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Model of competitive binding of vascular endothelial growth factor and placent al growth factor to VEGF receptors on endothelial cells

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Mac Gabhann, Feilim, and Aleksander S. Popel. Model of competitive binding of vascular endothelial growth factor and placental growth factor to VEGF receptors on endothelial cells. Am J Physiol Heart Circ Physiol 286: H153–H164, 2004. First published April 24, 2003; 10.1152/ajpheart.00254.2003.—Placental growth factor (PIGF) competes with vascular endothelial growth factor (VEGF) for binding to VEGF receptor (VEGFR)-1 but does not bind VEGFR2. Experiments show that PIGF can augment the response to VEGF in pathological angiogenesis and in models of endothelial cell survival, migration, and proliferation. This synergy has been hypothesized to be due to a combination of the following: signaling by PIGF through VEGFR1 and displacement of VEGF from VEGFR1 to VEGFR2 by PIGF, causing increased signaling through VEGFR2. In this study, the relative contribution of PIGF-induced VEGF displacement to the synergy is quantified using a mathematical model of ligand-binding to examine the effect on ligand-receptor complex formation of VEGF and VEGF acting together. Parameters specific to the VEGF-PIGF system are used based on existing data. The model is used to simulate in silico a specific in vitro experiment in which VEGF-PIGF interaction is observed. We show that, whereas a significant change in the formation of endothelial surface growth factor-VEGFR1 complexes is predicted in the presence of PIGF, the increase in the number of VEGFR2-containing signaling complexes is less significant; these results were shown to be robust to significant variation in the kinetic parameters of the model. Synergistic effects observed in that experiment thus appear unlikely to be due to VEGF displacement but to a shift from VEGF-VEGFR1 to PIGF-VEGFR1 complexes and an increase in total VEGFR1 complexes. These results suggest that VEGFR1 signaling can be functional in adult-derived endothelial cells.

angiogenesis; receptor-ligand interactions; kinetics; synergy; mathematical or computational model

VASCULAR ENDOTHELIAL GROWTH FACTOR (VEGF) and placental growth factor (PIGF) are two prominent members of the VEGF family of secreted proteins. They are released by cells as homodimeric glycoproteins of ~45 kDa (2, 17). They have distinct binding characteristics to the two primary VEGF-family receptors on endothelial cells: VEGF receptor (VEGFR)-1 binds both PIGF and VEGF, whereas VEGFR2 binds only VEGF.

VEGF is a known inducer of vascular permeability and angiogenesis in vivo and cultured endothelial cells respond to VEGF with increased survival, proliferation, and migration; there are several comprehensive reviews on the subject (38, 56). Adult PIGF-null mice demonstrate reduced pathological angiogenesis and vascular leakage (9, 35). PIGF is angiogenic in certain situations (59), is chemotactic for endothelial cells and monocytes in vitro (11), and can increase vascular permeability and survival in vivo (1, 41), but in many cases it acts by augmenting the VEGF-induced effect (43). PIGF increases the response of cultured endothelial cells to VEGF-induced survival, proliferation, and migration (9). In vitro assays of hydraulic conductivity and proliferation, PIGF increased the response to VEGF in a cell-type-specific and time-dependent manner (13). PIGF synergy is specific to VEGF; it does not augment basic fibroblast growth factor response.

Two pathways by which the addition of PIGF could impact on VEGF-induced effects have been suggested. First, PIGF, which binds VEGFR1 but not VEGFR2, may displace VEGF from VEGFR1 to VEGFR2: a concept known as ligand shifting (43). Second, the PIGF may play a more direct role signaling into the endothelial cell via VEGFR1. Evidence that this signaling takes place has been presented at least once (9); however, the issue of VEGFR1 signaling in endothelial cells remains controversial. Both of these pathways may occur at the same time, complicating the process of dissecting the functions of the various receptors. This study is concerned with quantifying the ligand shift effect, the displacement of VEGF by PIGF, with the use of a mathematical model. A particular set of recent experiments in which synergy was observed (9) is recreated in silico.

In these three in vitro angiogenesis-associated assays (for survival, proliferation, and migration) the cells examined were microvascular endothelial cells obtained from both wild-type and PIGF-null mice. The PIGF-null cells did not respond as strongly to VEGF as did those from wild-type mice, but the addition of exogenous PIGF along with VEGF restored the response in a dose-dependent fashion, suggesting that the wild-type endothelial cells produced sufficient PIGF to augment the VEGF response. For the PIGF-null cells, the VEGF-induced increase in migration rate was 3-fold; the addition of PIGF made it 16-fold. Proliferation rate increase by VEGF was 50% without and 280% with PIGF. Survival was only slightly improved by VEGF, with the apoptosis rate falling 7%; the addition of PIGF caused the rate to fall by 75%. In each of these cases, PIGF administered alone did not have a significant effect on the process but acted only in concert with VEGF. This study recreates in silico these assays performed on the PIGF-null cells. We compare the following two cases: 1) exogenous VEGF is added (PIGF− case) and 2) exogenous VEGF and PIGF are added together (PIGF+ case) to determine the relative importance of ligand shifting.

The three assays being modeled share a similar geometry. A layer of endothelial cells is in contact with a fluid layer containing serum and growth factors in a well of a 24-well plate. The well is
assumed to be symmetrical and we ignore the edge effects so that we examine only the variation in the vertical direction, that is, from the cell surface to the free surface of the fluid layer. At the start of the assay, 100 ng/ml of VEGF alone (PIGF− case) or 100 ng/ml each of VEGF and PIGF (PIGF+ case) were added to the media above the cultured cells. For the duration of the assay, the growth factors diffused toward the endothelial cells and bound to the cell surface receptors, from which they may be re-released into the medium or internalized: we model all of these transport processes. The assay measurement was a cell count at the completion of the assay. In the model, we noted instead the predicted concentration of ligand-receptor complexes formed on the endothelial cell surface. It is with the interpretation of these calculated values, and of their relationship to the outcome of the experimental assays that we are primarily concerned in this paper. It should be noted that this is the first computational model describing transport and binding of VEGF to its receptors. We have constructed this model to describe unsteady in vitro situations as a first step to modeling more complex, dynamic in vivo situations.

**Glossary**

- **V, P** Concentrations of VEGF and PIGF (M)
- **R1, R2** Concentrations of VEGFR1 and VEGFR2 (mol/cm²)
- **VR1, VR2** Concentrations of VEGF-VEGFR1, PIGF-VEGFR1, and VEGF-VEGFR2 (mol/cm²)
- **s** Insertion rate of surface species into endothelial cell membrane (mol/cm² s⁻¹)
- **k_{int}** Internalization rate of surface receptors and complexes (s⁻¹)
- **k_{on}** Kinetic rate of binding of volumetric species to surface receptor (M⁻¹ s⁻¹)
- **k_{off}** Kinetic rate of dissociation of volumetric species from surface receptor (s⁻¹)
- **Kₐ** Binding affinity (M)
- **D** Diffusivity (cm²/s)

**MODEL OF MULTILIGAND MULTIRECEPTOR INTERACTIONS**

To model the binding of VEGF family ligands to their cell surface receptors, we have constructed a set of coupled reaction-diffusion equations using the continuum approach, where the molecular species are represented in terms of concentrations. The equations necessary to describe the secretion, transport, binding, and internalization of ligands are similar to those formulated earlier (29, 46); they describe the secretion, transport, binding, and internalization of ligands and receptors on the endothelial cell surface. It is with the interpretation of these calculated values, and of their relationship to the outcome of the experimental assays that we are primarily concerned in this paper. It should be noted that this is the first computational model describing transport and binding of VEGF to its receptors. We have constructed this model to describe unsteady in vitro situations as a first step to modeling more complex, dynamic in vivo situations.

Two classes of species are represented in the model: volumetric (growth factors) and surface (receptors and ligand-receptor complexes). We assume symmetry in the well, that is, the concentrations of ligands and receptors are spatially uniform parallel to the plane of the cell surface; the equations are thus reduced to one spatial dimension (z) perpendicular to the cell surface. The evolution over time of the volumetric species concentrations (e.g., $\partial V/\partial t$) is represented by Eqs. 1 and 2, where $D_V$ and $D_R$ are the diffusivities of VEGF and PIGF (cm²/s), respectively. The assay is 24 h in duration; thus ligand degradation in the medium is neglected. No factors in the fluid that could bind the ligands, for example, soluble VEGFR1 or secreted extracellular matrix (ECM) proteins, are included at this point; their effect is investigated in calculations presented in the appendix.

The time evolutions of the surface receptors and complexes are represented by Eqs. 3–7, where $s_{R1}$ and $s_{R2}$ are the insertion rates of new receptors into the membrane (mol/cm² s⁻¹) and $k_{on}$ and $k_{off}$ are the effective on and off rates for the ligand-receptor binding interactions (M⁻¹ s⁻¹ and s⁻¹). We assume that receptor concentration is uniform over the cell surface with no receptor clusters being formed. We also assume that internalization is the only pathway to ligand and receptor degradation.

The boundary conditions for the volumetric species are Eqs. 8–10, where $q_V$ in Eq. 8 is the secretion rate of VEGF by the endothelial cells at the free surface. It should be noted that VEGF is released into the medium as a result of receptor binding and internalization. The effective on and off rates for the ligand-receptor binding interactions are also included in the model. $V_R$, $V_{PR}$, and $PR_1$. Other VEGF and PIGF receptors on endothelial cells, such as the neuropilins, are not considered in this study; their role is considered secondary to that of VEGF and VEGF receptor. The effect of heparan sulfate proteoglycans (HSPGs), which modulate VEGF-family binding, is assumed to be included in the effective rate constants.

Two classes of species are represented in the model: volumetric (growth factors) and surface (receptors and ligand-receptor complexes). We assume symmetry in the well, that is, the concentrations of ligands and receptors are spatially uniform parallel to the plane of the cell surface; the equations are thus reduced to one spatial dimension (z) perpendicular to the cell surface. The evolution over time of the volumetric species concentrations (e.g., $\partial V/\partial t$) is represented by Eqs. 1 and 2, where $D_V$ and $D_R$ are the diffusivities of VEGF and PIGF (cm²/s), respectively. The assay is 24 h in duration; thus ligand degradation in the medium is neglected. No factors in the fluid that could bind the ligands, for example, soluble VEGFR1 or secreted extracellular matrix (ECM) proteins, are included at this point; their effect is investigated in calculations presented in the appendix.

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The boundary conditions for the volumetric species are Eqs. 8–10, where $q_V$ in Eq. 8 is the secretion rate of VEGF by the endothelial cells (mol/cm² s⁻¹). At the cell surface (z = 0), the ligand is secreted by the endothelial cells and depleted by binding to the receptors. These PIGF-null cells secrete no PIGF; thus the corresponding term is not present in Eq. 9. At the free surface (z = h), the diffusive fluxes of VEGF and PIGF disappear.
Model Parameters

The existing experimental literature was researched for specific or representative values for the model parameters. The representative set of parameters used in this model is shown in Table 1, along with the ranges used for the sensitivity analysis. This is the first such compilation of these values.

The initial ligand concentration used in the assays is 100 ng/ml. For a 45-kDa ligand, this is equivalent to 2.22 nM. VEGF is initially present at this concentration; PIGF is initially present at this concentration only in the PIGF+ case. The number of receptors on the endothelial cell surface is heavily dependent on the cell type, tissue of origin, vessel of origin, and the activation state of the cell. Microvascular endothelial cells from the human colon demonstrated 79,900 VEGFR1 and 231,300 VEGFR2 receptors per cell (54). Endothelial cells from larger vessels may have fewer receptors; typical VEGFR1 and VEGFR2 populations for human umbilical vein endothelial cells (HUVECs) are 500–3,000 and 5,850–12,000 receptors/cell, respectively (6, 31, 39). Stimulated cells can also have increased receptor populations (7). For this experiment, the microvascular cells are assumed to have receptor populations of 80,000 and 230,000 for VEGFR1 and VEGFR2, respectively. Endothelial cell size is an important determinant of receptor concentration; for our purposes, size means the area of plasma membrane in contact with the fluid, to which ligands can bind. Endothelial cells from hamster arteries have effective (side-facing flow) surface areas of 1,000–1,550 μm² (21); the values for dog aortic cells were in the range of 400–700 μm² (30). Assuming a cell area of 1,000 μm², we estimate the initial receptor concentrations as 13.3 and 38.3 fmol/cm² for VEGFR1 and VEGFR2, respectively; because of the uncertainty of both the receptor populations and the cell size, a range of receptor concentrations about these values was examined.

The $K_d$ values of hVEGFA₁₆₅ to VEGFR1 and VEGFR2 on HUVECs have been determined in the ranges of 9–26 and 100–770 pM, respectively (6, 31, 39, 53). The measured values for the cell surface receptors on microvascular endothelial cells from human colon were 130 and 675 pM, respectively (54). The binding affinity of human PIGF for VEGFR1 on HUVECs was shown to be 230 pM (43), and the binding affinity of the mouse homologs of these molecules is assumed to be the same. The binding affinity of hVEGFA₁₆₅ to mouse VEGFR2 (mVEGFR2) in a cell-free system was shown to be 340 pM (28), and that is the value assumed here; the ratio of affinities for mVEGFR1 and mVEGFR2 is estimated to be in a similar ratio to the ratio for the human receptors, and thus the values of $K_d$ for hVEGFA₁₆₅/mVEGFR1 binding is taken to be 25 pM.

Because binding affinity is the ratio of the off and on rates, we use the affinity estimates as constraints on the values of these kinetic rates. Cell-free experiments using human ligand and immobilized human receptors measured values for the individual kinetic rates, $k_{on}$ and $k_{off}$. Justification for the suitability of using rates measured in such an experiment can be taken from the observation of equivalence of rates of binding to monomeric and predimerized VEGFR2 (12). For hVEGFA₁₆₅, the measured on rates were $4 \times 10^6$ M⁻¹s⁻¹ binding to human VEGFR1, $3.6 \times 10^6$ M⁻¹s⁻¹ binding to human VEGFR2, and $1.2 \times 10^6$ M⁻¹s⁻¹ binding to mVEGFR2 (12, 25, 51). The off rates from the same experiments were $3 \times 10^{-6}$, $134 \times 10^{-6}$, and $410 \times 10^{-6}$ s⁻¹, respectively. With the use of these rates and the constraint of the binding affinities, the rates for hVEGFA₁₆₅ and mouse mPIGF2 binding to mouse VEGF receptors were estimated. The values of $k_{off}$ were assumed to be $95 \times 10^{-6}$, $410 \times 10^{-6}$, and $345 \times 10^{-6}$ s⁻¹ and the values of $k_{on}$ were taken as $3.8 \times 10^6$, $1.2 \times 10^6$, and $1.5 \times 10^6$ M⁻¹s⁻¹ for hVEGFA₁₆₅-mVEGFR1, hVEGFA₁₆₅-mVEGFR2, and mPIGF2-mVEGFR1, respectively. Again, a range of values was examined. Although kinetic rates are obtained in cell-free systems and do not incorporate the effects of HSPGs on the cell surface, we examined the reported time course of binding of VEGF to HUVECs (6), and the kinetic rates obtained differed from those used here by a factor of four. As shown in the sensitivity analysis, this is insufficient to affect the conclusions of the model (see RESULTS).

The rate of internalization of ligand-receptor complexes was estimated from experiments in which the quantities of VEGF bound to microvascular cell surface and of internalized VEGF were measured over time after addition of VEGF to fluid over the cells (54). We have applied a simplified version of the model presented here for single growth factor and single receptor population to these data to obtain a value of $2.8 \times 10^{-4}$ s⁻¹ for the internalization rate of receptor-VEGF complexes. This value compares favorably with those obtained by estimation of instantaneous internalization rate ($4.3 \times 10^{-4}$ s⁻¹) of VEGF in HUVECs (6) and the measured internalization rates of basic fibroblast growth factor-receptor complexes ($2.7 \times 10^{-4}$ s⁻¹) (16, 47). A value for the internalization rate of unoccupied VEGF receptors was not available from the experimental literature; in some receptor systems, the rate of internalization of unoccupied receptors is lower than that of ligand-receptor complexes, e.g., by up to a factor of 10 for epidermal growth factor receptors (28). We assume a value of $10^{-5}$ s⁻¹ for the internalization rate of unoccupied VEGF receptors, and in the sensitivity analysis (see RESULTS) we note that, whereas the model is sensitive to increases in this parameter, this is due to the corresponding increase in the dependent insertion rates of receptors (see below).

Insertion rates of newly synthesized receptors were estimated from the receptor population and the internalization rates of the unoccupied receptors; in the absence of ligand, the internalization and receptor insertion rates at the cell membrane are balanced, to give a steady receptor population. Thus values for the insertion rates of 0.8 and 2.3

Table 1. Parameters used in the model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control Value</th>
<th>Range for Sensitivity Analysis</th>
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</thead>
<tbody>
<tr>
<td>$V_0$</td>
<td>2.22</td>
<td>0.001–100</td>
</tr>
<tr>
<td>$P_0$</td>
<td>0 or 2.22</td>
<td>0.001–100</td>
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<td>Initial receptor populations, $\times 10^{-15}$ mol/cm²</td>
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<td></td>
</tr>
<tr>
<td>$R_{1a}$</td>
<td>13.3</td>
<td>0.166–66</td>
</tr>
<tr>
<td>$R_{2a}$</td>
<td>38.3</td>
<td>0.166–66</td>
</tr>
<tr>
<td>Binding affinities, pM</td>
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<td></td>
</tr>
<tr>
<td>$k_{on,VR1}$</td>
<td>25</td>
<td>0.25–2.500</td>
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<tr>
<td>$k_{on,VR2}$</td>
<td>340</td>
<td>3.4–34,000</td>
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<tr>
<td>$k_{on,RP1}$</td>
<td>230</td>
<td>2.30–23,000</td>
</tr>
<tr>
<td>$k_{off,VR1}$</td>
<td>3.8</td>
<td>0.38–38</td>
</tr>
<tr>
<td>$k_{off,VR2}$</td>
<td>1.2</td>
<td>0.12–12</td>
</tr>
<tr>
<td>$k_{off,RP1}$</td>
<td>1.5</td>
<td>0.15–15</td>
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<td></td>
</tr>
<tr>
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<td>9.5–950</td>
</tr>
<tr>
<td>$k_{off,VR2}$</td>
<td>410</td>
<td>41.0–4,100</td>
</tr>
<tr>
<td>$k_{off,RP1}$</td>
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<td>Internalization rates, $\times 10^{-5}$ s⁻¹</td>
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<td>0.1–28</td>
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<tr>
<td>$s_{R1}$</td>
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<td></td>
</tr>
<tr>
<td>$s_{R2}$</td>
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<td></td>
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<tr>
<td>Diffusivity, $\times 10^{-6}$ cm²/s</td>
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<td>$D_v$</td>
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<td>0.1–10</td>
</tr>
<tr>
<td>$D_p$</td>
<td>1</td>
<td>0.1–10</td>
</tr>
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</table>

$V_0$ and $P_0$, initial concentrations of vascular endothelial growth factor (VEGF) and placental growth factor (PIGF); $R_{1a}$ and $R_{2a}$, initial concentration of VEGF receptors-1 and -2; $k_{on}$, kinetic rate of binding volumetric species to surface receptor; $k_{off}$, kinetic rate of dissociation of volumetric species from surface receptors; $k_{int}$, internalization rate of surface receptors and complexes; $D_v$, diffusivity of VEGF; $D_p$, diffusivity of PIGF.
receptors-cell⁻¹s⁻¹ for VEGFR1 and VEGFR2, or 1.3 × 10⁻¹⁹ and 3.8 × 10⁻¹⁹ mol·cm⁻²·s⁻¹, respectively, were calculated; these rates are similar to those of the EGFR system (48). We assume that this rate is constant during the experiment. The variation due to insertion rate was not examined because it is a dependent variable.

The diffusivity of the ligands in aqueous solution is estimated to be 10⁻⁶ cm²/s based on molecular weight (5). A range of values from 10⁻⁵ to 10⁻⁷ cm²/s was examined to estimate sensitivity to this parameter.

These PIGF-null cells do not secrete PIGF; however, they may secrete VEGF. We model zero VEGF secretion and discuss the effects of autocrine VEGF secretion by the endothelial cells later. The final parameter of importance is the height of media above the cells, h. This is estimated to be 1 mm, which represents 0.2 ml of media in one well of a 24-well plate. The sensitivity of the results to this parameter was also examined using a range of 10⁻¹–10 mm.

Solution

Equations 1–10 are a complete set of coupled, nonlinear partial and ordinary differential equations. The equations were nondimensionalized, and, using a fully implicit finite-difference scheme on a one-dimensional grid, we obtained a series of coupled nonlinear time-dependent algebraic equations that were solved iteratively at each time step. Iterations were complete when subsequent refinements resulted in a <10⁻¹⁰ fractional change. An adaptive time step size was used to decrease the total computation time required while still achieving convergence at early time points.

RESULTS

Model of In Vitro Assays

The molecular species of interest here are the ligand-receptor complexes as the gateways to cell signaling. In particular, the validity of the ligand shifting hypothesis rests on an increase in the number of VEGF-VEGFR2 complexes formed in the presence of PIGF and VEGF together. The model was applied to the case of addition of VEGF alone (PIGF− case) and then to that of addition of both VEGF and PIGF (PIGF+ case). The synergistic effects of the added PIGF were then investigated by examining the differences between these two cases.

The number of ligand-receptor complexes predicted by the model to be present on the cell surface during the assay is shown in Fig. 1A. The number of VEGFR1 complexes is a sum of VEGF-VEGFR1 and PIGF-VEGFR1 complexes. Growth factors bind rapidly to the unoccupied receptors early in the time course, and are then internalized. The number of complexes reaches a peak at 10 min (VEGFR1) and 25 min (VEGFR2) and then settles to a significantly lower complex concentration due to elevated internalization of occupied receptors relative to that of unoccupied receptors. At this scale, a difference in the number of occupied VEGFR1 receptors between the PIGF− and PIGF+ cases can be seen at early time points, and a smaller change in the number of occupied VEGFR2 receptors is discernable. The absolute change between the PIGF− and PIGF+ cases is shown in Fig. 1B. Three components are observed to change in complex formation in the PIGF+ case: an early transient increase (of <30 min), followed by a decrease in complexes caused by increased internalization of receptors, and finally a small elevation at later times. Transient increases of up to 14,000 VEGFR1 complexes per cell are predicted, with a maximal increase in VEGFR2 complexes of 6,000 per cell. The elevated complex formation at later times is due to decreased internalization of VEGF in the PIGF+ case, where less VEGF removed from the system keeps the receptor occupancy higher. The absolute increases of Fig. 1B are translated into percentage increases,
plotted in Fig. 1C. The model predicts the maximal increase in VEGFR2 complexes to be 5% and the total increase in VEGFR2 complex formation over the duration of the assay to be <1%.

In contrast, the predicted transient increase in the number of VEGFR1 complexes is more significant, as much as 43%; the formation of both PlGF-VEGFR1 and VEGF-VEGFR1 complexes is shown in Fig. 2A. This shows that the increase has two components: a decrease in VEGF-VEGFR1 formation and an increase in PlGF-VEGFR1 formation. The predicted percentage of VEGFR1 complexes that are PlGF containing are shown in Fig. 2B.

The in vitro assays being modeled demonstrated an increase in the efficacy of VEGF of 5- to 10-fold due to the addition of PlGF. The simulation results suggest that the observation of synergy in the in vitro assays was unlikely to be due to the displacement of VEGF by PlGF from VEGFR1 to VEGFR2 and signaling through VEGFR2 alone, the displacement giving only a transient 5% increase in VEGFR2 complex formation. They further predict a more significant increase in VEGFR1 complex formation and a change in the proportion of ligands bound to that receptor, suggesting a role for VEGFR1 signaling in the observed synergy and a change in the nature of VEGFR1 signaling on addition of PlGF. This supports the experimental findings of Carmeliet et al. (9), which demonstrated an abrogation of the PlGF synergy effects on addition of anti-VEGFR1 antibodies. Blocking that receptor should increase the VEGFR2 complex formation further by freeing more VEGF.

Effect of Growth Factor Concentration

The reason for the small change in VEGFR2 complex formation is that the ligand concentrations in this experiment are very high, higher than physiological concentrations and higher than the binding affinities of the receptors, which leads to high fractional occupancies of both receptors; the predicted fractional occupancies are shown in Fig. 3. Whereas VEGF may be displaced from VEGFR1, there is little unoccupied VEGFR2 for it to bind to. Thus we would expect ligand shifting by PlGF to be predicted at lower ligand concentrations, and, by simulating the conditions with different initial ligand concentrations, this is precisely what we see (Fig. 4A). Figure 4A shows the maximal percent change in VEGF-VEGFR2 complex formation for a range of initial ligand concentrations. The original conditions, corresponding to the in vitro assays modeled in Fig. 1, are marked in Fig. 4 by a solid circle. The effect of high ligand concentration on fractional occupancy is particularly important in explaining the small amount of shifting outside the early transient, when the receptors have had time to become occupied. The transient, on the other hand, is a measure of the rate at which the receptors become occupied by the ligands, which is directly dependent on the concentration of ligand available for binding; this is effectively double for VEGFR1 in the PlGF+ case, due to the presence of both ligands. In contrast, VEGFR2 does not bind PlGF, and there is a much smaller increase in the effective ligand concentration it sees. This small increase is due to the decrease in VEGF bound by VEGFR1 as it binds PlGF, but this increase is small relative to the excess of ligand already in place. The maximal fractional occupancies of the receptors for this range of initial conditions is shown in Fig. 4, B and C, demonstrating that significant ligand shifting occurs for high VEGFR1 occupancy and low VEGFR2 occupancy. This supports the interpretation that high VEGFR1 occupancy allows PlGF to displace large quantities of VEGF, raising the effective VEGF concentration in the medium, which in turn can increase binding to the abundant unoccupied VEGFR2 that are available under these conditions. In fact, the reason there is an observable change in the VEGFR2 complex formation is that the step change in ligand concentration, with only unoccupied receptors on the surface, causes a rapid depletion of the ligand close to the cell surface. This decreased local ligand concentration causes the sensitivity to PlGF. Were the diffusivity faster, the local ligand concentration would be maintained from the large pool of ligand above the cells instead of requiring time to equilibrate.

Figure 4 shows that high PlGF concentrations can cause large ligand shifts in low VEGF concentration situations. However, these high percentage changes can be misleading. Figure 5 shows that the predicted number of VEGF-VEGFR2 complexes formed is only a weak function of PlGF concentration and a strong function of VEGF concentration. These results suggest that experimental observations that high concentrations of PlGF can augment low VEGF concentrations
and elicit a response similar to a higher VEGF concentration (55) are not directly due to ligand shifting, as the displacement achieved by PlGF does not appear to cause sufficient VEGF-VEGFR2 formation to compensate for the decreased VEGF concentration. VEGFR1 complexes, in contrast, are increased in the low VEGF-high PlGF situation (data not shown). We would have to conclude at this point that the experimental finding of synergy in that experiment would also be due to VEGFR1 signaling rather than displacement of VEGF to VEGFR2.

Note that we have modeled the endothelial cells throughout as secreting no VEGF. Secretion would increase the concentration of VEGF in the fluid and thus further decrease the ligand shifting effects.

Effects of Soluble Receptors and ECM

It is important to consider the possibility that other factors are present in the assays that we excluded from the model, but which may have an impact on our interpretation of the results. It is possible, for example, that these endothelial cells are secreting factors that can sequester the growth factors. These factors include soluble VEGFR1 (sVEGFR1) and ECM components, to which both VEGFA165 and PlGF2 bind. To model the effect of these factors, the equations were extended to include these components, as volumetric species that could bind both PlGF and VEGF. The additional and modified equations required are detailed in the APPENDIX; additional parameters for binding were obtained and are listed in Table 2. The results of the simulations show that a thin layer of ECM proteins secreted and remaining close to the cell surface did not significantly increase the ligand shifting in this experiment, up to micromolar concentrations of ECM binding sites. Serum levels of sVEGFR1 in the healthy adult were reported to be 28 ng/ml, or 250 pM (32). This concentration of soluble receptor in the medium would cause a maximum change in VEGF-VEGFR2 formation of 8% (see Fig. 6). The change in VEGFR1 complex formation remains >40%. As the soluble receptor approaches the same concentration as the ligands, it is
shown in Fig. 7.

The sensitivity of the results of Fig. 1 to variation in model parameters used was also examined. The height of the fluid layer is directly related to the volume of medium-growth factor mixture added to the well. The effect of changing this height is to change the total quantity of ligand available for binding. The initial concentration is the same, but a lower total quantity can sequestering significant quantities of both, reducing the effective free ligand concentration available for surface binding, and thus reducing the fractional occupancies of the cell surface receptors and increasing the efficacy of the added PlGF in shifting VEGF. However, it is unlikely that the cells are secreting sVEGFR1 at the rates required to build up this level of concentration. Assuming that no sVEGFR1 was present at the beginning of the time period, secretion of sVEGFR1 would have to exceed 40 molecules·cell⁻¹·s⁻¹ to achieve this increase in VEGF-VEGFR2 complex formation.

Sensitivity Analysis

The uncertainty in some model parameters was noted earlier, due to both biological variability and uncertainty in measurement, and it is thus necessary to check the sensitivity of the model results to those parameters. In particular, the number of receptors on the endothelial cell surface was not known directly for the cell type used in the assays but was inferred from a similar cell type. The effect on PlGF-induced VEGF-VEGFR2 formation of variation in VEGFR1 populations is a similar cell type. The effect on PlGF-induced VEGF-VEGFR2 complex formation is shown in Fig. 7A; this uses the predicted maximal change in concentration of VEGFR1 becomes very large, it sequesters larger quantities of ligand at the same fractional occupancy. The results are similarly dependent on VEGFR2 concentration, an increase in that population increasing the initial depletion of ligand close to the surface. VEGFR1 and VEGFR2 concentrations higher than those used in the model have not been observed.

A decrease in cell size would lead to an increase in effective receptor concentration; however, the change required to see an effect is large. If the assumption of confluence implicit in the analysis were to be discarded, the loss of total cellular area would result in fewer receptors and, hence, lower total internalization of growth factor. This results in maintenance of higher ligand concentrations and thus decreases ligand shift.

The sensitivity of the results of Fig. 1 to variation in complex internalization rate is shown in Fig. 7B. Over a large range (10⁻⁶–10⁻³ s⁻¹), this internalization rate does not impact on the observed response of VEGF-VEGFR2 formation to PlGF. The same does not hold true for the internalization rate of unoccupied receptors, as increases in this rate must increase the constitutive insertion rate of receptors (see Model Parameters). A high turnover of receptors causes a greater depletion of ligand from the medium, and hence lowers fractional occupancies, which can lead to ligand shifting. For an unoccupied receptor internalization rate equal to the occupied receptor rate (which implies a receptor insertion rate increase of 28-fold), the increase in VEGFR2 ligation is 22%, and the increase in VEGFR1 ligation is still significantly (sevenfold) greater than the VEGFR2 increase.

The sensitivity of the results to variation in the other parameters used was also examined. The height of the fluid layer is directly related to the volume of medium-growth factor mixture added to the well. The effect of changing this height is to change the total quantity of ligand available for binding. The initial concentration is the same, but a lower total quantity can

Table 2. Additional parameters used in extended model

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding affinities, µM</td>
<td></td>
</tr>
<tr>
<td>$K_{d,V_{1M}}, K_{d,PM}$</td>
<td>0.5</td>
</tr>
<tr>
<td>On rates, $\times 10^3$ M⁻¹·s⁻¹</td>
<td></td>
</tr>
<tr>
<td>$k_{off,VM}, k_{off,PM}$</td>
<td>0.8</td>
</tr>
<tr>
<td>Off rates, $\times 10^{-3}$ s⁻¹</td>
<td></td>
</tr>
<tr>
<td>$D_{S1}, D_{S2}, D_{S3}$</td>
<td>0.65</td>
</tr>
<tr>
<td>VM, VEGF-extracellular matrix (ECM) complex; PM, PlGF-ECM complex; S1, soluble VEGFR1 (sVEGFR1); V31, VEGF-sVEGFR1 complex; P31, PlGF-sVEGFR1 complex.</td>
<td></td>
</tr>
</tbody>
</table>

VEGF-VEGFR2 complex formation as a marker of ligand shifting (other parameters are as for the original model). The displacement effect predicted at high VEGFR1 concentration reflects the fact that to observe shifting, the amount of VEGF displaced from the receptor must be large relative to the amount of VEGF already present in the medium. As the concentration of VEGFR1 becomes very large, it sequesters quantities of ligand at the same fractional occupancy. The results are similarly dependent on VEGFR2 concentration, an increase in that population increasing the initial depletion of ligand close to the surface. VEGFR1 and VEGFR2 concentrations higher than those used in the model have not been observed.

A decrease in cell size would lead to an increase in effective receptor concentration; however, the change required to see an effect is large. If the assumption of confluence implicit in the analysis were to be discarded, the loss of total cellular area would result in fewer receptors and, hence, lower total internalization of growth factor. This results in maintenance of higher ligand concentrations and thus decreases ligand shift.

The sensitivity of the results of Fig. 1 to variation in complex internalization rate is shown in Fig. 7B. Over a large range (10⁻⁶–10⁻³ s⁻¹), this internalization rate does not impact on the observed response of VEGF-VEGFR2 formation to PlGF. The same does not hold true for the internalization rate of unoccupied receptors, as increases in this rate must increase the constitutive insertion rate of receptors (see Model Parameters). A high turnover of receptors causes a greater depletion of ligand from the medium, and hence lowers fractional occupancies, which can lead to ligand shifting. For an unoccupied receptor internalization rate equal to the occupied receptor rate (which implies a receptor insertion rate increase of 28-fold), the increase in VEGFR2 ligation is 22%, and the increase in VEGFR1 ligation is still significantly (sevenfold) greater than the VEGFR2 increase.

The sensitivity of the results to variation in the other parameters used was also examined. The height of the fluid layer is directly related to the volume of medium-growth factor mixture added to the well. The effect of changing this height is to change the total quantity of ligand available for binding. The initial concentration is the same, but a lower total quantity can

Fig. 5. Number of VEGF-VEGFR2 complexes formed per cell, after the addition of both VEGF and PlGF. The original model conditions are noted (●). Experimental conditions that produce equivalent induction of gene expression in human umbilical vein endothelial cells (HUVECs) (55) are represented by □.

Fig. 6. Effect of soluble VEGFR1 on ligand shifting, maximal change in VEGF-VEGFR2 complex formation.

Fig. 7. Number of VEGF-VEGFR2 complexes formed per cell, after the addition of both VEGF and PlGF. The original model conditions are noted (●). Experimental conditions that produce equivalent induction of gene expression in human umbilical vein endothelial cells (HUVECs) (55) are represented by □.
lead to depletion in the concentration as the ligand molecules are internalized during the course of the experiment. Thus lowering the height, equivalent to using a smaller volume of medium, has a similar effect to increasing concentration of cell surface receptors.

The sensitivity to ligand concentration is dependent on the value of diffusivity and the values of the kinetic rates of binding: a lower value for diffusivity (or a higher value for kinetic rates) would increase the concentration gradients in the fluid, and thus the receptors would experience a lower effective ligand concentration, enlarging the effective ligand-shifting space shown in Fig. 4A. An order of magnitude decrease in diffusivity (to $10^{-7}$ cm$^2$/s) results in a maximal increase in VEGF-VEGFR2 complex formation of 21% and an increase in VEGFR1 complex formation of 72%. A range of variation in the binding affinities or the individual kinetic rates (see Table 1) did not increase the PlGF sensitivity significantly.

DISCUSSION

The results of the model developed here support the hypothesis that the observations of VEGF-PlGF synergy in cultured endothelial cells by Carmeliet et al. (9) are not the result of PlGF displacing VEGF to VEGFR2; eliminating this possibility leaves one with the conclusion that a change in the nature and/or quantity of VEGFR1 signaling due to PlGF binding is the mechanism by which synergy occurs. There are two significant differences between the two cases: the predicted brief increase in total VEGFR1 complex formation, which may result in changes in early signaling, and the shift from VEGF-VEGFR1 to PlGF-VEGFR1 formation, which is present throughout the time course of the experiment. Either or both may result in changes in signaling that account for the synergistic effects measured at the conclusion of the assay. PlGF administered alone was noted not to have an impact on the outcome of the experimental assays (9), and thus its synergy with VEGF is likely due to either 1) modulation of VEGFR2 activation or signaling by VEGFR1-dependent signals (discussed below) or 2) induction of VEGFR2 synthesis by VEGFR1-dependent signals. VEGF-induced upregulation of its receptors or of itself has been noted before (4, 45, 50).

The amount of ligand shifting is a direct consequence of the high growth factor concentrations administered in the assays: that ligand shifting is observed at all is due to transient ligand depletion close to the cell surface. Similar conclusions can be arrived at by examining three other experimental models of PlGF-VEGF synergy. In a recent gene expression study (55), the concentration of VEGF required to induce maximal gene expression in HUVECs was found to be 1 nM, but the same gene expression response was obtained by using 0.5 nM VEGF and 10 nM of a VEGFR1-selective VEGF mutant. These ligand concentrations are marked on Fig. 5 as solid squares; the concentrations of growth factors involved suggest little additional VEGFR2 complex formation. We would have to conclude that the experimental finding of synergy in that experiment would also be due to VEGFR1 signaling rather than displacement of VEGF to VEGFR2. High concentrations of PlGF (>100 pM) were shown to augment VEGF-induced proliferation at a submaximal VEGF concentration (<1 pM) in adrenal cortex capillary endothelial cells (43). However, the proliferation response was augmented much more than would be predicted in Fig. 4A and approached proliferation rates achieved at high VEGF concentrations (220 pM), which would be predicted by Fig. 5 to have considerably higher VEGFR2 complex formation. The time-dependent synergy observed in bovine aortic endothelial cells is also unlikely to be due to ligand shifting, again due to the high growth factor concentrations used (13).

The issue of PlGF synergy is intimately associated with the function of VEGFR1 on endothelial cells. In embryonic development, VEGFR1 appears to work as a dummy receptor, a VEGF sink, decreasing the amount of VEGF that is available to bind to VEGFR2: this coincides with a prevalence of the soluble form of VEGFR1 (9). Consistent with this, VEGFR1-null mice die at embryonic day 8.5 due to overproduction of endothelial cells (18), and mice in which VEGFR1 is expressed...
without its intracellular tyrosine kinase domain exhibit a defect in monocyte migration but not in embryonic vascular synthesis (23).

Signaling by the receptor in adult cells is a controversial issue; monocytes express VEGFR1 but not VEGFR2 and the receptor appears to transduce activation and migration signals (11). A signaling role in hematopoietic progenitor cells has also been demonstrated (10). In adult endothelial cells, a demonstration of active VEGFR1 signaling has proved elusive, because the tyrosine kinase activity of VEGFR1 is less than that of VEGFR2, but there is some indirect evidence. Were VEGFR1 acting as a dummy receptor to sequester VEGF, then antisense VEGFR1 should increase the signaling through VEGFR2. However, antisense VEGFR1 abrogated new capillary formation induced by VEGF in mouse as effectively as did antisense VEGFR2 (37). Tumors grew more slowly in mice that were null for the VEGFR1 tyrosine kinase domain than wild-type mice (22). Antibodies to VEGFR1 suppressed angiogenesis and tumor growth in mice (36, 49). However, in all of these cases, the explanation may be abrogation of VEGFR1 signaling in the monocytes (and hence inflammation), rather than in the endothelial cells. A diabody which blocks growth factor interactions with both receptors was shown to be more effective at inhibiting VEGF-induced migration and proliferation in cultured endothelial cells than antibodies to VEGFR2 alone (34), and an antibody for VEGFR1 blocked the synergistic effects of PlGF in the experimental assays modeled in this paper (9). VEGFR1 has been shown to transduce signals for actin reorganization (contributing to cell migration) in HUVECs (27), and VEGFR1 also downregulated VEGFR2 proliferation, via phosphatidylinositol 3-kinase 3-kinase (57) and VEGF-induced nitric oxide release, thus promoting stable capillary formation by HUVECs (8).

The present model describes an effect observed in cultured ECs in vitro, and while synergistic effects are also noted in vivo, it should be noted that extending the model directly to an in vivo situation is not straightforward. The concentrations of ligands added in the experimental assay (2.22 nM) may not be representative of physiological concentrations, but obtaining interstitial concentrations of ligands and the receptor populations of cells in situ is difficult. Measured tissue concentrations of VEGF range from 0.9 to 884 ng/g tissue (58); assuming an interstitial volume of 20%, this gives interstitial concentrations of ligands and the receptor populations of cells in situ is difficult. Measured tissue concentrations of VEGF range from 0.9 to 884 ng/g tissue (58); assuming an interstitial volume of 20%, this gives interstitial concentrations of 0.2–100 nM. However, this represents both free VEGF and VEGF sequestered in the ECM. Depending on the isoforms of VEGF prevalent in the tissue (40), the VEGF available for receptor binding may be a small percentage of the total (24). PlGF tissue concentrations in normal placenta were <4 ng/g tissue (~0.5 nM interstitial PlGF); however, choriocarcinoma tissue concentration was 116 ng/g tissue (42). Measured plasma concentrations of VEGF range from 0.5 to 2 pM and PlGF has been measured at 11 pM (14, 20, 26, 32, 33), and serum concentrations have been measured in the ranges of 0.5–4 and 1–11 pM, respectively (44, 52). At these lower concentrations, we cannot rule out ligand shifting as a part of the synergistic response (see Fig. 4A). In addition, the presence of soluble receptors and ECM proteins would lower diffusivity and change the transport characteristics of the growth factors, leading to increased possibility of ligand shifting (see Fig. 6). However, the number of receptors on the endothelial cell surface can vary over several orders of magnitude, even for the same cell type, due to cytokines, hypoxia, or other activating factors; the explanted cells for which receptor populations are measured likely overestimate the number in vivo due to overstimulation of the cells, decreasing likelihood of ligand shifting (see Fig. 7A). Experimental measurements of the receptor populations in vivo would be of great benefit in this analysis. The transients predicted for the in vitro model are due to a step change in ligand concentration; in vivo, there is a baseline secretion rate of growth factors, and changes in effective ligand concentration are gradual, due to an increase in secretion and subsequent diffusion to the endothelial cell surface. Thus the results of a similar analysis for the in vivo situation remain to be obtained.

The analysis in this study is based on a continuum model due to the high growth factor concentrations, and although this method has been used applied in previous studies of other ligand families (15, 19, 46), a stochastic model could be used to verify the range of applicability of the continuum assumptions when working with small concentrations of ligands and receptors, which appears to be the case in vivo. The analysis also relies on an assumed relationship between the formation of the ligand-receptor complexes and the generation of signals into the cell; we do not treat this signaling explicitly, using instead the formation of the complexes as a surrogate marker for the quantity of signaling. This relationship may not in fact be linear and may be tuned to small changes in the number of signaling complexes; information on the signaling from the receptors can be included in more accurately estimate the relative contributions of the receptors. One example of such signaling is an increase in the production of either or both of the VEGF receptors, due to signaling of VEGF-VEGFR2 complexes. Such an increase has the possibility of creating a significant feedback effect, which augments the increase in complex formation. This increase in the insertion rate of receptors can deplete the ligand growth factor in the medium further, decreasing the fractional occupancy and increasing the ligand shift. Because of the time required to synthesize and transport the new receptors to the surface, the early transient complex formation would be unchanged; however, an impact could be seen later in the time course.

This is the first model of VEGF transport and interactions with its receptors. To make the interpretation of the results tractable, we applied the model to an experiment with simple conceptual design and geometry. We have been able to reach conclusions that are of importance for understanding the mechanisms of interaction between VEGF and PlGF and their two receptors on the cell surface, VEGFR1 and VEGFR2. The model has limitations that could be relaxed in future studies. First, we considered only one isoform of VEGF, namely VEGFΔ65. There are at least six VEGFA splice isoforms in the human that compete for the same receptors and that have different binding affinities to the proteoglycans of the extracellular matrix. VEGF-PlGF heterodimers were also not included because their presence requires a cell type producing both VEGF and PlGF, but should be included in future models to be applied to the in vivo conditions. Second, there are additional cell surface VEGF receptors whose potential importance has recently been demonstrated: neuropilin-1 and -2 and VEGFR3. Third, explicit treatments of receptor dimerization and more complex, multireceptor interactions (including, for example, HSPGs) are additional features that may be impor-
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A layer of ECM is assumed to be laid between the cells and the medium; the height of this layer \(h_{\text{ECM}}\) was assumed to be 30 \(\mu\)m. The initial concentration of sVEGFR1, \(S_0\), was zero when secretion was included.

\[
M(t = 0, z < h_{\text{ECM}}) = M_0
\]

\[
S_1(t = 0, z) = S_{10}
\]  \(\text{(A1)}\)

\[
VM(t = 0, z) = PM(t = 0, z) = VS_1(t = 0, z) = \Psi S_1(t = 0, z) = 0
\]

These equations were solved using the parameters in Tables 1 and 2 and various initial concentrations of ECM sites, sVEGFR1, or secretion rates of sVEGFR1. Binding affinities for interactions with the ECM sites were estimated based on the capture of heparin-binding isoforms of VEGF (24), and kinetic rates were taken to be proportional to those for VEGF receptors. Binding affinities and kinetic rates for ligand interactions with sVEGFR1 were assumed to be identical with those for VEGFR1.

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REFERENCES


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