Vascular endothelial growth factor-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism

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Breslin JW, Gaudreault N, Watson KD, Reynoso R, Yuan SY, Wu MH. Vascular endothelial growth factor-C stimulates the lymphatic pump by a VEGF receptor-3-dependent mechanism. Am J Physiol Heart Circ Physiol 293: H709–H718, 2007. First published March 30, 2007; doi:10.1152/ajpheart.00102.2007.—Vascular endothelial growth factor (VEGF)-C plays an important role in lymphangiogenesis; however, functional responses of lymphatic vessels to VEGF-C have not been characterized. We tested the hypothesis that VEGF-C-induced activation of VEGF receptor (VEGFR)-3 increases lymphatic pump output. We examined the in vivo pump activity of rat mesenteric collecting lymphatics using intravital microscopy during basal conditions and during treatment with 1 nM recombinant VEGF-C, the selective VEGFR-3 agonist VEGF-Cys156Ser mutation (Cys156S; 1 nM), or 0.1 nM VEGF-A. Their specific responses were also analyzed during selective inhibition of VEGFR-3 with MAZ-51. Contractile frequency, end-diastolic diameter, end-systolic diameter, stroke volume index, pump flow index, and ejection fraction were evaluated. We also assessed arteriolar diameter and microvascular extravasation of FITC-albumin. The results show that both VEGF-C and VEGF-C156S significantly increased contraction frequency, end-diastolic diameter, stroke volume index, and pump flow index in a time-dependent manner. VEGF-A caused a different response characterized by a significantly increased stroke volume after 30 min of treatment. MAZ-51 (5 μM) caused tonic constriction and decreased contraction frequency. In addition, 0.5 and 5 μM MAZ-51 attenuated VEGF-C- and VEGF-C156S-induced lymphatic pump activation. VEGF-A caused vasodilation of arterioles, whereas VEGF-C and VEGF-C156S did not significantly alter arteriolar diameter. Also, VEGF-A and VEGF-C caused increased microvascular permeability, whereas VEGF-C156S did not. Our results demonstrate that VEGF-C increases lymphatic pumping through VEGFR-3. Furthermore, changes in microvascular hemodynamics are not required for VEGFR-3-mediated changes in lymphatic pump activity.

lymph flow; collecting lymphatics; endothelium

THE LYMPHATIC SYSTEM PLAYS a critical role in maintaining body fluid balance by returning excess interstitial fluid and solutes to the bloodstream while also serving as a conduit for lymphocytes to the lymph nodes (1). Lymphedema occurs when there is physical obstruction to flow such as surgical severing of lymphatic vessels or dysfunction of intrinsic lymphatic pump activity (46, 60). Understanding the mechanisms controlling lymph formation and flow is of particular interest for the prevention of lymphedema and is also applicable for improving our understanding of healthy processes such as wound healing and disease processes such as the development of diabetic foot ulcers or entry of metastatic cells into the lymphatic system.

Vascular endothelial growth factor (VEGF)-C promotes angiogenesis and lymphangiogenesis through activation of VEGF receptor (VEGFR)-2 and VEGFR-3, respectively. The importance of VEGFR-3 in the development and normal function of the lymphatic system has been highlighted in animal models as well as by the demonstration that mutation of the VEGFR-3 (FLT4) gene in humans is an underlying cause of primary (hereditary) lymphedema (11, 13, 14, 30, 35). The involvement of VEGFR-3 in ameliorating secondary lymphedema is also demonstrated in animal models showing that gene therapy with VEGF-C, or a VEGFR-3-selective mutant of VEGF-C (Cys156Ser mutation; VEGF-C156S; see Ref. 29), can reduce lymphedema by stimulating lymphangiogenesis (48, 63).

Although the aforementioned studies have addressed the importance of VEGF-C and VEGFR-3 in lymphangiogenesis and maintaining a functional lymphatic network, no previous studies have investigated the physiological characteristics of VEGFR-3 activation-mediated responses in the lymphatic system. VEGF-C is normally present in the circulation, with serum levels estimated to be as high as 10 ng/ml (0.6 nM) in healthy patients and even higher in patients who are obese or have cancer (34, 37, 41, 52, 54, 56, 57). Because other members of the VEGF family have vasoactive- and endothelial barrier-altering properties (4, 6, 10, 38, 61), we hypothesized that VEGF-C-induced activation of VEGFR-3 can alter the intrinsic, phasic pumping of collecting lymphatics. Here, we describe studies in which we examined rat mesenteric collecting lymphatics before and after administration of VEGF-C, the VEGFR-3-selective agonist VEGF-C156S (29), and a selective VEGF-3 inhibitor, MAZ-51 (31, 32). Furthermore, considering that VEGF-C can also activate VEGFR-2, which is known to mediate VEGF-A-induced vasodilation in arterioles and hyperpermeability in postcapillary venules (4, 6, 38, 61), we determined whether VEGF-C-induced changes in lymphatic tone and phasic activity may be associated with local changes in arteriolar diameter and microvascular leakage. We chose to study mesenteric lymphatics because they are representative of the splanchnic lymphatics, which contribute more than two-thirds of total body lymph flow and are an important lymphocyte homing site (5). In addition, these lymphatics are abundant and easily distinguished in the mesentery, making them conducive to intravital microscopy for real-time monitoring of pumping activity.

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MATERIALS AND METHODS

Recombinant human VEGF-C, VEGF-A, and the VEGF-C156S mutant were purchased from R&D Systems (Minneapolis, MN). MAZ-51 was purchased from EMD/Calbiochem (San Diego, CA). All other chemicals, unless otherwise specified, were obtained from Sigma (St. Louis, MO).

Animal preparation. Protocols using Sprague-Dawley rats were approved by the University of California Davis Institutional Animal Care and Use Committee and were in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals and the guidelines of the Animal Welfare Act. Male rats (n = 49) weighing 250–500 g were anesthetized with an intramuscular injection of urethane (1.75 g/kg). The left jugular vein was cannulated for infusion of solutions, and the right carotid artery was cannulated for continuous monitoring of blood pressure through a pressure transducer and DigiMed Blood Pressure Analyzer Model 400 (Micro-Med, Louisville, KY). A midline laparotomy was performed, and a section of mesentery was exteriorized over an optical stage for microscopic observation. The exteriorized mesentery was superfused with lactated Ringer USP (Baxter Healthcare, Deerfield IL) at 37°C, 4 ml/min, and body temperature was maintained at 37°C with a heating pad (Fine Science Tools, North Vancouver, BC). Animals received a continuous intravenous infusion of lactated Ringer solution at a rate of 0.004 ml·min⁻¹·100 g body wt⁻¹ to replenish normal fluid loss during the experiment (64).

Intravital microscopy. Collecting lymphatics ranging 30–180 μm in diameter and exhibiting contractile activity were observed using a Nikon Eclipse E600FN Microscope equipped with a Photometrics

Table 1. Baseline lymphatic pump characteristics

<table>
<thead>
<tr>
<th>No. of rats studied</th>
<th>Control</th>
<th>VEGF-C</th>
<th>VEGF-C156S</th>
<th>VEGF-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of lymphatics studied</td>
<td>11</td>
<td>8</td>
<td>8</td>
<td>4</td>
</tr>
<tr>
<td>CF, contractions /min</td>
<td>15</td>
<td>17</td>
<td>19</td>
<td>8</td>
</tr>
<tr>
<td>EDD, μm</td>
<td>13.6 ± 3.2</td>
<td>10.7 ± 1.2</td>
<td>13.6 ± 1.4</td>
<td>13.0 ± 2.6</td>
</tr>
<tr>
<td>ESD, μm</td>
<td>92.1 ± 6.0</td>
<td>89.1 ± 10.2</td>
<td>82.7 ± 8.2</td>
<td>80.7 ± 5.3</td>
</tr>
<tr>
<td>SVI, μm²</td>
<td>71.4 ± 6.4</td>
<td>60.2 ± 7.4</td>
<td>59.4 ± 5.7</td>
<td>56.9 ± 5.1</td>
</tr>
<tr>
<td>PFI, μm³/min</td>
<td>10,400 ± 1,870</td>
<td>16,050 ± 3,598</td>
<td>12,280 ± 3,221</td>
<td>10,387 ± 1,532</td>
</tr>
<tr>
<td>EF</td>
<td>0.40 ± 0.06</td>
<td>0.55 ± 0.04</td>
<td>0.48 ± 0.03</td>
<td>0.43 ± 0.08</td>
</tr>
</tbody>
</table>

Values are means ± SE. VEGF, vascular endothelial growth factor; CF, contraction frequency; EDD, end-diastolic diameter; ESD, end-systolic diameter; SVI, stroke volume index; PFI, pump flow index; EF, ejection fraction.
CF and Lymphatic Pump Function

We first evaluated the pattern and time course of VEGF-C- and VEGF-C156S-mediated changes in diameter in rat mesenteric collecting lymphatics. These lymphatic vessels are typically found in parallel with arterioles and venules, are surrounded by adipose cells in rat mesentery, but are also often found at the edge of adipose regions or traversing the translucent regions of mesenteric tissue. Figure 1 shows a typical collecting lymphatic in a translucent region of rat mesentery adjacent to an adipose-rich region. These collecting lymphatic vessels are anatomically distinguished as segmental and having one-way valves that minimize backflow. Most importantly, mesenteric collecting lymphatics often display spontaneous phasic contractile activity, with obvious diastolic and systolic phases (Fig. 1, A and B). A representative tracing of lymphatic vessel diameter vs. time during baseline conditions and after the addition of 1 nM VEGF-C156S is shown in Fig. 1C.

Fig. 2. VEGF-C treatment alters lymphatic diameter and contraction frequency. The time courses of changes in contraction frequency (A), end-diastolic diameter (B), and end-systolic diameter (C) during continuous suffusion with 1 nM VEGF-C, 1 nM VEGF-C156S, 0.1 nM VEGF-A, or sham control are shown. *P < 0.05 vs. sham control at the same time point. For the VEGF-C group, n = 17 lymphatics studied; for the VEGF-C156S group, n = 19; for the VEGF-A group, n = 8; and for the sham control group, n = 15.

RESULTS

We first evaluated the pattern and time course of VEGF-C- and VEGF-C156S-mediated changes in diameter in rat mesenteric collecting lymphatics. These lymphatic vessels are typically found in parallel with arterioles and venules, are surrounded by adipose cells in rat mesentery, but are also often found at the edge of adipose regions or traversing the translucent regions of mesenteric tissue. Figure 1 shows a typical collecting lymphatic in a translucent region of rat mesentery adjacent to an adipose-rich region. These collecting lymphatic vessels are anatomically distinguished as segmental and having one-way valves that minimize backflow. Most importantly, mesenteric collecting lymphatics often display spontaneous phasic contractile activity, with obvious diastolic and systolic phases (Fig. 1, A and B). A representative tracing of lymphatic vessel diameter vs. time during baseline conditions and after the addition of 1 nM VEGF-C156S is shown in Fig. 1C.
mean baseline values for various parameters studied did not significantly differ between groups and are provided in Table 1.

We assessed VEGF-C- and VEGF-C156S-induced changes in rat mesenteric lymphatics over a 60-min time period (Fig. 2). Treatment with 1 nM VEGF-C or VEGF-C156S caused a significant elevation in CF, starting 1 min after the initiation of treatment and lasting until the 30-min time point (Fig. 2 A). CF was not altered by the sham treatment or by treatment with 0.1 nM VEGF-A. In addition to this positive chronotropic response, we also observed that VEGF-C and VEGF-C156S cause lymphatic vasodilation. VEGF-C caused an increase in EDD within 1 min after the start of treatment, which remained significantly elevated through the 20-min time point, whereas VEGF-C156S caused a significant increase first observed at 10 min and then throughout the remainder of the time course. Sham treatment caused no significant change in EDD throughout the time course, and VEGF-A caused only a slight increase starting at the 20-min time point (Fig. 2 B). VEGF-C also caused a significant increase in ESD at the 1-min time point, and there was a sustained (although statistically insignificant) elevation in ESD for the remainder of the time course with both VEGF-C and VEGF-C156S treatment compared with sham (Fig. 2 C). VEGF-A did not cause any significant changes in ESD.

To further understand the response to VEGF-C treatment, we determined how these changes alter the pumping activity of rat collecting lymphatics (Fig. 3). The SVI, which is indicative of the amount of lymph that flows per lymphangion contraction, was significantly elevated 10 min after the initiation of VEGF-C156S treatment and was slightly, but insignificantly, elevated in the VEGF-C group. Sham treatment produced no response (Fig. 3 A). Although no individual time point in the VEGF-C group was significantly different from sham, the mean maximum change in SVI during VEGF-C treatment (208.5 ± 28.3% increase; mean ± SE) vs. baseline was highly significant (P < 0.001). VEGF-A also caused an elevation in SVI, but at later time points. VEGF-C and VEGF-C156S both produced significant increases in the PFI, peaking, respectively, at the 1- and 10-min time points, whereas VEGF-A and sham treatment caused no significant change (Fig. 3 B). These increases indicate an elevated amount of lymph pumped by the lymphangion per minute, analogous to cardiac output in the heart. EF, which is an indicator of pumping efficiency, is based on the amount of lymph pumped per contraction relative to the total amount of lymph in the lymphangion. VEGF-C, VEGF-C156S, and VEGF-A did not significantly alter EF (Fig. 3 C), indicating no change in the efficiency of lymphatic pump function. With regard to VEGF-C and VEGF-C156S, this latter result was because of the fact that EDD and ESD were elevated uniformly.

To further determine that the VEGF-C156S-induced activation of collecting lymphatics was because of VEGFR-3 activation, we used an inhibitor of the VEGFR-3 tyrosine kinase, MAZ-51 (31). Treatment with 5 μM MAZ-51, a dose shown to inhibit VEGFR-3 activity (31), caused significant reductions in basal CF, EDD, and ESD, whereas 0.5 μM MAZ-51 did not

Table 2. Baseline arteriolar and venular diameters measured

<table>
<thead>
<tr>
<th></th>
<th>VEGF-C</th>
<th>VEGF-C156S</th>
<th>VEGF-A</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of animals studied</td>
<td>5</td>
<td>5</td>
<td>3</td>
</tr>
<tr>
<td>Arteriolar diameter</td>
<td>13.7 ± 1.1</td>
<td>13.6 ± 1.7</td>
<td>14.1 ± 0.4</td>
</tr>
<tr>
<td>Venular diameter</td>
<td>20.3 ± 0.9</td>
<td>19.9 ± 0.7</td>
<td>19.0 ± 1.5</td>
</tr>
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</table>

Values are means ± SE. Units for diameter are μm.
alter these parameters (Fig. 4). Both doses of MAZ-51 inhibited VEGF-C-induced changes in lymphatic pumping (Fig. 4). MAZ-51 inhibited the maximal positive chronotropic and dilation responses to VEGF-C and VEGF-C156S in a dose-related manner (Fig. 5).

Treatment with 5 μM MAZ-51 also caused significant decreases in both basal SVI and basal PFI, whereas 0.5 μM MAZ-51 caused no significant changes from baseline (Fig. 6, A and B). However, both doses of MAZ-51 inhibited the VEGF-C- and VEGF-C156S-induced increases in SVI and PFI in a dose-related fashion (Fig. 6, C and D).

**Fig. 4.** Inhibition of VEGFR receptor (VEGFR)-3 affects baseline lymphatic pumping and blocks VEGF-C-induced lymphatic phasic activity. The time courses of changes in contraction frequency (A), end-diastolic diameter (B), and end-systolic diameter (C) during continuous suffusion with the VEGFR-3 inhibitor MAZ-51 (0.5 or 5 μM), followed by the addition of 1 nM VEGF-C are shown. *P < 0.05 vs. baseline (BL). For the 0.5 μM MAZ-51 group, n = 12 lymphatics studied and n = 10 for the 5 μM MAZ-51 group.

**Fig. 5.** Inhibition of VEGFR-3 blocks VEGF-C-induced changes in lymphatic pump activity. The maximal changes in contraction frequency (A), end-diastolic diameter (B), and end-systolic diameter (C) in response to 1 nM VEGF-C or 1 nM VEGF-C156S in the absence or presence of the VEGFR-3 inhibitor MAZ-51 (0.5 or 5 μM) are shown. Bars on top indicate suffusion of agents. *P < 0.05 and **P < 0.01 vs. VEGF-C treatment in the absence of MAZ-51. †P < 0.05 and ††P < 0.01 vs. VEGF-C156S treatment in the absence of MAZ-51. The number of lymphatics studied in each group is as follows: VEGF-C alone (n = 17), VEGF-C + 0.5 μM MAZ-51 (n = 12), VEGF-C + 5 μM MAZ-51 (n = 10), VEGF-C156S alone (n = 19), VEGF-C156S + 0.5 μM MAZ-51 (n = 5), and VEGF-C156S + 5 μM MAZ-51 (n = 5).
We next evaluated whether the lymphatic pump changes in response to VEGF-C and VEGF-C156S may be associated with increased microvascular leakage, which could potentially create an edemagenic stress that may alter lymphatic pump activity (9). Increased microvascular leakage can occur when there is elevated blood flow, which is controlled by resistance arterioles, or elevated microvascular permeability in venules (7). Therefore, we examined the diameter of arterioles and microvascular extravasation from venules before and during treatment with VEGF-C or VEGF-C156S (Table 2). VEGF-A served as a positive control for both parameters (4). Neither 1 nM VEGF-C nor VEGF-C156S caused a significant change in arteriolar diameter, whereas 0.1 nM VEGF-A caused a significant vasodilation (Fig. 7A). On the other hand, VEGF-C caused an elevation in the extravasation of FITC-albumin that, although statistically significant at only one time point (30 min), was only slightly less than the significant increase in extravasation elicited by VEGF-A. VEGF-C156S caused no significant change in FITC-albumin extravasation (Fig. 7B).

**DISCUSSION**

In this study, we demonstrate for the first time that VEGF-C produces a time-dependent change in lymphatic phasic activity. Treatment with either wild-type VEGF-C, which can activate VEGFR-2 and VEGFR-3, or the mutant VEGF-C156S, a selective VEGFR-3 agonist, caused a rapid increase in CF, EDD, SVI, and PFI in rat mesenteric collecting lymphatic vessels. On the other hand, VEGF-A, which can activate VEGFR-2 but not VEGFR-3, caused a very different response characterized by a significant but delayed increase in SVI. We also show that the selective VEGFR-3 inhibitor MAZ-51, in a dose-related fashion, can inhibit VEGF-C or VEGF- and C156S-induced changes in lymphatic phasic activity and can even significantly decrease baseline lymphatic pump function. Furthermore, we demonstrate that the VEGF- and C156S-induced changes in lymphatic pumping are not associated with alterations of arteriolar diameter or microvascular permeability. Taken together, these data indicate that VEGF-C increases lymphatic CF, dilation, and pump flow through its action on VEGFR-3. In addition, there is the novel finding that inhibition of baseline lymphatic phasic activity by MAZ-51 implicates the possible involvement of VEGF-3 in the physiological regulation of lymphatic contractile activity.

VEGF-C is widely expressed in many adult human tissues (28); however, its physiological activity on lymphatic vessels was not characterized previously. To begin an assessment of the physiological role of VEGF-C, one important initial objective we had was choosing a relevant dose of VEGF-C to study. Several groups have reported serum levels of VEGF-C in healthy patients to be roughly 10 ng/ml (0.6 nM; see Refs. 34, 41, 52, 54), although it is worth noting that other groups have reported serum levels in healthy humans up to 4 to 10 times lower (37, 56, 57). Importantly, VEGF-C levels are higher than normal in obese patients (54) or those with lung cancer, esophageal cancer, and cervical cancer (34, 37, 41, 56, 57). We used 1 nM VEGF-C in our study. Assuming that the aforementioned serum levels are accurate, we speculate that 1 nM VEGF-C probably reflects a physiological interstitial concentration in tissues that express VEGF-C. Notably, the physiological stimulus for normal VEGF-C production in vivo remains to be characterized. One potential candidate stimulus is elevated interstitial fluid pressure, supported by evidence that VEGF-C expression is higher in tissues that are swollen because of experimental lymphedema (47) as well as in certain tumors, which are also known to have high interstitial fluid pressures (26).
where Io is the extraluminal fluorescence intensity and Ii is intensity in the baseline. 

P venular lumen during continuous iv infusion of FITC-albumin. 

In addition, elevated VEGF-C expression and lymphatic hyperplasia occur during experimental secondary lymphedema in a mouse tail model in which initial lymphatic vessels exhibit poor draining of interstitial fluid, with evidence of leakiness and backflow (47). One explanation for this apparent inconsistency is that VEGF-C could have additional important actions other than lymphangiogenesis to improve drainage and reduce lymphedema. Our finding that VEGF-C elevates pump activity of collecting lymphatics may thus explain why VEGF-C therapy may reduce lymphedema despite the presence of undesired lymphatic hyperplasia.

Another important unresolved clinical issue involving VEGF-C is the finding that tumors with VEGF-C overexpression tend to be more metastatic (34), although tumor lymphangiogenesis has not been convincingly demonstrated in humans (26). The functional lymphatic vessels of the tumor margin are thought to be sufficient for lymphatic metastases and have elevated surface area in VEGF-C-expressing tumors, presumably providing a greater opportunity for entry of cancer cells (45). Based on our current data, we propose an additional, alternative explanation in which VEGF-C-induced elevations in lymphatic pumping may enhance the process of interstitial flow from the tumor to lymphatic vessels, facilitating the entry of cancer cells.

We show that VEGF-C increases both lymphatic vessel diameter and CF. To begin to evaluate the mechanism of this response, we examined the relative contributions of the two known receptors of VEGF-C, namely VEGFR-2 and VEGFR-3. To evaluate whether VEGFR-3 activation contributes to this response, we used the VEGF-C mutant VEGF-C156S, which does not bind to VEGFR-2. Our finding that VEGF-C156S causes a roughly similar response as VEGF-C indicates that VEGFR-3, and not VEGFR-2, mediates the VEGF-C-induced increase in lymphatic phasic activity in collecting lymphatics. This is supported by the findings that VEGF-A, which activates VEGFR-1 and VEGFR-2, did not cause the same type of response in collecting lymphatics and that the VEGFR-3 inhibitor MAZ-51 attenuated the responses to VEGF-C and VEGF-C156S. We also show that the highest dose of MAZ-51 we tested, 5 μM, caused a decrease in lymphatic pump activity. At 5 μM, MAZ-51 inhibits tyrosine autophosphorylation of VEGFR-3 but does not affect tyrosine kinase activity and phosphorylation of VEGFR-2, the angiopoietin receptor Tie2, epithelial growth factor receptor, erythroid leukemia viral oncogene homolog 2, insulin-like growth factor-I receptor, or fibroblast growth factor receptor 1 in vitro. Nevertheless, it is important to note that this compound, like any pharmacological agent, may also have undocumented effects on other tyrosine kinases (31, 32). Assuming that MAZ-51 is specifically inhibiting VEGFR-3 at this dose when superfused over rat mesentery, the demonstration that 5 μM MAZ-51 decreased lymphatic pump parameters from baseline in a precisely opposite manner as VEGF-C or VEGF-C156S supports the notion that VEGFR-3 plays a physiological role in controlling the contraction of collecting lymphatics.

Several reports indicate myogenic responses to pressure and flow in lymphatics, as well as endothelium-dependent regulation of both the phasic and tonic aspects of contraction (17, 18, 22, 33, 39, 42–44, 55, 58, 59). Elevated interstitial pressure can serve as a driving force for fluid and solutes to cross the microscopic one-way primary valves thought to be present in the endothelium of initial lymphatics (15, 40). Thus one possible mechanism we investigated was whether VEGF-C-induced increases in lymphatic pumping might be because of elevated interstitial fluid pressure caused by changes in vascular hemodynamics. Arteriolar dilation, venular permeability, or

Fig. 7. Evaluation of arteriolar diameter and microvascular permeability upon stimulation of VEGFR-2 and VEGFR-3. A: luminal diameter (Lum Dia) of mesenteric arterioles during treatment with 1 nM VEGF-C (n = 5), 1 nM VEGF-C156S (n = 5), and 0.1 nM VEGF-A (n = 3). Luminal diameter is expressed as %control (basal) condition. B: changes in microvascular permeability during treatment with the same doses of VEGF-C (n = 5), VEGF-C156S (n = 5), or VEGF-A (n = 3), as determined by the intensity ratio L/I, where L is the extraluminal fluorescence intensity and I is intensity in the venular lumen during continuous iv infusion of FITC-albumin. *P < 0.05 vs. baseline.

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a combination of both could potentially increase flux of fluid and solutes into the interstitium, and proinflammatory agents such as formyl-Met-Leu-Phe can increase lymph flow by such a mechanism (8, 20). The response to VEGF-A in collecting lymphatics involved a small but sustained elevation in lymphatic EDD starting 20 min after the initiation of VEGF-A treatment, as well as a significant increase in the SVI at 30 and 45 min. Although it is possible that the response we observed in collecting lymphatics to VEGF-A could be because of direct activation of VEGFR-2 on lymphatic endothelial cells, because the response developed more slowly than the VEGF-A-induced increases in arteriolar dilation and plasma leakage, it appears more likely to be the result of elevated lymph formation. Increases in lymphatic CF, EDD, and SVI are typically observed in response to increased lymph formation (8, 9), and the latter two characteristics were observed with VEGF-A treatment. The absence of an associated increase in CF during VEGF-A treatment could possibly be because of the fact that VEGF-A stimulates production of nitric oxide, a signal that suppresses phasic activity (17, 18, 43, 58, 59). VEGF-A has been shown to cause dilation of preconstricted lymphatics (thoracic duct) ex vivo via a mechanism involving nitric oxide (3). Although a direct comparison between our study and that study is difficult because the models utilize lymphatics from distinct anatomical regions, potentially having varied contractile activity (16), and different conditions (isolated and preconstricted vs. in vivo), both studies show that VEGF-A can influence lymphatic pumping, either through a direct or indirect mechanism.

Unlike VEGF-A, VEGF-C and VEGF-C156S caused minimal changes in arteriolar diameter, suggesting that increased blood flow and convective plasma filtration are not likely to be involved in the VEGF-C-induced lymphatic contractile response. Also, although VEGF-C caused increased extravasation of FITC-albumin, VEGF-A caused a higher degree of extravasation at 1 min without associated rapid changes in CF or diameter in collecting lymphatics at the same time point. The rapid increases in CF and diameter observed in lymphatics are thus not likely a response to increased plasma leakage. This was confirmed by experiments with VEGF-C156S and suggests direct chronotropic and dilatory effects of VEGF-C activation. Plasma leakage mediated by VEGF-C-induced activation of VEGFR-2 (23) could still potentially contribute to the observed response, but its relative contribution to the observed changes in lymphatic pumping appears to be insignificant because VEGF-C156S produced roughly similar changes in lymphatic pump parameters as VEGF-C. The impact of microvascular leakage on VEGFR-3-mediated changes in lymphatic pumping in tissues with higher degrees of edematous stress remains to be determined. Additionally, another issue that merits further investigation is whether the combined effects of VEGF-C156S produced a net increase, decrease, or no change in interstitial fluid pressure over time.

One interesting aspect of VEGF-C-induced increases in phasic activity is that the CF steadily declines toward baseline after the initial increase, possibly reflecting a negative feedback mechanism, whereas lymphatic diameter tends to remain elevated. This response was observed with both VEGF-C and VEGF-C156S, and it is currently unclear why diameter remains elevated. Comprehensive studies in which lymphatic luminal pressure is measured, as well as utilizing isolated lymphatic vessels, in which pressures and flow can be tightly controlled, will be required to elucidate the mechanism underlying this phenomenon.

VEGFR-3 is predominantly expressed on lymphatic vessels in most tissues, with exceptions such as expression in corneal epithelial cells and in activated macrophages found in tumors and atherosclerotic plaques (12, 51). Because collecting lymphatics responded so quickly to VEGF-C in our model, we speculate that the response is mediated through VEGFR-3 on lymphatic endothelial cells. The mechanism of communication from lymphatic endothelial cells to the smooth muscle layer in VEGFR-3-mediated changes in lymphatic pump activity remains to be characterized comprehensively. Potential mediators for study include nitric oxide and endothelin, which have been shown to respectively signal relaxation and constriction, in a similar manner as in blood vessels (18, 21, 42, 43, 49, 53). VEGF-C caused both positive chronotropic activity and also an increase in diameter, which presents an interesting challenge because previously characterized agents that increase phasic contractions usually also increase tone, and vice versa (2, 12, 58, 59). Considering this and the fact that VEGF-C also stimulates other complex cellular responses such as altered gene expression (62), we speculate that the lymphatic contractile response to VEGFR-3 activation involves multiple signaling pathways to regulate phasic and tonic contractility.

In this study, we used an in vivo model to observe lymphatic pump function. Therefore, it is important to note that neurogenic and metabolic factors, cytokines, and extrinsic forces are present. Because of the potential for differential responses between males and females (25, 50), we limited the current study to male rats. In addition, because we used pharmacological agents, we cannot exclude the possibility of nonspecific effects of these agents. We attempted to minimize this possibility by using previously published doses in the lower range at which the agents are selective for the intended receptors. We also used time-matched controls for all experiments.

In summary, we demonstrate a novel response to VEGF-C-induced VEGFR-3 activation in lymphatic vessels that is characterized by positive chronotropic activity and lymphatic dilation, resulting in elevated lymphangion stroke volume and output. This finding highlights the importance of this endothelial receptor in the regulation of lymphatic contractility and also presents VEGFR-3 as a potential pharmacological target to specifically alter lymphatic function.

GRANTS

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