the HCASMC conditioned medium did not stimulate endothelial cell migration (**Fig. 3*A*** and data not shown).

We next wanted to see whether the lymphatic endothelium is also able to recruit PCs/SMCs in vivo. Whole mount immunofluorescence staining for SMC actin (SMA) together with simultaneous imaging of functional lymphatic vessels by FITC-dextran injection demonstrated that the collecting lymphatic vessels in mouse skin were indeed coated by SMA-expressing cells, although the coverage was sparser than in the arteries and veins (Fig. 3*B*, arrows). By contrast, the initial lymphatic capillaries, which were revealed by anti-VEGFR-3 whole mount immunofluorescence staining, were SMA negative (Fig. 3*C*, arrowheads).

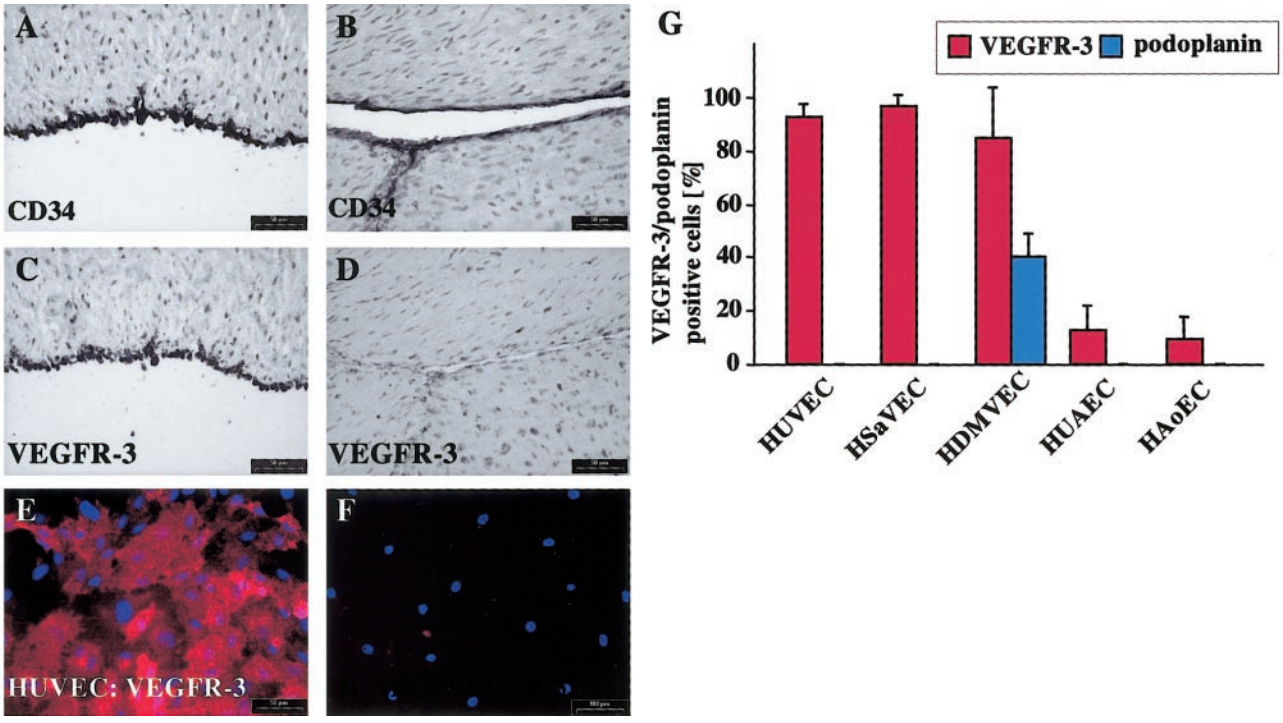

Figure 4. Human umbilical vein endothelial cells (ECs) but not umbilical artery ECs express VEGFR-3 in vivo and in vitro. Integrity of the analyzed EC monolayers in vivo was visualized by immunostaining of paraffin embedded human umbilical veins (*A*) or arteries (*B*) for the EC marker CD34, which is expressed on venous and arterial ECs (dark DAB staining in panels *A, B*). VEGFR-3 expression was similarly analyzed in panels *C* and *D* in tissue sections or by immunofluorescence (red) in the corresponding cultured ECs (*E, F*). The quantitative analysis of VEGFR-3-positive cells in panel *G* shows that >90% of HUVEC and HSaVEC are VEGFR-3 positive, whereas HUAEC and HAoEC remain VEGFR-3 negative. HDMVEC express at least some VEGFR-3 in most of the cells, although they contain both blood vascular and lymphatic ECs as reflected by podoplanin expression in ~40% of the cells (see also ref 19).

Contact with SMCs down-regulates endothelial VEGFR-3 expression

The collecting lymphatic vessels covered by SMCs stained only weakly for VEGFR-3 compared with the lymphatic capillaries (data not shown; see ref 28). This suggested that SMC contact may down-regulate VEGFR-3 expression in vivo. Further evidence supporting the hypothesis that the SMC coating may regulate VEGFR-3 expression was obtained from the histochemical staining of human umbilical cord vessels. The umbilical artery was negative for VEGFR-3, which was, however, expressed in the umbilical vein (**Fig. 4*A–D***). The corresponding cells, HUAECs and HUVECs retained their typical VEGFR-3 expression patterns in cell culture (Fig. 4*E, F*). A similar asymmetric arteriovenous expression pattern for VEGFR-3 was observed in human aortic endothelial cells and human saphenous vein endothelial cells (Fig. 4*G*), although neither cell type expresses VEGFR-3 in vivo (data not shown).

To determine whether contact with SMCs down-regulates VEGFR-3 expression, we used an endothelial cell-SMC coculture system that mimics the 3-dimensional assembly of the vessel wall (21). Indeed, when HUVECs were cocultured with SMCs in spheroids, VEGFR-3 expression was completely down-regulated (**Fig. 5*D***). In contrast, culture of HUVECs as spheroids alone or in combination with fibroblasts did not affect VEGFR-3 expression (Fig. 5*E, F*). Specific down-regulation of VEGFR-3 but not, for example, the endothelial cell adhesion molecule CD31 was confirmed by Western blot of cell lysates (Fig. 5*G*).

DISCUSSION

Our present study shows that the lymphangiogenic growth factor VEGF-C mediates proliferation and cell shape/migration by using distinct receptor signals in LECs. Furthermore, VEGFR-2 signals up-regulated Ang-2 mRNA levels in both LECs and BECs, but BMP-2 mRNA levels only in BECs, indicating that the gene expression programs activated in response to stimulation of the same receptor tyrosine kinase differ in these two cell types. We also demonstrate that LECs recruit SMCs both in vitro and in vivo and that contact with SMCs down-regulates endothelial VEGFR-3 expression. These results highlight the intrinsic differences between BEC and LEC responses to the VEGFs and identify a pericellular regulatory mechanism for VEGFR-3 down-regulation in endothelial cells.

Activation of VEGFR-3 has been shown to be sufficient for the growth, survival, and migration of cultured LECs and for lymphangiogenesis in vivo (6, 19). However, the specificities of the VEGFR-2 and VEGFR-3 signal transduction mechanisms have not been compared in more detail in cultured LECs. Although both VEGFR-2 and VEGFR-3 transduce proliferation signals in LECs, changes in cell morphology and motility were mediated by VEGFR-2. Our results furthermore dem-

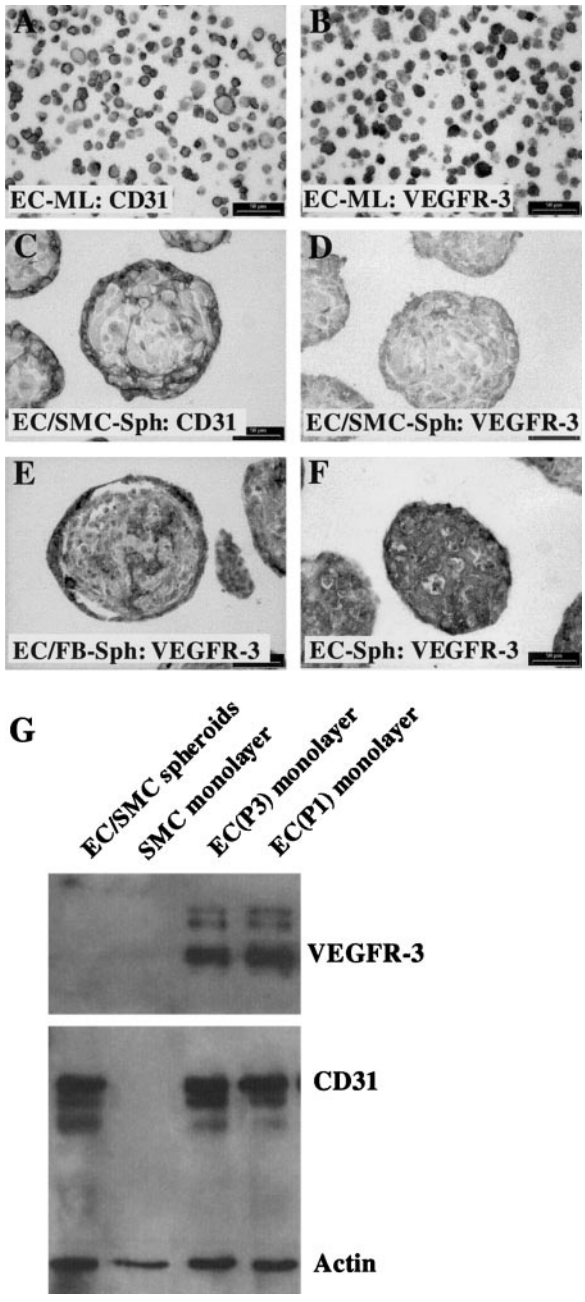

Figure 5. Down-regulation of VEGFR-3 expression in HUVECs by contact with SMCs. Endothelial VEGFR-3 expression was visualized by immunostaining (dark DAB staining) of paraffin sections of ECs cultured as a monolayer (ML) culture (*B*) or spheroids (Sph) (*D–F*). Immunostaining for the EC marker CD31 (monolayer-grown cells in panel *A*) shows that ECs form a surface monolayer on top of a core of SMCs in coculture spheroids (*C*). Most ECs express VEGFR-3 if cultured as a monolayer (*B*) or as spheroids (*F*); coculture with fibroblasts (FB) does not affect endothelial VEGFR-3 expression, whereas coculture with SMCs down-regulates VEGFR-3 expression (*E*). Western blot analysis (*G*, top) of lysates of ECs cultured as monolayer (EC-P1: HUVEC passage 1; EC-P3: HUVEC passage 3) shows a strong VEGFR-3 expression. No VEGFR-3 expression is detected in lysates of SMC or EC/SMC coculture spheroids. Blots were reprobed and analyzed for actin (total loading control) and CD31 as EC-specific loading control (*G*, bottom).

onstrate that BECs and LECs have differences in the transduction of VEGFR-2 signals, since VEGFR-2 stimulation resulted in the up-regulation of BMP-2 only in the BECs but Ang-2 in both cell types. Recent studies have shown that BECs and LECs represent differentiated cell lineages without evidence of interconversion between the two (19, 29). BMP-2 has been shown to potently induce SMC chemotaxis and to synergize with PDGF in the recruitment of perivascular cells (17). Therefore, the induction of BMP-2 expression in BECs but not LECs is consistent with the in vivo situation, as endothelial cells in blood vessels are completely covered by periendothelial cells. N-cadherin, a molecule shown to be important for endothelial-SMC interactions (30), has been found to be expressed exclusively in the BECs (31).

Although LECs expressed very little BMP-2, they secrete other factors that strongly induced SMC chemotaxis. One such factor could be PDGF-BB, which is expressed by both BECs and LECs (T. Makinen, unpublished data). SMA-positive cells were indeed detected around the collecting lymphatic vessels in vivo, but their coating was much less dense than that of the blood vessels. In contrast to blood capillaries that have associated PC/SMCs, lymphatic capillaries were SMA negative. This is in line with the lesser role of periendothelial cells in the lymphatic capillary network.

We found that SMC interaction down-regulates VEGFR-3 expression in endothelial cells. Our previous results from reporter mice heterozygous for VEGFR-2/LacZ, VEGFR-3/LacZ, and Tie-1/LacZ have indicated that VEGFR-3 is strongly expressed in the initial lymphatic capillaries, whereas the collecting lymphatic vessels containing valves express predominantly VEGFR-2 and Tie-1 (28, 32). The present data suggest that this differential receptor expression pattern is due to transcriptional changes resulting from contacts between the endothelial cells and SMCs. In adults, arteries and veins are generally negative for VEGFR-3, while fenestrated capillaries of several organs show weak expression (33). In the blood vasculature, SMCs induce endothelial cell quiescence and make them less responsive to angiogenic stimuli. Analogous mechanisms may operate in the lymphatic vessels, as SMCs down-regulate VEGFR-3, thus decreasing its signaling capacity, which is required for the induction of lymphatic vessel growth. Likewise, we have shown that the phenotype of the collecting lymphatic vessels expressing VEGFR-2 is normal in the K14-VEGF-C transgenic mice that have severe hyperplasia of the VEGFR-3-positive skin lymphatic capillaries (28).

VEGF-C is perhaps the most potent stimulator of lymphangiogenesis, and stimulation of human primary LECs with VEGF-C strongly increased Ang-2 expression via VEGFR-2. This indicates that in addition to its role in initiating angiogenesis, Ang-2 may also play a role in lymphangiogenesis. The recently reported phenotype of Ang-2 knockout mice (34) supports this interpretation. Besides defective postnatal angiogenesis and blood vessel remodeling, Ang-2 deficient mice suffered from hypoplasia of the lymphatic capillaries in the skin and in the small intestine (34). Also, the mesenteric collecting lymphatic vessels in these mice were nonfunctional and poorly covered by SMCs (34). Ang-2 thus seems to be required for the proper development of the lymphatic vessels and may be involved in the regulation of the lymphatic endothelial–periendothelial cell interactions. Similar to what has been proposed for BECs (11, 25), LECs may be made more responsive to lymphangiogenic stimuli by Ang-2/Tie-2 signals. However, Ang-2 does not appear to mediate LEC survival since recombinant Ang-2 did not affect serum withdrawal-induced apoptosis in the LECs (authors' unpublished data).

Stimulation of VEGFR-3 alone by VEGF-C156S did not result in increased Ang-2 expression or morphological changes in the LECs. Indeed, analysis of lymphangiogenesis induced by VEGF-C156S and VEGF-C in a variety of in vivo models (28) has shown that VEGF-C156S-induced lymphangiogenesis involves less lymphatic sprouting than lymphangiogenesis induced by VEGF-C (28). As VEGF-C156S failed to up-regulate Ang-2 mRNA levels or to cause morphological changes in the LECs in vitro, it may be that Ang-2 expression is required specifically for efficient lymphatic sprouting in vivo.

Collaboration between the VEGF and angiopoietin growth factor families seems to be required not only for the proper formation of the blood vascular system, but also for the development and function of the lymphatic vascular system. While VEGFR-3 mediated signals are essential for growth and maintenance of the lymphatic vessels (35, 36), signals mediated by Tie-2 are needed for the subsequent remodeling and maturation of these vessels (34). These results have important implications for the development of gene therapy approaches for treating lymphatic hypoplasia and dysfunction as they suggest that single factor therapy will not be sufficient for the generation of stable and functional lymphatic vessels. FJ

We thank Dr. Dontscho Kerjaschki (Vienna, Austria), Dr. Gerd Zimmer (Hannover, Germany), and Dr. David G. Jackson (Oxford, UK) for antibodies and Sanna Karttunen, Kaisa Makkonen, Mari Helanterä, Paula Hyvärinen, and Tapio Tainola for excellent technical assistance. This study was supported by the Finnish Cancer Organizations, the Farmos Research Foundation, the Finnish Cultural Foundation, the Human Frontier Science Program, the EU Biomed Program (QLG1-CT-2001-01172), and by the Schwerpunktprogramm Angiogenese (SPP1069) of the Deutsche Forschungsgemeinschaft (DFG Au83/3-2 to H.A.).

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Received for publication March 10, 2003.
Accepted for publication July 7, 2003.