Interactions of VEGF isoforms with VEGFR-1, VEGFR-2, and neuropilin in vivo: a computational model of human skeletal muscle

Feilim Mac Gabhann and Aleksander S. Popel

Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, Maryland

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Mac Gabhann F, Popel AS. Interactions of VEGF isoforms with VEGFR-1, VEGFR-2, and neuropilin in vivo: a computational model of human skeletal muscle. Am J Physiol Heart Circ Physiol 292: H459–H474, 2007. First published September 15, 2006; doi:10.1152/ajpheart.00637.2006.—The vascular endothelial growth factor (VEGF) family of cytokines is involved in the maintenance of existing adult blood vessels as well as in angiogenesis, the sprouting of new vessels. To study the proangiogenic activation of VEGF receptors (VEGFRs) by VEGF family members in skeletal muscle, we develop a computational model of VEGF isoforms (VEGF121, VEGF165), their cell surface receptors, and the extracellular matrix in in vivo tissue. We build upon our validated model of the biochemical interactions between VEGF isoforms and receptor tyrosine kinases (VEGFR-1 and VEGFR-2) and nonsignaling neuropilin-1 coreceptors in vitro. The model is general and could be applied to any tissue; here we apply the model to simulate the transport of VEGF isoforms in human vastus lateralis muscle, which is extensively studied in physiological experiments. The simulations predict the distribution of VEGF isoforms in resting (nonexercising) muscle and the activation of VEGF signaling. Little of the VEGF protein in muscle is present as free, unbound extracellular cytokine; the majority is bound to the cell surface receptors or to the extracellular matrix. However, interstitial sequestration of VEGF165 does not affect steady-state receptor binding. In the absence of neuropilin, VEGF121 and VEGF165 behave similarly, but neuropilin enhances the binding of VEGF165 to VEGFR-2. This model is the first to study VEGF tissue distribution and receptor activation in human muscle, and it provides a platform for the design and evaluation of therapeutic approaches.

vascular endothelial growth factor; cytokine; growth factor; mathematical model

Vascular endothelial growth factor (VEGF) is a critical family of cytokines involved in the process of angiogenesis (or neovascularization), the outgrowth of new microvessels from existing vasculature (30, 36). VEGF family members are diffusible secreted dimers that bind to and activate receptor tyrosine kinases on endothelial cells and stimulate proliferation and migration. They are also involved in the chemotactic guidance of the nascent sprouts. The involvement of VEGF in the angiogenic process identifies it as a target for proangiogenic therapy in skeletal muscle. The increase of muscle vascularization is a possible route for the delivery of oxygen is reduced because of the loss or partial loss of functional vasculature (15, 32). To study the effects of VEGF on muscle vascularization, we first investigate the behavior of VEGF and VEGF receptors (VEGFRs) in healthy, resting muscle.

Although the action of VEGF on cells in vitro has been extensively studied (30, 36), it is difficult to measure the same parameters (receptor activation, for example) in vivo. Here, we build a model of VEGF transport in skeletal muscle, to understand the distribution and availability of VEGF in human tissue, as well as the activation of VEGFR-1 and VEGFR-2, the primary receptor tyrosine kinases for VEGF on blood microvascular endothelial cells. The VEGF family consists of four genes in humans, and of these VEGF-A is the best studied. Each of the genes encodes multiple splice isoforms that can generate proteins of varying lengths. The two most abundant isoforms of VEGF-A are VEGF121 and VEGF165. The additional 44 amino acids of the longer isoform encode a domain that binds neuropilin-1 as well as a heparin-binding domain that allows the extracellular matrix (ECM) to sequester that isoform (while VEGF121 remains freely diffusible).

The complexity of the interactions between the various members of the VEGF family and their receptors (including ligand-independent receptor-receptor coupling interactions) is difficult, if not currently impossible, to analyze without the benefit of a computational model. This is especially true in vivo, where it is difficult to make many of the measurements (in regard to receptor binding, for example) that can be performed in vitro. The model takes the measurements that can be made, and a detailed kinetic network, and predicts the concentrations of molecular species that cannot currently be experimentally measured. In addition to better understanding physiological behavior, establishing a detailed model of the skeletal muscle VEGF system would allow us to test therapeutic interventions in silico.

We have previously published models of the interactions between VEGF family members and VEGFR-1 and VEGFR-2 in vitro (19, 20), as well as a validated model of the biochemical interaction network of VEGF121, VEGF165, VEGFR-2, and neuropilin-1 (18). That study demonstrated that simulations of the interactions between VEGF165, VEGFR-2, and neuropilin agreed with all published results of VEGF165 and VEGF121 binding to and activation of VEGFR-2 on endothelial cells. Here we extend that interaction network to in vivo tissue, by including the ECM as well as multiple cell types (myocytes and endothelial cells) and the geometry of the tissue.

The model is general and could be applied to any tissue. In this study we focus on the human vastus lateralis muscle, widely studied in physiological investigations, particularly in relation to the effects of exercise on muscle vascularization (4, 8, 9, 33). Despite uncertainty in the values of some of the parameters, we can make several conclusions about the in vivo functioning of the VEGF system.

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METHODS

Glossary

- $[V_{121}]$: Concentration of unbound VEGF$_{121}$ or VEGF$_{165}$ in the interstitial space
- $[V_{165}]$: Concentration of unbound glycosaminoglycan (GAG) chains in ECM, endothelial basement membrane (EBM), or myocyte basement membrane (MBM)
- $[M_{ECM}]$: Concentration of unbound VEGF$_R$-1, VEGF$_R$-2, or neuropilin-1 on endothelial cell surface
- $[M_{EBM}]$: Concentration of unbound VEGF bound to GAG chains
- $[M_{MBM}]$: Concentration of cell surface VEGF$_{165}$ bridging VEGF$_R$-2 and neuropilin
- $q_{V,m}$: Secretion rate of VEGF from myocytes
- $k_{on}$, $k_{off}$: Kinetic rates of binding and unbinding
- $k_c$: Coupling rate of neuropilin-bound or VEGF$_R$-bound VEGF to a second receptor
- $k_{disso}$: Dissociation rate of two receptors
- $s_{R1, R2, N1}$: Insertion rate of new receptors into the membrane
- $k_{int}$: Internalization rate of receptors

Development of Computational Model

VEGF transport and binding. We constructed a model of VEGF transport and interactions with its receptors in skeletal muscle in vivo (Fig. 1). The skeletal muscle is composed of three primary elements: myocytes (muscle fibers), blood vessels, and the interstitial space between them. This interstitial space contains a proteoglycan-based matrix and is divided into three regions: the ECM and two denser basement membranes, one surrounding the myocytes (MBM) and the other on the abluminal surface of the endothelial cells of blood vessels (EBM). VEGF is secreted by myocytes and binds to cell surface receptors on endothelial cells; in the case of VEGF$_{165}$, it may also be sequestered by VEGF binding sites in the ECM and basement membranes.

The signaling ligand-receptor complexes formed by VEGF$_{121}$, VEGF$_{165}$, and their receptors are shown in Fig. 1, B and C, along with the interactions between the ligands and receptors. Both VEGF$_{121}$ and VEGF$_{165}$ bind to VEGFR-1 and VEGFR-2:

- $V_{121} + R_1 ⇄ V_{121}R_1$
- $V_{165} + R_1 ⇄ V_{165}R_1$
- $V_{165} + R_2 ⇄ V_{165}R_2$

In addition, VEGF$_{165}$ binds to neuropilin-1:

- $V_{165} + N_1 ⇄ V_{165}N_1$

VEGF$_{165}$-VEGF$_R$-2 and VEGF$_{165}$-neuropilin-1 can diffuse laterally on the cell membrane, and VEGF$_{165}$ binds to the complementary receptor to couple VEGF$_R$-2 and neuropilin-1 together:

- $V_{165}R_2 + N_1 ⇄ V_{165}R_1 N_1$
- $V_{165}N_1 + R_1 ⇄ V_{165}R_1 N_1$

While VEGF$_R$-2 and neuropilin-1 do not bind each other but are coupled by VEGF$_{165}$, VEGF$_{165}$ binds directly to neuropilin-1, and this complex is permissive for binding VEGF$_{121}$ but not VEGF$_{165}$:

- $R_1 + N_1 ⇄ R_1 N_1$
- $V_{165} + R_1 N_1 ⇄ V_{121}R_1 N_1$
- $V_{121}R_1 N_1 + R_2 ⇄ V_{121}R_2 N_1$

The interactions between VEGF$_{165}$ and the interstitial binding sites are:

- $V_{165} + M_{ECM} ⇄ V_{165}M_{ECM}$
- $V_{165} + M_{EBM} ⇄ V_{165}M_{EBM}$
- $V_{165} + M_{MBM} ⇄ V_{165}M_{MBM}$

Computational model. We assume that the concentration of free (unbound) VEGF is uniform across the interstitial space. The Dankohler number (ratio of diffusion time to reaction time) is significantly less than 1, indicating that diffusion is significantly faster than the kinetics of binding to and unbinding from ECM glycosaminoglycan (GAG) chains or cell surface receptors. The possible formation of diffusion-limited VEGF gradients will thus not be studied here; instead our model represents average VEGF in the interstitium and average receptor binding on the cell surface. Spatial variability in binding due to gradient formation may be important for angiogenic signaling in vivo (17) but is not dealt with here. Thus the system is described by a set of coupled nonlinear ordinary differential equations. For interstitial proteins these equations are as follows:

\[
\begin{align*}
\frac{d[V_{121}]}{dt} &= q_{V_{121,m}} - k_{on,VR}[V_{121}] \cdot [R_1] + k_{off,VR}[V_{121}R_1] - k_{on,VR2}[V_{121}] \cdot [R_2] + k_{off,VR2}[V_{121}R_2] - k_{on,VR1}[V_{121}] \cdot [R_1 N_1] + k_{off,VR1}[V_{121}R_1 N_1] \\
\frac{d[V_{165}]}{dt} &= q_{V_{165,m}} - k_{on,VM}[V_{165}] \cdot [M_{ECM}] + k_{off,VM}[V_{165} M_{ECM}] - k_{on,VM}[V_{165}] \cdot [M_{EBM}] + k_{off,VM}[V_{165} M_{EBM}] \\
&\quad - k_{on,VM}[V_{165}] \cdot [M_{MBM}] + k_{off,VM}[V_{165} M_{MBM}] - k_{on,VR}[V_{165}] \cdot [R_1] + k_{off,VR}[V_{165} R_1] \\
&\quad - k_{on,VR2}[V_{165}] \cdot [R_2] + k_{off,VR2}[V_{165} R_2] - k_{on,VM}[V_{165}] \cdot [N_1] + k_{off,VM}[V_{165} N_1] \\
\frac{d[M_{ECM}]}{dt} &= - k_{on,VM}[V_{165}] \cdot [M_{ECM}] + k_{off,VM}[V_{165} M_{ECM}] \\
\frac{d[M_{EBM}]}{dt} &= - k_{on,VM}[V_{165}] \cdot [M_{EBM}] + k_{off,VM}[V_{165} M_{EBM}] \\
\frac{d[M_{MBM}]}{dt} &= - k_{on,VM}[V_{165}] \cdot [M_{MBM}] + k_{off,VM}[V_{165} M_{MBM}] \\
\frac{d[V_{165} M_{ECM}]}{dt} &= k_{on,VM}[V_{165}] \cdot [M_{ECM}] - k_{off,VM}[V_{165} M_{ECM}] \\
\frac{d[V_{165} M_{EBM}]}{dt} &= k_{on,VM}[V_{165}] \cdot [M_{EBM}] - k_{off,VM}[V_{165} M_{EBM}] \\
\frac{d[V_{165} M_{MBM}]}{dt} &= k_{on,VM}[V_{165}] \cdot [M_{MBM}] - k_{off,VM}[V_{165} M_{MBM}]
\end{align*}
\]
where \( q \) is secretion rate and \( k_{on} \) and \( k_{off} \) are kinetic rates of binding and unbinding, respectively. Although we note VEGF as secreted from myocytes, VEGF secreted from other sources, e.g., endothelial cells, if significant, could be included in the same term by adding all the sources of VEGF together. For cell surface receptors we have the following equations:

\[
\frac{d[R_1]}{dt} = s_{R1} - k_{on,R1}[R_1] - k_{on,VR1}[V_121] \cdot [R_1] + k_{off,VR1}[V_121] \cdot [R_1] + k_{off,VR}[V_{165}] \cdot [R_1] - k_{on,VR1}[V_{165}] \cdot [R_1] - k_{off,VR}[V_{165}] \cdot [R_1] - k_{dissoc}[R_1][N_1] + k_{dissoc}[R_1][N_1]
\]

\[
\frac{d[R_2]}{dt} = s_{R2} - k_{on,R2}[R_2] - k_{on,VR2}[V_121] \cdot [R_2] + k_{off,VR2}[V_121] \cdot [R_2] + k_{off,VR}[V_{165}] \cdot [R_2] + k_{off,VR2}[V_{165}] \cdot [R_2] - k_{on,VR2}[V_{165}] \cdot [R_2] - k_{off,VR2}[V_{165}] \cdot [R_2] - k_{dissoc}[R_2][N_1] + k_{dissoc}[R_2][N_1]
\]

\[
\frac{d[N_1]}{dt} = s_{N1} - k_{on,N1}[N_1] - k_{on,VPN}[V_{165}] \cdot [N_1] + k_{off,VPN}[V_{165}][N_1] - k_{on,VR2,N1}[V_{165}][R_2] \cdot [N_1] + k_{off,VR2,N1}[V_{165}][R_2] \cdot [N_1] + k_{dissoc}[V_{165}][N_1][R_1]
\]

Each of the VEGF binding sites—cell surface receptors as well as interstitial GAG chains—comes into equilibrium with the VEGF concentration in the interstitium when the system has reached steady state. The coupled set of ordinary differential equations is solved for appropriate values of the parameters (below) to find the steady-state concentrations of all molecular species. Steady state here represents resting muscle; the effects of the exercising muscle on the dynamics of VEGF secretion and transport will not be considered here.

**Model Parameters**

The parameters required for simulation of this model fall into three categories: geometry, kinetic rates, and initial concentrations; they are given in Tables 1–3.

**Geometry.** The model is general and applicable to any tissue. In this study, we use the geometric parameters typical for skeletal muscle, in particular human vastus lateralis muscle, a tissue extensively studied in human physiologic experiments, particularly those involving exercise, as summarized in Table 1. The extracellular water fraction of this tissue is taken to be 8.4% (cm^3/cm^3), based on 7.9 ml/100 g wet muscle (16, 38) and a muscle density of 1.06 g/ml (23, 35). Of this, the capillary blood volume is taken to be 1.4% (cm^3/cm^3) (34).

The capillary-to-fiber ratio in human vastus lateralis has been measured as 1.38 and the cross-sectional area of fibers as 5,232 \( \mu m^2 \) for sedentary men (8), although muscle fiber size and capillary density in the vastus lateralis vary with age, sex, and exercise training (4, 8, 9, 33, 42). However, to match the measured vascular space, this gives a characteristic capillary diameter of 8.6 \( \mu m \) and a microvascular density of 240 capillaries/mm^2 (when adjusted for extracellular space). The capillary size is larger than measured capillary diameters (5–7.5 \( \mu m \)) in human skeletal muscle (11, 41), while the density is too low to deliver required oxygen to the tissue according to the analysis of McGuire and Secomb (22). Thus we assume that the fiber cross-sectional area was overestimated because of muscle contraction and other artifacts of tissue handling (as suggested in Ref. 22) but maintain the measured capillary-to-fiber ratio. From this, we calculate the capillary density and size based on the vascular volume of 1.4% (cm^3/cm^3). For example, for a fiber cross-sectional area of 3,500 \( \mu m^2 \), the microvascular density would be 360 capillaries/mm^2, with a 7-\( \mu m \) characteristic diameter; for 3,000-\( \mu m^2 \) fiber area, the microvascular density would be 360 capillaries/mm^2, with a 7-\( \mu m \) characteristic diameter. We take the latter value. This diameter is consistent with measurements of capillary diameter (11, 41), and the density is consistent with the oxygen consumption analysis (22).

The nonextracellular volume is composed of the skeletal muscle fibers and the endothelial cells themselves. The endothelial cells are assumed to have a thickness of 0.5 \( \mu m \) (consistent with measurements; Ref. 11) surrounding the vessels and thus occupy a volume of 0.46% (cm^3/cm^3). The remaining volume—91.14% (cm^3/cm^3)—is composed of fibers.

The muscle fibers are not cylindrical. The measured perimeter of the fibers is \( \sim 14 \)% greater than that predicted for a cylinder of equivalent cross-sectional area (8). Thus, in calculating the surface area of the fibers, a correction of 1.14 is applied to the cylindrical surface area determined from the volume fraction of fibers. The fibers have a perimeter of 222 \( \mu m \), and the surface area of fibers per unit volume is 674 cm^2 fiber surface/cm^3 tissue.

Similarly, the blood vessels are not cylindrical. The abluminal surface area of capillaries is affected by the elliptical shape of the vessel and bulging caused by the nucleus. As an upper limit, the perimeter to cross-sectional area ratio has been measured for tumor microvessels (which have been noted to be more irregular than “normal” muscle vessels) as 1.23 (28, 29). For the muscle microvessels, we assume a surface area correction of 10% (1.10), and the surface area of microvessels per unit volume is 109 cm^2 endothelial cell surface/cm^3 tissue. The results of our simulations are not significantly affected by a change in the surface area correction (data not shown).

Both the vessels and the muscle fibers have associated basement membranes. The thickness of these was measured in resting human vastus lateralis as 42 and 24 \( \mu m \), respectively (21). These thicknesses are used to calculate the volumes of each type of basement membrane in the tissue (Table 1). The remaining interstitial space is designated as ECM.
Muscle fibers are long, multinucleate cells. We divide these into smaller structures (similar to mononuclear cell size) by applying the concept of the myonuclear domain (MD), the volume of the fiber under the control of a single nucleus (1). More specifically for this study of cell surface receptors, we use the term to refer to the surface area of the fiber cell membrane under the control of each nucleus. Secretion from the fibers is thus given in units of molecules per MD per second, rather than molecules per fiber per second. One study of nuclear density in human vastus lateralis gave a MD of $1,100 \mu m^2$, with 94 nuclei per millimeter of length of a fiber of cross section 3,600 $\mu m^2$ and a perimeter correction of 15% (elderly male subjects) (12). Soleus muscles of healthy human males, with 150 nuclei/mm and cross-sectional areas of 2,000 $\mu m^2$, have a MD of $\sim 1,200 \mu m^2$ (27).

We assume that young, healthy male vastus lateralis would have higher density of nuclei (120/mm) and calculate a MD of $1,850 \mu m^2$.

These geometric parameters are incorporated into the kinetic parameters and concentrations of the model (Tables 1–3), and the results are therefore tissue specific. Tissue geometry changes result in changes to the kinetic parameters and concentrations in the model and affect predictions of both the VEGF distribution and VEGFR occupancy.

**Kinetics.** The kinetic rates for VEGF isoform binding to and unbinding from VEGFRs and neuropilins are based on experimental measurements and similar to those used in previous models (18) and are summarized in Table 2. The ECM binding sites for VEGF are a diverse group of proteins and proteoglycans with different affinities.

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**Fig. 1.** Vascular endothelial growth factor (VEGF) transport in skeletal muscle. **A:** the tissue is characterized by long muscle fibers of (approximately) constant cross section and a capillary network in the interstitial space between the fibers. VEGF is secreted by the muscle fibers into the interstitial space, which is composed of 3 sections: the basement membranes (BM) surrounding the muscle fibers and blood vessels and the extracellular matrix (ECM). VEGF$_{165}$ binds to (and is sequestered by) glycosaminoglycan (GAG) chains in the interstitial space; VEGF$_{121}$ does not. Both isoforms bind to the cell surface signaling receptors, although the pattern of binding is different for the 2 isoforms. **B** and **C:** VEGF isoform interactions with VEGF receptor (VEGFR)-1 (**C**), VEGFR-2 (**B**), and neuropilin-1. VEGF$_{121}$ and VEGF$_{165}$ are the 2 most abundant isoforms of VEGF in human tissue. Both isoforms bind VEGFR-1 and VEGFR-2, receptor tyrosine kinases. Only VEGF$_{165}$ binds to neuropilin-1 and to the GAG chains on the proteoglycans of the ECM and BM. VEGF$_{165}$ can bind to both VEGFR-2 and neuropilin-1 simultaneously and thus can couple these receptors together on the endothelial cell surface (with rate $k_c$). VEGFR-1 and neuropilin-1 interact directly, and this complex is permissive for VEGF$_{121}$ binding to VEGFR-1 but not VEGF$_{165}$.
Table 1. Geometric parameters of human vastus lateralis muscle

<table>
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<tr>
<th>Skeletal Muscle Characteristic</th>
<th>Value</th>
<th>Unit</th>
<th>Reference</th>
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<tr>
<td>Muscle fibers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cross-sectional area of 1 fiber</td>
<td>3,000</td>
<td>µm²</td>
<td>See text</td>
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<tr>
<td>Perimeter of 1 fiber</td>
<td>222</td>
<td>µm</td>
<td>See text</td>
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<tr>
<td>Capillary-fiber ratio</td>
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<td>Capillary density</td>
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<tr>
<td>Muscle fiber density</td>
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<td>Fibers/mm² tissue</td>
<td>Calculated</td>
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<td>Volume fractions</td>
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<td></td>
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<tr>
<td>Interstitial space</td>
<td>7%</td>
<td>cm³/cm³ tissue</td>
<td>16, 38</td>
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<td>Fibers</td>
<td>91.14%</td>
<td>cm³/cm³ tissue</td>
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<tr>
<td>Microvessels</td>
<td>1.86%</td>
<td>cm³/cm³ tissue</td>
<td>Calculated</td>
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<tr>
<td>Of which vascular space</td>
<td>1.4%</td>
<td>cm³/cm³ tissue</td>
<td>34</td>
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<td>Microvessels</td>
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<tr>
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<td>6.52</td>
<td>µm</td>
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<td>Thickness of endothelial cell</td>
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<td>µm</td>
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<td>Cross-sectional area of 1 microvessel</td>
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<tr>
<td>Perimeter of 1 microvessel</td>
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<td>Surface areas</td>
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<td>Muscle fibers</td>
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<td>cm²/cm³ tissue</td>
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<tr>
<td>Microvessels</td>
<td>109</td>
<td>cm²/cm³ tissue</td>
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<td>Basement Membranes (BMs)</td>
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<tr>
<td>Thickness of muscle fiber BM</td>
<td>24</td>
<td>nm</td>
<td>21</td>
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<tr>
<td>Basement membrane volume (muscle fiber)</td>
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<td>Thickness of microvessel BM</td>
<td>43</td>
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<td>BM volume (muscle fiber)</td>
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<td>Extracellular matrix volume</td>
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<td>Myonuclear domain surface area</td>
<td>1,850</td>
<td>µm²</td>
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Table 2. Kinetic parameters of VEGF system

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<th>Parameter</th>
<th>Measured Parameter</th>
<th>Value</th>
<th>Unit</th>
<th>Tissue Model</th>
<th>Value</th>
<th>Unit</th>
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<tbody>
<tr>
<td>VEGF binding to VEGFR-1</td>
<td>k_on</td>
<td>3×10⁷</td>
<td>M⁻¹ s⁻¹</td>
<td>4.3×10⁻¹</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k_off</td>
<td>10⁻³</td>
<td>s⁻¹</td>
<td>2.3×10⁻³</td>
<td>pmol/cm³ tissue</td>
<td></td>
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<tr>
<td>VEGF binding to VEGFR-2</td>
<td>k_on</td>
<td>10⁷</td>
<td>M⁻¹ s⁻¹</td>
<td>1.4×10⁻¹</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
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</tr>
<tr>
<td></td>
<td>k_off</td>
<td>10⁻³</td>
<td>s⁻¹</td>
<td>7.0×10⁻³</td>
<td>pmol/cm³ tissue</td>
<td></td>
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<tr>
<td>VEGF₁₆₅ binding to NRP-1</td>
<td>k_on</td>
<td>3.2×10⁶</td>
<td>M⁻¹ s⁻¹</td>
<td>4.6×10⁻²</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k_off</td>
<td>10⁻³</td>
<td>s⁻¹</td>
<td>2.2×10⁻²</td>
<td>pmol/cm³ tissue</td>
<td></td>
</tr>
<tr>
<td>VEGF₁₆₅ binding to GAGs</td>
<td>k₁₀</td>
<td>4.2×10⁵</td>
<td>M⁻¹ s⁻¹</td>
<td>6.0×10⁻³</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k₂₀</td>
<td>10⁻²</td>
<td>s⁻¹</td>
<td>1.7</td>
<td>pmol/cm³ tissue</td>
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<tr>
<td>Coupling of NRP-1 and VEGFR-2</td>
<td>k₁₁</td>
<td>3.1×10¹³</td>
<td>(mol/cm³)⁻¹ s⁻¹</td>
<td>2.8×10⁻¹</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
<td></td>
</tr>
<tr>
<td></td>
<td>k₂₁</td>
<td>10⁻³</td>
<td>s⁻¹</td>
<td>9.2×10⁻¹</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
<td></td>
</tr>
<tr>
<td>VEGF₁ coupling to NRP-1</td>
<td>k₁₁</td>
<td>10¹⁴</td>
<td>(mol/cm³)⁻¹ s⁻¹</td>
<td>9.2×10⁻¹</td>
<td>(pmol/cm³ tissue)⁻¹ s⁻¹</td>
<td></td>
</tr>
<tr>
<td>VEGFR internalization</td>
<td>k₁_{free}</td>
<td>2.8×10⁻⁴</td>
<td>s⁻¹</td>
<td>2.8×10⁻⁴</td>
<td>s⁻¹</td>
<td></td>
</tr>
</tbody>
</table>
Table 3. **VEGF concentration and receptor densities for skeletal muscle**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Measured Parameter</th>
<th>Value</th>
<th>Unit</th>
<th>Tissue Model</th>
<th>Value</th>
<th>Unit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Free VEGF concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human vastus lateralis, rest (13)</td>
<td>1</td>
<td>pM</td>
<td></td>
<td></td>
<td>7.0×10⁻⁵</td>
<td>pmol/cm³ tissue</td>
</tr>
<tr>
<td>Total VEGF tissue concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human vastus lateralis, rest (4, 8, 9, 33)</td>
<td>1–2</td>
<td>pg/µg protein</td>
<td>3.4–6.9</td>
<td>pmol/cm³ tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR1 tissue concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human vastus lateralis, rest (4, 9, 33)</td>
<td>1.6–1.8</td>
<td>pg/µg protein</td>
<td>1.1–1.2</td>
<td>pmol/cm³ tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VEGFR2 tissue concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human vastus lateralis, rest (4, 9, 33)</td>
<td>0.33–0.5</td>
<td>pg/µg protein</td>
<td>0.24–0.34</td>
<td>pmol/cm³ tissue</td>
<td></td>
<td></td>
</tr>
<tr>
<td>NRPI tissue concentration</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Not available; test a range</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.018–0.18</td>
<td>pmol/cm³ tissue</td>
</tr>
<tr>
<td>ECM binding site density</td>
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<td></td>
<td></td>
<td></td>
<td>1,000–100,000</td>
<td>No/EC</td>
</tr>
<tr>
<td>Vessel BM</td>
<td>0.75</td>
<td>µM</td>
<td></td>
<td></td>
<td>51</td>
<td>pmol/cm³ tissue</td>
</tr>
<tr>
<td>Myocyte BM</td>
<td>13</td>
<td>µM</td>
<td></td>
<td></td>
<td>6</td>
<td>pmol/cm³ tissue</td>
</tr>
<tr>
<td>Myocyte BM</td>
<td>13</td>
<td>µM</td>
<td></td>
<td></td>
<td>21</td>
<td>pmol/cm³ tissue</td>
</tr>
</tbody>
</table>

Conversion of receptor densities to tissue concentrations is based on relationship described in Table 2 and the surface area of an endothelial cell (EC; 1,000 µm²). VEGF concentration is normalized based on the entire interstitial space, because it diffuses throughout: 7×10¹⁰ (pmol/cm³ tissue)/(mol/l interstitial space). VEGF binding sites in extracellular matrix (ECM) and BMs are based on those volumes: 6.8×10¹⁵ (pmol/cm³ tissue)/(mol/l ECM), 4.6×10⁶ (pmol/cm³ tissue)/(mol/l EBM), and 1.6×10¹⁵ (mol/cm³ tissue)/(mol/l MBM), where EBM is endothelial BM and MBM is myocyte BM. The effect of tissue geometry is thus included in the model through both the concentrations (this table) and the kinetic parameters (Table 2). Conversions from pg/mg protein are based on 155 mg protein/g tissue (10) and 45-kDa VEGF, 210-kDa VEGFR, and 240-kDa VEGFR2. Numbers in parentheses are reference numbers.

An effective binding affinity is used for the ensemble of sites. VEGF binding affinity to the GAG chains on the proteoglycans of the ECM chains is of a similar order to that of bFGF, and therefore we use similar binding site density and affinity (7).

Concentrations. The mean concentration of unbound VEGF has been determined by microdialysis of resting human vastus lateralis to be 0.6–1.3 pM (13). This is consistent with microdialysis measurements of VEGF in normal breast tissue (0.4–0.8 pM; Ref. 5); other human tissues have yet to be investigated with this method. The concentration of VEGF in fluid (ascitic fluid can be assumed to be of similar composition to interstitial fluid; in cancer, the VEGF concentrations are consistent with VEGF measurements of extracted nonmalignant circulatory fluid: 2–3 pM (13)). This is consistent with microdialysis measurements of VEGF in normal breast tissue (0.4–0.8 pM; Ref. 5); other human tissues have yet to be investigated with this method. It is also consistent with VEGF measurements of extracted nonmalignant cirrhotic or tuberculous ascites: 2–3 pM (ascitic fluid can be assumed to be of similar composition to interstitial fluid; in cancer, the VEGF concentration in fluid can be up to a hundredfold higher; Refs. 6, 45). We assume that a steady-state concentration of 1 pM VEGF should be attained in our simulations. Two different isoforms of VEGF are studied here, freely diffusible VEGF₁₂₁ and matrix-binding VEGF₁₆₅, and we run each simulation twice, each assuming one isoform being secreted by itself. Muscle secretes both isoforms, and so the net behavior of VEGF in the muscle lies between the two extremes. We examine the effect of simultaneous secretion of the two isoforms in Fig. 11, described in **Simultaneous Secretion of VEGF Isoforms**. The 1 pM VEGF interstitial concentration is thus VEGF₁₂₁, VEGF₁₆₅, or the sum of the two, depending on the simulation.

Because there is uncertainty in some of the parameters, e.g., receptor densities, and because the secretion rate of VEGF from myocytes in vivo has not been measured, we use a different secretion rate for each simulation to maintain the 1 pM free VEGF concentration at steady state. The secretion rate is shown in the relevant figure for each simulation. Although VEGFR-1, VEGFR-2, and neuropilin have all been identified as being expressed on the endothelial cells of the vasculature, quantitative estimates of the receptor density in human vastus lateralis have been published only for VEGFR-1 and VEGFR-2 (4, 9, 33). For those receptors, the measured range is equivalent to 10,000–20,000 and 50,000–100,000 receptors per endothelial cell (based on 155 mg protein/g tissue; Ref. 10). In addition, these measured values do not discriminate between intracellular and cell surface receptors and, in the case of VEGFR-1, secreted receptors (we do not include soluble VEGFR-1 in this model; no information was available on the concentration in tissue). We assume that the number of receptors on each endothelial cell is 10,000 for VEGFR-1 and VEGFR-2, but we also examine several expression levels of all three receptors—starting with these estimates—to estimate the effect of receptor density on VEGF distribution.

**Model Solution and Simulations**

Simulations of VEGF transport in skeletal muscle were developed based on the model described above. The secretion rate for each simulation was adjusted to achieve a steady-state free (unbound) VEGF concentration of 1 pM. We consider the secretion of VEGF₁₂₁ and VEGF₁₆₅ separately for most simulations. The coupled ordinary differential equations were solved with an adaptive step-size Runge-Kutta integration scheme (5th-order accuracy) implemented in Fortran and run on a desktop PC.

**RESULTS**

**VEGF Internalization Capacity**

At steady state, secretion of VEGF must be balanced by the internalization by the receptors. The maximum internalization capacity, I_{max}, is determined by

\[ I_{max} = k_{int,C}[VR]_{max} \]

but since

\[ [VR]_{max} = [R]_{total}(k_{int,R}/k_{int,C}) \]

and

\[ [R]_{total} = s_Rk_{int,R} \]

then

\[ I_{max} = s_R \]

i.e., the total internalization capacity is equal to the insertion rate of receptors. The same conclusion would be arrived at from a mass balance on VEGFRs. The maximum secretion rate q_{max} must not exceed the maximum internalization rate, I_{max}. If
both $q_{\text{max}}$ and $I_{\text{max}}$ (or $s_R$) are expressed in tissue volume units (mol·cm$^{-3}$·tissue·s$^{-1}$), the numerical values are equal. Expressing $q_{\text{max}}$ and $s_R$ in “local” secretion or internalization units (molecules/cell/s) for this tissue,

$$q_{\text{max}} = \frac{\text{myocyte surface area per cm}^3 \text{ of tissue}}{\text{myonuclear domain surface area}}$$

$$= \frac{\text{microvessel surface area per cm}^3 \text{ of tissue}}{\text{endothelial cell surface area}}$$

$$= \frac{674 \text{ cm}^2/\text{cm}^3}{109 \text{ cm}^2/\text{cm}^3} = 6.15 \cdot 10^{-5} \text{ cm}^2$$

we obtain

$$q_{\text{max}} = 0.3s_R$$

$$q_{\text{max}} = 8.4 \cdot 10^{-3}[R_{\text{total}}]$$

where $s_R$ and $q_{\text{max}}$ are measured in molecules per endothelial cell per second and molecules per MD per second, respectively, and $R_{\text{total}}$ is measured in molecules per endothelial cell. If the secretion rate exceeds this upper bound, then no steady state can exist in the system and the concentration of VEGF will continue to build up over time. It is possible that this occurs in pathological situations, or for short times (e.g., during exercise), but in resting muscle we will assume that a balance will be maintained and that the secretion rate does not exceed the internalization capacity of the tissue. The effect of high VEGF secretion rate approaching the internalization capacity of the tissue is illustrated in Fig. 2, as the steady-state concentration of VEGF becomes asymptotically high.

**Secretion Rate of VEGF and Receptor Density Determine VEGF Concentration**

The free (unbound) VEGF concentration is determined by the balance between VEGF secretion and VEGF internalization (Fig. 2). For these simulations, only VEGFR-2 is expressed on the endothelial cells, and either VEGF$_{121}$ or VEGF$_{165}$ is secreted by the myocytes. Not all receptors are expressed on the vessels of each tissue, and thus the single-receptor case is relevant to certain tissues in addition to being informative in advance of the consideration of multiple receptor expression.

A higher secretion rate from the myocytes results in higher VEGF concentration; higher receptor density increases the binding of VEGF to the cell surface and subsequent internalization into the cell, and thus decreases interstitial VEGF. It is of particular note that for the same secretion rate the unbound interstitial VEGF is the same for both VEGF$_{121}$ and VEGF$_{165}$. Even though VEGF$_{165}$ binds to the proteoglycans of the ECM, this does not affect the unbound VEGF concentration at steady state. The total (free and GAG bound) interstitial VEGF$_{165}$ concentration is 47-fold higher (in the simulation of this tissue) than that of VEGF$_{121}$. Increasing proteoglycan (or GAG) density or affinity for VEGF (or increased basement membrane thickness) would increase the amount of VEGF$_{165}$ held in the matrix. It takes longer to establish a steady state for VEGF$_{165}$, as the matrix acts as a capacitor or reservoir for VEGF and must be “filled” before the steady state can be reached.

The similarity between the isoforms in this simulation can be understood by considering the transport of VEGF at steady state. The secretion of VEGF from the muscle fibers must be balanced by the internalization into the endothelial cells. In effect, we have a continuous flux through the system as VEGF is moved across the interstitial space. The ECM, being in equilibrium with the free VEGF, offers no significant impediment to the net transport across the gap. Because the binding affinities of VEGF$_{121}$ and VEGF$_{165}$ for VEGFR-2 are the same, the same concentration of each VEGF isoform is required to bind the same number of receptors; the receptor-bound VEGF population is then internalized.

The measured steady-state free VEGF concentration in resting human vastus lateralis is 0.6–1.3 pm (13). We assume that a steady-state concentration of 1 pm of VEGF$_{121}$ or VEGF$_{165}$ should be attained in our simulations. The secretion rate required to achieve 1 pm at steady state depends on the receptor density (Fig. 2, gray line). Because there is uncertainty in some of the parameters, e.g., receptor densities, and because the secretion rate of VEGF from myocytes in vivo has not been measured, we use a different secretion rate for each simulation to maintain the 1 pm free VEGF concentration at steady state. An alternative way to study this system would be to maintain a constant secretion rate throughout the analysis; however, using the VEGF concentration to determine the secretion rate for each case gives us a better picture of the resting muscle. Starting from the predicted secretion rate, it would be possible to determine the impact of therapeutic interventions (e.g., increasing VEGF secretion or receptor density) or exercise on VEGF distribution, but that is not studied here.

The secretion rates predicted for myocytes by the simulation are consistent with measured VEGF secretion rates from cells in vitro: 0.01 molecule·cell$^{-1}$·s$^{-1}$ for cells derived from rat tibialis anterior (37), 0.48 molecule·cell$^{-1}$·s$^{-1}$ for C$_2$C$_{12}$ myo-
blasts (43), and $3.6 \times 10^{-8}$ to $2.3 \times 10^{-6}$ pmol·cm$^{-3}$·tissue·s$^{-1}$ for cells from various other tissues (47) (compare to Fig. 2).

**Distribution of VEGF in Tissue**

Although for a VEGFR-2-expressing vasculature the same secretion rate of VEGF$_{165}$ and VEGF$_{121}$ will lead to the same steady-state free VEGF concentration (Fig. 2), the total amount of VEGF in the tissue will be different because of the binding of VEGF$_{165}$ to the GAGs in the interstitial space. Thus the distribution of VEGF (free, bound to cell surface receptors, and bound to the matrix) is different for the two isoforms (Fig. 3). VEGF$_{121}$ is primarily endothelial cell surface receptor bound, and the proportion that is free decreases as the receptor density increases (Fig. 3A). VEGF$_{165}$, on the other hand, has a lower total proportion free (although the ratio of receptor bound to free is the same as for VEGF$_{121}$) and a high proportion, 20–90% depending on the receptor density, bound to the ECM (Fig. 3B). Of this matrix-bound VEGF, 8% is bound to the basement membrane surrounding the microvessels and 27% to the basement membrane surrounding the muscle fibers.

**Distribution of VEGF in Tissue**

- Figure 3. VEGF distribution in skeletal muscle with microvasculature expressing VEGF-2. The majority of VEGF—of either isomer—is bound to cell surface receptors (light gray) or matrix (dark gray; VEGF$_{165}$ only). Unbound VEGF decreases as the receptor density increases. The total quantity of extracellular (free, matrix bound, and receptor bound) VEGF for each case is shown in Fig. 4C. Note the 3 scales for VEGF receptor density; they are shown here for comparison but are not repeated in subsequent figures.

**Coexpression of VEGFR-1 and VEGFR-2**

For muscle tissue such as human vastus lateralis that has microvasculature expressing both VEGFR-1 and VEGFR-2, there is again no difference between the VEGF isoforms in the secretion rate from the myocytes required to attain 1 pM steady state interstitial VEGF (Fig. 4A) or the fractional occupancy of either VEGFR (Fig. 4B). The increase of VEGF secretion as the receptor density increases (Fig. 4A) does not alter the fractional occupancy of the receptors (Fig. 4B); this results from the fact that the fractional occupancy is determined by the VEGF concentration available for binding and the receptor binding affinity, and not the secretion rate. The addition of VEGFR-1, a higher-affinity receptor for VEGF, results in competition between these two receptors for VEGF binding, but the fractional occupancy is unchanged (the secretion rate at steady state must be higher to balance the increased binding). The predicted fractional occupancies of both receptors is <2.5%. This is discussed and examined further in [Fractional Occupancy of Receptor Tyrosine Kinases](#).

There are differences, however, in the total VEGF content of the tissue and the distribution of VEGF isoforms in the tissue. As the receptor density increases, the percentage of VEGF in the tissue that is surface bound increases significantly (Fig. 4D). This is accompanied by a convergence between the total amounts of VEGF$_{165}$ or VEGF$_{121}$ in the tissue (Fig. 4C). As more VEGF is bound to the cell surface, the matrix becomes a less important determinant of the VEGF content of the tissue. The free and matrix-bound VEGF content of the tissue for each simulation can be calculated from Fig. 4D: the free VEGF$_{121}$ concentration is all of the non-receptor-bound VEGF, whereas free VEGF$_{165}$ is ~2% of the non-receptor-bound VEGF.

This effect of VEGFR-1 expression on the distribution of VEGF in muscle that expresses 10,000 VEGFR-2/endothelial cell is shown in more detail in Fig. 5. The presence of this receptor at densities within the measured range for this muscle decreases the “reservoir” of VEGF bound to the matrix.

**Coexpression of VEGFR-2 and Neuropilin-1**

The presence of neuropilin on endothelial cells has been demonstrated to increase VEGF$_{165}$ binding and activation of VEGFR-2 in vitro (18, 39, 40, 44). In our in vivo simulations, increasing neuropilin density increases the fractional occupancy of VEGFR-2 (Fig. 6B), whereas the binding of VEGF$_{121}$ is unaffected. Even a low density of neuropilin (1,000/endothelial cell) results in a significant increase in the binding of VEGF$_{165}$ to VEGF-2. The lower the density of VEGF-2, the more significant the effect of neuropilin. A higher secretion rate is required to balance this increased VEGFR-2 binding and internazilization (Fig. 6A), and the increased binding of VEGF to VEGF-2 further shifts the VEGF distribution away from interstitial space and toward the microvessel cell surface (Fig. 6, D and E).

**Coexpression of VEGFR-1, VEGFR-2, and Neuropilin-1**

The interaction between VEGFR-1 and neuropilin—which form a complex that prevents the binding of VEGF$_{165}$ to either receptor—results in additional differences in signaling between the isoforms. The presence of neuropilin on a cell that expresses both VEGFR-1 and VEGFR-2 (at a VEGFR-2 density
of 10,000/endothelial cell) does not change VEGF signaling through either VEGFR-1 or VEGFR-2, but does result in an increase in VEGF165 binding to VEGFR-2 and a decrease in VEGF165 binding to VEGFR-1 (Fig. 7, A and B). These simultaneous shifts in the receptor-binding profile of VEGF may be synergistic in terms of downstream signaling, as there is recent evidence suggesting that VEGF-VEGFR-1 signaling may modulate or inhibit VEGFR-2 signaling (2, 31, 46).

The change in the binding to receptors affects the total VEGF content of the tissue (Fig. 7D) and VEGF distribution (Fig. 7, E and F). It also alters the total internalization of VEGF, and therefore the secretion rate to balance it (Fig. 7C). However, the shape of these curves is not as intuitive as for the previous figures. For low VEGFR-1 density on the endothelial cells of the tissue, a low concentration of neuropilin prevents VEGF165 binding to VEGFR-1 and increasing neuropilin increases the binding to VEGFR-2. For higher VEGFR-1 density, more of the neuropilin is involved in forming complexes with VEGFR-1 and thus a higher neuropilin density is required to enhance VEGF165 binding to VEGFR-2. There is a characteristic “dip” in the curves: this is the point at which neuropilin is effectively inhibiting binding to VEGFR-1 (decreasing secretion rate required and VEGF concentration) but is not present in sufficient quantities to enhance binding to VEGFR-2 (which would increase the required secretion rate and VEGF concentration).

The effect of variations in both VEGFR-1 and neuropilin density can be seen more clearly in Fig. 8; VEGF-2 density is 10,000/endothelial cell for these simulations. The contour graphs show the result of simultaneous variation of both receptors for VEGF165 secretion; the line graphs represent tissues that express no VEGFR-1 or no neuropilin (Fig. 8, A–E, left and bottom, respectively). The results for VEGF121 are equivalent to the results for VEGF165 in the absence of neuropilin. The secretion rate is a function primarily of receptor

![Fig. 4. VEGFR-1-VEGFR-2 coexpression increases tissue VEGF but not fractional occupancy. A: VEGF secretion rate (for 1 pM unbound extracellular VEGF121 or VEGF165 concentration). B: fractional occupancy of VEGFR-1 and VEGFR-2. Variation in the density of either receptor does not change the occupancy. C: total extracellular (unbound, receptor bound, and matrix bound) concentration of VEGF. D: % of cell surface-bound VEGF. Notation as in C.](http://ajpheart.physiology.org/)

![Fig. 5. VEGF distribution in skeletal muscle with microvasculature expressing VEGFR-1 and VEGFR-2. VEGFR-2 density is 10,000/EC. High receptor concentrations decrease the proportion of VEGF bound to matrix (dark gray, VEGF165 only). Unbound VEGF also decreases as the receptor density increases. The total quantity of extracellular (free, matrix bound, and receptor bound) VEGF for each case is shown in Fig. 4C.](http://ajpheart.physiology.org/)
Fig. 6. Neuropilin coexpression increases VEGF₁₆₅ bound to VEGFR-2. VEGFR-1 is not expressed. A: VEGF secretion rate (for 1 pM extracellular unbound VEGF₁₂₁ or VEGF₁₆₅ concentration). B: fractional occupancy of VEGFR-2. C: total extracellular (unbound, receptor bound, and matrix bound) concentration of VEGF. D: % of cell surface-bound VEGF. Notation as in C. E: VEGF distribution in skeletal muscle with microvasculature expressing VEGFR-2 and neuropilin-1. VEGFR-2 density is 10,000/EC.

Fig. 7. Neuropilin coexpression decreases VEGF₁₆₅ bound to VEGFR-1 (10,000 VEGFR-2/EC). A: fractional occupancy of VEGFR-1. B: fractional occupancy of VEGFR-2. C: VEGF secretion rate (for 1 pM extracellular unbound VEGF₁₂₁ or VEGF₁₆₅ concentration). D: total extracellular (unbound, receptor bound, and matrix bound) concentration of VEGF. E: % of cell surface-bound VEGF. Notation as in C. F: VEGF distribution in skeletal muscle with microvasculature expressing VEGFR-1, VEGFR-2 and neuropilin-1. VEGFR-1 density is 10,000/EC.
density (Fig. 8C), as the receptors increase the total amount of VEGF bound and internalized into the endothelial cells, unless VEGFR-1 and neuropilin-1 are present in close to equal quantities; then they bind to each other and are not significantly involved in VEGF₁₆₅ binding to VEGFR-2. The competition between VEGFR-1 and VEGFR-2 for VEGF binding is governed by neuropilin (Fig. 8, A and B); in the absence of neuropilin, the occupancies of VEGFR-1 and VEGFR-2 are not determined by receptor density. High neuropilin results in high VEGFR-2 occupancy and low VEGFR-1 occupancy, but an excess of VEGFR-1 over neuropilin will result in neuropilin binding VEGFR-1 rather than VEGF₁₆₅ and decreasing its effects. The distribution of VEGF in the tissue follows the VEGF secretion and correlates with receptor density (Fig. 8, D and E).

Fractional Occupancy of Receptor Tyrosine Kinases

The fractional occupancy of the receptor tyrosine kinases VEGFR-1 and VEGFR-2 was predicted by these simulations to be quite low, ~2% and 1%, respectively, in the absence of neuropilin (Fig. 4B). The impact of receptor density on the fractional occupancy of each of the receptors is given in more detail in Fig. 9. VEGFR-2 is present at 10,000/endothelial cell; VEGFR-1 and neuropilin densities are shown. The solid lines in Fig. 9, A and B, represent iso-VEGFR-1 lines; the dotted lines represent iso-neuropilin lines. The fractional occupancies are plotted against the secretion rate that is required to obtain a steady-state free VEGF concentration of 1 pM.

While the presence of neuropilin can lead to increased fractional occupancy of VEGFR-2 (Fig. 9B), the fractional occupancy of VEGFR-1 decreases almost to zero (Fig. 9A). This raises some interesting questions about the function of receptor occupancy and sensitivity to increased VEGF concentrations (see Discussion). It should be noted that at high neuropilin concentrations, the solid (iso-VEGFR-1) lines converge, as the effect of VEGFR-1 density is decreased.

VEGF Distribution and VEGF Secretion Rate

VEGF tissue content and VEGF distribution demonstrate a dependence on VEGF secretion rate (Fig. 9C), falling along a single line irrespective of the receptor densities. This implies that the receptor densities do not independently determine the

Fig. 8. VEGFR-1 and neuropilin-1 balance and tissue VEGF effects (10,000 VEGFR-2/EC). Contour graphs show results for VEGF₁₆₅ only. Line graphs accompanying each contour graph represent simulation results for zero VEGFR-1 expression (left) and zero neuropilin expression (bottom). Units for contour graphs are the same as for line graphs. A: fractional occupancy of VEGFR-1. B: fractional occupancy of VEGFR-2. C: VEGF secretion rate (for 1 pM extracellular unbound concentration of VEGF₁₂₁ or VEGF₁₆₅). D: total extracellular (unbound, receptor bound, and matrix bound) concentration of VEGF. E: % of cell surface-bound VEGF.
VEGF distribution in the tissue; rather, they determine the VEGF secretion rate required to maintain the VEGF interstitial concentration (1 pM in this case). It is the secretion rate that determines the VEGF distribution within the tissue.

Matrix VEGF Binding Site Density

The GAGs of the ECM and basement membranes do not have an impact on the steady-state binding of either VEGF isoform to VEGFR-1 (Fig. 10A) or VEGFR-2 (Fig. 10B). Decreasing the matrix affinity (increasing the numerical value of the dissociation constant) does not affect the VEGF secretion rate (Fig. 10C) but does decrease the total VEGF content (Fig. 10D) by decreasing the matrix-bound fraction of VEGF (Fig. 10, E and F). Decreasing the matrix density would have the same effect (data not shown). This reservoir of VEGF may have significant impact on the dynamics of VEGF binding to its receptors and represents a potential source (through proteolytic cleavage) of additional receptor-binding VEGF.

Simultaneous Secretion of VEGF Isoforms

Human muscle and other tissues express multiple isoforms of VEGF. In mouse skeletal muscle, VEGF164 mRNA is ~10-fold more abundant than VEGF120 (26) (mouse VEGF isoforms are 1 amino acid shorter than human). Simulations of VEGF121 and VEGF165 simultaneous secretion (Fig. 11) demonstrate that a 10-to-1 ratio of VEGF165 to VEGF121 results in similar binding profiles (Fig. 11, A and B) and VEGF distribution (Fig. 11, E and F) as VEGF165 alone. Only at high neuropilin densities (100,000/endothelial cell) are the fractional occupancies of the receptors (Fig. 11, A and B) and the total secretion rate of VEGF (Fig. 11C) significantly affected by the presence of VEGF121 at a level of 10% or less of total VEGF. That is, at lower neuropilin densities, VEGF121 would have to make up a higher proportion of the VEGF present to observe changes in receptor occupancy.

DISCUSSION

The model described here and the simulations performed are the first to provide an insight into the activation of VEGFRs in human tissue. Building on a validated model of the biochemical interactions between the VEGF isoforms and their receptors (18), we introduce ECM sequestration of VEGF165 and the impact of the geometry of the tissue on the secretion and internalization of VEGF. The model developed is general and can be applied to other tissues; here it is applied to the resting human vastus lateralis. This tissue was chosen because it is the subject of many physiological studies in humans and is the typical model tissue for the effects of exercise on vasculature changes in skeletal muscle (4, 8, 9, 33). Using geometric parameters, VEGF concentrations, and VEGFR densities typical of this tissue, we investigated the steady-state activation of VEGFRs as well as the distribution of VEGF within the tissue and the effects of receptor density on these results. There are also therapeutic applications for this work, since inducing angiogenesis in peripheral arterial disease (PAD) may be a promising therapeutic strategy (15, 32).

The simulations predict that in the absence of neuropilin expression on the vasculature of skeletal muscle, VEGF165 and VEGF121 behave similarly at steady state: for a given free VEGF concentration, fractional receptor activation is the same, even as the relative densities of VEGFR-1 and VEGFR-2 are varied. Of course, the absolute value of the receptor activation, e.g., number of active receptors per cell, is increased as the receptor density increases, but the fractional occupancy is the same.

There is uncertainty over whether fractional occupancy or absolute receptor activation is the more meaningful determinant of VEGF signaling. It is likely that the absolute number of
Fig. 10. VEGF sequestration in the ECM does not affect steady-state cell surface binding (10,000 VEGFR-2, 10,000 VEGFR-1 per EC). GAG, glycosaminoglycan chains of interstitial proteoglycans to which VEGF binds. Increasing affinity is represented by lower values (i.e., affinity decreases to the right). A: fractional occupancy of VEGFR-1. B: fractional occupancy of VEGFR-2. C: VEGF secretion rate (for 1 pM extracellular, unbound VEGF concentration). D: total extracellular (unbound, receptor bound, and matrix bound) concentration of VEGF. E: % of cell surface-bound VEGF. Notation as in C. F: VEGF distribution in skeletal muscle with microvasculature expressing VEGFR-1, VEGFR-2, and neuropilin-1. 10,000 Neuropilin-1/EC.

Fig. 11. Balance of VEGF isoform secretion (10,000 VEGFR-2, 10,000 VEGFR-1 per EC). A: fractional occupancy of VEGFR-1. B: fractional occupancy of VEGFR-2. C: VEGF secretion rate (for 1 pM extracellular, unbound total VEGF concentration). D: total extracellular (unbound, receptor bound, and matrix bound) concentration of VEGF. E: % of cell surface-bound VEGF. Notation as in C. F: VEGF distribution in skeletal muscle with microvasculature expressing VEGFR-1, VEGFR-2, and neuropilin-1. 10,000 Neuropilin-1/EC.
activated receptors will determine the level of signaling transduced at any given time. However, the fractional occupancy gives additional information, in particular concerning the responsiveness of the system to changes in the concentration of the extracellular signal. VEGFR-1 and VEGFR-2 are predicted (in the absence of neuropilin) to have low fractional occupancies—2% and 1%, respectively. For a cell expression of 10,000 receptors, this is only 100–200 active receptors per cell, or 1 active receptor in each 5–10 μm² of cell membrane. The sparseness of this activation raises questions about the responsiveness of the cell to spatial stochastic fluctuations in which receptors are active, especially for a receptor family that is implicated in chemotactic responses. No in vivo experimental evidence is yet available to test this prediction of low fractional occupancy in the resting state, but the presence of VEGF concentrations below the $K_d$ for VEGF-VEGFR binding would suggest that this is the case.

The presence of neuropilin changes this considerably for VEGFR-2. The fractional occupancy is increased significantly without a change in the extracellular VEGF₁₆₅ concentration. This explains why the measured interstitial VEGF concentration is significantly lower than the binding affinity for the receptor tyrosine kinases, which is otherwise unexpected for typical signal-sensor pairs. This could be a mechanism to keep interstitial VEGF concentration low—and to render the cell typical signal-sensor pairs. This could be a mechanism to keep interstitial VEGF concentration low—which increases the binding of neuropilin, because the two receptors couple directly together and this prevents VEGF₁₆₅ (but not VEGF₁₂₁) binding to VEGFR-1.

This low predicted fractional occupancy of VEGFR-1, along with much experimental evidence that suggests that the kinase activation of VEGFR-1 is weak (even in the absence of neuropilin), suggests that the role of VEGFR-1 should be reexamined. Although there are cases in which VEGF-VEGFR-1 signaling appears to be functional (2, 3, 19), it is possible that its function as an “occupier” of neuropilin (binding to it and preventing it from binding VEGF₁₆₅ or coupling to VEGFR-2) may be as important. In this sense, VEGFR-1 works as an anti-VEGFR-2 signaling agent, occupying neuropilin-1 that would otherwise be increasing the binding of VEGF₁₆₅ to VEGFR-2. This is in contrast to the often-cited “decoy receptor” hypothesis (24), in which VEGFR-1 is viewed as a nonfunctional sequestering site to reduce the availability of VEGF to VEGFR-2. In our simulations, the presence of VEGFR-1 is not predicted to change the binding of VEGF to VEGFR-2 by sequestering VEGF, in agreement with our previous study (19), in which the presence of placental growth factor, a VEGFR-1-specific ligand, did not significantly affect VEGF-VEGFR-2 binding. In the absence of neuropilin, VEGFR-1 does not affect VEGF-2 activation. In the presence of neuropilin, it continues to sequester VEGF₁₂₁ but does not alter VEGF₁₂₁ binding to VEGFR-2.

In this steady-state study, the ECM and basement membranes do not modulate VEGF binding to VEGFRs. However, the sequestration of VEGF₁₆₅ by the GAG chains in the interstitial space represents a significant reservoir for VEGF that can be released (for example, by matrix metalloproteinases—not included in this study). The capacity of this reservoir is determined by the proteoglycan density and binding affinity. Whether being sequestered or proteolytically released, VEGF-receptor binding dynamics may be significantly affected by the ECM.

The geometry of the tissue under investigation here is included in the model: interstitial space, fiber volume and surface area, and microvessel volume and surface area. These are incorporated into the kinetic parameters and concentrations of the model, and the results are therefore tissue specific. Both VEGF distribution and VEGFR occupancy are affected by changes in the tissue geometry.

These simulations give predictions for VEGF distribution and receptor activation in vivo, which are difficult to measure experimentally. There are additional components that could be added in the future to make the model more complete. For example, we do not consider the presence of soluble VEGF₁-1 (sVEGFR-1), because we do not yet have experimental estimates for its quantity in human or other muscle. The effect of sVEGFR-1 would be similar to that of the ECM in sequestering VEGF, except that sVEGFR-1 would sequester both isoforms. This would not significantly affect the outcome of our study, because at steady state sequestration away from the surface does not affect receptor binding in resting muscle.

In addition, VEGFR dimerization is not explicitly included in this model (the kinetic parameters used include dimerization implicitly). Dimerization would not affect VEGF binding to its receptors but may have an impact on downstream signaling. In addition, the formation of VEGFR-1-VEGFR-2 heterodimers may result in significantly different signal transduction (14, 25). The interaction of neuropilin with such heterodimers is not known.

Disturbance to the functionality of the vasculature in skeletal muscle can result in impaired ability to exercise, as the delivery of oxygen, which may be enough for the resting muscle, becomes insufficient as the consumption rate increases. This may be due to upstream blockage of vessels (e.g., PAD; Ref. 15) or to the long-term effects of disease, inactivity, and/or aging (4, 8, 9, 33).

The studies presented here can be extended to include the effects of therapeutic interventions, e.g., the induction of higher VEGF secretion or the induction of receptor expression, either by gene delivery or as a secondary effect of long-term training. In addition, the transient behavior of VEGFR activation that takes place during transient upregulation of VEGF (e.g., during and immediately following exercise) can be investigated.

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GRANTS

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