Vascular Endothelial Growth Factor (VEGF) and VEGF-C Show Overlapping Binding Sites in Embryonic Endothelia and Distinct Sites in Differentiated Adult Endothelia

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Abstract—Vascular endothelial growth factor (VEGF) is a key modulator of angiogenesis during development and in adult tissues, whereas the related VEGF-C has been shown to induce both lymphangiogenesis and angiogenesis. To better understand the specific functions of these growth factors, we have here analyzed their binding to sections of mouse embryonic and adult tissues and compared the distribution of the bound growth factors with the expression patterns of the 3 known members of the VEGF receptor family as well as with neuropilin-1, a coreceptor for VEGF165. Partially overlapping patterns of VEGF and VEGF-C binding were obtained in embryonic tissues, consistent with the expression of all known VEGF receptors by vascular endothelial cells. However, the most striking differences of binding were observed in the developing and adult heart, in which VEGF decorated all vessels, whereas strong VEGF-C signals were obtained only from epicardial vessels. In the lymph nodes, VEGF and VEGF-C showed distinct binding patterns in agreement with the differential location of their specific receptors. These results show that both VEGF-C and VEGF target embryonic blood vessels, whereas a more selective binding of VEGF-C occurs to its lymphatic vascular receptor in certain adult tissues. Our results suggest that VEGF and VEGF-C have both overlapping and distinct activities via their endothelial receptors. (Circ Res. 1999;85:992-999.)

Key Words: angiogenesis ■ vascular endothelial growth factor receptor ■ lymphatic vessel

Endothelial cells arise from the differentiation of mesodermal progenitors extraembryonally in the blood islands and within the embryo as angioblastic precursors that aggregate into endothelial strands and cords. This differentiation process is termed vasculogenesis. After the primary vascular plexus has been formed, more endothelial cells and capillaries are generated by angiogenesis, which involves the sprouting or splitting of preexisting vessels.1 Whereas vasculogenesis is mainly restricted to embryogenesis, angiogenesis continues to operate throughout life in female reproductive functions, wound healing and angiogenesis-dependent pathological conditions such as tumor growth. Endothelial cells form several types of specialized vessels in different parts of the body, and their growth and differentiation are regulated by polypeptide growth factors and their receptors. Five endothelial cell-specific receptor tyrosine kinases are known, including 3 receptors for vascular endothelial growth factors (VEGFs) and a receptor for angiopoietins, Tie-2, as well as the related orphan receptor Tie-1.2–4 These receptors and their ligands have been shown to play important roles in vasculogenesis, angiogenesis, and vascular remodeling.1,5

The VEGF family has recently been expanded and is presently composed of at least 5 members, VEGF, placenta growth factor (PIGF), VEGF-B, VEGF-C, and VEGF-D.4,6–11 In addition, 3 viral homologues, collectively called VEGF-E, are encoded by the genome of different Orf virus strains.12–15 These growth factors display a substantial degree of homology within the cystine knot motif, which forms the binding loops for the VEGF receptors (VEGFRs). VEGF binds both VEGFR-1/Flt-1 and VEGFR-2/KDR,4 whereas PIGF and VEGF-B bind exclusively to VEGFR-1.16,17 VEGF-C and VEGF-D bind both VEGFR-2 and VEGFR-3/Flt4 and constitute a subgroup within the VEGF family characterized by N- and C-terminal extensions flanking the VEGF homology domain.4–11,18 VEGF-E binds selectively to VEGFR-2.13–15 Recently, a non–tyrosine kinase receptor, neuropilin-1 (NP-1), was identified to bind the VEGF165 isoform via its exon 7-encoded sequences.19 Also PIGF-2, VEGF-B, and VEGF-ES22 interact with NP-1.15,20,21 In response to VEGF165, NP-1 binding was shown to enhance the proliferation and migration of endothelial cells synergistically with VEGFR-2.19 However, so far, no data have been reported on possible effects of NP-1 in concert with the other receptor tyrosine kinases.

Differences between the phenotypes of the VEGF and VEGFR-2/Flik-1–deficient embryos have suggested the existence of another VEGFR-2 ligand.22–24 VEGF-C is expressed...
early in development\textsuperscript{25} and is capable of inducing endothelial, but not hematopoietic, cell differentiation of cells from the posterior mesoderm of gastrulation-stage quail embryos.\textsuperscript{26} Furthermore, VEGF-C has been shown to induce lymphangiogenesis when overexpressed in the basal layer of the epidermis, indicating an in vivo specificity toward VEGFR-3.\textsuperscript{27} On the other hand, recombinant VEGF-C also promotes angiogenesis when applied to early chorioallantoic membrane of chicks, to mouse cornea or to ischemic hindlimbs of rabbits.\textsuperscript{28,29} Its receptor, VEGFR-3, was also implicated in angiogenesis, because mouse embryos with a genetically targeted inactivation of VEGFR-3 died of cardiovascular failure at stage E10.5.\textsuperscript{5} These results indicate that VEGFR-3 plays an important role during blood vascular development before the formation of the lymphatic system. Thus, depending on the spatial and temporal expression patterns of the receptors, VEGF-C is likely to play a dual role both as an angiogenic and a lymphangiogenic growth factor.

Our aim with this study was to clarify the in vivo binding pattern of VEGF and VEGF-C during mouse development and in certain adult tissues. For comparison, the expression patterns of the VEGFRs and NP-1 were determined by in situ hybridization. The results lead us to conclude that in adult tissues, in which VEGFR-3 is largely confined to the lymphatic vasculature, the binding of VEGF-C occurs preferentially to this receptor, whereas in the developing embryos the binding sites include almost all vessel structures.

**Materials and Methods**

**Receptor-Binding Analysis Using Iodinated Growth Factors**

Recombinant human (rh) VEGF\textsubscript{165} (R&D systems) or the 21-kDa mature form of human VEGF-C (residues 103 to 215), produced and purified as described,\textsuperscript{30} were labeled with \textsuperscript{125}I using the Iodo-Gen reagent (Pierce), and the free iodine was removed by gel filtration in PD-10 columns (Pharmacia). The specific activities were $2.2 \times 10^5$ cpm/ng and $1.0 \times 10^5$ cpm/ng for rhVEGF and rhVEGF-C, respectively. The iodinated growth factors were tested for specific binding using NIH-VEGFR-1 and PAE-VEGFR-3 cells.\textsuperscript{17,30} Samples of mouse tissues were frozen immediately and kept at $-70 \degree$C. Frozen sections were cut at 7 \textmu m from E8.5 and E12.5 mouse embryos, adult lymph nodes, and heart. The sections were mounted onto silane-coated slides and stored in airtight boxes at $-70 \degree$C. After thawing, the sections were incubated for 30 minutes at room temperature in the blocking solution (MEM [GIBCO], 0.5 mg/mL BSA, 20 mmol/L HEPES [pH 7.4], 1 mmol/L PMSF, and 4 \mu g/mL leupeptin). The blocking buffer was then removed and the sections were covered by a droplet of the same buffer containing 10 pmol/L \textsuperscript{125}I-labeled rhVEGF or 10 pmol/L \textsuperscript{125}I-labeled rhVEGF-C. To define nonspecific binding, adjacent sections were incubated in the same concentration of iodinated growth factor in the presence of 1 nmol/L of the corresponding nonlabeled growth factor. Cross-competition of
binding was assessed in the presence of 1 nmol/L rhVEGF-C for 

de-VEGF or 1 nmol/L rhVEGF for 
ed-VEGF-C binding. After a 90-minute incubation in a humidified chamber at room temperature, the sections were rinsed for 5×3 minutes on ice, once with the binding buffer and 4 times with PBS. Sections were then fixed for 10 minutes in 2% paraformaldehyde, 2% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.4), rinsed for 2 to 5 seconds in dH2O, and dried at room temperature for 2 hours. The dried sections were covered with NTB-2 emulsion (Eastman Kodak Co.) and stored at 4°C for 2 weeks, developed, and stained.

In Situ Hybridization

In situ hybridization was performed for sections of E8.5 and E12.5 mouse embryos and adult lymph node. The samples were fixed with 4% paraformaldehyde for ×20 hours before dehydration and paraffin embedding. The mouse VEGF-C antisense RNA probe was generated as described. Radiolabeled RNA was synthesized using T7 RNA-polymerase and 

dS-labeled UTP (Amersham). Mouse VEGFR-3 antisense and sense RNA probes were synthesized in a similar manner from linearized pGEM-3Z(f+) plasmid containing the previously described VEGFR-3 cDNA insert. Mouse VEGFR-2 RNA probe was made from linearized pGEM-3Z(f+) plasmid containing an EcoRI fragment covering bp 1958 to 2682, kindly provided by Dr. J. Rossant (Samuel Lunenfeld Research Institute, Mount Sinai Hospital, Toronto, Ontario, Canada). VEGFR-1 antisense and sense probes were generated from linearized pCR2.1-TOPO plasmids containing bp 1438 to 2090. The template for murine NP-1 probe containing bp 1774 to 2936 of the mNP-1 (GenBank accession No. d50086; a kind gift from Dr. Hajime Fujisawa, Division of Biological Science, Nagoya University, Japan) was generated by polymerase chain reaction and cloned into pCR2.1-TOPO vector (Invitrogen) in both orientations. Antisense and sense probes were generated using T7 polymerase. For the embryo sections, the high-stringency wash was for 45 minutes at 65°C in 4× SSC containing 30 mmol/L DTT. For adult sections, the high-stringency wash was for 60 minutes at 65°C in 4× SSC containing 30 mmol/L DTT for the VEGF-C probe and in 2× SSC containing 30 mmol/L DTT for the VEGFR-3 probe. The slides were exposed for 28 days except for VEGFR-1 and NP-1, which were exposed for 35 days, developed, and stained with hematoxylin.

Results

Distribution of VEGF-C and VEGF Binding Sites in Mouse Embryos

As shown for the E8.5 embryos in Figure 1A and 1D, iodinated VEGF-C bound to the head mesenchyme (m) and extraembryonally to the endothelium of the venous lacunae (vl), the venous sinusoids (s), and the trophoblastic giant cells (arrowheads) partially fused to the Reichert membrane. The ectoplacental cone (arrow) and amnion (am) were devoid of signal (Figure 1A and 1D and data not shown). These patterns are coincident with the distribution of the VEGFR-3 mRNA at this developmental time point.25,31 The other receptor for VEGF-C, VEGFR-2, was also expressed in the yolk sac (ys), amnion, and allantois (al) (Figure 1E, Figure 2A and 2B, and data not shown). Within the embryo, the VEGFR-2 mRNA was detected in putative angioblasts of the head mesenchyme (m) and in the endocardium (e). Interestingly, although VEGF-C binding was intense in the endothelium of the venous lacunae, the surrounding cells were devoid of VEGF-C transcripts as analyzed by in situ hybridization. Cross-competition of the VEGF-C receptor binding signal with excess nonradioactive VEGF did not result in significant differences of the VEGF-C binding pattern, whereas excess nonradioactive VEGF-C abolished all binding (data
not shown). These results, together with the obtained binding pattern of VEGF-C extraembryonally, support the conclusion of a preferential binding of this factor to VEGFR-3.

The VEGF binding sites in E8.5 embryos were also localized to the head mesenchyme and extraembryonally to sites of VEGF-C binding, although to a lesser extent to the venous lacunae than for VEGF-C (Figure 1B). VEGF bound strongly to the proximal part of the ectoplacental cone (arrow) where VEGFR-1 was expressed (Reference 33 and data not shown). VEGF binding sites were also found in the amnion and part of the yolk sac, in structures that were positive for VEGFR-2, VEGFR-1, and NP-1 mRNAs (Figure 1E through 1G and Figure 2A through 2F). At this stage, VEGFR-1, like VEGFR-2, was expressed in the head mesenchyme, dorsal aorta, endocardium, and vascular structures of the allantois (Figure 2A through 2D). The mRNA for NP-1 was particularly abundant in the amnion, allantois, yolk sac, and head mesenchyme (Figures 1G and 2E and 2F). Unlike for VEGF-C, no binding of VEGF was observed to the trophoblastic giant cells. Cross-competition with nonradioactive VEGF-C did not affect the VEGF binding patterns obtained, whereas nonradioactive VEGF blocked all binding sites (Figure 1C).

Binding of radiolabeled VEGF-C to E12.5 embryos was found to occur in the subcutaneous vascular plexus and capillaries of the developing brain and neck regions (Figure 3A). The most striking signal, however, originated from the mesenchyme of the metanephric area (mn in Figure 3C). Silver grains also accumulated in the tongue, in the nasopharyngeal and jugular (j) regions, in the intervertebral vessels (iv), and in the umbilical cord (uc) (Figure 3A). These patterns coincide with VEGFR-3 expression in E12.5 embryos.

Figure 3. Binding of radiolabeled VEGF-C and VEGF and expression of VEGFR-3 mRNA in E12.5 embryos analyzed by in situ hybridization. A, Binding of iodinated VEGF-C to the metanephric area, intervertebral vessels (iv), corpus striatum, and cerebral artery. B, VEGF binding to the heart, the umbilical cord, the intersomitic vessels (iv), and the cerebral artery. C through F, Magnifications of A and B. C, VEGF-C binding to the metanephric area. D, VEGF binding to the metanephric area. E, Heart shows VEGF-C binding to the ventricular myocardium (ve). F, Intensive VEGF binding to the ventricular and atrial (at) myocardium and the endocardium (arrow). G, Faint VEGF-3 signal is detected in the epicardium. H, Bright-field image of the same section. mn indicates metanephric area; cs, corpus striatum; ca, cerebral artery; h, heart; uc, umbilical cord; ve, ventricular myocardium; j, jugular area; lu, lung; ct, endocardial cushion tissue; and ep, epicardium. A and B, bar=1 mm; C through F, bar=0.1 mm; G and H, bar=0.1 mm.
central nervous system and the heart (Figure 4A and Reference 31). VEGF-C bound to the main cerebral artery (ca) and to the brain capillaries and the corpus striatum (cs) (Figure 3A), which also expressed VEGFR-2 (Figure 4A). In the developing heart, the epicardium (ep, in Figure 3E) contained abundant binding sites for VEGF-C, whereas the endocardium, which expressed VEGFR-1, VEGFR-2, and NP-1 (Figure 2A, 2C, and 2E) but not VEGFR-3 (Figure 3G and 3H), did not give a VEGF-C binding signal. The atrium (at) and the endocardial cushion tissue (ct) were negative (Figure 3E). Most of the binding sites for VEGF-C in embryonic tissues overlap with sites of VEGF-3 mRNA distribution, suggesting that binding of VEGF-C mainly occurs to VEGFR-3.

VEGF bound to the vascular network of several developing organs in the E12.5 embryos, such as the lung (lu), the intestine, and most prominently the heart (h) (Figure 3B). Intense binding was localized to the intersomitic vessels, umbilical vessels, and cerebral artery. Smaller capillaries in the neck, spinal cord, brain, tongue, and nasopharyngeal area showed specific VEGF binding. In general, at this stage, the binding patterns were coincident with VEGFR-1 and VEGFR-2 in situ hybridization signals, which occurred in almost all vessels, although VEGFR-2 signals were more prominent in the capillaries throughout the embryo (Figure 4A and 4B). The VEGF binding to the heart was intense in the ventricle (ve) and the atrium, whereas the endocardial cushion tissue was negative. The VEGF binding signal was strong in the endocardium and myocardium, where it bound to myocardial vessels and to cardiac myocytes (Figure 3F). Notably, VEGFR-1, VEGFR-2, and NP-1 transcripts were all present in the heart and in the choroid plexus (cp) (Figure 4A through 4D). Strong expression of NP-1 was found in the developing spinal cord (sp) (Figure 4C).
VEGF-C and VEGF Binding Sites in Adult Lymph Nodes and Heart

In the lymph nodes, VEGF-C binding was localized to the paracortical area around the germinal centers (gc), where the efferent lymph vessels emerge (Figure 5A and 5B) and where VEGFR-3 is expressed as demonstrated by in situ hybridization (Figure 5C and 5D). The germinal centers of the lymph nodes, containing actively proliferating B cells, were negative for VEGF-C binding as well as for VEGFR-3 mRNA. Addition of an excess of nonradioactive VEGF-C to the binding assay abolished the signals (data not shown). VEGF binding sites were restricted to the vessels of the medullary region (mr) (Figure 5E and 5F), and addition of excess nonradioactive VEGF abolished the specific binding (data not shown).

In the adult heart, iodinated VEGF-C bound to extended, irregular, and collapsed vessels in the epicardium (arrows), which on the basis of their morphology and lack of red blood cells could be identified as lymphatic vessels (Figure 6A and 6B and data not shown). Very few smaller vessels and

Figure 5. A through F, Binding of radio-labeled VEGF-C and VEGF to sections of adult lymph node, compared with the localization of VEGFR-3 transcripts. A and B, VEGF-C binding to the paracortical areas shown in darkfield and bright-field photographs. C and D, VEGFR-3 mRNA expression in the paracortex, detected by in situ hybridization (ish). E and F, VEGF165 binding to the vessels of the medullary region. gc indicates germinal center, and mr, medullary region. Bar=0.1 mm.

Figure 6. Binding of VEGF-C and VEGF to sections of adult heart. ep indicates epicardium; my, myocardium; and en, endocardium. A and B, VEGF-C bound to the epicardium (arrow). Inset shows the result of a blocking experiment with nonradioactive VEGF-C. C and D, VEGF165 binding to vessels in the myocardium (arrows) and to smaller capillaries; also, the endocardium contains binding sites. Bar=0.1 mm.
capillaries in the myocardium (my) bound VEGF-C weakly, and no binding occurred to the endocardium. The endothelium of the heart valves exhibited a discernible amount of bound VEGF-C (data not shown). Unlike VEGF-C, VEGF bound to more regular, round-shaped vessels in the myocardium (arrows) and, to a greater extent, to the myocardial capillaries (Figure 6C and 6D). The endocardial endothelium, as well as the heart valves, exhibited specific signals. Again, the specificity of the binding was evident from nonradioactive ligand competition experiments (inset, Figure 6A and 6B, and data not shown).

Discussion
This study demonstrates the in vivo localization of receptors for VEGF-C and VEGF in mouse embryos and in some adult organs and gives insight into the roles of these 2 growth factors during vascular development and in adult vessels.

The binding patterns of VEGF-C and VEGF were similar in tissues of E8.5 embryos, but some differences were found in the distribution of binding sites extraembryonally. The binding patterns were in good correlation with the localization of the VEGFR mRNAs. VEGF-C, but not VEGF, bound to the giant cells partially fused to the Reichert membrane, where VEGFR-3 and VEGFR-2 are expressed, whereas VEGFR-1 was not detected in this area. In contrast, binding of VEGF but not VEGF-C was detected in the amnion, perhaps explained by the presence of VEGFR-1, VEGFR-2, and NP-1 and the absence of VEGFR-3 (our unpublished data and References 31 and 33). Some binding sites for VEGF-C were found in the yolk sac, where VEGFR-1, VEGFR-2, VEGFR-3, and NP-1 are expressed (our unpublished data; see also Figure 2). VEGF-C did not bind to the ectoplacental cone, whereas VEGF bound to its proximal but not to its distal part. The similarity of the VEGF and VEGF-C binding patterns reflect the coexpression of the receptors at this stage, and the inability of the factors to cross-compete for binding indicate that VEGF-C bound preferentially VEGFR-3 and that main binding of VEGF occurs to VEGFR-1. However, we cannot exclude the presence of an as-yet-unknown receptor for VEGF-C.

Jakeman et al.14 have performed a VEGF binding assay similar to the study described here, on rat sections, and the estimated receptor affinities were found to be in agreement with VEGFR-1 binding (KD=16 to 35 pmol/L). As VEGF is known to have a 5- to 10-fold higher affinity to VEGFR-1 than to VEGFR-2,35,36 the binding at the concentrations used also in our studies probably occurs preferentially to VEGFR-1. However, the intense VEGF binding pattern observed in E12.5 embryos, especially in the heart, cannot fully be explained by binding to VEGFR-1 only. It is possible that the strong VEGF binding to cardiac myocytes can be attributed to NP-1, the receptor for the axonal chemorepellent semaphorin III.37,38 NP-1 has been reported to play a role in the development of the cardiovascular system and limbs in addition to its involvement in axonal guidance in the developing nervous system.39,40 In the mouse heart, NP-1 is expressed in the cardiac myocytes, endocardial cells, and blood vessels, in the latter presumably by both endothelial and mesenchymal cells.39 The fact that no significant changes in the VEGF binding pattern were obtained on cross-competition with VEGF-C may be explained by the inability of VEGF-C to compete for the binding to VEGFR-1 and NP-1.

In the adult lymph nodes, VEGF-C binding sites were localized to the hilus and to the paracortex, a region occupied by T lymphocytes and rich in lymphatic sinuses that enter the lymph node. The lymphatic sinuses also gave an intense signal for VEGFR-3 mRNA. No VEGF-C binding occurred to the cortical region of the lymph node, which is a B lymphocyte–rich area. In contrast, VEGF bound to vessel structures in the medullar region. High endothelial venules, which are the main pathway for lymphocyte entry into the lymph nodes and extravasation into tissues, exist mainly in the paracortical area of the lymph node and have been found to be positive for VEGFR-3.31 From these data and previously published results, according to which preferential binding of VEGF-C occurred to lymphatic vessels and of VEGF to all discernible vessels in human skin,41 we can conclude that these growth factors bind selectively to endothelia adapted to serve specialized functions in differentiated vessels of adult organs.

In the adult heart, strong signals from the bound VEGF-C were localized to irregular and collapsed pericardial vessels, and only weak signals were obtained from the myocardial vessels. VEGFR-3 mRNA was expressed in the epicardial vessels but not in myocardial capillaries,42 suggesting that most of the binding of VEGF-C occurs to VEGFR-3 in adult heart. Labeled VEGF, on the other hand, bound to the endothelium of several types of vessels and to a higher extent to the myocardial capillaries. This suggests that VEGF binds to VEGFR-1 in the myocardial vessels, which have been reported to express this receptor, whereas both VEGFRs could be targets of binding in myocardial and epicardial capillaries in which they are coexpressed.43 NP-1, as discussed above for the E12.5 embryo, could also provide VEGF binding sites in the adult heart.

Taken together, our present results show that both VEGF-C and VEGF target embryonic blood vessels, whereas a more selective binding of VEGF-C occurs to its lymphatic vascular receptor in certain adult tissues. Our results help to explain why VEGF and VEGF-C have both overlapping and distinct activities via their endothelial receptors. Assessment of the binding sites and receptor expression should now also be carried out in pathological conditions, such as tissue ischemia.

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