A multiscale computational model predicts distribution of anti-angiogenic isoform VEGF_{165b} in peripheral arterial disease in human and mouse

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Angiogenesis is the growth of new blood vessels from pre-existing microvessels. Peripheral arterial disease (PAD) is caused by atherosclerosis that results in ischemia mostly in the lower extremities. Clinical trials including VEGF-A administration for therapeutic angiogenesis have not been successful. The existence of anti-angiogenic isoform (VEGF_{165b}) in PAD muscle tissues is a potential cause for the failure of therapeutic angiogenesis. Experimental measurements show that in PAD human muscle biopsies the VEGF_{165b} isoform is at least as abundant if not greater than the VEGF_{165a} isoform. We constructed three-compartment models describing VEGF isoforms and receptors, in human and mouse, to make predictions on the secretion rate of VEGF_{165b} and the distribution of various isoforms throughout the body based on the experimental data. The computational results are consistent with the data showing that in PAD calf muscles secrete mostly VEGF_{165b} over total VEGF. In the PAD calf compartment of human and mouse models, most VEGF_{165a} and VEGF_{165b} are bound to the extracellular matrix. VEGF receptors VEGFR1, VEGFR2 and Neuropilin-1 (NRP1) are mostly in ‘Free State’. This study provides a computational model of VEGF_{165b} in PAD supported by experimental measurements of VEGF_{165b} in human and mouse, which gives insight of VEGF_{165b} in therapeutic angiogenesis and VEGF distribution in human and mouse PAD model.

Angiogenesis is the process of new blood vessel formation from the pre-existing microvessels. Members of vascular endothelial growth factor (VEGF) superfamily critically but differentially regulate angiogenesis in normal physiological and pathophysiological conditions including exercise, ischemic cardiovascular diseases, and cancer\textsuperscript{1}. The VEGF family includes five ligands VEGF-A, VEGF-B, VEGF-C, VEGF-D and PlGF (Placental growth factor), and five receptors VEGFR1, VEGFR2, VEGFR3, NRP1 (neuropilin-1) and NRP2 (neuropilin-2). Among the members of VEGF family, VEGF-A and VEGFR2 are considered to be potent pro-angiogenic molecules. However, recent identification of VEGF_{xxy} isoforms has changed the classical paradigm of VEGF-A:VEGFR2 function in regulation of angiogenesis\textsuperscript{2}.

Alternate splicing in the 8\textsuperscript{th} exon of VEGF-A results in the formation of sister families: pro-angiogenic VEGF_{xxx} (VEGF_{165a}, in human) isoform (xxx denotes number of amino acids) containing an amino acid sequence 'CDKPRR' and anti-angiogenic VEGF_{165b} isoform containing an amino acid sequence 'PLTGKD' in their C-terminus, respectively. The positively charged cysteine and arginine residues (CDKPRR) in pro-angiogenic VEGF-A isoform facilitate the binding of VEGF_{165a} to VEGFR2 and NRP1 to induce a conformational change and internal rotation of intracellular domain and maximal activation of VEGFR. However, replacement of cysteine and arginine residues with neutral lysine and aspartic acid in VEGF_{xxx} isoform was predicted to result in partial

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VEGFR2 activation that cannot induce torsional rotation required for autophosphorylation and downstream signaling. Hence, the balance between VEGF165a and VEGF165b levels may play a crucial role in promoting angiogenesis especially in ischemic cardiovascular diseases such as peripheral arterial disease (PAD) or coronary artery disease (CAD).

PAD is caused by atherosclerosis, which results in ischemia most frequently in the lower extremities. Clinical trials including exogenous VEGF-A administration to activate VEGFR2 dependent therapeutic angiogenesis were not successful. While suboptimal delivery or dosage might be the contributing factors, induction of VEGF165b in ischemic muscle could compete with pro-angiogenic VEGF165a isoform for binding sites on VEGFR2 to decrease VEGFR2 activation. The mechanism of VEGF165b binding to VEGFR2 suggests the potential reason for the failure of therapeutic angiogenesis in VEGF-A clinical trials. Currently, the balance between VEGF 165b and VEGF165a isoforms that can modulate VEGFR2 activation and angiogenic signaling in the ischemic skeletal muscle of PAD patients is not fully understood.

We have previously reported experimental evidence that VEGF165b levels are significantly higher in biopsies of PAD patients 3. Kikuchi et al. measured the ratios of VEGF 165b versus VEGF 165a in serum (by western blot analysis) and peripheral blood mononuclear cells (PBMCs, by mRNA) in PAD patients, which are 4:1 and 8:1, respectively 4. Currently there is no study measuring the ratio of VEGF 165b and total VEGF 165 in human PAD muscle biopsies. The predominance of VEGF165b in human muscles provides the potential mechanism why the clinical trials of VEGF therapeutic angiogenesis

Results
In this study, we use both experimental and computational approaches to predict the secretion rate of VEGF165b and receptor occupancy of VEGFR1 and VEGFR2 in PAD. In practice, it is not possible to measure the secretion rates of VEGF in different tissues in vivo. These important physiological parameters could be only estimated or calculated from the model. This is the first computational model to investigate and account for the experimental ratios of VEGF165b and total VEGF165 in human PAD muscle biopsies. The predominance of VEGF165b in human muscles provides the potential mechanism why the clinical trials of VEGF therapeutic angiogenesis
have failed. Based on the measurement of ratio of VEGF_{165b} and total VEGF_{165} in muscle biopsies, we use the three-compartment model to predict the secretion rates of VEGF_{165b} in different tissues. This model enables prediction of receptor occupancy with VEGF_{165b} and other isoforms in human and mouse.

**Specificity and sensitivity of VEGF-A and VEGF_{165b} antibodies.** VEGF-A ELISA detected both recombinant VEGF_{165a} and VEGF_{165b} with equal sensitivity and specificity. This result indicates that the actual

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**Figure 3.** Concentrations of VEGF_{165a} and VEGF_{165b} plotted against the secretion ratio of VEGF_{165b} to total VEGF_{165} isoforms in normal (left column), blood (middle column) and disease compartments (right column). Each row represents the variation of secretion ratio of VEGF_{165b} to total VEGF_{165} in (A) normal, (B) blood, and (C) disease compartment.

**Figure 4.** Local sensitivity analysis for VEGF_{165a}, VEGF_{165b}, and VEGF_{121}. The results show that VEGF_{165a} in the disease compartment and VEGF_{165b} in the blood compartment are more sensitive to VEGFR2 than VEGFR1, whereas VEGF_{121} in the disease and blood compartment is sensitive to both VEGFR1 and VEGFR2. VEGF_{165a}, VEGF_{165b}, VEGF_{121} are not sensitive to NRP1. The sensitivity analysis demonstrates the importance of VEGF_{165b}-VEGFR2 binding.
measurement of pro-angiogenic VEGF-A by VEGF-A antibodies have been overestimated due to its cross reactivity with anti-angiogenic VEGF165b isoforms. However, VEGF165b antibody raised against the unique 6 amino acid sequence (SLTRKD) used in ELISA specifically detected only recombinant VEGF 165b but not recombinant VEGF165a at any concentrations indicating that a ratio of VEGF165b:total VEGF-A measurements will be needed to determine the actual amount of pro-angiogenic VEGF-A isoforms in biological samples (Fig. 1).

Three-compartmental models of PAD: motivation, assumptions and simulations. In our western analysis we observed a significant increase in total VEGF 165 levels in ischemic muscle compared to non-ischemic at day 3 and day 7 post hindlimb ischemia (HLI). However, the levels of VEGF165b were also significantly higher at day 3 and day 7 post HLI. The ratio VEGF165b:VEGF-A (from the densitometry values of western blot analysis) showed a ~4 fold induction in the fraction of VEGF165b in total VEGF-A in ischemic muscle at day 7 post HLI compared to non-ischemic (i.e. VEGF165b isoforms constitute ~80% of total VEGF-A) in experimental PAD muscle compared to control (Fig. 2).

We used the above experimental results in our model to predict the concentrations of VEGF165b and VEGF165a in the calf muscles. Kikuchi et al. measured the ratios of VEGF165b versus VEGF165a in serum (protein) and peripheral blood mononuclear cells (PBMCs, mRNA) in PAD patients, which were reported as 4:1 and 8:1, respectively4. Hoier et al. measured the interstitial total VEGF protein concentration (i.e. including both VEGF165a and VEGF165b) in the thigh skeletal muscle of PAD patients as 69 ± 21 and 190 ± 78 pg/ml in rest and active exercise, respectively. We convert these numbers to molar concentrations as 69 pg/ml · 1000 ml/l · 1 mole/46000 g = 1.5 pM and 190 pg/ml = 4.1 pM, respectively, based on the molecular weight 46 kDa for VEGF homodimers7. We have constrained the model so that the predictions of steady-state concentration of VEGF 165b and total VEGF165 are within an experimentally observed range (e.g. 0–10 pM) in any of the three compartments to be consistent with the experimental data.

However, because of the sparseness of experimental data it is not possible to identify all the unknown parameters from the available data. Thus, we choose an initial set of secretion rates based on our previous analyses and perform the sensitivity analysis to determine a range of secretion rates constrained with available experimental data, both our own and others. Note that future experiments may provide additional data that could allow further constraining the solutions; presently, the analysis is heuristic aiming at stimulating further experiments.

Prediction of secretion rates of VEGF165b and secretion ratio of VEGF165b over total VEGF in three compartments. The values of secretion rate of VEGF in normal and blood compartments are adopted from the tumor angiogenesis model8. The secretion rate in PAD calf compartment is set up the same as the secretion rate in the normal compartment. The initial set of secretion rates of total VEGF including VEGF165a, VEGF165b and VEGF121 is assumed as 0.02, 0.031 and 0.02 molecules/cell/s, respectively, in all the three compartments (normal, blood and
The secretion ratio of VEGF121 over total VEGF (VEGF165a + VEGF165b + VEGF121) is assumed to be 10%, i.e. 0.002, 0.0031 and 0.002 in normal, blood and disease compartments, respectively. We scan the ratio of secretion rate of VEGF165b over total VEGF165 (i.e. the sum of VEGF165a and VEGF165b) in the three compartments from 0 and 1 in 0.1 increments. When the ratio of VEGF165b over total VEGF165 is 0.5 in all three compartments (i.e. secretion rates of VEGF165a and VEGF165b have equal values 0.009, 0.01395 and 0.009 in normal and disease compartments, respectively), the concentration of VEGF165b in the normal compartment reaches 31 pM. When the ratio of VEGF165b over total VEGF165 in all three compartments is 10%, the concentration of VEGF165b in the normal compartment drops to 6.5 pM, which is within the experimentally observed range (<10 pM).

We can also change the secretion ratio of VEGF165b/total VEGF165 in each of the three compartments individually. We plot the predictions for steady-state concentrations of VEGF165a and VEGF165b in Fig. 3A. We follow the same strategy in Fig. 3B,C, except we vary the secretion ratio of VEGF165b/total VEGF165 in the blood compartment for B and the disease compartment for C, while the total secretion rates are fixed and the ratios are fixed at 0.1 in the other compartments. The model predicts that the concentration of VEGF165b remains at approximately 6.5 pM, which is within the experimentally observed range (<10 pM).

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Sensitivity analysis of VEGF_{165a}, VEGF_{165b} and sVEGFR1. We use SimBiology to perform a local sensitivity analysis, which quantifies how changes in a given parameter value influence predicted concentrations of interest.
We calculate the normalized sensitivity coefficient $\frac{\partial Y}{\partial X}$ based on the algorithm$^9$, where $Y$ is the species VEGF$_{165a}$, VEGF$_{165b}$, and VEGF$_{121}$, and $X$ is the three VEGF receptors VEGFR1, VEGFR2 and NRP1 in the disease compartments; $[X]$ and $[Y]$ denote the molar concentrations. Figure 4 shows that VEGF$_{165a}$ in the disease compartment and VEGF$_{165b}$ in the blood compartment are more sensitive to VEGFR2 than VEGFR1, whereas VEGF$_{121}$ in the disease and blood compartment is sensitive to both VEGFR1 and VEGFR2. VEGF$_{165a}$, VEGF$_{165b}$, VEGF$_{121}$ are not sensitive to NRP1. The sensitivity analysis in Fig. 4 demonstrates the importance of VEGF$_{165b}$-VEGFR2 binding.

**Tissue VEGF distribution and VEGFR occupancy in human three compartment model.**

We show the steady-state distribution of VEGF ligands and their receptors for each tissue in Fig. 5A,B, respectively. The y-axis represents the percentage of each species in x-axis in each VEGF ligand (VEGF$_{165a}$, VEGF$_{165b}$, and VEGF$_{121}$) in Fig. 5A and each receptor (VEGFR1, VEGFR2, NRP1, and sVEGFR1) in Fig. 5B. In PAD calf, most VEGF$_{165a}$ is bound to the ECM and parenchymal basement membrane (PBM) (55% and 17%, respectively); most VEGF$_{165b}$ is also bound to ECM and PBM (62% and 20%, respectively). Most VEGF$_{121}$ isoform is bound to VEGFR1 and NRP1 as the VEGF$_{121}$:VEGFR1:NRP1 complex in the disease (48%) and normal (51%) compartment. Regarding receptor occupancy, in the normal compartment, the three receptors VEGFR1, VEGFR2 and NRP1 are in the free states, except the VEGF$_{121}$:VEGFR1:NRP1 complex (33%) and VEGF$_{165a}$:VEGFR2:NRP1 (49%). In the PAD calf compartment, most receptors are in the free states, except the complexes VEGF$_{165a}$:VEGFR2:NRP1 (20%) and VEGFR1:NRP1 (18%). Most sVEGFR1 are bound to ECM, endothelial basement membrane (EBM) and PBM (62%, 15% and 19%, respectively). Our model in PAD resembles the similar distribution of VEGF and VEGFR occupancy in tumor$^{16}$.

**Table 2. Geometric parameters for the PAD calf compartment in human and mouse.**

| Human PAD geometric parameters | Value | Units | Resources |
|-------------------------------|-------|-------|-----------|
| Compartment volume           | 738   | cm$^3$ | Combined volume of lateral gastrocnemius, medial gastrocnemius, soleus muscles$^{10}$ |
| Fluid volume in ECM           | 31.87%| cm$^3$/cm$^3$ tissue | Calculated from ref. 14 |
| Fluid volume in EBM           | 0.45% | cm$^3$/cm$^3$ tissue | Calculated from ref. 14 |
| Fluid volume in PBM           | 0.58% | cm$^3$/cm$^3$ tissue | Calculated from ref. 14 |
| Muscle fiber cell surface area | 417   | cm$^2$/cm$^3$ tissue | Calculated from ref. 14 |
| Muscle fiber cross-sectional area | 3,464 | μm$^2$ | Calculated from refs 21–23 |

| Mouse PAD geometric parameters | Value | Units | Resources |
|-------------------------------|-------|-------|-----------|
| Compartment volume           | 0.01  | cm$^3$ | 6 |
| Fluid volume in ECM           | 28.92%| cm$^3$/cm$^3$ tissue | 6 |
| Fluid volume in EBM           | 0.097%| cm$^3$/cm$^3$ tissue | 6 |
| Fluid volume in PBM           | 0.68% | cm$^3$/cm$^3$ tissue | 6 |
| Muscle fiber cell surface area | 713.68| cm$^2$/cm$^3$ tissue | Based on mouse myocytes$^{17}$ |
| Muscle fiber cross-sectional area | 2500 | μm$^2$ | Based on mouse myocytes$^{17}$ |

Figure 7. **Three-compartment model of VEGF in peripheral arterial disease.** 121: VEGF$_{121}$, 165a: VEGF$_{165a}$, 165b: VEGF$_{165b}$, N: neuropilin-1. 1: soluble and membrane-bound VEGFR1. 2: VEGFR2. GAG: glycosaminoglycan. α2M: alpha-2 macroglobulin.
VEGF distribution and VEGFR occupancy in murine three compartment model. Constructing a computational model of mouse VEGF distribution is necessary to make use of the experimental measurements in hindlimb ischemia (HLI) models in mice where numerous studies have been conducted. In the mouse, VEGF isoforms are shorter by one amino acid, e.g., VEGF164a and VEGF120, but VEGF165b. Construction of the three-compartment model in mouse can help identify the relative concentration of VEGF164a and VEGF165b in the different compartments, and predict VEGF receptor occupancy. We replace the geometric parameters in the three-compartment model from human to mouse. These geometric parameters of mouse are described in the tumor xenograft model and in the mouse two-compartment model. The results of VEGF distribution and VEGFR occupancy are shown in Fig. 6(A, B), respectively. Figures 5(A) and 6(A) show that VEGF165b (human and mouse) mostly bind to the ECM and rarely exist as free ligands in both human and mouse. VEGF164a and VEGF165b bind to PBM with higher percentage in mouse than the corresponding isoforms in human. The VEGF receptor occupancies in Figs 5(B) and 6(B) show the similar trend between mouse and human. However, there is lower percentage of free VEGFR1 and VEGFR2 in human (Fig. 5B) than in mouse (Fig. 6B). This result is due to the higher percentage of VEGF121:VEGFR1:NRP1 and VEGF165a:VEGFR2:NRP1 in human than the corresponding complexes in mouse.

Discussion
We previously developed the three-compartment models of VEGF in tumor and in PAD. The anti-angiogenic form of VEGF165b has not been included in any of the previous computational models; this is the first study to include VEGF165b in our human PAD model. This is also the first study that compares human PAD and mouse hindlimb ischemia (HLI) models. By changing the geometric parameters in human PAD calf muscles and adding the kinetics equations of VEGF165b, we predict the VEGF165b level in the PAD calf, blood and normal tissue compartments. We summarize the predictions in Table 1. Our computational model shows that the ratio of VEGF165b/VEGF165a, in PAD is higher than 1 (1.70, 2.10 and 1.41 in disease, blood and normal compartments, respectively). This prediction is consistent with the experimental measurements of VEGF165b, as the predominant isoform of VEGF in PAD. The ratio of VEGF165b/total VEGF165 in the disease compartment of our model is

| Receptors        | Value   | Units   | References |
|------------------|---------|---------|------------|
| R1: Abluminal EC (normal) | 3,750 receptors/EC | Extrapolated from receptor density on normal ECs, accounting for different cell surface areas |
| R2: Abluminal EC (normal) | 300 receptors/EC | 24, 25    |
| R1: Abluminal EC (Disease) | 3,750 receptors/EC | 24        |
| R2: Abluminal EC (Disease) | 300 receptors/EC | 24        |
| N1: Abluminal EC (Disease) | 34,500 receptors/EC | 24        |

Table 3. Number of cell surface receptors VEGFR1, VEGFR2 and NRP1. Units of values: dimers/EC in VEGFR1 and VEGFR2 and dimer/EC in NRP1; EC: endothelial cell.
slightly lower than the experiment in human biopsies; however, it should be noted that our predictions are for VEGF concentration in the interstitial fluid in the normal and diseased tissues, whereas the measurements represent the bulk tissue including intracellular components.

We do not include intrinsic heterogeneity that arises from the stochastic nature of biochemical reactions because we consider relatively large concentrations (pM). It may be more relevant to consider extrinsic heterogeneity (i.e., due to variations in protein concentrations)\(^1^5\). Others have included heterogeneity in the receptor distributions\(^1^6\) and found that variability in receptor expression can influence the response to anti-VEGF cancer treatment. The focus of the present work is to investigate and quantify how VEGF\(_{165b}\) molecular interactions influence overall VEGF distribution in human and mouse. This is the first model to incorporate the VEGF\(_{165b}\) isoform.

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(3 \times 10^7\) M\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{on}\) | \(10^{-2}\) s\(^{-1}\) | 26,27 |
| \(K_d\) | 33 pM | 26,27 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(10^7\) M\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{on}\) | \(10^{-2}\) s\(^{-1}\) | 26,27 |
| \(K_d\) | 100 pM | 26,27 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(3.2 \times 10^6\) M\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{on}\) | \(10^{-3}\) s\(^{-1}\) | 26,27 |
| \(K_d\) | 312.5 pM | 26,27 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(4 \times 10^3\) M\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{on}\) | \(10^{-2}\) s\(^{-1}\) | 26,27 |
| \(K_d\) | 23.8 pM | 26,27 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | 25 M\(^{-1}\)s\(^{-1}\) | Calculated |
| \(k_{on}\) | \(10^{-4}\) s\(^{-1}\) | Assumed |
| \(K_d\) | 4.0 \(\mu\)M | 28 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(2.4 \times 10^7\) M\(^{-1}\)s\(^{-1}\) | Calculated |
| \(k_{on}\) | \(10^{-2}\) s\(^{-1}\) | Assumed |
| \(K_d\) | 0.42 \(\mu\)M | 28 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(3 \times 10^7\) M\(^{-1}\)s\(^{-1}\) | Assume, based on VEGF binding to VEGFR1 |
| \(k_{on}\) | \(10^{-2}\) s\(^{-1}\) | Assumed |
| \(K_d\) | 33 pM | Assumed |

| Value | unit | References |
|-------|------|------------|
| \(k_{c}\) | \(10^4\) (Mol/cm\(^2\))\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{off}\) | \(10^{-2}\) s\(^{-1}\) | 26,27 |

| Value | unit | References |
|-------|------|------------|
| \(k_{c}\) | \(3.1 \times 10^{11}\) (Mol/cm\(^2\))\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{off}\) | \(10^{-3}\) s\(^{-1}\) | 26,27 |
| \(k_{c}\) | \(10^4\) (Mol/cm\(^2\))\(^{-1}\)s\(^{-1}\) | 26,27 |
| \(k_{off}\) | \(10^{-3}\) s\(^{-1}\) | 26,27 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(5.6 \times 10^5\) M\(^{-1}\)s\(^{-1}\) | Calculated |
| \(k_{on}\) | \(10^{-2}\) s\(^{-1}\) | Assumed, based on VEGFR1 coupling to NRPI |
| \(K_d\) | 1.8 \(nM\) | 29 |

| Value | unit | References |
|-------|------|------------|
| \(k_m\) | \(4.2 \times 10^6\) M\(^{-1}\)s\(^{-1}\) | Assumed, based on VEGF binding to GAGs |
| \(k_{on}\) | \(10^{-3}\) s\(^{-1}\) | Assumed |
| \(K_d\) | 24 pM | Assumed |

Table 4. Kinetic parameters.
and we focus on the issues related to the expression of this isoform. Future work can incorporate variability in protein levels.

This study is also important for computational modeling of pharmacokinetics and pharmacodynamics (PK/PD) of administering VEGF165b-antibody as a potential pro-angiogenic therapeutic. Current VEGF antibodies including bevacizumab bind both total VEGF (VEGF165a, VEGF165b and VEGF121). We propose to introduce an antibody to VEGF165b as a PAD therapeutic to stimulate angiogenesis. This strategy will be explored in upcoming computational and experimental studies.

Conclusions

We applied the three-compartment model of VEGF distribution to predict the concentration of VEGF isoforms, including VEGF165b, in the body in peripheral arterial disease. The experimental data show that the expression level of VEGF165b is higher than VEGF165a in the human biopsies and the computational model results are consistent with these measurements. The secretion ratio of VEGF165b to total VEGF165 is estimated as 1 in the disease compartment, and 0.1 in the blood and normal compartments. Our predictions support the importance of VEGF165b in PAD, and provide a foundation for therapeutic inhibition of VEGF165b in PAD in the future. This multiscale model can also provide a basis for simulating the pharmacokinetics of VEGF165b antibody in PAD in human and mouse models.

Methods

ELISA to measure VEGF165b antibody sensitivity. VEGF-A and VEGF165b (R&D) Dual sandwich ELISAs were used to determine the sensitivity and specificity of VEGF-A and VEGF165b antibodies in differentiating recombinant pro-angiogenic VEGF-A (VEGF165) and anti-angiogenic VEGF-A (VEGF165b) isoforms. Recombinant VEGF165a and VEGF165b isoforms were serially diluted at 1000, 500, 250, 125 pg/ml concentrations and standard VEGF-A and VEGF165b ELISA were performed according to manufacturer instructions on all the samples to determine the sensitivity and specificity of VEGF-A and VEGF165b Mice.

Murine Model of Hindlimb Ischemia. Hindlimb ischemia (HLI) induced by femoral artery ligation and resection was used as an experimental model of human PAD. All animal experiments were approved by the University of Virginia Animal Care and Use Committee and conformed to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health. HLI was performed on 8- to 12-week-old age and sex matched C57Bl/6 mice as described previously11,12. Briefly, mice were anesthetized by a combination of ketamine and Xylazine (ketamine 90 mg/kg and xylazine 10 mg/kg) and femoral artery was ligated and resected from just above the inguinal ligament to its bifurcation at the origin of saphenous and popliteal arteries. The inferior epigastric, lateral circumflex, and superficial epigastric artery branches were also ligated.

Table 5. Transport parameters.

|                          | Value    | Unit     | References |
|--------------------------|----------|----------|------------|
| Permeability between normal and blood compartments |          |          |            |
| VEGF                     | $4.0 \times 10^{-8}$ | cm/s     | 27         |
| sVEGFR1                  | $1.5 \times 10^{-4}$ | cm/s     | 30         |
| VEGF:sVEGFR1 complex     | $1.5 \times 10^{-8}$ | cm/s     | 30         |
| Permeability between disease and blood compartments |          |          |            |
| VEGF                     | $4.0 \times 10^{-7}$ | cm/s     | Assumed, based on high permeability in PAD |
| sVEGFR1                  | $3.0 \times 10^{-7}$ | cm/s     | Assumed    |
| VEGF:sVEGFR1 complex     | $1.5 \times 10^{-7}$ | cm/s     | Assumed    |
| Clearance                |          |          |            |
| VEGF                     | $1.1 \times 10^{-3}$ | s$^{-1}$ | Calculated, based on half-life |
| sVEGFR1                  | $5.0 \times 10^{-6}$ | s$^{-1}$ | 30         |
| VEGF:sVEGFR1 complex     | $3.0 \times 10^{-4}$ | s$^{-1}$ | 30         |
| α2M                      | $3.9 \times 10^{-5}$ | s$^{-1}$ | 31         |
| VEGF:α2M complex         | $3.9 \times 10^{-5}$ | s$^{-1}$ | Assumed, based on α2M |
| α2M_int                  | $3.9 \times 10^{-3}$ | s$^{-1}$ | 32         |
| VEGF:α2M_int complex     | $3.9 \times 10^{-3}$ | s$^{-1}$ | Assumed, based on α2M_int |
| Degradation              |          |          |            |
| sVEGFR1                  | $1.9 \times 10^{-4}$ | s$^{-1}$ | Assumed, based on VEGF |
| VEGF:sVEGFR1 complex     | $1.9 \times 10^{-4}$ | s$^{-1}$ | Assumed, based on VEGF |
| Synthesis                |          |          |            |
| α2M                      | $3.5 \times 10^{10}$ | Molecules/cm$^3$ tissue/s | Calculated |
| α2M_fast                 | $1.9 \times 10^{10}$ | Molecules/cm$^3$ tissue/s | Calculated |

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Antibodies. VEGF<sub>165b</sub> antibody was purchased from Millipore (Clone 56/1, Cat No: MABC595), VEGF-A antibody was purchased from Santa Cruz Biotech (Cat No: SC-7269) and β-Actin was purchased from Sigma (Cat No: A2103).

Western blotting, Densitometry and Statistics. Mice were sacrificed with an overdose of anesthesia and non-ischemic and ischemic gastrocnemius muscle samples were collected at day 3 and day 7 post HLI. Tissue was homogenized in RIPA with protease inhibitor cocktail. Equal amounts of protein were resolved by Sodium Dodecyl Sulphate-Poly Acrylamide Gel Electrophoresis, transferred onto nitro cellulose membranes and western blotted against VEGF<sub>165b</sub> VEGF-A and Actin by chemiluminescent method. Bands on the film were scanned and quantified by densitometric analysis of the band intensity by NIH image-J (1.6) software. Densitometric values were plotted on Graph PAD Prism 6 or 7 and analyzed for statistical significance. One-way ANOVA with Dunnetts post–test was used to check statistical significance. p < 0.05 considered statistically significant.

Three-compartment models for human and mouse. The model is comprised of three components: normal tissue (representing all tissues and organs except the diseased calf and blood), blood, and diseased calf of PAD (Fig. 7). For clarity, we use the notation VEGF<sub>165a</sub> to denote the pro-angiogenic isoform; we refer to the combination of VEGF<sub>165a</sub> and VEGF<sub>165b</sub> as total VEGF<sub>165</sub>. VEGF<sub>165a</sub>, VEGF<sub>165b</sub> and VEGF<sub>121</sub> are secreted by myocytes and possibly stromal cells in the normal tissues and by calf muscles in PAD, respectively, and also by endothelial cells in all compartments. VEGF receptors (VEGFR1 and VEGFR2) and co-receptor neuropilin-1 (NRP1) are localized on the surfaces of endothelial and parenchymal cells. We include soluble VEGFR1 and glycosaminoglycan (GAG) chains in the interstitial space of normal and calf compartments, and alpha-2-macroglobulin (α2M) in the blood. These soluble factors bind VEGF and can be present in high concentrations; therefore, it is important to include them in the model. Geometric parameters are used to characterize the compartment and enable conversion of the concentrations from units used in the model (moles/cm<sup>3</sup> tissue) to more standard units (molarity). The geometric parameters in the normal and blood human compartments are described in ref. 17 where applications to cancer are considered. The geometric parameters in the diseased calf compartment are described in ref. 14. The geometric parameters for mouse compartments are described in refs 6 and 13. We list the details of geometric parameters used in our model in Table 2.

Binding of VEGF<sub>165a</sub>, VEGF<sub>165b</sub> and VEGF<sub>121</sub> to VEGFR1 and VEGFR2. The molecular interactions between VEGF isoforms and their receptors are illustrated in Fig. 8. VEGFR1 and VEGFR2 are the transmembrane receptors that bind to VEGF<sub>165a</sub>, VEGF<sub>165b</sub> and VEGF<sub>121</sub>. VEGF<sub>165b</sub> has the equivalent binding to VEGFR2 as VEGF<sub>165a</sub>, and functions as a competitive inhibitor of the major downstream effects of VEGF<sub>165a</sub>- VEGF<sub>165a</sub> and VEGF<sub>165b</sub> are the two glycoproteins with heparin binding domain that can bind the extracellular matrix. VEGF<sub>121</sub> is a freely diffusible protein lacking a heparin-binding domain. VEGF<sub>165b</sub> could bind to VEGFR1 and VEGFR2 but cannot bind the co-receptor NRP1 because it lacks exon 8a.19 The densities of cell receptors VEGFR1, VEGFR2 and NRP1 are listed in Table 3 based on available in vivo and in vitro experimental data. The kinetic parameters are listed in Table 4. The model is described in terms of 80 ordinary differential equations (ODE) and is presented in the Supplementary File.

We assume that the total secretion rate of VEGF isoforms to be 0.02 molecules/cells/s in the disease and normal compartment, and 0.031 in the blood compartment (see Results for the details). The model equations were implemented in MATLAB R2014b (MathWorks, Natick, MA) using the SimBiology toolbox and were solved with the Sundials solver.

Transport parameters. Molecular species are transported between compartments via microvascular permeability (k<sub>j</sub>) and lymphatic drainage (kJ) as shown in Fig. 7. All isoforms of unbound VEGF and soluble VEGFR1 (sVEGFR1) in the tissue compartments are subject to proteolytic degradation (k<sub>deg</sub>) and are removed from the blood via plasma clearance (c<sub>p</sub>). We list the transport parameters in Table 5.

Sensitivity analysis. The sensitivity analysis is implemented using Matlab SimBiology toolbox. The time-dependent sensitivities of the species states are calculated with respect to species initial conditions and parameter values in the model. The sensitivity is calculated using ∂Y/∂X, which determines how changes in parameter X influences the output Y by taking the partial derivatives.

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Author Contributions
L.H.C., V.C.G., B.H.A. and A.S.P. proposed the idea; L.H.C. and G.C. implemented the simulations; V.C.G. and M.H.C. conducted the experiments; L.H.C., V.C.G., S.D.F., B.H.A. and A.S.P. edited the paper. The authors thank Drs. Hojjat Bazzazi and Feilim Mac Gabhann for critical comments and suggestions.

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