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Endothelin-1 cooperates with hypoxia to induce vascular-like structures through vascular endothelial growth factor-C, -D and -A in lymphatic endothelial cells

Emirena Garrafa [b], Valentina Caprara [a], Valeriana Di Castro [a], Laura Rosanò [a], Anna Bagnato [a,*], Francesca Spinella [a,*]

[a] Molecular Pathology, Regina Elena Cancer Institute, Rome, Italy
[b] Microbiology Section, Department of Experimental Medicine, University of Brescia, Italy

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ABSTRACT

Aims: Lymphangiogenesis refers to the formation of new lymphatic vessels and is thought to constitute conduits for the tumor cells to metastasize. We previously demonstrated that endothelin (ET)-1 through its binding with ET_B receptor (ET_BR) expressed on lymphatic endothelial cells (LEC), induced cell growth and invasiveness. Since vascular endothelial growth factor (VEGF)-A/-C/-D, and hypoxia play key role in lymphatic differentiation, in this study we investigated the involvement of these growth factors and hypoxia in ET-1-induced lymphangiogenesis.
Main methods: Real time PCR and ELISA were used to quantify VEGF-A/-C/-D. LEC morphological differentiation was analyzed by tube formation assay on Matrigel.
Key findings: Hypoxia, as well as ET-1, induced an increase in VEGF-A/-C and -D expression that was reduced in the presence of a selective ET_BR antagonist, BQ788, and enhanced when ET-1 was administered under hypoxic conditions. We analyzed the role of hypoxia on LEC morphological differentiation, and found that hypoxia increased the formation of vascular-like structures on Matrigel and that in combination with ET-1 this effect was markedly enhanced. The use of specific antibodies neutralizing VEGF-A, or recombinant VEGFR-3/(Flt-4)/ Fc that block VEGF-C/-D, inhibited the effect of ET-1 as well that of hypoxia.
Significance: These results demonstrated that ET-1 and hypoxia act, at list in part, through VEGF to induce lymphangiogenic events and that these two stimuli may cooperate to induce VEGF-A/-C/-D expression and lymphatic differentiation. These data further support the role of ET-1 as potent lymphangiogenic factor that relies on the interplay with hypoxic microenvironment and with VEGF family members.

Introduction

Metastasization is the leading cause of cancer mortality. Although numerous studies have identified genes and mechanisms that modulate the metastasization process, our knowledge about how cancer cells initially gain access to vascular and/or the lymphatic system is limited (Alitalo et al., 2005). The lymphatic spreading can occur by invasion of tumor cells in pre-existing lymphatic vessels and in intratumoral networks of newly formed lymphatic network induced by tumor cells, inflammatory and stromal cells (Albrecht and Christofori, 2011). The identification of mechanisms that control lymphatic vessel growth is likely to contribute to the development of therapies aimed at preventing lymphatic metastases. Because of their common origin, lymphangiogenesis, as well as angiogenesis, relies on the interplay of several angio- and lymphoangiogenic factors and receptors (Mäkinen et al.,

2007). Members of the vascular endothelial growth factor (VEGF) family, VEGF-C and VEGF-D, are so far the best-characterized lymphangiogenic factors that bind the VEGF receptor-3 (VEGFR-3). In normal adult tissues, VEGFR-3 expression is largely restricted to the lymphatic endothelium, where it promotes lymphatic endothelial cell proliferation (LEC), migration, and survival (Mäkinen et al., 2001). In addition to VEGF-C and VEGF-D, VEGF-A also stimulates lymphatic growth in several experimental systems, and the activation of its receptor VEGFR-2 results into an increase of lymphatic metastasization (Cursiefen et al., 2004; Zeng et al., 2006; Halin et al., 2007). Preclinical and clinical studies indicate that these signaling molecules regulate concomitantly blood and lymphatic vessels suggesting a relationship between angio- and lymphangiogenesis, and that the concomitant blockade of these has an additive effect to inhibit tumor growth (Tvorogov et al., 2010; Albrecht and Christofori, 2011; Witte et al., 2011). We previously demonstrated that the endothelin (ET-1) axis promotes the growth and differentiation of LEC purified from human lymph nodes *in vitro* and *in vivo* providing the first evidence that ET-1 axis may act also as a lymphangiogenic mediator (Spinella et al., 2009). Moreover, it has been recently suggested

* Corresponding authors at: Laboratory of Molecular Pathology, Regina Elena National Cancer Institute, Via Delle Messi D'Oro 156, Rome, Italy.
E-mail addresses: bagnato@ifo.it (A. Bagnato), spinella@ifo.it (F. Spinella).

that up-regulation of ET-1 may be responsible for podoplanin-induced tumor lymphangiogenesis (Cueni et al., 2010). The ET-1 family consists of three small structurally related, 21-aa vasoactive peptides (ET-1, ET-2, and ET-3), and two G-protein-coupled receptors namely ET$_A$R and ET$_B$R (Morbidelli et al., 1995; Davenport and Maguire, 2006; Thorin and Webb, 2010). ET genes are readily inducible by growth factors, oncogenes, cytokines and hypoxia, and have been implicated in the pathophysiology of a wide range of human tumors, including ovarian carcinoma and melanoma (Nelson et al., 2003; Grimshaw, 2007; Rosanò et al., 2009; Spinella et al., 2010a). ET-1 family members are also expressed by lymphatic endothelial cells and the coordinated action of ET$_B$R and ET$_A$R, expressed in the endothelium and in smooth muscle cells, respectively, modulates lymphatic vascular tone (Reeder and Ferguson, 1996; Marchetti et al., 1997). Recently, we demonstrated that the activation of ET$_B$R by ET-1 in LEC is also implicated in mediating various stages of lymphangiogenesis, including matrix-metalloproteinase (MMP) activation, a prerequisite for LEC invasion (Spinella et al., 2010b). ET-1-induced effects on LEC are abolished by ET$_B$R antagonists, suggesting that this receptor may represent a potential target to inhibit lymphangiogenesis (Spinella et al., 2009, 2010b). Among microenvironmental components, hypoxia represents one of the principal angiogenic stimuli, and it has been proposed to be also implicated in controlling lymphangiogenesis (Irigoyen et al., 2007; Ota et al., 2007; Voss et al., 2010). We previously demonstrated that ET-1 may share with hypoxia a similar molecular mechanism through which regulates VEGF-A expression and that under hypoxic conditions this effect was increased (Spinella et al., 2002). Moreover, in LEC we observed that hypoxia increased hypoxia-inducible factor (HIF)-1α, the principal transcription factor regulating VEGF-A expression, supporting the role of hypoxia as inducer of lymphangiogenesis (Spinella et al., 2009). Here we show that exposing LEC to ET-1 or under hypoxic condition led to the increase in VEGF-C, -D and -A production, and that this effect is amplified when ET-1 is added under hypoxic conditions. At functional level, ET-1 and hypoxia significantly increase the number and the length of vascular-like tubules formed by LEC. Moreover neutralization of VEGF-A and/or VEGF-C and -D results in a marked reduction in ET-1- as well hypoxia-induced formation of a lymphatic endothelial networks. These results demonstrating the role of ET-1 on inducing lymphangiogenesis and in potentiating hypoxia effect provide new insight in the mechanism by which ET-1 may interact with microenvironment component and regulate 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 non-infectious or neoplastic conditions. Lymph nodal LEC were isolated as previously described (Garrafa et al., 2005, 2006; Spinella et al., 2009). Cells were grown on collagen type I-coated flasks (5 μg/cm^2; Boehringer Mannheim) in EGM-2 MV (Lonza, Basel Switzerland). To expose cells to hypoxia, a modular incubator was used with an atmosphere setting of 5% CO_2, 95% N_2, and 1% O_2. ET-1 (Peninsula Laboratories, Belmont, CA) was used at 100 nM, and VEGF-C was used at 50 ng/ml (R&D Systems, Minneapolis, MN). When BQ788 1 μM (Peninsula Laboratories), 100 ng/ml anti-VEGF-A or recombinant VEGFR-3 (Flt-4)/Fc (R&D Systems) were tested, they were added 30 min before the stimuli.

Quantitative real-time-PCR

Total RNA was isolated using the Trizol (Invitrogen, Grand Island, NY, USA) according to the manufacturer's protocol. Five micrograms of RNA was reversed transcribed using SuperScript® VILOis™ cDNA synthesis kit (Invitrogen). Quantitative real-time-PCR was performed by using LightCycler rapid thermal cycler system (Roche Diagnostics,

Mannheim, Germany) according to the manufacturer's instructions. Reaction was performed in 20 μl volume with 0.3 μM primers, by using LightCycler-FastStart DNA Master Plus SYBR Green mix (Roche Diagnostics) from 1 μl cDNA under the following conditions: denaturation at 95 °C for 5 min; melting at 95 °C for 10s; annealing between 58 °C for 10s; extension at 72 °C for 10s. Primers used were as follows: VEGF-C (forward) 5′-GTGTCCAGTGTAGATGAACTC-3′ and (reverse) 5′–ATCTGTAGACGGACACACATG-3′; VEGF-D (forward) 5′-GTTGCAATGAA-GAGAGCCTT-3′ and (reverse) 5′-TCCCATAGCATGTCAATAGG-3′; VEGF-A (forward) 5′-CCTCAGTGGGCACACACACTCC-3′ and (reverse) 5′-CGAAAC-CATGAACTTTCTGC-3′; cyclophilin-A (forward) 5′-TTCATCTGCACTGCCA-AGAC-3′ and (reverse) 5′-TGGAGTTGTCCACAGTCAGC-3′. The number of each gene-amplified product was normalized to the number of cyclophilin-A amplified product and expressed as copy numbers over cyclophilin-A ($\times 10^{-3}$).

ELISA

The VEGF-A protein levels in the conditioned media were determined in duplicate by ELISA using the Quantikine Human VEGF immunoassay kit (R&D Systems). The sensitivity of the assay is <5.0 pg/ml. Intra-assay and interassay variations were 5.4% and 7.3%, respectively. Human VEGF-C Quantikine ELISA Kit (R&D System) was used for VEGF-C level determination in LEC media. The sensitivity of the assay is 13.3 pg/ml. Intra-assay and interassay variations were 6.9% and 9.6%, respectively

In vitro tube formation assay

Tube forming activity was analyzed as previously described (Spinella et al., 2009). In brief, 50 μl of Matrigel (Cultrex, basal membrane extract, Trevigen, 12.7 mg/ml) was dropped onto each well of a 96-well plate and allowed to solidify for 1 h at 37 °C in humidified 5% CO_2 incubator. One to 5×10^4 LEC were plated in the presence of 100 nM ET-1 or 50 ng/ml VEGF-C, and the cells were cultured under normoxia or hypoxia. When 100 ng/ml anti-VEGF-A or recombinant VEGFR-3 (Flt-4)/Fc was tested they were added 30 min before the stimuli. Formation of tube-like structures was photographed after 24 h using light microscopy and quantified by counting the number of tubules intersections and the length of tubes in 10 randomly chosen fields using Scion image analysis program. Magnification 200×.

Statistical analysis

Results were the means ± SD from at least five sets of replicated experiments, and in each set, measurements were made in triplicate for real-time PCR or duplicate for ELISA and capillary-like structure formation assays. Data were analyzed by one-way ANOVA followed by Tukey's multiple comparison tests. Data were sampled from population with identical SDs as tested by Barlett method and followed Gaussian distribution as tested by Kolmogorov–Smirnov methods. p Values were significant at $p < 0.05$ using PRISM software (GraphPad Software, Inc., San Diego, CA).

Results

ET-1 and hypoxia induce VEGF-A, -C and-D expression and production

VEGF-A, VEGF-C and VEGF-D represent the principal mediators of lymphangiogenesis, and recent data argue for a more relevant role for hypoxia in controlling their expression in lymphatic (Irigoyen et al., 2007; Ota et al., 2007; Spinella et al., 2009). Here we monitored the capacity of ET-1 and hypoxia to cooperatively increase the expression of these growth factors. Highly purified human LEC were stimulated with ET-1 and/or cultured under hypoxic conditions for 6 or 24 h, and mRNA or conditioned media were analyzed by real time RT-PCR, or ELISA, respectively. ET-1, as well as hypoxia, significantly

increased the mRNA of VEGF members (VEGF-D mRNA $n=6$; $p<0.01$ versus control; Fig. 1A) (VEGF-C and -A mRNA $n=6$; $p<0.001$ versus control; Fig. 1B and C) and protein levels ($n=5$; $p<0.001$ versus control; Fig. 1D). To investigate whether ET-1 may influence hypoxic response cells were pretreated with a selective ET_BR antagonist, BQ788, and exposed to hypoxic conditions. As shown in Fig. 1, in the presence of BQ788 the capacity of hypoxia to up-regulate VEGF-D, -C and -A mRNA levels was attenuated (VEGF-D mRNA $n=6$; $p<0.05$ versus hypoxic control, Fig. 1 A) (VEGF-C and -A mRNA $n=6$; $p<0.001$ versus hypoxic control). Moreover, simultaneous stimulation of LEC with ET-1 and hypoxia resulted in a further increase in VEGF-D, -C and VEGF-A mRNA ($n=6$; $p<0.001$ versus ET-1; Fig. 1A–C), and protein levels compared to ET-1 alone ($n=5$; $p<0.001$; Fig. 1D). Taken together, these results suggest that ET-1 may induce VEGF-A, -C and -D expression, and production in LEC and interact with hypoxia to amplify these effects on lymphangiogenic mediators.

ET-1 induces lymphangiogenesis through VEGF-A and VEGF-C production

To investigate the functional significance of ET-1- and hypoxia-induced VEGF-A, VEGF-C and -D we neutralized these growth factors and monitored the capacity of LEC to form vascular-like structures on Matrigel, an assay that permits to measure cell adhesion, migration, protease activity, and tubule formation capacity. Treatment with ET-1 or hypoxia induced the formation of tube-like structures compared to the control cells (Fig. 2A). To investigate whether ET-1 and hypoxia may cooperate in the formation of vascular-like structures, LEC were simultaneously stimulated with hypoxia and ET-1. In the presence of anti-VEGF-A, or in the presence of recombinant human VEGFR-3 (Flt4)/Fc chimera protein (VEGFR-3/Ig), which has been shown to bind to both VEGF-D and VEGF-C and to block ligand/receptor binding (Su et al., 2006), the effect of ET-1 as well as that of hypoxia was reduced (Fig. 2A) indicating that VEGF-C and VEGF-A are involved in mediating ET-1 and hypoxia actions. Quantitative analysis demonstrated that ET-1 induced a significant increase in the number of intersection between the tube-like structures and in their length compared to the control ($n=10$; $p<0.001$) (Fig. 2B). In the presence

of anti-VEGF-A or rhVEGFR-3 (Flt4)/Fc lower number of intersection and length of tube-like structures were monitored (Fig. 2B). Therefore, we next combined recombinant VEGFR-3/Fc and anti-VEGF-A. In these conditions the number and the length of tube-like structures was reduced approximately 60% compared to ET-1 alone ($p<0.001$; $n=10$) (Fig. 2B), further supporting the notion that ET-1 may act in a VEGF-mediated mechanism in controlling lymphangiogenesis. Similar results were observed when LEC were pretreated with anti-VEGF-A or recombinant VEGFR-3 and cultured under hypoxic conditions (Fig. 2A). There were lower numbers of intersections between tubes and their length was smaller in the presence of anti-VEGF-A or recombinant VEGFR-3/Fc ($p<0.001$; $n=10$) than in hypoxia (Fig. 2B). When neutralizing antibodies were combined, the effect of hypoxia was even more inhibited (Fig. 2B), suggesting that both VEGF-A and VEGF-C are required by hypoxia to induce tube-like structures on LEC. As positive control we monitored the effect of VEGF-C and VEGFR-3/Fc on vascular-like structure formation of LEC under normoxic conditions. The effect of VEGF-C was completely impaired by recombinant VEGFR-3/Fc (Fig. 3A and B). These results indicate that ET-1 and hypoxia act through a common mechanism that involves VEGF-mediated signaling pathway and that both stimuli may cooperate to enhance lymphangiogenesis.

Discussion

ET-1 acts as a tumorigenic as well as an angiogenic factor, and recent reports demonstrated that the activation of ET_BR in lymphatic endothelial cells led to a potent lymphangiogenic effect. ET-1 has been demonstrated to induce LEC proliferation, growth and differentiation highlighting the potential role of ET_BR as a molecular target to control lymphangiogenesis (Spinella et al., 2009; Cueni et al., 2010). In this study we further investigated the molecular mechanism through which ET-1 regulates LEC behavior. Moreover, the role the interaction between ET-1 and hypoxia increases cellular responsiveness was also addressed. We found that ET-1, as well as hypoxia, increased VEGF-A, VEGF-C and -D expression that was further enhanced when ET-1 was administered under hypoxic conditions. By monitoring the capacity of
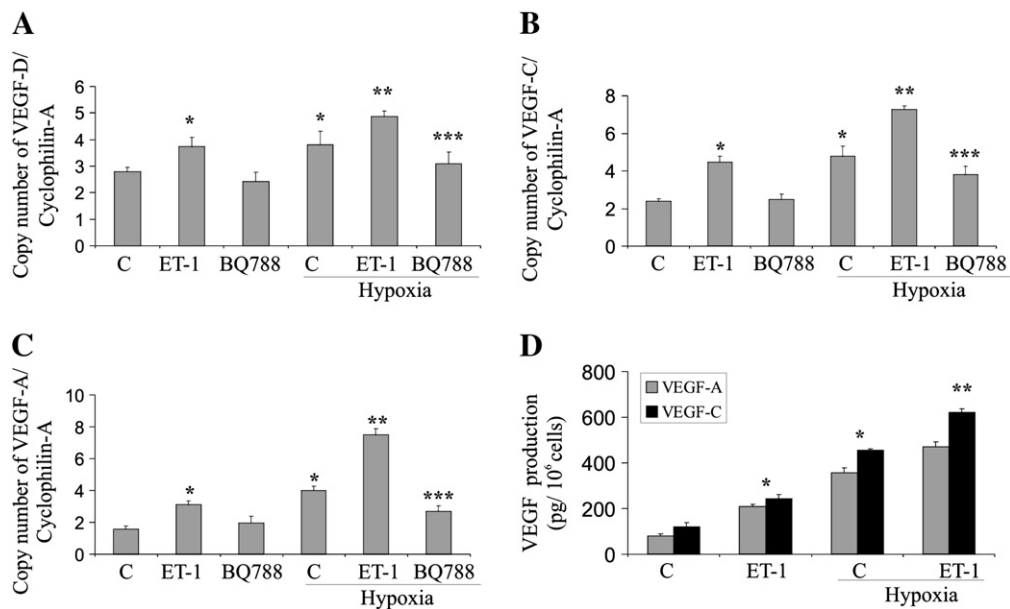
Fig.1. ET-1 and hypoxia cooperate to induce VEGF-A, VEGF-C and -D in LEC through ET_BR. Highly purified human LEC were stimulated with 100 nM ET-1 or with 1 µM BQ788 and cultured under normoxia or hypoxic conditions. After 6 h total RNA was extracted and VEGF-D (A), VEGF-C (B) or VEGF-A (C) transcript levels were quantified by real time RT-PCR. Results are expressed as copy numbers of VEGF-D, VEGF-C or VEGF-A transcripts over cyclophilin-A. Bars, $\pm$SD ($n=6$). In A * $p<0.01$ compared to control in normoxia, ** $p<0.001$ compared to ET-1, and *** $p<0.05$ compared to hypoxic control. In B and C * $p<0.001$ compared to control, ** $p<0.001$ compared to ET-1, and *** $p<0.01$ compared to hypoxic control. (D) Cells were stimulated with 100 nM ET-1 and/or cultured under normoxia or hypoxic conditions for 24 h, and conditioned media was analyzed for VEGF-C or VEGF-A production by ELISA. Bars, $\pm$SD. ($n=5$). *, $p<0.001$ compared to control in normoxia; **, $p<0.001$ compared to control in hypoxia.
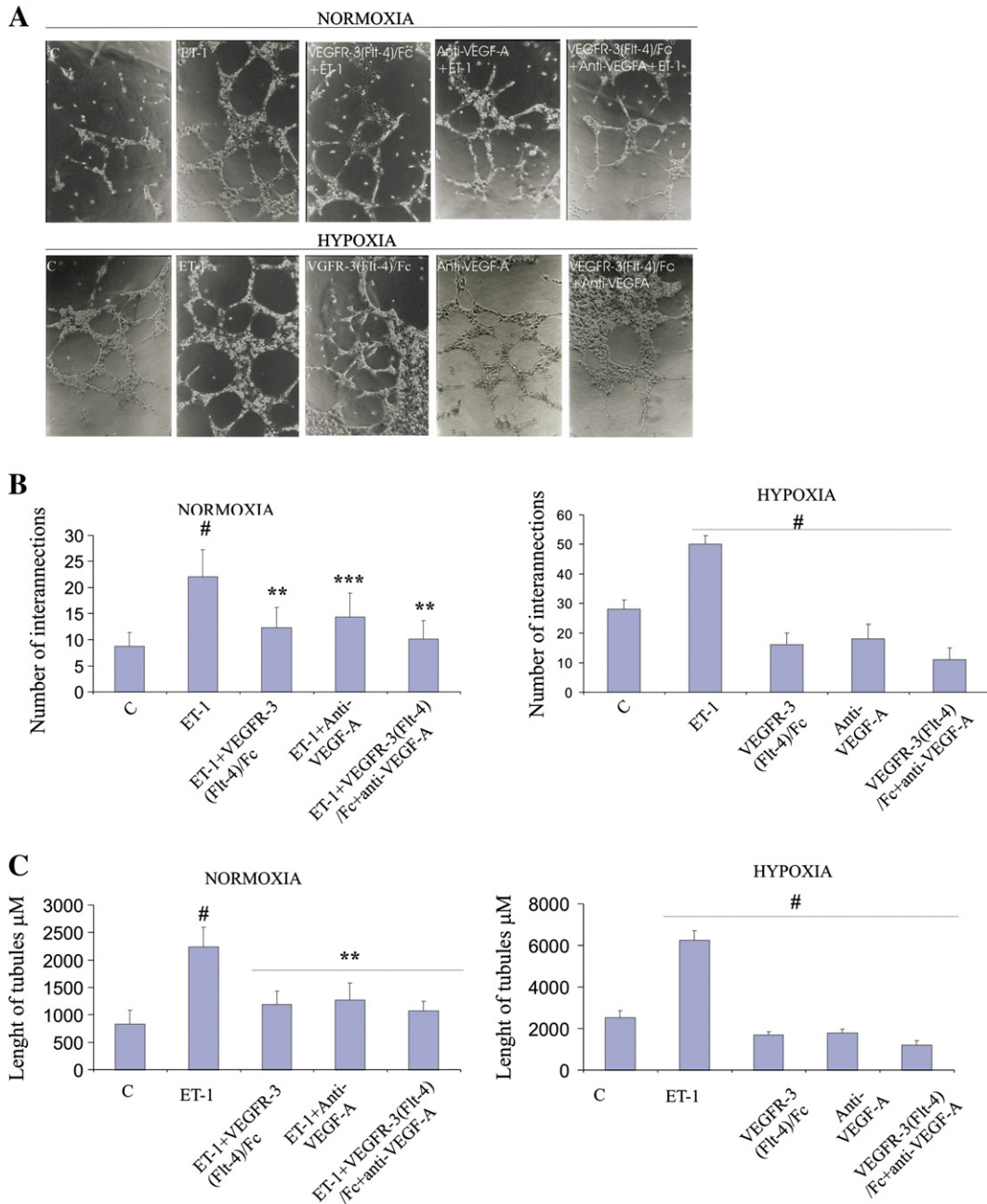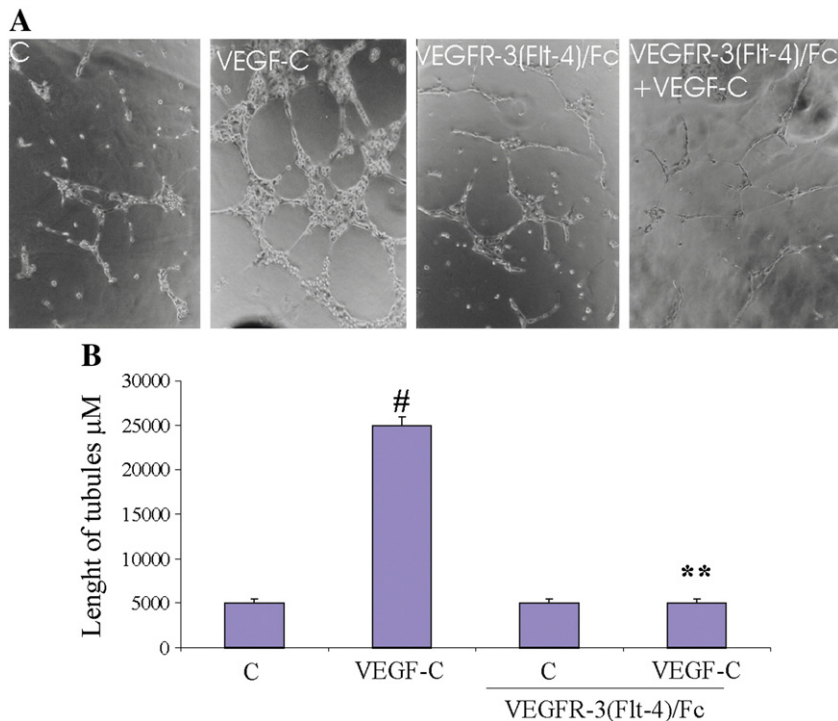
Fig. 2. ET-1 and hypoxia induce tube like structure formation through VEGF members.Tube-like structure formation was monitored in LEC seeded on Cultrex. (A) Representative images from three independent experiments of LEC treated with 100 nM ET-1 alone or in combination with anti-VEGF-A and/or recombinant VEGFR-3/Fc for 24 h in normoxia, or cultured under hypoxic condition. Quantification of cell intersection number (B) and tubule length (C) of experiment described in A. Data shown in B, (left panel) bars, $\pm$SD. #, $p < 0.001$, compared to the control; **, $p < 0.001$ compared to the ET-1. ***, $p < 0.01$ compared to ET-1. Data shown in B (right panel). Bars, $\pm$SD. #, $p < 0.001$, compared to the control. Data shown in C (left panel). Bars, $\pm$SD. #, $p < 0.001$, compared to the control; **, $p < 0.001$ compared to the ET-1. Data shown in C (right panel) panel. Bars, $\pm$SD; #, $p < 0.001$, compared to the control.

highly purified human LEC to form vascular-like structures on 3 dimensional cell culture, we found that ET-1 cooperates with hypoxia through VEGF-A, VEGF-C and -D to induce a rich network of interconnected tube-like structures. Recently several new mediators of lymphangiogenesis have been identified that act through a direct mechanism or through VEGF family members (Cao et al., 2006; Kang et al., 2009; Larrieu-Lahargue et al., 2010; Sawane et al., 2011). Although VEGF-C and VEGF-D represent the principal mediators of lymphangiogenic process during development and under pathological condition, VEGF-A has been also demonstrated to stimulate this process due to its capacity to bind VEGFR-3 expressed on LEC (Koch et al., 2011). On the other hand VEGF-C and VEGF-D may bind VEGFR-2 also expressed by LEC and activate lymphatic cells (Goldman et al., 2007). Here we describe that ET-1 induced VEGF members. To investigate more deeply their involvement in ET-1-

induced lymphangiogenesis, we selectively neutralized VEGF-A and/or VEGF-C and -D by anti-VEGF-A and by human recombinant VEGFR-3. In this condition endogenous as well as the ET-1-induced VEGF-A or VEGF-C and -D are virtually inactive. When we tested the capacity of ET-1 to induce LEC growth, differentiation and invasiveness by a 3 dimensional test, we found that inactivation of VEGF-A or VEGF-C significantly, but not completely, reduced the effect of ET-1. Interestingly, when VEGF-A, VEGF-C and -D were simultaneously neutralized, the effect of ET-1 was only partially reduced, indicating that ET-1, besides being a direct modulator of lymphangiogenesis, may also act in a VEGF-dependent manner. On this regard we previously demonstrated that the induction of VEGF-A was significantly induced after 6 h of stimulation while ET-1 through ET_BR rapidly activated MAPK and AKT, intracellular signaling molecules implicated in transducing

Fig. 3. VEGF-C induces tube like structure formation through VEGFR-3 LEC were seeded on Cultrex, pretreated with recombinant VEGFR-3/Fc and stimulated with 50 ng/ml VEGF-C. Tube-like structure formation was monitored and photographed after 24h (A). Tube formation was quantified by measuring tubule length (B). Data shown in (A) are representative images from three independent experiments. Data shown in (B) Bars, ±SD. #, $p<0.001$, compared to the C, **, $p<0.001$, compared to the VEGF-C.

proliferation and differentiation processes (Spinella et al., 2009). These data suggest that a direct involvement of ET_BR-mediated signaling pathway is implicated in the early events after ET-1 stimulation, while ET-1-induced VEGF members may occur later to prolong the effect of ET-1. Recent studies have also shown that hypoxia, besides regulating neoangiogenesis, can modulate lymphangiogenesis. Genome wide analysis of LEC exposed to low oxygen conditions indicated that hypoxia is capable of increasing VEGF-C, and VEGF-A mRNA (Irigoyen et al., 2007; Ota et al., 2007). In addition, in LEC hypoxia has been shown to enhance stabilization of HIF-1α, the main transcription factor controlling VEGF-A expression. In this context, we demonstrated that ET-1, not differently from hypoxia, triggered an accumulation of HIF-1α followed by an HIF-1α-mediated upregulation of VEGF-C and VEGF-A, suggesting that ET-1 axis and hypoxia may act on HIF-1 α-dependent machinery to promote lymphangiogenesis via VEGF family members (Spinella et al., 2009). Our findings demonstrating that hypoxia regulates VEGF-A, VEGF-C and -D, and that blockade of these growth factors inhibits hypoxia-induced vascular-like structures in LEC further support that like angiogenesis, hypoxia increases lymphangiogenesis through VEGF-family members. Overall, our observations demonstrate that ET-1 and hypoxia stimulate VEGF-A, VEGF-C and -D expression, and these factors have addictive effects on the formation of vascular-like structures. The mechanisms underlying the cooperation between ET-1 and hypoxia are not well defined. In our study, the additive actions of ET-1 and hypoxia on VEGF member expression and vessel formation reflect the independence between their individual signaling mechanisms. Interestingly, we found that blockade of ET_BR by BQ788 lowered the hypoxia-induced VEGF-A and VEGF-C expression, suggesting that the effects of hypoxia may be, at least in part, mediated by ET-1/ET_BR axis. It is also interesting to note that, besides VEGF, ET-1 is an HIF-1α target gene; therefore under hypoxic conditions ET-1 levels it may be also increased and, in turn, it contributes to VEGF increase suggesting a more complex regulation between hypoxia, VEGF, and ET-1 that modulates lymphangiogenic responses. This hypothesis needs to be further investigated to obtain more information on the complexity of lymphangiogenic process especially during pathological condition such as tumor in which the interplay between hypoxia microenvironment and tumor cells permits a selective growth advances for cancer cells (Guise, 2010).

Conclusions

Similar to angiogenesis, our results demonstrate that the process of lymphangiogenesis is finely tuned by the interplay of multiple hypoxia-regulated growth factors, such as VEGF members and ET-1 axis with the hypoxic microenvironment. In view of the correlation between tumor expression of ET-1 axis and lymphatic metastasis (Wülfing et al., 2003), the present results raise the possibility that ET_BR activation contributes to tumor progression by direct effects on tumor cells and on LEC disclosing a yet unidentified regulatory mechanism, which relies on the involvement of tumor microenvironment.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.lfs.2012.03.033.

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