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Systems biology of pro-angiogenic therapies targeting the VEGF system

Feilim Mac Gabhann,

Institute for Computational Medicine and Department of Biomedical Engineering, Johns Hopkins University, Baltimore, MD 21218

Amina A Qutub,

Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Brian H Annex, and

Division of Cardiovascular Medicine, Department of Medicine and Robert M. Berne Cardiovascular Research Center, University of Virginia School of Medicine, Charlottesville, VA 22908

Aleksander S Popel

Department of Biomedical Engineering, Johns Hopkins University School of Medicine, Baltimore, MD 21205

Feilim Mac Gabhann: feilim@jhu.edu; Amina A Qutub: aminaq@jhu.edu; Brian H Annex: annex@virginia.edu; Aleksander S Popel: apopel@jhu.edu

Abstract

Vascular endothelial growth factor (VEGF) is a family of cytokines for which the dysregulation of expression is involved in many diseases; for some excess VEGF causes pathological hypervascularization, while for others VEGF-induced vascular remodeling may alleviate ischemia and/or hypoxia. Anti-angiogenic therapies attacking the VEGF pathway have begun to live up to their promise for treatment of certain cancers and of age-related macular degeneration. However, the corollary is not yet true: in coronary artery disease and peripheral artery disease, clinical trials of pro-angiogenic VEGF delivery have not, so far, proven successful. The VEGF and VEGF-receptor system is complex, with at least five ligand genes, some encoding multiple protein isoforms and five receptor genes. A systems biology approach to designing pro-angiogenic therapies, using a combination of quantitative experimental approaches and detailed computational models, is essential to deal with this complexity and to understand the effects of drugs targeting the system. This approach allows us to learn from unsuccessful clinical trials and to design and test novel single therapeutics or combinations of therapeutics. Among the parameters that can be varied in order to determine optimal strategy are dosage, timing of multiple doses, route of administration, and the molecular target.

Keywords

Drug efficacy; Drug transport; Physiome; Protein interaction networks; Systems medicine

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Major clinical problems follow systemic atherosclerosis that occludes blood flow in coronary artery disease (CAD), peripheral artery disease (PAD) and ischemic stroke. These diseases affect approximately 16.8, 8 and 6.5 million people living in the US, respectively [50]. For these diseases, techniques to increase the blood flow around vascular occlusions to the tissue by inducing angiogenesis, arteriogenesis or increased collateral pathways have

been clinically tested (for detailed review, see [35]), typically falling into one of three categories: physical interventions e.g. mechanical stimulation or transmyocardial laser injury [1,34,93]; cell-based, e.g. local implantation of autologous bone marrow cells [73,93,94]; and growth factor-based, e.g. gene delivery of pro-angiogenic cytokines to the target tissue, the most studied being VEGF [32,53,79] and FGF [24,25,85]. So far, however, growth-factor based pro-angiogenic clinical trials for CAD or PAD have not proven successful (Sidebar 1).

Sidebar 1

VEGF-targeting clinical trials in CAD and PAD

The known pro-angiogenic clinical trials involving VEGF, listed below, have not yet successfully yielded an approved drug. For more detail and other trials, see the following reviews: [2,26,35,36,³⁹,86,92,97,103]. IC, intracoronary; IV, intravascular; IM, intramuscular; IA, intra-arterial; CAD, coronary artery disease; PAD, peripheral artery disease; EC, endothelial cell.

CAD

VEGF₁₆₅ protein, IC infusion, Mild defect reduction [31]

VEGF₁₆₅ protein, IV infusion, No clear effect [33]

VEGF₁₆₅ protein, IC+IV infusion (VIVA trial), No clear effect [32]

AdVEGF₁₆₅, IC infusion (KAT trial), Improved perfusion [28]

AdVEGF₁₂₁, IM injection (REVASC trial), Improved exercise duration [88]

VEGF₁₆₅ plasmid, IM injection (Euroinject One trial), No functional improvement [41]

VEGF₁₆₅ plasmid, IM injection (NORTHERN trial), No functional or perfusion improvement [89]

PAD

VEGF₁₆₅ plasmid, IM injection, Collaterals, Edema [6,7]

AdVEGF₁₂₁, IM injection, Tolerated, Improved EC function [80,81]

SB-509 (ZFP-VEGF plasmid), IM injection, Increased circulating progenitor cells [64]

AdVEGF₁₆₅, IA injection, Increased vascularity [61]

AdVEGF₁₂₁, IM injection (RAVE trial), No functional improvement [79]

VEGF₁₆₅ plasmid, IM injection (Groningen trial), No improvement in amputations [45]

Ad2-HIF1 α -VP16 (WALK trial), IM injection, No functional improvement [13]

When considering collateral formation and endothelial growth it is critical to remember that ischemic diseases frequently occur as a result of or within the context of vascular risk factors, e.g. hypercholesterolemia, diabetes and hypertension. These complicating factors make it even more important that drug design be comprehensive, rational, and optimized for the individual patient or group of patients.

In this review we outline the pro-angiogenic therapeutic possibilities of the VEGF family. Ligands of this family are strong cytokines that induce robust effects on the microvasculature [103]; they are widely studied and have been subjected to numerous clinical trials [35]; they form a complex network of interactions that are not amenable to

single-agent reductive investigation [59]; and they have been successfully targeted by inhibitory anti-angiogenic therapies (Sidebar 2), suggesting that pro-angiogenic strategies are possible, with the correct formulation and design of the therapy.

Sidebar 2

The gap between approved anti-angiogenic therapies and pro-angiogenic therapies targeting VEGF system

Several anti-angiogenic drugs targeting the VEGF-VEGFR system have been approved for various cancers or for wet AMD, while no pro-angiogenic formulations have been approved. This gives us the opportunity to compare pathways targeted by the pro-angiogenesis and anti-angiogenesis compounds using systems biology and computational modeling approaches, to find common traits of the successful and unsuccessful therapies.

Wet AMD: pegaptanib (Macugen), aptamer for VEGF₁₆₅; ranibizumab (Lucentis), antibody for VEGF

Cancer: bevacizumab (Avastin), antibody for VEGF; tyrosine kinase inhibitors sunitinib (Sutent) or sorafenib (Nexavar); blockade of VEGF secretion via EGFR by gefitinib (Iressa) or erlotinib (Tarceva)

Systems biology may improve therapeutic design

Clinical trials to increase expression of one or even multiple VEGF ligands in CAD and PAD have not succeeded (Sidebar 1). For a multicellular physiological mechanism as complex as microvascular remodeling, with multiple cytokine families involved [59, 76], a more nuanced approach is needed. In particular, quantitation is a core requirement for the design of new therapies. This is where systems biology – a synergistic combination of computational models and quantitative biological experiments – is most important. Without extensive measurement of in vitro and in vivo mRNA, proteins and signaling pathways, as well as anatomical and physiological parameters, computational models may not yield meaningful results; likewise, without the ability to integrate the wealth of experimental data into models, therapeutic design is somewhat blind.

By establishing a solid quantitative foundation, the systems biology approach allows the prediction of the most promising target, and can optimize dosing, timing and even combinatorial delivery of multiple agents. This approach allows the rapid analysis of multiple conditions and in-vivo studies can then establish which set of modeled conditions is most appropriate. By allowing researchers to test many approaches in silico, the success rate of the translation to in vivo drug or gene delivery can be improved.

In addition, we aim to ultimately identify subgroups of the patient population who will respond well to different therapies. Just as trastuzumab (Herceptin) works only for those with HER-2-positive breast cancer, so too the individual variability in ischemic diseases (e.g. baseline VEGF ligand/receptor expression) can be used to design specific therapies for the affected subpopulation. In ischemia, or during exercise training, multiple elements of the VEGF pathway exhibit altered expression [51,95]; we can use systems biology to test therapies that mimic these multifactorial changes.

Among the quantitative experimental approaches useful for systems biology, characterization of cytokine networks include microarrays (for expression data), proteomic and phosphoproteomic arrays (for protein concentrations) and mass spectrometry. A subset of the data is incorporated into the computational models as parameters or inputs; the

remainder is reserved for comparison to model outputs (validation). The computational models are therefore designed to be molecularly-detailed; that is, they specifically include all the molecules involved to enable comparisons to experimental data. Models may be developed *ab initio* or use one of many modeling platforms to create and simulate experimental conditions, including BioNetGen [17], VCell [66], SBMLToolbox [42], ECell [91] and others.

It is important to note that Systems Biology is not an end in itself; one of the primary goals in creating and using computational models such as those described in this review is to generate new, and most importantly, *testable* hypotheses to guide scientific progress. As with any model system, including cell culture and pre-clinical animal models, predicted optimal treatments must be extensively tested for safety, efficacy and more. In addition, as hypotheses are tested, both positive and negative outcomes are instructive. Model-building is, in a sense, never complete, and negative outcomes inform additions, revisions and improvements to the model, to then generate improved hypotheses. However, predicted optimal treatments might not be arrived at – or might have taken much longer to generate – without the computational models.

The pro-angiogenic VEGF-VEGFR axis

The VEGF-VEGFR system is a largely paracrine signaling network in which hypoxic or otherwise activated parenchymal or stromal cells secrete ligands that diffuse to and bind receptors that are likely located primarily on the surface of endothelial cells.

Transcriptional regulation of VEGF and VEGFR

The angiogenic balance (Figure 1A) is maintained by pro- and anti-angiogenic proteins, of which the VEGF family is one part. VEGFA was the first gene shown to display haploinsufficiency [10, 18], and two- to three-fold overexpression of the gene is also embryonic lethal [63]; thus its expression appears to need tight control during development. Expression of the VEGF and VEGFR genes is controlled by a number of transcription factors. The best-studied of these are the hypoxia-inducible factors, HIF-1 and HIF-2 [77, 99]. Low local oxygen levels lead to increased stability and activity of the HIFs, which translocate to the nucleus, bind to the HREs (hypoxia response elements) upstream of the various VEGF and VEGFR genes (Figure 2), and activate transcription. HIF-2 shares the same hydroxylation cofactors and functions in a similar oxygen-sensing manner to HIF1, but the two isoforms are expressed differentially in different tissues and during disease [74]; HIF2 also appears to play a dominant role in mammalian development [62, 72].

In addition, non-HIF hypoxia-responsive transcription factors (e.g. PGC-1 α /ERR α) can control expression of VEGF family members [3]. In cancer, there are also hypoxia-independent VEGF-upregulation pathways downstream of oncogenes such as K-ras, however these are less relevant for our purposes here [65]. Recent studies point to the cross-talk between hypoxia-responsive transcription factors (e.g., PGC-1 α and HIF1) and suggest mechanisms as to how this cross-talk could affect VEGF [68] For a comprehensive review of VEGF gene regulation, see [22].

Complexity of the VEGF and VEGFR families

The VEGF family consists of at least five genes encoding ligands (*vegfa* thru *-d*, *plgf*), three receptor genes (*vegfr-1* thru *-3*) and two co-receptor genes, the Neuropilins (*npr-1* and *-2*). These genes can be expressed at different levels and can be differentially regulated biologically [12]. In addition, each of these genes can result in translation of multiple proteins due to alternate mRNA splicing [46]. This confers on certain ligands but not others the ability to bind to extracellular matrix proteins or co-receptors; and results in both

membrane-bound (signaling) and non-membrane bound (nonsignaling or sequestering) receptors (Figure 3). The binding interactions between all the VEGF ligands and all the receptors are a complex web of competing and non-competing reactions (Figure 3). Upstream, there are multiple HIFs and other transcription factors; downstream, receptors may heterodimerize and each receptor-ligand-receptor combination has a different phosphorylation profile, initiating a large number of alternate signaling pathways.

The major problem with this level of complexity is not only the creation of a computational model, but also selecting the models and data needed for validation. Many experiments focus on and measure only one or two or three of the VEGF isoforms, while there are many more (Figure 3). How do we include incomplete results such as these in our model? The number of parallel measurements now required for comparison with the model predictions becomes high and high-throughput systems biology approaches are necessary.

Individual variability increases the complexity

As we approach the era of personalized medicine, it is becoming increasingly clear that inter-individual differences – whether due to genetic polymorphisms, environmental history, cellular damage, coincident diseases or diet – can vastly change not just the prognosis but also the prescription. As two examples, the pre-existing vascular structure can determine the extent of injury following ischemic arterial blockade [4,11,16,29,60,90], and expression of ligands and/or receptors can be altered by disease background [27]. Inbred mouse strains or genetically modified mice can serve as a reproducible pre-clinical analog of inter-individual variability. Specific examples for ischemic diseases include mouse models of diabetes, hypercholesterolemia and hypertension. Again, a systems biological approach seeks out, measures, and exploits exactly these details in order to: (a) further validate the predictive models; and (b) generate individualized treatment plans.

Therapeutic targets in the VEGF-VEGFR axis

While most tested strategies have targeted VEGF ligands, if we consider the pro-angiogenic axis (Figure 1B), spanning stimuli (e.g., hypoxia, cytokines, cell transformation), transcription factors (e.g. hypoxia inducible factor, HIF), VEGF ligands, VEGF receptors, and downstream signaling, these are all potential targets for therapeutic intervention. The goal with combined computational-experimental systems biology approaches is to answer the dual questions: which of these therapies is most likely to work, and for whom?

HIF1 α as a therapeutic target

Possible pro-angiogenic targets: gene delivery of HIF; ZFP-VEGF; HIF hydroxylation cofactors—Due to the extensive and well-defined control network that surrounds the HIFs, numerous methods for targeting its regulation have been put forward. While most studies have focused on downregulating HIF1 in cancer, a growing number focus on upregulation of HIF, and by extension, increased VEGF production. These pro-angiogenic strategies include gene delivery of HIF and modulation of cofactors in HIF hydroxylation.

Preclinically, adeno-associated virus (AAV) delivery of HIF1 α to mouse tibialis anterior skeletal muscle [71] resulted in increased capillary sprouting, VEGF-dependent endothelial proliferation and increases in overall limb perfusion. Intramuscular injections of an adenoviral vector expressing a constitutively active HIF1 α hybrid (AD2/HIF1 α /VP16) increased capillary density and reduced vascular leakage in a diabetic rat model of hindlimb ischemia [40]. However, clinical testing of the AD2/HIF1 α /VP16 construct in human PAD showed no functional benefit [13]. An engineered zinc finger protein transcription factor

(ZFP-VEGF) that upregulated all VEGF-A isoforms also showed promising preclinical results in mice [14,49,104] but has not progressed beyond Phase I clinical testing in PAD [64]. The failure of these two agents suggests that even upregulating multiple VEGF isoforms is not a solution, reinforcing that ligands alone should not be the target.

Beyond targeting HIF1 directly, pro-angiogenic strategies include targeting the HIF1 hydroxylation pathway. By blocking hydroxylation, HIF1 cannot bind to von Hippel Lindau protein, and hence cannot be ubiquitinated and degraded. The main enzymes involved in hydroxylation are the prolyl hydroxylase domains (PHD1, PHD2, PHD3). Each is expressed differentially across tissues and cell types, as well as localized differentially within individual cells. The different isoforms also have different binding rates to HIF1 and unique sensitivities to oxygen levels. A recent study tested electrotransfer of plasmids encoding for short hairpin RNAs (shRNA) directed against HIF1, PHD1, PHD2, and PHD3 as a pro-angiogenic strategy in mice with femoral artery ligation [52]. Encouragingly, when PHD2 and PHD3 were silenced in this study, increases were seen in capillary density (1.8 and 2.1 fold, respectively) and foot perfusion (1.2 and 1.4 fold, respectively) in mice with hindlimb ischemia.

As with the VEGF family, the complexity of the HIF1 system and its inconsistent therapeutic success, can be approached and better understood quantitatively through the use of models. To this end, extensive computational modeling of the HIF family has taken place [15,43,75,77,78,105]. Benefits of these modeling efforts towards pro-angiogenic drug development are twofold: (1) highlighting novel and/or multiple pathways to target therapeutically and (2) quantitatively predicting the relative degree and timing of HIF regulation required to achieve desired therapeutic effects.

Intracellular models detailing the kinetics of HIF1 hydroxylation open up the possibility of targeting multiple cofactors in silico. Beyond the PHDs, the intracellular hydroxylation cofactors that control HIF responses to oxygen include CoCl_2 , ascorbate (vitamin C), deoxoglutarate and iron. In silico results predict that modification or depletion of ascorbate or iron may enhance HIF1 accumulation, however nonspecifically [77]. Models also assess the degree to which accumulation of succinate (a byproduct of the hydroxylation reaction and a TCA cycle intermediate) can block HIF1 hydroxylation and subsequent degradation [78]. A recent model predicts how the protein FIH (factor inhibiting HIF1) quantitatively impacts HIF1 activation and genetic regulation of VEGF [15]. Other molecules interfering with this pathway include reactive oxygen species (ROS) and hydrogen peroxide in particular. Therapeutically, such as in ischemia, increases in ROS could upregulate HIF1 and may induce angiogenesis; however care must be taken not to cross a threshold beyond which ROS causes cell damage and even apoptosis. Computational predictions of HIF correlated to hydrogen peroxide levels during and postischemia can be used to guide therapeutic regimes and tight regulation of HIF1 [75].

Predictions from the computational models of the HIF1 pathway and knowledge from ongoing HIF1-targeted clinical trials in pro-angiogenic systems medicine could be optimized when coupled with experiments and models of the VEGF system.

VEGF-A isoforms as therapeutic targets

Possible pro-angiogenic targets: gene delivery of VEGF₁₆₅, VEGF₁₂₁—Targeted (intramuscular, intracoronary) or systemic (intravenous) delivery of VEGF-A isoforms as genes or proteins have comprised the majority of clinical trials to this point (Sidebar 1). Other cytokines, including bFGF, have also been delivered, but these have not produced better results [24, 25, 85]. Systems biology allows us to ask why these treatments have not been successful, using a quantitative perspective.

Using three-dimensional computational models of VEGF transport in skeletal muscle, we have shown that VEGF delivery alone – whether by gene or cell-based delivery – can increase the average activation of VEGFR2 in the tissue, but does not significantly increase the interstitial gradients of VEGF [38,54–56]. In contrast, exercise – the current therapy for PAD, which increases both ligand and receptor expression – was predicted to increase both VEGFR2 activation and VEGF gradients; thus VEGF-only therapies may stimulate endothelial activation through VEGFR2, but fail to provide sufficient directional information to guide neovascularization.

The ability of exogenous VEGF to increase VEGFR2 activation comes, however, at a price. Using a single-compartment model of skeletal muscle, we showed that there is an accompanying increase in the ligation of VEGFR1, modulating VEGFR2 activation via its own signaling pathways [57]. This concomitant increase in competing pro- and anti-angiogenic signals suggests that the impact of VEGF depends on the relative expression levels of each of the receptors before and after the intervention, and may indicate why VEGF-A isoforms have not been effective targets. Below we suggest methods to circumvent this problem.

An alternate possibility is that delivery of VEGF genes to the tissue or blood stream has been inefficient and transient. Sustained local delivery of VEGF DNA by hydrogels has demonstrated improvements in mouse perfusion [44], but it remains to be seen whether this translates to human therapy. Computational modeling can assess the predicted difference between: delivery of genes and recombinant proteins; single, multiple and continuous dosing; local and systemic administration.

Taking this further to multi-compartment models of VEGF transport throughout the body, we noted that an increase in VEGF production in one tissue did not significantly alter VEGF concentration in other tissues unless vascular permeability was very high [87,101]. Surprisingly, a sequestering agent for VEGF ligands (sVEGFR1) did not result in a decrease in VEGF concentration in the plasma, but in fact increased it due to a shuttling effect [100,101]. Counterintuitive results such as these are precisely why systems biology is so important to the study of therapeutics.

Other ligands as therapeutic targets

Possible pro-angiogenic targets: gene delivery of PlGF; VEGF-B; VEGF-C—A system as interconnected as the VEGF family has many levers (Figure 3). Rather than using VEGF-A isoforms to increase angiogenic signaling, it may be beneficial to target other ligands such as PlGF and VEGF-B, which bind to VEGFR1 but not VEGFR2 [20]. PlGF-VEGFR1 complexes are differentially phosphorylated compared to VEGF-VEGFR1 [5], and appear to be pro-angiogenic. In our models, administration of PlGF both increases pro-angiogenic signaling while simultaneously cannibalizing the modulatory VEGFR1 signaling [57,58]. We will note this ability to tip both sides of the angiogenic balance (Figure 1A) in the positive direction later for receptor-targeting therapies also, but ligands are considered easier to deliver. Notably, plasma PlGF levels are not elevated in PAD patients, while VEGF levels are [19], suggesting that there is room for additional PlGF activity.

To our knowledge, results of clinical trials in CAD or PAD using these alternate VEGF family ligands have not yet been published, though negative results for VEGF-C plasmid delivery caused that trial to be halted [103].

Matrix components as therapeutic targets

Possible pro-angiogenic targets: protease activation; integrin signaling—The extracellular matrix is centrally involved in vascular remodeling. Along with sequestering certain isoforms of VEGF, it communicates directly with endothelial cells through integrins, some of which modulate the signaling of growth factor receptors [21]. Pro-angiogenic signaling may be augmented by release of sequestered VEGF isoforms, for example by proteases [30], though truncated isoforms appear to encourage endothelial proliferation at the expense of vessel branching [48], and thus it is not yet clear whether proteases are pro-angiogenic on balance.

VEGFRs and NRPs as therapeutic targets

Possible pro-angiogenic targets: gene delivery of VEGFR2, NRP1; siRNA to VEGFR1—Upregulation of VEGF receptors during exercise is predicted to significantly increase both the intracellular signaling and the extracellular VEGF gradient [38,54–56]. This suggests that delivery of receptors could be an effective pro-angiogenic therapy. While VEGF increases signaling of both VEGFR1 and VEGFR2, our models predict that increased endothelial expression of VEGFR2 cannibalizes VEGF from VEGFR1, thus having two synergistic impacts on pro-angiogenic expression [57]. Similarly, NRP1 increases the binding of certain isoforms of VEGF to VEGFR2, while blocking their binding to VEGFR1. Combination therapies – concomitant upregulation of both ligand and receptor – would be predicted to be even more effective in increasing the pro-angiogenic signal at the expense of anti-angiogenic signals.

Ligands that diffuse from one cell to another can be delivered in multiple ways: as a protein via intratissue or intravascular infusion; as a gene expression vector with or without cell-specific promoters. For membrane-bound receptors, on the other hand, protein delivery would not be successful. In addition, for receptors location is central to function, and thus gene delivery vectors must include cell-specific promoters in order to target the expression to the correct cell type (typically, endothelial cells). Several efficient endothelial-specific promoters exist (e.g. CD31 or Tie2) and are currently used for labeling. Transient expression of delivered genes may pose a problem, however this is equally an obstacle for ligand gene delivery.

Genetically engineered stem cells also may offer a delivery mechanism for membrane-bound receptors, as well as ligands, to a localized area. Recently increased local VEGF ligand expression in mouse ischemic hindlimb was achieved through the implantation of scaffolds of mesenchymal stem cells that had been made to express hVEGF gene via nonviral, biodegradable nanoparticles [102].

In view of the predicted large response to VEGFR delivery and predicted sensitivity of VEGF delivery to baseline receptor expression, it is clearly important to use experimental systems biology techniques to quantify receptor expression in vivo. Although this is possible using, for example, technetium-labeling, new high-throughput methods are needed. Quantitative characterization of receptor content would also be important for future progress of personalized medicine.

To our knowledge, VEGF receptor delivery has not entered clinical testing for CAD, PAD or IS.

Potential therapeutic targets downstream from VEGFRs

Possible pro-angiogenic targets: phosphatase inhibitors, signaling kinases—

The complex web of intracellular signaling downstream of VEGF receptor activation has been the subject of many studies and reviews [69,83]. Clinical trials of drugs blocking or stimulating receptor phosphorylation and signaling are underway in cancer, but not in ischemic diseases. This is a fertile area for drug design, as along with transducing downstream effects, many signaling pathways also result in the feedback up- or down-regulation of ligands and receptors. Thus, a drug-initiated 'virtuous circle' is possible, reinforcing pro-angiogenic physiological signals that may be too weak to help in the disease state.

Cell-cell communication as therapeutic target

Possible pro-angiogenic targets: dll4-notch signaling—Not all endothelial cells exposed to pro-angiogenesis levels of VEGF form tip cells and sprout from nascent vessels. In fact, correct spacing of blood vessels would be important for improved network topology. Control of vessel sprouting is accomplished by the Delta (Dll)-Notch system: VEGF-VEGFR2 signaling increases Dll4 expression, which binds to Notch1 on the neighboring endothelial cell, repressing VEGFR2 expression and upregulating VEGFR1 in that cell, resulting in VEGF insensitivity and quiescence adjacent to tip cell formation [8,37]. For pro-angiogenesis therapies, however, it is not clear how to employ this pathway: repression would increase the density of vessel sprouting, however this can result in inefficient vascularization without functional benefit.

Cadherins have been shown to associate with VEGF receptors in cell-surface junctional complexes [96]. VEGFR2 signaling disrupts cell-cell junctions by phosphorylation and internalization of VE-cadherin, increasing permeability. This is also a feedback cycle as VE-cadherin association can prevent VEGFR2 endocytosis and increase perimembrane dephosphorylation of the receptor [47]. Disrupting VE-cadherin may play a viable supporting role in promoting new vessel formation; however it is likely that this process is more relevant to VEGF's permeability functions than to new vessel growth.

Promotion of neovessel stability

While the VEGF family is centrally involved in endothelial cell mobilization, tip cell specification and chemotaxis, newly formed vessels are vulnerable to regression unless they undergo further specialization. In addition, VEGF delivery can result in hypervascular growth, or growth of abnormal vessels [70]; similar vessels are typically observed in tumors [67]. Thus, VEGF family targeting for the induction of angiogenesis may require accompanying or temporally-delayed cofactors that increase the stability or maturity of the new vessel, such as angiopoietin-I (Ang I) [82], or the recruitment of pericytes, through cytokines such as platelet-derived growth factor (PDGF) [23] or transforming growth factor beta (TGF β) [98].

Off-target and side effects

Along with therapeutic predictions based on the impact of a strategy on a target, combined experimental-computational systems biology approaches also allow us to pre-emptively ask important questions such as: What happens if the VEGF therapy goes to tissues that do not need it? What happens if increase in VEGF receptors occurs on cells other than ECs? What are the long-term impacts of alterations in vascular permeability?

Future outlook

While the current state of knowledge allows us to make reasonable predictions about which elements in the VEGF pathways to target, and how to deliver therapies, the cross-talk between VEGF and other pathways opens even more possible avenues to explore [97]. For example, it has been suggested that inhibition of the Notch pathway may stimulate VEGFR2 expression and thus increase sensitivity to VEGF [9]. Methods of delivery can also go beyond intramuscular and intravenous plasmids or proteins, with promising preclinical results now emerging from controlled-release hydrogels containing VEGF [44,84]. Cell-based therapies, either those that overexpress VEGF, or delivery of endothelial precursor cells, continue to be studied as candidate future VEGF-targeted therapies. We have primarily focused on methods for increasing and decreasing promoters of angiogenesis, e.g. delivery of VEGF or of VEGF-sequestering agents. There are other options (Figure 1A) including upregulation of endogenous inhibitors (anti-angiogenesis) or repression/secretion of endogenous inhibitors (pro-angiogenesis). That is certainly an opportunity for further investigation.

Conclusion

Clinical trials targeting the VEGF family in ischemic disease have so far focused on the ligands, and these trials have not succeeded. By using a combination of quantitative biology and computational models, we can not only suggest reasons for the failure of ligand-directed therapies, but also predict more effective targets, e.g., the VEGF receptors. For all of the therapies described here, both present and future, the further parallel development of experimental quantitation and computational modeling is central to increasing the probability of successful therapies being tested and approved.

Sidebar 3

Complexity of the VEGF-VEGFR axis

Systems biology and systems medicine use both quantitative biology and computational modeling to deal with the combinatorial complexity of the VEGF system. For a more detailed discussion, see [59].

Transcription factors (TFs): At least three hypoxia-dependent TFs; also non-hypoxia-dependent TFs; multiple co-factors involved in activation or stability of the TFs.

Ligands: five human VEGF genes; up to 7 alternately spliced isoforms per gene; secreted protein is homo- or heterodimeric; diffusible or presented by extracellular matrix.

Proteolytic processing: sequestered ligands can be freed by proteolytic cleavage of the matrix or of the ligand itself; in addition, soluble receptor forms can be cleaved from membrane-bound receptors.

Receptors: three VEGFR genes; membrane-bound and truncated soluble isoforms; receptors can homo- or heterodimerize.

Co-receptors: two Neuropilin co-receptor genes; membrane-bound and truncated soluble isoforms; multiple cell-surface heparan sulfate proteoglycan co-receptors.

Receptor Context/Location: abluminal vs. luminal (polarization); filopodial vs cell body (for sprouting cells); cadherin and integrin association; signaling by intracellular VEGF receptors; non-endothelial cell expression.

Ligand-Receptor complexes: each ligand isoform has different patterns of binding to each of the receptors; each ligand-receptor pair produces different receptor phosphorylation profiles.

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Figure 1. Overview of the HIF-VEGF-VEGFR angiogenic axis

A, The angiogenic balance is maintained by pro- and anti-angiogenic proteins. To push the system towards angiogenesis, either an increase in the pro-angiogenic factors or a decrease in (or inhibition of) anti-angiogenic factors can be employed. Exercise does both by increasing expression of both the ligands and the pro-angiogenic receptor expression, thus decreasing binding of VEGF to anti-angiogenic or modulatory receptors. B, Stimuli such as hypoxia, mechanical stress and other cytokines can cause an increase in expression or stability of transcription factors (TFs), notably HIF-1 and HIF-2, but also PGC-1 and others. Upon binding to the hypoxia response element (HRE) or other upstream regulatory element on the ligand genes, these TFs increase production and secretion of one or more of the many VEGF ligands, including isoforms of VEGF-A and PlGF. Following diffusion through the extracellular matrix, and possibly communication between tissues via the blood, the ligands bind to receptors on endothelial cells, initiating pro-angiogenic signals. Contextual stimuli such as cell-communication and cell-matrix interactions can further modify the response to VEGF signals.

Figure 2. Transcriptional regulation of VEGF

While HIF binding to the HRE is the best-studied part of the *vegfa* promoter system, several other transcriptional activators and co-activators are known, both hypoxia-dependent and – independent. The hypoxia-independent pathways are often dysregulated in angiogenic tumors. Note that *vegfa* was the first gene shown to display haploinsufficiency, and thus its expression appears to normally be tightly regulated, particularly during development.

Figure 3. VEGF and VEGF receptor proteins and their interactions

The mRNA products of *vegfr* and *plgf* gene expression are spliced into multiple isoforms. The protein products have different profiles of binding to each of the five cell-surface receptors in the *vegfr* and *npr* families. In addition, the receptors themselves have alternate splice forms that are truncated and non-cell-surface associated. Typically, these soluble forms of the receptors bind the same ligands as the full-length receptor.

Figure 4. Sample results from computational models of VEGF gene therapy

As noted in the text, clinical trials involving gene delivery of VEGF ligands, or of ligand-upregulating transcription factors, have not been successful in treating ischemic diseases. Using a computational model of VEGF transport in skeletal muscle, we predict that VEGF ligand upregulation results in simultaneous upregulation of both pro-angiogenic VEGFR2 activation (VEGFR2*) and modulatory VEGFR1 activation (VEGFR1*). By calculating the change in the ratio of the competing VEGFR2 and VEGFR1 signals following treatment, we note that there is actually a slight decline in this anti-angiogenic metric (yellow bar). Interestingly, this result is independent of the relative strengths of the VEGFR kinases. In contrast, by increasing VEGFR2 or NRP1 expression, or by decreasing VEGFR1 expression, the model predicts lower increases in VEGFR2 activation, but decreases in VEGFR1 activation, resulting in a substantial improvement in the signaling ratio pro-angiogenic metric. Data for this graph was taken from unpublished results, based on models developed in [57].