Endothelin-1 Stimulates Lymphatic Endothelial Cells and Lymphatic Vessels to Grow and Invade

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Abstract

The lymphatic vasculature is essential for tissue fluid homeostasis and cancer metastasis, although the molecular mechanisms involved remain poorly characterized. Endothelin-1 (ET-1) axis plays a crucial role in angiogenesis and tumorigenesis. Here, we first report that ET-1 acts as a lymphangiogenic mediator. We performed *in vitro* and *in vivo* studies and show that lymphatic endothelial cells produce ET-1, ET-3, and express the endothelin B receptor (ET_BR). In these cells, ET-1 promotes proliferation, invasiveness, vascular-like structures formation, and phosphorylation of AKT and p42/44 mitogen-activated protein kinase through ET_BR. In normoxic conditions, ET-1 is also able to up-regulate the expression of vascular endothelial growth factor (VEGF)-C, VEGF receptor-3, and VEGF-A, and to stimulate hypoxia-inducible factor (HIF)-1α expression similarly to hypoxia. Moreover, HIF-1α silencing by siRNA desensitizes VEGF-C and VEGF-A production in response to ET-1 or hypoxia, implicating HIF-1α/VEGF as downstream signaling molecules of ET-1 axis. Double immunofluorescence analysis of human lymph nodes reveals that lymphatic vessels express ET_BR together with the lymphatic marker podoplanin. Furthermore, a Matrigel plug assay shows that ET-1 promotes the outgrowth of lymphatic vessels *in vivo*. ET_BR blockade with the specific antagonist, BQ788, inhibits *in vitro* and *in vivo* ET-1–induced effects, demonstrating that ET-1 through ET_BR directly regulates lymphatic vessel formation and by interacting with the HIF-1α–dependent machinery can amplify the VEGF-mediated lymphatic vascularization. Our results suggest that ET-1 axis is indeed a new player in lymphangiogenesis and that targeting pharmacologically ET_BR and related signaling cascade may be therapeutically exploited in a variety of diseases including cancer. [Cancer Res 2009;69(6):2669–76]

Introduction

Although the lymphatic vascular system plays an essential role in the maintenance of normal fluid homeostasis as well as in the pathogenesis of several human diseases, such as tumor metastasis, the molecular mechanisms that regulate lymphangiogenesis remain poorly characterized. Identification of lymphatic endothelial specific markers, such as the transcriptional factor Prox-1, podoplanin, and lymphatic vessel endothelial hyaluronan receptor-1 (LYVE-1), and the recent studies using mouse genetic tools have greatly increased our understanding of how lymphatic endothelial cell (LEC) growth and differentiation are regulated (1). Lymphatic vessels seem to originate from a subset of venous endothelial cells, which commit to the LEC lineage. Because of their common origin, lymphangiogenesis as well as angiogenesis relay on the interplay of several growth factors and receptors. Members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, are thus far the best characterized lymphangiogenic factors that bind the VEGF receptor-3 (VEGFR-3) specifically expressed on LEC, and transduce proliferation and differentiation responses (2–5). In addition to VEGF-C and VEGF-D, also VEGF-A stimulates lymphatic growth in several experimental systems, and the activation of its receptor VEGFR-2 seems to be required for LEC organization into functional capillaries (6–9). Other growth factors, such as fibroblast growth factor-2, hepatocyte growth factor, angiopoietin-1/-2, and platelet-derived growth factor, have been shown to be involved in the developmental and pathologic formation of lymphatic vessels (3, 10). However, a number of lymphangiogenic factors remain as-yet-unidentified. The endothelin (ET) axis is composed of three isopeptides namely ET-1, ET-2, and ET-3 and two G-protein coupled receptors, ET A receptor (ET_AR) and ET_BR, which are expressed in endothelial cells, tumor cells, fibroblasts, and macrophages (11). Experimental and preclinical studies have shown that overexpression of ET-1 axis occurs in a variety of malignancies and that ET-1 selective receptor antagonists could represent potential antitumor agents (11, 12). ET-1 axis, in fact, contributes to cancer progression by several mechanisms including mitogenesis, cell survival, invasiveness, and angiogenesis (13–15). Regarding this latter effect, it has been shown that ET-1 acting on ET_BR can directly modulate endothelial cell proliferation, migration, invasiveness, and vascular differentiation, and can also act indirectly through the induction of VEGF (16–22). ET-1–induced angiogenic effects are abolished by ET_BR antagonists, suggesting that this receptor may represent a potential target to inhibit angiogenesis (23–25). Moreover, in corneal neovascularization, ET-1/ET_BR expression has been detected on endothelial cells of ingrown blood vessels, pointing to an involvement of ET-1 axis in corneal angiogenesis (26). Interestingly, many proteins that were originally shown to be relevant in nervous system formation, such as ET_BR that is expressed in migratory neural crest cells (27), have also been recently implicated in vascular system development (28). These observations have primarily been made for blood vessel formation but are becoming to be extended to lymphatic vessel development as well (29).

Among microenvironmental components, hypoxia, one of the principal angiogenic stimuli, is also able to control lymphangiogenesis. Hypoxia has been shown to stimulate expression of

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VEGF-family members in LEC (30, 31), but the role of hypoxia-inducible factor-1α (HIF-1α) in lymphatic system function and development has not been previously examined. HIF-1α is the transcriptional factor that conveys signaling elicited by hypoxia and growth factors, activating the transcription of genes that are involved in crucial aspects of angiogenesis, including *VEGF, erythropoietin,* and *ET-1* (32, 33).

As determined using comparative transcriptional profiling studies, *ET_BR* was expressed at much higher levels in cultured LEC then in blood vascular endothelial cells (5.3-fold; ref. 34), indicating that strong ET_BR expression is characteristic for the lymphatic phenotype *in vitro*. Moreover *ET-1* is one of the most up-regulated genes in tumor-associated LEC compared with normal-derived LEC (35), highlighting the new role of ET-1 axis in lymphangiogenesis. Although correlation between ET-1/ET_BR expression and lymphovascular invasion has been reported in human breast cancers (36), no evidence has been thus far gathered as to whether ET-1 axis promotes lymphangiogenesis. To address this issue, we used highly purified LEC population from human lymph nodes. We show that LEC produce ETs and express functional ET_BR, which in response to ET-1 activates signaling pathways, leading to early and late events of lymphangiogenesis. Although the key role of VEGF family members in lymphangiogenesis has been established, few studies have been addressed to analyze the induction of VEGF expression on LEC. In this study, we show that ET-1 selectively through ET_BR enhances VEGF-A, VEGF-C, and VEGFR-3 expression. In tumor microenvironment, ET-1 signaling has also been reported to induce HIF-1α accumulation (18, 20). Here, we show that in LEC ET-1, through ET_BR activation, mimics cellular hypoxia to induce HIF-1α, resulting into enhanced VEGF-A and VEGF-C expression. Moreover, lymphangiogenesis assay *in vivo* shows that ET-1 induces formation of lymphatic vessels and that this effect is blocked by an ET_BR selective antagonist. Our *in vivo* analysis shows that ET_BR is expressed on LYVE-1–positive endothelial cells lining lymphatic vessels. As several small molecules that target ET-1 receptors have already entered clinical testing, the question of whether such agents might also affect lymphangiogenesis and metastasis has taken on particular importance. Our *in vitro* and *in vivo* results showing that a specific ET_BR antagonist is able to inhibit ET-1–induced lymphangiogenic activities suggest that ET_BR blockade could effectively impair lymphangiogenesis.

Materials and Methods

Lymphatic endothelial cell purification and cell cultures. Human lymph node specimens were obtained according to the guidelines of Helsinki Declaration from patients undergoing surgical procedures for noninfectious or neoplastic conditions. Lymph nodal LEC were isolated from tissues lacking histopathologic changes by double immunomagnetic purification as previously described (37, 38). Cells were cultures in EGM plus VEGF-C (25 ng/mL) and grown in a humidified atmosphere at 37°C and 5% CO_2. When the cells were exposed to hypoxia, oxygen deprivation was carried out in an incubator with 1%O_2, 5%CO_2, and 94% N_2 and cells were growth for 24 h. Human endothelial cells were isolated from human umbilical vein (HUVEC), as previously described (21), and grown on collagen type I–coated flasks (5 μg/cm^2; Boehringer Mannheim).

Flow cytometric analysis. To determine the degree of LEC purification, cells were detached from flasks by trypsinization, and resuspended in PBS containing 1% fetal bovine serum. Cell suspension was then incubated for 30 min at 4°C with rabbit anti-human CD31 (BD Biosciences) and with mouse anti-human podoplanin (RELIATech). Binding of Ab was detected by using R-Phycoerythrin–conjugated anti-rabbit IgG (BD Biosciences) and anti-mouse Ig, respectively. Negative controls were represented by cells incubated with phycoerythrin isotype matched normal IgG (BD Biosciences). Cells were analyzed with FACSCalibur and data acquisition was performed with CellQuest software (BD Biosciences).

Immunocytochemistry and immunohistochemical analysis. Immunocytochemical studies were performed on LEC and HUVEC seeded on glass slides coated with type I collagen, fixed in cold 4% paraformaldehyde in PBS (pH 7.4), for 10 min at room temperature. After washing with PBS, cells were incubated with 10% goat serum (Invitrogen) to block aspecific binding, then incubated for 90 min at 37°C in appropriate dilution of the following primary antibody (Ab): murine MoAb to CD31 (Dako), ULEX-1, KDR (Santa Cruz Biotechnology), LYVE-1, podoplanin, and Prox-1 (RELIATech) and CD44 (Novocastra Laboratories), with rabbit antisera to the Von Willebrand factor (vWf; RELIATech). After two washings with PBS, cells were incubated for 45 min at room temperature with 1:300 diluted cyanine dye–labeled goat anti-mouse or goat anti-rabbit IgG (Jackson Immunoresearch). Cells were then mounted with Fluorosave (Calbiochem) and photographed using a Zeiss Axiophot-2-microscope. As negative control of primary polyclonal antisera, the incubation with the primary Ab was omitted, whereas for murine MoAb, isotype-matched normal IgG were used. The evaluation of staining was done by 2 independent investigators at least in 5 fields (×20), and scored as no stain (−), weak heterogeneous stain (+/−), positive homogeneous stain (++), intense homogeneous stain (+++), and not tested (Nt).

Normal human lymph nodes removed during mammoplast were snap frozen in liquid nitrogen. Four-micrometer cryostat sections were obtained and, after fixation in absolute acetone for 10 min, were used for double immunofluorescence analysis using MoAb D2-40 to podoplanin (Signet), and rabbit polyclonal Ab to ET_BR. Goat anti-mouse (Alexa Fluor 488 labeled; Molecular Probes) and goat anti-rabbit (Alexa Fluor 594 labeled; Molecular Probes) were used as secondary Ab. Negative control stain was represented by sections in which the incubation with the primary Ab was substituted by isotype matched mouse IgG or normal rabbit IgG. Fluorescence signals were analyzed by recording stained images using a CCD camera (Zeiss) and IAS2000/H1software (Delta Sistemi).

LEC transfection. Serum-starved LEC were transfected with 100 nmol/L siRNA duplexes against HIF-1α (SMART pool) or with scrambled mock siRNA (Dharmacon). The transfection was done using Lipofectamine reagent (Invitrogen) according to manufacturer's protocol. Cell medium were replaced with fresh serum-free endothelial cell growth 48 h later and exposed to ET-1 or vehicle for 24 h.

Reverse transcription-PCR. Reverse transcription-PCR (RT-PCR) was performed using a Superscript One-Step RT-PCR System (Invitrogen) according to the manufacturer's instructions. The primers sets were as follows: ET-1, 5′-TGCTCCTGCTCGTCCCTGATGGATAAAGAG-3′ and 5′-GGTCACATAACGCTCTCTGGAGGGCTT-3′; ET-3, 5′-TGATCTAGGTTCA-TGGAGCCG-3′ and 5′-GCCAAAACTCTCCAAAACCA-3′; ET_AR, 5′-CACTGG-TTGGATGTGTAATC-3′ and 5′-GGAGATCAATGACCACATAG-3′: ET_BR, 5′-TCAACACGGTGGTGTCCTGC-3′ and 5′-ACTGAATAGCCACCAATCTT-3. VEGF-C, 5′-GACTCAACAGATGGATTCC-3′ and 5′-GGGCAGGTTCTTTTA-CAT-3′; VEGF-A, 5′-TCGGGCCTCCGAAACCATG-3′ and 5′-GCGCA-GAGTCTCCTCTTC; VEGFR-3, 5′-CTACAAAGACCCCGACTACG-3′ and 5′-GAGGGCTCTTTGGTCAAGCA-3′; glyceraldehyde-3-phosphate dehydrogenase was used as an internal control and the primer sets used was 5′-TGAAGGTCGGTGTCAACGGA-3′ and 5′-GATGGCATGGACTGTGGTCAT-3′. The cDNA was amplified for 35 cycles of a denaturation step at 94°C for 1 min; a primer annealing step at 54°C for 30 s and an extension step at 72°C for 1 min. The PCR products were analyzed by electrophoresis on a 2% agarose gel containing ethidium bromide.

Western blotting analysis. Whole cell lysates were subjected to SDS-PAGE and analyzed by immunoblotting using Ab to ET-1; ET_BR and ET_AR (Alexis); VEGF-C, VEGFR-3, and VEGF-A (Santa Cruz Biotechnology); and HIF-1α (Transduction Laboratory), phosphorylated and unphosphorylated forms of p42/44 mitogen-activated protein kinase (MAPK), and AKT (Cell Signaling). Blottings were developed with the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). The membranes were

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Table 1. Differential expression of lymphatic and blood vascular markers on LEC and HUVEC by immunocytochemistry

LEC markers	LEC	HUVEC
LYVE-1	+++*	−
Podoplanin	++	−
Prox-1	+++	−
Blood vascular markers	**LEC**	**HUVEC**
CD31	++	+++
vWf	+/−	+++
Ulex-1	+++	+++
KDR	Nt	+++
CD44	++	+++

Abbreviation: vWf, Von Willebrand factor.
*−, no stain; +/−, weak heterogeneous stain; ++, positive homogeneous stain; +++, intense homogeneous stain; Nt, not tested.

reprobed with anti–β-actin to assure the equal amount of protein (Oncogene; CN Biosciences, Inc.).

ELISA. Subconfluent LEC were serum starved for 24 h and cultured for the indicated times. The conditioned medium was then collected, centrifuged, and stored in aliquots at −20°C. ET-1 release was measured in triplicate on microtiter plates by an ELISA kit (R&D Systems) according to the manufacturer's instructions. ET-1 was measured in the range 0 to 120 pg/mL. The sensitivity was <1.0 pg/mL. The VEGF protein levels in the conditioned medium were determined in duplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D System). The sensitivity of the assay was <5.0 pg/mL. Intra-assay and interassay variations were 5.4% and 7.3%, respectively.

Thymidine incorporation assay. Cells were seeded in 96-well plates at ∼80% confluence (1×10^4 cells per well) and incubated in serum-free medium for 24 h to induce quiescence. Different concentrations of ET-1 alone or in combination with BQ788 (1 μmol/L) were added for 24 h, and 1 μCi of [methyl-^{3}H]thymidine (6.7 Ci/mmol; DuPont New England Nuclear Research Products) was added to each well. Six hours later, culture medium were removed, and cells were washed thrice with PBS, fixed with 10% trichloroacetic for 15 min, washed twice with 100% ethanol, and solubilized in 0.4 N sodium hydroxide. The cell-associated radioactivity was then determined by liquid scintillation counting. Responses to all treatments were assayed in sextuplicate, and results were expressed as the medium of three separate experiments.

Chemoinvasion assay. Chemoinvasion was assessed using a 48-well–modified Boyden's chamber (NeuroProbe) and 8 μm pore polyvinyl pyrrolidone–free polycarbonate Nucleopore filters (Costar) as previously described (21). The filters were coated with an even layer of 0.5 mg/mL Matrigel (Becton Dickinson). The lower compartment of chamber was filled with chemoattractant (ET-1 100 nmol/L), and/or BQ788 (1 μmol/L). LEC (2×10^6 cells/mL) were harvested and placed in the upper compartment (55 μL per well). After 18 h of incubation at 37°C, the filters were removed, stained with Diff-Quick (Merz-Dade), and the migrated cells in 10 high-power fields were counted. Each experimental point was analyzed in triplicate.

Tube formation assay. Tube forming activity was analyzed on LEC seeded on basal membrane extract (100 μL of Cultrex BME; Trevigen) at 4°C were layered onto prechilled 48-well plates and cultured in low-serum condition (0.5% FCS), until solidification. LEC at 80% confluence were cultured for 24 h in the presence of ET-1 (100 nmol/L) or VEGF-C (25 ng/mL). Cells were observed after 24 h under an inverted microscope.

Matrigel plug assay. Lymphangiogenesis was evaluated *in vivo* using a Matrigel plug assay, as described previously (10, 39). Male C57BL/6 mice (age 5-wk, 10 per group; Charles River Laboratories) were handled according to the institutional guidelines under the control of the Italian Ministry of Health. Mice were s.c. injected with 0.5 mL Matrigel containing PBS (control), 0.8 μmol/L ET-1 alone or in combination with 8 μmol/L BQ788. The Matrigels surrounded by murine tissue were removed 10 d after implantation, and snap frozen in liquid nitrogen. Indirect immunoperoxidase staining was carried out on acetone-fixed 4-μm frozen sections. The avidin-biotin assays were done using the Vectastain Elite kit (Vector Laboratories). Mayer's hematoxylin was used as nuclear counterstain. Sections incubated with isotype-matched IgG or normal IgG served as negative controls. Lymphatic vessels were identified using an antimouse LYVE-1 rabbit polyclonal Ab (RELIATech). To confirm the identity of lymphatic vessels, Matrigel sections were stained with anti–Prox-1 (RELIATech; data not shown). The evaluation of lymphatic vessel density in Matrigel was assessed by two independent observers on a ×200 magnification. At least five fields in different sections that displayed the highest density of LYVE-1 cells arranged into vascular structures were counted.

Statistical analysis. Results are representative of at least three independent experiments each performed in triplicate. All statistical analyses were assessed using a two-tailed Student's t test.

Results

Purified lymphatic cells express ET-1 and ET$_B$R. Highly purified LEC population from lymph nodes, analyzed by flow cytofluorimetry, displayed the cell membrane surface expression of the CD31 and podoplanin endothelial markers in over 98% of cells (Supplementary Fig. S1*A*). Analysis of these cells was also performed by immunocytochemistry in comparison with HUVEC. The results of this evaluation are summarized in Table 1. LEC expressed all the lymphatic lineage markers LYVE-1, Prox-1, and podoplanin (Supplementary Fig. S1*B*). These markers were either not expressed or expressed at very low levels by HUVEC, which were positive for the blood vascular lineage markers CD31, Von Willebrand factor, ULEX-1, CD44, and KDR (Supplementary Fig. S1*B*; Table 1). The phenotype of cultured LEC closely resembles that observed in the tissue sections of lymph node vasculature (data not shown), demonstrating that cultured LEC maintain

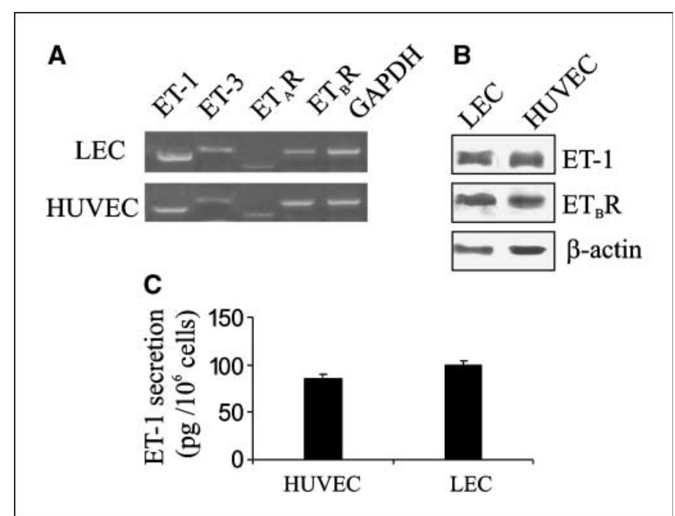

Figure 1. ET-1 axis expression in human LEC and HUVEC. ET-1, ET-3, ET$_A$R, and ET$_B$R expression in LEC and HUVEC were analyzed by RT-PCR (*A*) and Western blotting (*B*). Medium from serum-starved LEC and HUVEC were analyzed after 24 h for ET-1 production by ELISA (*C*). *Columns,* mean; *bars,* SD.

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in vitro the original expression levels of the specific markers. Although lymphatic and hematic systems play different roles and express distinct markers, it has become clear that they share molecular mechanisms during embryonic development and vascular renewal as well that are triggered by several growth factors (1). The expression profiles of ET family members, ET-1, ET-3 and their receptors, ET_AR and ET_BR, were analyzed by RT-PCR analysis and Western blotting in LEC and HUVEC. High levels of ET-1, ET-3, and ET_BR mRNA were detected in LEC, whereas ET_AR mRNA level was barely detectable (Fig. 1*A*). Likewise, Western blotting analysis showed the expression of ET-1 and ET_BR proteins in LEC (Fig. 1*B*). Similar results were obtained in HUVEC that expressed specifically ET_BR and ET-1 (Fig. 1*A* and *B*). Finally, serum-starved LEC conditioned medium were analyzed for ET-1 production by ELISA. As shown in Fig. 1*C*, LEC actively produced and secreted ET-1 by an extent comparable with that secreted by HUVEC. These data show that LEC express ET_BR and release ET-1.

ET-1 through ET_BR promotes LEC proliferation, activation of intracellular signaling molecules, invasion, and branching morphogenesis. Lymphangiogenesis is a complex cellular process that occurs via proliferation, migration, and differentiation of LECs. To investigate whether ET-1 had *in vitro* lymphangiogenic activity, we performed proliferation, migration, and capillary-like tube formation assays in LEC. ET-1 significantly increased [^{3}H]-thymidine incorporation in a dose-dependent manner. Addition of 0.1 nmol/L up to 100 nmol/L ET-1 induced a marked and significant increase in LEC proliferation compared with untreated control. This effect was completely abrogated in the presence of the specific ET_BR antagonist BQ788 (Fig. 2*A*), demonstrating that ET-1 through ET_BR induces LEC proliferation, an essential step for lymphangiogenesis. Consistent with ET_BR-driven mitogenic effects, ET-1 stimulated a time-dependent phosphorylation of intracellular signaling molecules such as p42/44 MAPK and AKT (Fig. 2*B*). The p42/44 MAPK and AKT phosphorylation, which was induced within 5 minutes after stimulation and was still detectable until 30 minutes (Fig. 2*B*), was blocked by BQ788. We next examined the ability of ET-1 to promote LEC invasion. Chemoinvasion assay showed that treatment with ET-1 induced a 6-fold increase compared with the untreated LEC (Fig. 2*C*), whereas this induction was significantly reduced in the presence of BQ788. To further confirm that ET-1 has *in vitro* lymphangiogenic activity, we examined the capacity of ET-1 to promote the capillary-like tube formation of LEC. Under serum-starved condition, LEC formed a small number of capillary-like structures (Fig. 2*D*). In the presence

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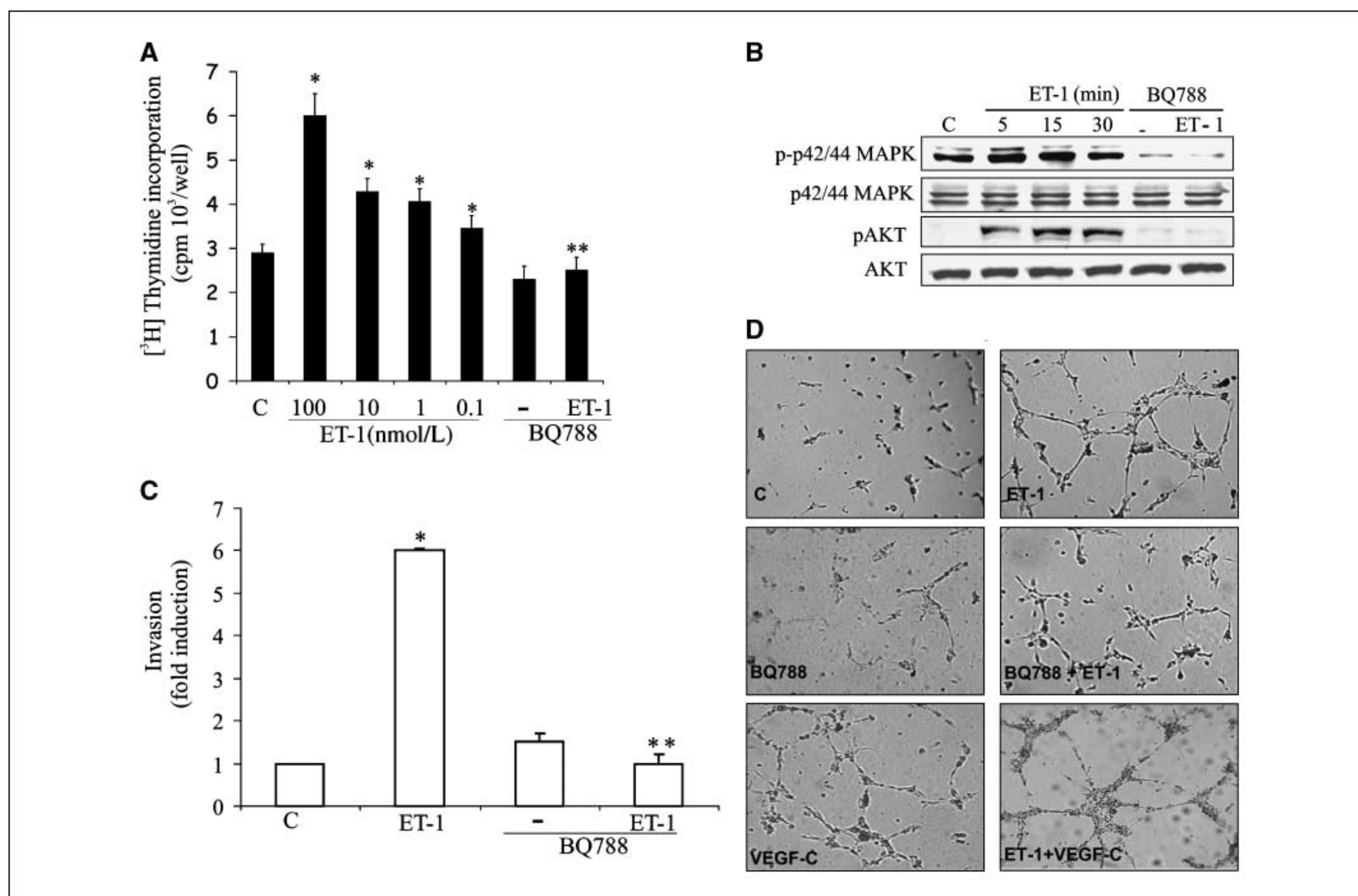

Figure 2. ET-1 through ET_BR increases LEC proliferation, invasiveness, capillary-like tube formation, and intracellular signaling pathway activation. *A,* cell proliferation was measured by [^{3}H]-thymidine incorporation assay in serum-starved LEC treated for 24 h with different concentrations of ET-1 or with 1 μmol/L BQ788 alone or in combination with ET-1 (100 nmol/L). *Columns,* mean; *bars,* SD. *, $P < 0.005$ compared with control; **, $P < 0.004$ compared with ET-1. *B,* phosphorylation of the p42/44 MAPK and AKT was analyzed by Western blotting in human LEC stimulated with ET-1 (100 nmol/L) at different times or in combination with 1 μmol/L BQ788 at 5 min. *C,* LEC invasion was measured using a Boyden's chamber invasion assay in cells treated with ET-1 (100 nmol/L) and/or with BQ788 (1 μmol/L). *Columns,* mean; *bars,* SD. *, $P < 0.004$ compared with control; **, $P < 0.005$ compared with ET-1. *D,* tube forming activity was tested on LEC seeded on basal membrane extract and cultured in low-serum conditions (0.5% FCS: control, *C*), or treated with ET-1 (100 nmol/L), and/or with BQ788 (1 μmol), and/or with VEGF-C (25 ng/mL). Photographs were taken 24 h later (magnification, ×200).

of ET-1, the cells became elongated, forming thin cords of interconnecting structures. BQ788 reverted the effect of ET-1 resulting in abrogation of capillary-like structures. In the presence of VEGF-C, LEC were able to form tube-like structures by an extent similar to that observed in the presence of ET-1. Furthermore, coaddition of ET-1 and VEGF-C resulted in a marked increase in the formation of capillary-like structures, compared with that observed with either ET-1 or VEGF-C alone, suggesting that ET-1 cooperates with VEGF-C to enhance LEC differentiation into vascular structures (Fig. 2*D*). These data show that ET-1 exerts *in vitro* lymphangiogenic activity by promoting early and late lymphangiogenic events specifically through ET_BR-driven downstream signaling pathways.

ET-1 induces VEGF-C, VEGFR-3, and VEGF-A expression through ET_BR. VEGF-C/VEGFR-3 system, as well as VEGF-A, have recently been identified as key molecules that are involved in lymphangiogenesis (40). Because we previously showed that ET-1 stimulates angiogenesis by inducing VEGF-A secretion from cancer cells (20, 23, 24), in this study, we investigated whether ET-1 may regulate the expression of VEGF-C/VEGFR-3 and VEGF-A in primary LEC cultures. As revealed by RT-PCR, serum-starved LEC expressed VEGF-C, VEGF-A, and VEGFR-3 mRNA. This expression increased when cells were stimulated with ET-1 for 6 hours, whereas preincubation of LEC with BQ788 resulted in a complete block of ET-1–induced effects (Fig. 3*A*). The increased mRNA expression was paralleled by the induction of VEGF-C, VEGFR-3, and VEGF-A protein levels, which were blocked in the presence of BQ788 (Fig. 3*B*). As measured by ELISA, ET-1 stimulation resulted in a 4-fold increase in VEGF-A production levels compared with untreated cells that were significantly inhibited by BQ788 (Fig. 3*C*), demonstrating that ET-1/ET_BR is able to increase VEGF family members in LEC.

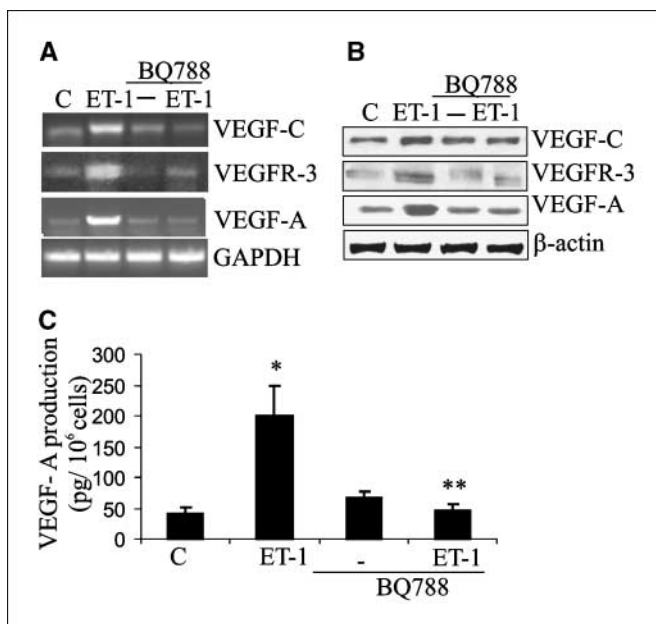

Figure 3. ET-1 increases VEGF-C, VEGFR-3, and VEGF-A expression through ET_BR. *A,* VEGF-C, VEGFR-3, and VEGF-A mRNA expression was analyzed in serum-starved LEC treated for 6 h with ET-1 (100 nmol/L) alone or in combination with BQ788 (1 μmol/L) by RT-PCR. GAPDH expression was used as loading control. *B,* VEGF-C, VEGFR-3, and VEGF-A protein levels were analyzed by Western blotting in serum-starved LEC treated for 16 h with ET-1 (100 nmol/L) alone or in combination with BQ788 (1 μmol/L). β-actin was used as loading control. *C,* VEGF-A secretion in the conditioned medium of LEC treated as in *B* was analyzed by ELISA. *Columns,* mean; *bars,* SD. *, $P < 0.005$, compared with control; **, $P < 0.001$ compared with ET-1.

ET-1 acts through HIF-1α to induce VEGF-C and VEGF-A expression. Despite the large evidence that blood endothelial cells respond to hypoxia (32, 33), little is known about the effects of low oxygen levels on LEC behavior. To asses whether exposure of LEC to low oxygen could modify the protein expression pattern of various VEGF members, we cultured LEC under hypoxic conditions. The protein expression of VEGF-A and VEGF-C increased 24 h after exposure to hypoxia to an extent similar to that induced by ET-1 in normoxic conditions (Fig. 4*A*). To study more in-depth the molecular mechanism by which ET-1 induces VEGF-C and VEGF-A, we analyzed the role of ET-1 and hypoxia on HIF-1α, the master transcriptional factor controlling VEGF production (32, 33). As revealed by Western blotting, under normoxic conditions, LEC expressed barely detectable levels of HIF-1α, whereas ET-1 drastically induced HIF-1α protein accumulation that paralleled the increase in VEGF-C and VEGF-A levels (Fig. 4*B*). BQ788 inhibited the ET-1–induced HIF-1α protein expression, indicating that this effect progress selectively through an ET_BR-mediated pathway (Fig. 4*B*). Next, we transfected LEC with siRNA for HIF-1α, and monitored the levels of VEGF-C, VEGF-A, and HIF-1α in response to ET-1 or hypoxia. Selective inactivation of HIF-1α resulted in the inhibition of ET-1– and hypoxia-induced VEGF-C, VEGF-A, and HIF-1α expression (Fig. 4*C*), whereas scramble siRNA (scRNAi) transfection did not alter the response of LEC to both stimuli. Interestingly, ET-1–induced HIF-1α was similar to that induced by hypoxia (Fig. 4*C*). Likewise, ELISA showed that the production of VEGF-A in the ET-1–treated scRNAi LEC, as well as in cell cultured under hypoxic conditions, increased up to 4-fold over the control, whereas this induction was significantly reduced in HIF-1α silenced LEC (Fig. 4*D*). Altogether, these findings show that ET-1, as well as hypoxia, induce VEGF-C and VEGF-A expression through HIF-1α, indicating that ET-1 represents an inducer of HIF-1α equipotent to hypoxia and that in lymphatic structures this transcriptional factor mediates the ET-1–induced VEGF-C and VEGF-A expression.

ET_BR is expressed by lymphatic vessels *in vivo*. To investigate whether ET_BR is also expressed by lymphatic vessels *in situ*, we performed double-immunofluorescence analyses of normal human lymph nodes, using antibodies against ET_BR and against the lymphatic marker podoplanin in lymphatic vessels. As shown in Fig. 5*A*, podoplanin only stained thin-walled, erythrocyte-free (i.e., lymphatic) vessels, whereas ET_BR stained both blood and LECs. Merge image showing double immunofluorescence analysis clearly shows colocalization of ET_BR and podoplanin in lymphatic vessels. These data further show that ET_BR is expressed in lymphatic vessels and that ET-1 may directly regulate lymphatic behavior via activation of ET_BR.

ET-1 promotes *in vivo* lymphangiogenesis. Because ET-1 stimulated invasion and differentiation of LEC *in vitro*, we investigated whether ET-1 axis may also induce lymphangiogenesis *in vivo*. To this end, we implanted Matrigels containing ET-1 alone or in combination with BQ788, or PBS into the back s.c. tissue of mice. Immunostaining of Matrigels containing ET-1 with anti-mouse LYVE-1 identified pronounced lymphatic vessel formation (12 ± 3, compared with control) within discrete areas at the boundaries with the murine surrounding tissue (Fig. 5*B*). These structures were absent in Matrigel controls and in those containing ET-1 and BQ788. These results indicate that ET-1 selectively through the ET_BR acts as a lymphangiogenic factor *in vivo*.

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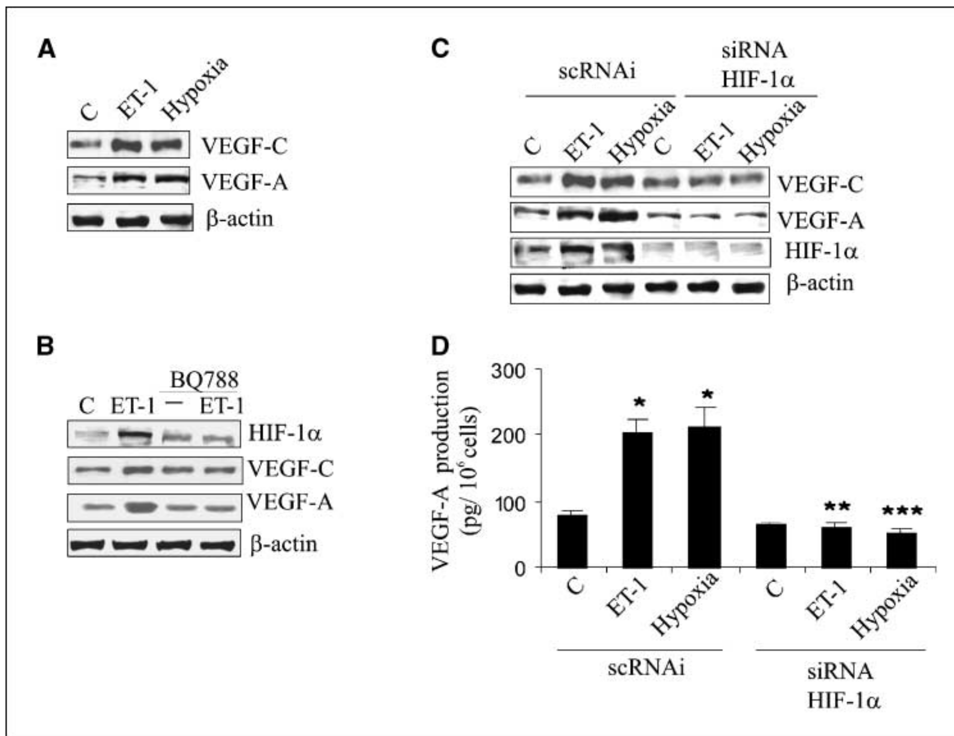

Figure 4. ET-1 and hypoxia increase VEGF-A and VEGF-C via HIF-1α expression. *A,* VEGF-C and VEGF-A protein levels were detected by Western blotting in serum-starved LEC treated with ET-1 (100 nmol/L) or exposed to hypoxia. β-actin was used as loading control. *B,* HIF-1α, VEGF-C, and VEGF-A expression was analyzed by Western blotting in LEC stimulated with ET-1 (100 nmol/L) alone or in combination with BQ788 (1 μmol/L). β-actin was used as loading control. *C,* scRNAi or siRNA HIF-1α transfected-LEC were stimulated with ET-1 (100 nmol/L) or cultured under hypoxic condition, and whole cell lysates were monitored for VEGF-C, VEGF-A, and HIF-1α expression by Western blotting. *D,* conditioned medium of cells transfected and treated as in *C* was analyzed for VEGF-A secretion by ELISA. *Columns,* mean; *bars,* SD. *, $P < 0.004$, compared with control; **, $P < 0.002$ compared with ET-1 in scRNAi transfected cells; ***, $P < 0.005$ compared with hypoxia in scRNAi-transfected cells.

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Discussion

ET-1 acts as a tumorigenic as well as an angiogenic factor (16–21, 23, 24). Members of ET-1 axis are frequently overexpressed in many solid tumors, and their expression has been correlated with increased lymphatic dissemination (11, 12, 36), suggesting that ET-1 axis may be involved in both angiogenesis and lymphangiogenesis. In the present study, we show that ET-1 promotes *in vitro* and *in vivo* lymphangiogenesis by stimulating the growth and differentiation of LEC through a mechanism mediated by ET_BR expressed by lymphatic endothelium. To the best of our knowledge, this study is the first to establish that ET-1 is a lymphangiogenic mediator.

Several report have shown that ETs have potent mitogenic and migratory effect on endothelial cells (16, 21, 24). In this study, we showed that highly purified LEC actively produce and secrete ET-1, which, after binding to ET_BR, induces LEC proliferation, and

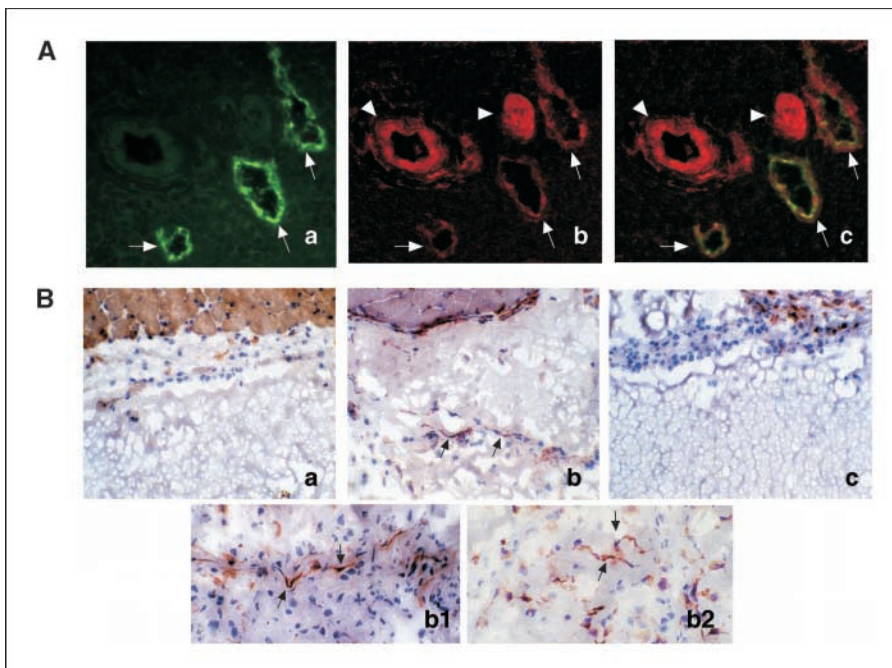

Figure 5. ET-1 promotes *in vivo* lymphangiogenesis. *A,* double-immunofluorescence analysis of normal human lymph node revealed a coexpression of the lymphatic-specific marker podoplanin (*green, a*) with ET_BR (*red, b*; *c, merged pictures*). *White arrows,* podoplanin-positive/ET_BR-positive structures (lymphatic vessels); arrowheads, podoplanin-negative/ET_BR-positive structures (blood vessels). Original magnification, ×40. *B,* 10 d after s.c. injection of Matrigels containing PBS (*a*), ET-1 (*b, b1, b2*), or BQ788+ET-1 (*c*) in mice, the implants were immunostained with anti-mouse LYVE-1. Whereas in PBS containing Matrigels (*a*) as well as in those containing ET-1+BQ788 (*c*), no LYVE-1–positive capillary structures are present, in implants containing ET-1 (*b*), such structures (*arrows*) are observed at the boundaries between Matrigel and the murine surrounding tissues (*b1* and *b2* show two different fields). *a, b*, and *c,* ×16; *b1* and *b2,* ×25 original magnification.

invasion concomitantly with activation of p42/44 MAPK and AKT pathways. Moreover, ET-1 promotes the formation of tube-like structures by an extent similar to that induced by VEGF-C and, in concert with this, potentiates VEGF-C–induced morphogenic effects, demonstrating that ET-1 may control LEC differentiation into vascular structures *in vitro*. Remarkably, a small molecule–specific antagonist of ET_BR blocks cell proliferation, invasion, and capillary-like tube formation induced by ET-1, demonstrating a direct mechanism mediated by ET_BR on lymphatic growth and differentiation. Furthermore, we provide evidence that ET-1 axis can mediate lymphangiogenesis also by an indirect mechanism, as shown by the capacity of ET-1 to increase the expression of the selective lymphangiogenic factor VEGF-A, VEGF-C, and VEGFR-3. Recent studies have also shown that hypoxia, beside regulating neoangiogenesis, can modulate lymphangiogenesis (30, 31). The expression of HIF-1α has been shown to correlate with lymph node metastasis (41, 42), and genome wide analysis of LEC exposed to low oxygen conditions indicates that hypoxia is capable of increasing VEGF-C, VEGF-D, VEGF-A, and HIF-1α mRNA (30). In addition to the classic hypoxia-mediated induction of HIF-1α, different growth factors, including ET-1, have been shown to enhance HIF-1α accumulation in tumor cells (18, 20, 43, 44). In this context, we show that ET-1, not differently from hypoxia, triggers an accumulation of HIF-1α followed by an HIF-1α–mediated up-regulation of VEGF-C and VEGF-A, indicating that ET-1 axis and hypoxia can act on HIF-1α–dependent machinery to promote lymphangiogenesis via VEGF family members. Similar to angiogenesis, our *in vitro* results show that the complex process of lymphangiogenesis is finely tuned by the interplay of multiple hypoxia-regulated growth factors, such as VEGF-C and VEGF-A and ET-1 axis. This regulation includes both early events, such as increased cell proliferation, and late events, such as invasion and differentiation into vascular cords, that may cooperatively contribute to the development of lymphangiogenesis. Furthermore, we also show that ET_BR is expressed in podoplanin-positive lymphatic vessels of human lymph nodes, and that in Matrigel plug neovascularization assay, ET-1 selectively through ET_BR promotes the outgrowth of lymphatic vessels *in vivo*. Immunohistochemical analysis showing ET_BR expression, besides podoplanin-positive cell, also on endothelial cell lying blood vessel further supports the hypothesis that ET-1 axis may regulate contextually blood and lymphatic system in agreement with several other angiogenic factors (40).

Recent studies have shown that inflammatory cells, such as tumor-associated macrophages (TAM), are recruited to tumors to stimulate lymphangiogenesis by a wide range of tumor cell–derived cytokines and growth factors, including VEGF-C and VEGF-D (45). Interestingly TAM release also ETs and express both ET receptors. Stimulation of TAM with ETs lead to chemotaxis and production of other molecules that can influence lymphangiogenesis (43, 46). In this scenario, we hypothesized that ET-1 can directly stimulate lymphangiogenesis and indirectly via VEGF-A or VEGF-C. Concurrently TAM, which are educated to enter tumor hypoxic areas where lymphatic vascularization is needed, release lymphangiogenic regulators, including VEGF family members and ETs, providing an alternative or complementary mechanism whereby TAM can promote lymphangiogenesis. This issue, however, will require further investigation.

In view of the correlation between tumor expression of ET-1 axis and lymphatic metastasis (36) together with the recent gene expression profile identifying *ET-1* as one of the significantly up-regulated genes in LEC isolated from metastatic lymph node (35), the present results raise the possibility that ET-1 contributes to tumor progression by promoting hypoxia-mediated lymphangiogenic signaling disclosing a yet unidentified regulatory mechanism, which relays on the involvement of tumor microenvironment.

Although pharmacologic inhibition of lymphangiogenesis may be clinical relevant in a variety of conditions, the number of available drugs is limited (47, 48). In this context, the present study indicates that small molecules targeting ET_BR are potential candidates in the treatment of lymphatic-associated diseases, as well as tumor spreading, in view of their capacity to block common pathways of lymphangiogenesis and angiogenesis, which represent important routes of metastatization.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

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