Endothelial Nitric Oxide Synthase Mediates Lymphangiogenesis and Lymphatic Metastasis

Johanna Lahdenranta, Jeroen Hagendoorn, Timothy P. Padera, Tohru Hoshida, Gregory Nelson, Satoshi Kashiwagi, Rakesh K. Jain, and Dai Fukumura

Abstract

Lymphatic metastasis is a critical determinant of cancer prognosis. Recently, several lymphangiogenic molecules such as vascular endothelial growth factor (VEGF)-C and VEGF-D were identified. However, the mechanistic understanding of lymphatic metastasis is still in infancy. Nitric oxide (NO) plays a crucial role in regulating blood vessel growth and function as well as lymphatic vessel function. NO synthase (NOS) expression correlates with lymphatic metastasis. However, causal relationship between NOS and lymphatic metastasis has not been documented. To this end, we first show that both VEGF receptor-2 and VEGF receptor-3 stimulation activate eNOS in lymphatic endothelial cells and that NO donors induce proliferation and/or survival of cultured lymphatic endothelial cells in a dose-dependent manner. We find that an NOS inhibitor, L-NMMA, blocked regeneration of lymphatic vessels. Using intravital microscopy that allows us to visualize the steps of lymphatic metastasis, we show that genetic deletion of eNOS as well as NOS blockade attenuates peritumor lymphatic hyperplasia of VEGF-C–overexpressing T241 fibrosarcomas and decreases the delivery of metastatic tumor cells to the draining lymph nodes. Genetic deletion of eNOS in the host also leads to a decrease in T241 tumor cell dissemination to the lymph nodes and macroscopic lymph node metastasis of B16F10 melanoma. These findings indicate that eNOS mediates VEGF-C–induced lymphangiogenesis and, consequently, plays a critical role in lymphatic metastasis. Our findings explain the correlation between NOS and lymphatic metastasis seen in a number of human tumors and open the door for potential therapies exploiting NO signaling to treat diseases of the lymphatic system.

Introduction

Nitric oxide (NO) mediates an array of physiologic and pathologic processes including the formation and function of blood vessels as well as the growth and dissemination of tumors via blood vessels (1). There are three isoforms of NO synthase (NOS): neuronal NOS, inducible NOS (iNOS), and endothelial NOS (eNOS). In blood vessels, NO derived from eNOS mediates vascular function, angiogenesis, and vessel maturation (1). Perivascular NO gradients can normalize structural and functional abnormalities of tumor vasculature (2). eNOS is also expressed in the lymphatic system (3) and affects microlymphatic fluid flow by acting on collecting lymphatics (4). A positive correlation between NOS expression/activity in tumor tissues and lymphatic metastasis has been shown in head and neck, thyroid, breast, stomach, gallbladder cancers (reviewed in ref. 1), and melanoma (5). Blocking NO signaling through soluble guanylate cyclase has also been recently shown to reduce UVB irradiation–induced lymphatic hyperplasia (6). These studies suggest that NO signaling mediates lymphangiogenesis in vivo. However, it remains unknown whether NOS plays a causal role in lymphangiogenesis during wound healing and lymphatic metastasis (7).

The lymphatic vascular system is a network of endothelium-lined vessels that absorbs and transports interstitial fluid, in addition to lymphocytes and antigen-presenting cells, to lymph nodes (8). The lymphatic system also transports metastatic cancer cells to lymph nodes (9). Vascular endothelial growth factor (VEGF)-C and D, acting via activation of the lymphatic endothelial VEGFR-3, were identified as the first lymphangiogenic factors (reviewed in ref. 10). Tumors overexpressing VEGF-C exhibit increased lymphatic vessel density and lymphatic hyperplasia, and an increased rate of lymphatic metastasis as a result of abnormal formation and enlargement of peritumor lymphatics (9, 11, 12). In this study, we explore whether NOS plays a causal role in VEGF-C–induced lymphangiogenesis, lymphatic hyperplasia, and lymphatic metastasis in this setting.

Here, we use in vitro and in vivo models to dissect the role of NO on lymphangiogenesis. First, we assess the ability of VEGFR-2 and VEGFR-3 ligands to activate eNOS in cultured lymphatic microvascular endothelial cells (LEC) and the ability of NO to stimulate the growth of LECs grown in culture. Next, we assess the effect of NOS-blockade with L-NMMA on lymphangiogenesis in collagen implants in a model of dermal regeneration in the mouse tail. Finally, we assess the effect of pharmacologic or genetic blockade of NO on peritumor lymphatic hyperplasia in VEGF-C–overexpressing T241 fibrosarcomas and B16F10 melanomas implanted in the mouse ear. In this model, we also quantify the number of metastatic tumor cells arriving in the draining lymph node or, alternatively, the presence of macroscopic metastasis. Our results provide the first direct evidence that eNOS mediates VEGF-C–induced lymphangiogenesis, peritumor lymphatic hyperplasia, and lymphatic metastasis.

Materials and Methods

Cells, antibodies, and growth factors. Neonatal Human Dermal LECs were obtained from Cambrex. LECs were cultured in complete EGM-2 MV media on human fibronectin–coated (1 μg/cm²; BD Biosciences) flasks.
T241 fibrosarcoma cell line stably overexpressing VEGF-C and engineered to constitutively express green fluorescent protein (GFP; T241-VEGF-C-GFP) has been described (9). Akt, Phospho-Akt (Ser473), p42/p44, Phospho-p42/p44 (Thr202/Tyr204), and Phospho-eNOS (Ser1177) antibodies were from Cell Signaling Technologies (used 1:1,000 for Western Blot-analysis), eNOS and iNOS antibodies from BD Transduction Laboratories (used 1:2,500 for Western Blot analysis and 1:200 in PBS and for iNOS) (18), MECA-32 (used 1:200 for immunohistochemical (IHC) analysis) antibody from BD Pharmingen, LYVE-1 antibody [used 1:2,000 for IHC analysis] from Upstate Cell Signaling Solutions, and proliferating cell nuclear antigen [ready-to-use solution, used 1:5 for IHC analysis] antibody from DAKO. Recombinant human (rh) VEGF-A, VEGF-C wild-type (WT), and VEGF-C (Cys565Ser) were from R&D Systems.

Cell proliferation assay. LECs were seeded in fibronectin-coated (1 µg/cm²; Sigma) microtiter wells (3,500 cells per well) and starved in EGM-2 media supplemented with hydrocortisone, ascorbic acid, gentamicin, amphotericin-B (according to manufacturer’s instructions), and 1% fetal bovine serum (FBS; starving media) for 16 to 20 h. Medium was changed to full medium (EGM-2 MV containing 5% FBS) or starving medium containing increasing concentrations of (7)1-[1-(2-amino-2-naphthoyl)-N-(2-deoxy-(2-ammonioethyl)amino)]diazen-1-ium-1,2-diolate (DETA-NONOate) or [N-(2-d oxo-α,β-γ-thre suggestion (11), authored by Dr. Melody Swartz and colleagues (14) that allows intravital microscopy of lymphatic vasculature in living animals. Using a combination of intravital microscopy and peritumor lymphangiography, we were able to visualize lymphatic hyperplasia and lymphatic metastasis in vivo. These findings contribute to our understanding of the pathophysiology of lymphatic diseases and provide new insights into the mechanisms underlying lymphatic hyperplasia and lymphatic metastasis.

Results and Discussion

VEGF-C activates eNOS in lymphatic endothelial cells through P38 pathway. Activation of VEGFR-2 increases production of NO in blood vascular endothelial cells via activation of eNOS (18). A number of intracellular molecules mediate eNOS activation in blood vascular endothelial cells, including calcium, the P38/Akt/heat-shock protein 90, and phospholipase C-γ (reviewed in ref. 19). Interestingly, both the P38/Akt cascade (20) and phospholipase C-γ (21) are induced by VEGF-C/VEGFR-3 signaling, suggesting a possible downstream dependence of VEGFR-3 signaling on nitric oxide. To determine whether activation of VEGFR-3 by VEGF-C and the following downstream signaling events can lead to activation of eNOS in LECs, we tested the activation of eNOS in response to...
angiogenic and lymphangiogenic stimuli in LECs. First, LECs were stimulated with full growth media VEGF-A, VEGF-C, or VEGF-C156S (VEGFR-3–specific variant; ref. 22). We examined eNOS activation by Western blot analysis with a phospho-eNOS–specific (Ser1177) antibody, and found eNOS to be activated in response to VEGF-A and VEGF-C stimulation. In addition, the VEGFR-3–specific form of VEGF-C (VEGF-C156S) led to eNOS activation, establishing that VEGFR-3 activation alone is sufficient for the activation of eNOS in LECs (Fig. 1A).

Intracellular molecules mediating VEGF-C–induced eNOS activation in LECs have remained unidentified. Because PI3K/Akt signaling pathway is activated upon VEGFR-3 stimulation in LECs (20), we tested whether this pathway has a role in the activation of eNOS in lymphatic endothelial cells. We examined Akt activation by Western blot analysis with a phospho-Akt (Ser473)-specific antibody, and found, as anticipated, that Akt was activated in response to VEGF-A, VEGF-C, and VEGF-C156S stimulation (Fig. 1A). In addition, pretreatment of LECs with Wortmannin, a PI3K inhibitor, before the growth factor stimulation was able to partially block the activation of eNOS by preventing Akt activation (Fig. 1A), establishing that eNOS activation through VEGFR-2 and VEGFR-3 signaling is dependent on PI3K-mediated Akt activation. In contrast, MAP/ERK kinase 1/2 Inhibitor U0126 did not block eNOS activation in LECs (Fig. 1A). Because p42/p44 has been indicated in the eNOS activation, we examined its activation in LECs by Western blot analysis with a phospho-p42/p44 (Thr202/Tyr204)-specific antibody, and found that p42/p44 was activated in response to VEGF-C and VEGF-C156S stimulation (Fig. 1A).

In summary, we show that stimulation of LECs through either VEGFR-2 or VEGFR-3 leads to a PI3K-dependent activation of eNOS. However, there might be other downstream signaling pathways from VEGFR-2 and VEGFR-3 that further modulate eNOS activity.

**NO donors stimulate lymphatic endothelial cell proliferation and/or survival.** We assessed the effects of two structurally...
different slow-releasing NO donors on LEC proliferation and/or survival in vitro. LECs were subjected to increasing concentrations of either DETA NONOate (t1/2 of release 20 hours) or Glyco-SNAP-2 (t1/2 of release 24 hours). After 72 hours, proliferation of LECs was assessed by a colorimetric assay with WST-1. DETA-NONOate and Glyco-SNAP-2 significantly induced proliferation and/or survival of LECs in a dose-dependent manner from 50 to 500 μmol/L (P < 0.05; Fig. 1B). Glyco-SNAP-2 had no significant effect on cell proliferation at 1,000 μmol/L, possibly due to Glyco-SNAP-2 toxicity to LECs (Fig. 1B). In agreement with the recent report (5), these data suggest that NO stimulates the proliferation and/or survival of microvascular lymphatic endothelial cells.

NOS inhibition blocks lymphangiogenesis in dermal regeneration mouse-tail model. We next assessed the effect of NOS inhibition on lymphangiogenesis using a dermal regeneration model in mouse tail (14). This model allows intravital microscopy of lymphangiogenesis (9) in a collagen construct where growth of new lymphatic vessels occurs from the distal to proximal direction between days 25 and 60 after implantation and depends on VEGF-C/VEGFR-3-signaling (23). A circumferential segment of skin and subcutis was removed and filled with type I collagen (14). To obtain high-resolution images of angiogenic and lymphangiogenic vessels in the collagen construct, we performed lymphangiography and MPLSM in Tie2 promoter driven-GFP (Tie2P-GFP)/FVB mice (constitutively expressing GFP in blood endothelial cells) between days 35 and 70 (Fig. 2A). Consistent with previous observations (14), lymphatic sprouting occurred in the distal margin of the construct at day 35 and subsequently expanded in the proximal direction (Fig. 2B). At day 60, newly regenerated lymphatics had formed in the characteristic hexagonal pattern of the mouse-tail and multiple small sprouts (Fig. 2C), and nearly reached the proximal margin. Lymphatic regeneration detected by lymphangiography was heterogeneous, with the number of hexagon segments between 10% and 70% of that in normal tail skin. The newly formed lymphatics did not expand after day 70 and were stable for up to one year (Fig. 2D). Consistent with previous work Tie2P-GFP expression was not detectable on the microlymphatics of these mice (24), possibly because Tie2 promoter activity in lymphatics was below the detection limit of the GFP reporter by multiphoton microscopy (25).

To assess whether NOS is involved in lymphangiogenesis, we performed lymphangiography and intravital microscopy in mice that received an NOS inhibitor, L-NMMA, between days 21 and 60 after implantation of the collagen construct (n = 4) and mice that received vehicle (PBS) alone (n = 6). Although 83.3% of the control mice exhibited growth of new lymphatics by 60 days after the wound creation, none of the mice treated with an NOS inhibitor, L-NMMA, showed functional lymphatics in the implanted collagen construct at the same time (P < 0.05, Fisher’s exact test; Fig. 2E and F). Instead, fluorescent tracer diffused through certain areas of the regenerating region (data not shown). The lymphatics distal and proximal to the construct seemed to function normally and none of the mice developed edema. Because the blood vasculature in this...
model is completely regenerated within 21 days of collagen implantation (14), the effect of NOS inhibition on angiogenesis did not contribute to this study.

These data suggest that NOS mediates lymphangiogenesis in the model of dermal regeneration. Interestingly, in agreement with our findings, a previous study showed administration of cavatratin, which blocks eNOS activity, reduces intratumor density of VEGFR-3–expressing cells (26). Possibly, this was due to an antilymphangiogenic effect of eNOS-blockade, although this study did not further specify whether these cells were lymphatic endothelial cells (7).

**Pharmacologic and genetic inhibition of eNOS attenuates peritumor lymphatic hyperplasia.** We have shown that overexpression of VEGF-C in T241 fibrosarcomas leads to hyperplasia of the peritumor lymphatic vessels without affecting growth of the primary tumor (9, 11). Observed lymphatic hyperplasia was associated with an increased lymph flow rate and increased delivery of metastatic tumor cells in the cervical lymph node compared with wild-type tumors (9, 11). Using a pharmacologic NOS inhibitor, L-NMMA, as well as a genetic inactivation of eNOS, we investigated the role of NOS in peritumor lymphatic hyperplasia in VEGF-C–overexpressing tumors.

We used a model that allows in vivo functional lymphangiography and multiphoton microscopy of peritumor lymphatics and the draining lymph node on each step of lymphatic metastasis (9). Nude mice were implanted with T241 fibrosarcoma cells constitutively expressing VEGF-C and GFP (T241-VEGF-C-GFP). Mice received L-NMMA (n = 8) or PBS (n = 8) beginning 7 days after tumor implantation. Before the start of NOS inhibition, the mean lymphatic vessel diameter of peritumor lymphatics was 88.3 μm ± 3.8 μm as determined by lymphangiography. After 7 days of L-NMMA treatment (14 days after tumor implantation), the mean lymphatic vessel diameter did not increase in the L-NMMA–treated group, whereas a 46% increase was observed in controls (94 μm ± 137 μm, P < 0.05; Fig. 3 A, B, and C). There was no difference in tumor size between the L-NMMA and control groups after 7 days of treatment (Table 1). LYVE-1 IHC confirmed that peritumor lymphatics in L-NMMA treated animals had a significantly smaller perimeter than controls (P < 0.05; Table 1). In agreement with our previous studies (4), peritumor lymphatics in L-NMMA treated animals had a significantly smaller perimeter than controls (P < 0.05; Table 1). In agreement with our previous studies (4), peritumor lymphatics expressed eNOS (Fig. 3 D and E) but not iNOS (Supplementary Fig. S1). The peritumor lymphatics in L-NMMA–treated animals contained fewer proliferating endothelial cells per vessel cross-section than controls (P < 0.05; Table 1). In addition, intratumor blood vessel endothelial cell density in these tumors assessed by MECA-32 immunohistochemistry was not affected by this L-NMMA treatment schedule (Table 1). Thus, the effect of NO on lymphatic vessels cannot be explained solely by a secondary phenomenon of the effect on blood vessels in our experimental models.

To delineate that the observed reduction in lymphatic hyperplasia was attributable to the inhibition of the endothelial isof orm of NOS, eNOS/−/− (n = 6) or WT (57Bl/6) mice (n = 6) were implanted with T241-VEGF-C-GFP tumors. Tumor growth rates
Interestingly, the peritumor lymphatic diameters in the L-NMMA–
hyperplasia when compared with

were similar in eNOS−/− and WT mice (data not shown). When
tumors reached ~80 mm³ in size (7–8 days after implantation),
peritumoral lymphatics were visualized by lymphangiography.
Normal ear lymphatic vessels had similar diameters in WT and
eNOS−/− animals (74.2 ± 4.2 μm and 78.5 ± 3.5 μm; P = 0.5)
whereas WT animals had significantly more peritumoral lymphatic
hyperplasia when compared with eNOS−/− mice (lymphatic
diameters, 116.1 ± 3.9 μm and 98.4 ± 6.2 μm; P < 0.05; Fig. 3D).
Interestingly, the peritumor lymphatic diameters in the L-NMMA–
treated and eNOS−/− animals were similar to that in non–VEGF-C–
overexpressing tumors, VEGFR-3–neutralizing antibody treated
VEGF-C–overexpressing tumors (9) or the tyrosine kinase inhibitor
cediranib (27). Taken together, these results show that eNOS-
blockade attenuates peritumor lymphatic endothelial cell proliferation
and peritumor lymphatic vessel hyperplasia associated with
VEGF-C–overexpressing tumors. Similar results of L-NMMA
treatment and eNOS null mice experiments suggest that the effect
of L-NMMA is dominated by ordinal eNOS inhibition and not eNOS
uncoupling in our study.

In addition to NO production by lymphatic endothelial cells,
other sources of NO production are possible. Tumor cells can
produce NO through genetic and environmental up-regulation of
NOSs (1). However, our immunohistochemical analysis suggests
that tumor cells do not produce eNOS or iNOS appreciably in the
model used in this study. Macrophages and fibroblasts can be
activated in tumors and lead to iNOS induction and NO
production. Another potential source of NO in the tumors is blood
vascular endothelium. NO produced by these cells may provide a
lymphangiogenic signal required for lymphatic regeneration and
peritumor lymphatic hyperplasia. Blockade of this source of NO
would also lead to the results seen in our experiments.

Interestingly, several recent studies suggest that iNOS-derived NO
may increase production of VEGF-C and/or VEGF-D in tumor cells
and, thus, contribute to lymphangiogenesis and lymph node
metastasis (28–30). Because VEGF-C is under a constitutive
expression and is produced at much higher levels than in the
mock-transduced cells in our experiments, iNOS-derived NO is
unlikely to influence the levels of tumor cell–produced VEGF-C.

Nevertheless, through differential effects on tumor cells, stroma,
and vasculature, NO has a complex roles in carcinogenesis, tumor
progression, and metastasis (1).

**NOS inhibition decreases arrival of metastatic tumor cells in the
lymph node and macroscopic metastasis.** Peritumor
lymphatic hyperplasia induced by VEGF-C increases the
opportunity of tumor cells to enter the lymphatics (9, 31) and increases
intralymphatic transport of fluid to the lymph node (9, 32).
Reduction of peritumor lymphatic hyperplasia by NOS-blockade
could thus lead to decreased delivery of tumor cells to lymph node
and, thus, fewer lymph node metastases. To test this hypothesis, we
imaged metastatic T241-VEGF-C-GFP cells that spontaneously
spread to the cervical lymph node from ear tumors using
multil新兴 microscopic imaging (Fig. 4A). We first used L-NMMA to
inhibit NO activity and found that number of GFP-positive tumor
cells in the draining cervical lymph node at day 14 after tumor
implantation was significantly decreased in L-NMMA-treated
(tumors 7 days to 14) compared with control animals
(P < 0.05; Fig. 3B). Similar decreases in tumor cell arrival
were previously observed in the same tumor model with
VEGFR-3–
neutralizing antibody treatment and the tyrosine kinase inhibitor
cediranib (9, 27). Next, we imaged metastatic T241-VEGF-C-GFP
cells spreading to the cervical lymph node from ear tumors in
wild-type and eNOS−/− mice. When tumors had reached ~80 to
90 mm³ in size, the number of GFP-positive tumor cells in the
draining cervical lymph node after tumor implantation was
significantly decreased in eNOS−/− animals (n = 8) compared with
wild-type animals (n = 6; 107.5 ± 38.1 and 330.3 ± 75.4 tumor cells/
lymph node; P < 0.05, Mann-Whitney U test; Fig. 3B). These data
suggest that inhibition of peritumor lymphatic hyperplasia by NOS-
blockade or genetic deletion of eNOS decreases the number of
metastatic cells arriving in the lymph node.

Finally, we used a syngeneic B16F10 tumor model in eNOS−/−
and wild-type mice to address whether genetic deletion of eNOS in
the lymphatic vessels affects peritumor hyperplasia and the
formation of clinical metastasis in a tumor model without artificial
overexpression of VEGF-C: eNOS−/− or wild-type mice were
implanted with B16F10 tumors. When tumors reached ~80 to
100 mm³ in size, primary tumors were resected and processed for
immunohistochemical analysis. Fourteen days after resection of
the primary tumors, the number of draining cervical lymph nodes with
metastatic lesions was significantly higher in wild-type mice than
in eNOS−/− mice (9/14 LN positive and 2/14 LN positive; P < 0.05,
Fisher’s exact test; Fig. 4C). Although statistically nonsignificant,
we observed a trend toward smaller diameters of LYVE1+ peritumor
lymphatic vessels in eNOS−/− mice when compared with wild-type
mice (22.6 ± 2.9 μm and 26.2 ± 1.6 μm; P = 0.3; Fig. 4D). Diameters of
peritumoral blood vessels were similar in eNOS−/− and wild-type
mice (6.4 ± 0.7 μm and 5.8 ± 0.4 μm; P = 0.4).

In summary, we show that inhibiting eNOS activity either
genetically or pharmacologically significantly reduces the number
of tumor cells arriving to the tumor draining lymph node and
the subsequent formation of macroscopic metastasis. Because NOS
inhibition reduced the number of cancer cells delivered to the
draining lymph nodes, our data suggest that NO participates in
the earliest steps of lymphatic metastasis. As NOS inhibition can
also decelerate tumor growth, likely through an angiogenic
mechanism, the use of NOS inhibition may prove a useful adjuvant
therapy for metastatic disease. Both the further spread of cancer
cells from the tumor bed to the lymph node and the growth of
those cells in lymph nodes could be reduced by NOS inhibition.

<p>| Table 1. Effect of NOS blockade on lymphatic and blood vessels in T241-VEGF-C-GFP tumors |
|-------------------------------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Control</th>
<th>L-NMMA</th>
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<tr>
<td>Lymphatic vessel diameter (μm)</td>
<td>137 ± 12</td>
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<td>(IVM)</td>
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<tr>
<td>Lymphatic vessel perimeter (μm)</td>
<td>386 ± 27</td>
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<td>(histology)</td>
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<td>Proliferating lymphatic endothelial</td>
<td>3.1 ± 0.6</td>
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<td>cell (cells/vessel section)</td>
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<tr>
<td>Blood vessel density (no of endothelial cell nuclei per 20 μm² field)</td>
<td>64 ± 11</td>
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<tr>
<td>Tumor volume (mm³)</td>
<td>64 ± 8</td>
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Note: Animals received L-NMMA or PBS treatment starting 7 d after
VEGF-C–overexpressing T241 fibrosarcoma implantation into the
mouse ear. Fourteen days after the tumor implantation, peripheral ear
lymphangiography was performed followed by immunohistochemical
analyses of blood vessel density, lymphatic vessel perimeter, and
lymphatic endothelial cell proliferation.

*P < 0.05 compared with the corresponding controls, Student’s t test.
We show here for the first time a causal relationship between NO, lymphangiogenesis, and lymphatic metastasis. It is attractive to think that treatments that block NO production and signaling could simultaneously block tumor-associated angiogenesis and reduce lymphatic metastasis. Future investigations will need to determine the importance of the relative contributions of VEGFR-2 and VEGFR-3 and the different NOS isoforms (eNOS, iNOS, neuronal NOS) to lymphangiogenesis and lymphatic metastasis. In addition, determining the target cells producing NO will be important in therapeutic design. Our novel findings add NO to the growing list of lymphangiogenic molecules and open the door for potential therapies exploiting NO signaling to treat diseases of the lymphatic system, including lymphatic metastasis and lymphedema.

References


Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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