Angiogenesis
Where Do We Stand Now?

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The concept of modulating blood vessel growth—stimulation or inhibition—to serve a desired clinical goal has an enormous intellectual appeal. It can be used to relieve ischemia in tissues with compromised arterial blood supply, improve edema in areas of impaired lymphatic drainage, and inhibit the growth of tumors. Other applications involve facilitating reendothelialization and inhibiting neointima formation after vascular injury, preventing the progression of atherosclerotic plaque, and promoting "vascular health" in general. The least-explored application of this concept involves modulating body and organ size by regulating the endothelial cell mass.

All of these areas received a significant, albeit varied, amount of attention in past decades but only recently have we begun approaching clinical applications. Indeed, the first antiangiogenic agent has been approved by the US Food and Drug Administration for the treatment of certain types of cancer, and it is hoped that the emergence of therapeutically useful angiogenesis-promoting agents is not far behind. This article examines some of our current understanding of the regulation of blood vessel growth in mature adult tissues and the various therapeutic applications of this knowledge. Obviously, a review of this size cannot cover such a vast field in any amount of detail, and the author regrets not citing primary references for many of the facts and concepts that are mentioned.

Angiogenesis, Arteriogenesis, Vasculogenesis

In the cardiovascular field, there is a tendency to think of new vessel growth occurring almost exclusively in the setting of ischemia. Although ischemia is clearly an important stimulus for such an event, it is not the only one. The spectrum of physiological and pathophysiological processes in which blood vessel growth occurs ranges from tissue hypertrophy (eg, left ventricular pressure overload) to wound healing (eg, postangioplasty restenosis) to inflammation (eg, coronary atherosclerotic plaque), among others. It is, therefore, important to consider a specific blood vessel growth paradigm in the context of its biological milieu.

The process of new blood vessel growth is frequently referred to as angiogenesis. Because this term has also come to denote a specific biological process that does not encompass the entire spectrum of events that can result in new blood vessel development, it is used here only in a narrow sense and blood vessel growth is referred to in general as neovascularization. As currently understood, neovascularization is the result of several processes, including angiogenesis, arteriogenesis, and, potentially, vasculogenesis (Figure 1). The term *angiogenesis* describes the sprouting of new capillaries from postcapillary venules,[1] and in adults, it is stimulated mainly by tissue hypoxia via activation of hypoxia-inducible factor (HIF)-1α expression. HIF-1α activates the transcription of numerous genes, including vascular endothelial growth factor (VEGF), VEGF receptors flt-1 and neuropilin-1, and angiopoietin-2, among others.[2] Angiogenesis leads predominantly to the development of capillaries, although the formation of larger-size vessels has also been noted in certain animal models. An important issue related to ischemia-induced angiogenesis is whether even a large increase in the capillary bed size can be effective in increasing overall blood flow to the tissue in the presence of flow-limiting lesions in the proximal arterial conduit.

In contrast, *arteriogenesis* refers to the process of maturation or perhaps de novo growth of collateral conduits[3,4] that are frequently of a sufficient diameter to be visualized angiographically.[5] Arteriogenesis typically occurs outside the area of ischemia in response to local changes in shear stress–induced accumulation of blood-derived mononuclear cells at the sites of arterial stenosis. Our understanding of the ensuing sequence of events is rather sketchy, but it seems to involve the release of a number of growth factors including fibroblast growth factors (FGF), platelet-derived growth factors (PDGF), and VEGF[3,6] in addition to CXC cytokines. Because arteriogenesis leads to the formation of arterial conduits, its ability to fully restore blood flow is considerable.

One important and hotly debated issue related to arteriogenesis is whether collateral development occurs de novo, similar to angiogenesis, or whether it represents remodeling and enlargement of preexisting vascular channels. Angiographic studies in rodent hindlimb ischemia models clearly demonstrate remodeling of preexisting vessels.[3] Whether this is specific to rodents or whether an equally extensive collateral tree exists in humans is not clear at this time. One important development has been the advent of micro-CT

Neovascularization

Angiogenesis   Arteriogenesis   Vasculogenesis

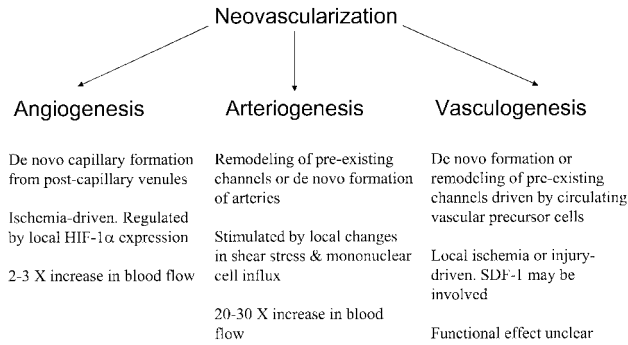| Angiogenesis	Arteriogenesis	Vasculogenesis
De novo capillary formation from post-capillary venules	Remodeling of pre-existing channels or de novo formation of arteries	De novo formation or remodeling of pre-existing channels driven by circulating vascular precursor cells
Ischemia-driven. Regulated by local HIF-1α expression	Stimulated by local changes in shear stress & mononuclear cell influx	Local ischemia or injury-driven. SDF-1 may be involved
2-3 X increase in blood flow	20-30 X increase in blood flow	Functional effect unclear

Figure 1. Neovascularization: 3 processes that can result in formation of new vessels in adult tissues. SDF-1 indicates serum-derived factor-1; HIF-1α, hypoxia-inducible factor-1α.

analysis, which allows far more accurate assessment of collateral response than has been available (Figure 2). It is also interesting to note that the collateral response tends to be overexuberant, with only a few of the newly formed collaterals surviving long-term.

The role of preexisting collateral channels in the heart is far less well established. Early studies failed to show the existence of coronary-to-coronary artery connection in normal human hearts[7,8]; however, functional studies show that up to 25% of patients with coronary stenosis demonstrate the existence of significant collateral flow when challenged with a temporary coronary occlusion.[9]

Finally, *vasculogenesis* is the process of an in situ formation of blood vessels from circulating endothelial progenitor cells (EPCs) and vascular progenitor cells.[10,11] The functional significance of vasculogenesis in the setting of ischemia in coronary or peripheral circulation has not been established conclusively. Remarkably, the reported spectrum of benefit ranges from significant[12] to none at all.[13] Clearly, this area requires much additional investigation.

It should be noted that the choice of a model to study a particular neovascularization event may give undue prominence to one or the other processes mentioned above. Thus, in the case of tissue injury, angiogenesis is likely to be the predominant process, with little arteriogenesis taking place; however, in the case of a common femoral artery ligation, arteriogenesis will predominate at the site of ligation, whereas angiogenesis will predominate in the ischemic distal bed. Finally, in the case of a lethally irradiated mouse receiving a bone marrow transplant, vasculogenesis will predominate at the sites of injury.

Biology of New Vessel Growth

As already mentioned, 2 principal stimuli are thought to stimulate vessel growth: local tissue ischemia or hypoxia

stimulates angiogenesis and some ill-defined factors, including shear stress, stimulate arteriogenesis. In addition, even more ill-defined stimuli induce the release of endothelial progenitor cells from the bone marrow that may contribute to vasculogenesis.

The best understood of these processes in molecular terms is hypoxia-induced angiogenesis. The oxygen tension in tissue is sensed by the proline hydroxylase-HIF system. HIF is a transcription factor that regulates a master genetic program that controls many forms of energy homeostasis at the cellular and systemic levels including glycolysis (local energy production), erythropoiesis (blood oxygen delivery), and angiogenesis (blood flow regulation), among many others.[14] HIF-1 is a heterodimer of the HIF-1α and HIF-1β chains, both of which are capable of directly binding to DNA.

HIF-1β (also known as aryl hydrocarbon nuclear translocator) is a stable subunit the concentration of which remains stable under most conditions. In contrast, HIF-1α has a short (<5 min) half-life under normal conditions because of ongoing degradation via a proteasome-dependent pathway. The newly translated HIF-1α protein is posttranslationally modified and immediately tagged for degradation by prolyl hydroxylase–containing enzymes that require oxygen as a cofactor.[15] Once modified, HIF-1α is immediately tagged for degradation by the von Hippel–Lindau (VHL) protein. The absence of VHL-mediated HIF-1α degradation that occurs in the VHL syndrome results in excessive VEGF production and facilitated tumor development.[16] In the absence of oxygen, however, prolyl hydroxylation and the subsequent VHL-tagged proteasome-mediated HIF-1α degradation is impaired, resulting in a rapid increase in its intracellular levels.

Among many genes induced by HIF-1α, the genes directly involved in angiogenesis include most prominently the VEGF family of genes, angiopoietins, and the inducible form of nitric oxide synthase (iNOS). The VEGF family comprises 5 closely related genes: VEGF-A, -B, -C, -D, and PlGF. Of these, the "founding" member, VEGF-A, also known as a vascular permeability factor, comes in several isoform "flavors" (VEGF-A_{204}, -A_{189}, -A_{165}, -A_{145}, and -A_{121}) that differ by their amino acid length and, most importantly, their ability to bind cellular heparan sulfates. The latter feature is critical to VEGF biology. Thus, higher-molecular-weight isoforms VEGF-A_{204} and -A_{189} bind to heparan sulfates so tightly that they have no ability to diffuse through the extracellular matrix. In contrast, VEGF-A_{121} does not bind heparan sulfates at all and as a result shows wide diffusibility. The middle member, VEGF-A_{165}, preserves some degree of heparan sulfate binding, reducing its diffusibility but at the same time increasing its ability to stimulate VEGF receptors. VEGFs are highly involved in all aspects of angiogenesis and are critical
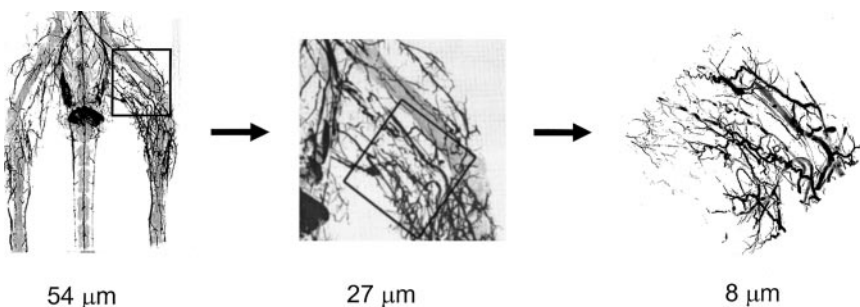
Figure 2. Micro-CT images of arteriogenesis in a mouse hindlimb ischemia model at 3 different resolutions. Note extensive development of corkscrew-shaped vessels. Courtesy of W. Li and J.P. Singh, Eli Lilly & Co.

54 μm   27 μm   8 μm

to this process. As a result, VEGF levels are tightly regulated and even minor changes (on the order of 50% of baseline levels) can have profound physiological effects. For a detailed review of VEGF biology, the reader is referred to several excellent recent reviews.[17,18] PlGF is a particularly interesting member of the VEGF family because of its predominantly arteriogenic effects and its ability to release EPC from the bone marrow.[19,20]

Angiopoietins are a family of 4 genes involved in the regulation of vessel stability and remodeling. Whereas VEGF-induced angiogenesis causes vessels to be more permeable (as described above), Ang-1 opposes this effect and induces vessels to tighten their endothelial permeability barriers.[21] This likely occurs by enhancing the interaction between endothelial cells, pericytes, and the surrounding matrix. Unlike the biology of VEGF, biology of angiopoietins, including regulation of their expression and interaction between various family members, is poorly understood.[22] As a result, few attempts have been made to use angiopoietins as therapeutic angiogenesis agents.

Unlike angiogenesis, the regulation of arteriogenesis does not depend on local tissue hypoxia. Rather, shear stress and local activation of endothelium seem to play critical roles,[23] although other as yet undefined factors may play roles as well. The activation of endothelial surfaces induces, in an NFκB-dependent fashion, the activation of numerous adhesion molecules including selectins, vascular cell adhesion molecules, intercellular cell adhesion molecules, and many others. The activation induces an influx of blood-derived mononuclear cells capable of secreting a number of cytokines and growth factors including FGFs and PDGF, as well as numerous matrix-degrading enzymes.[3]

FGFs are perhaps the best-studied family of arteriogenic growth factors; the family includes 23 different members.[24] These proteins differ in their ability to activate various FGF receptors that include 4 tyrosine kinase members as well as a heparan sulfate–carrying syndecan-4[24] and in their spatial and temporal distribution. FGFs with the most pronounced angiogenic activity include FGF-1, -2, -4, and -5. Unlike VEGFs, FGFs are potent inducers of cell growth and migration but have little effect on vessel permeability. Also unlike VEGF, FGF activity is regulated predominantly not at the level of the growth factor expression but at the level of FGF receptor(s) expression and activation in target tissues.[25,26]

Other contributors to arteriogenic response include PDGF and hepatocyte growth factor, each having various structural isoforms and specific receptors. The complexity of their respective interactions is still largely uncharacterized, although several discovered models of angiogenic synergy between particular growth factor combinations have been studied for their clinical relevance.[27,28]

Finally, the release of bone marrow–derived EPC may play a role in vasculogenesis. The subject of EPC is too complex to cover in this article, and the reader is referred to several recent publications.[12,28,29] EPC release from bone marrow increases in certain pathological conditions, including acute myocardial infarction, congestive heart failure, and certain forms of systemic diseases, and may be facilitated by agents such as stromal cell–derived factor-1.[29–32]

Coronary and Peripheral Artery Disease

During the past decade numerous clinical trials have tested the concept of therapeutic angiogenesis in various coronary artery disease (CAD) and peripheral arterial disease (PAD) patient subsets. Despite claims of success in early small open-label trials, to date all double-blind randomized placebo-controlled trials failed to conclusively show a clinical benefit.[32] This state of affairs raises a number of issues about our ability to translate a vast universe of highly positive studies in a variety of animal models[33,34] into clinical practice. Assuming that the underlying premise of therapeutic angiogenesis—the ability to induce vascular growth to compensate for insufficient blood supply to the heart or the lower limb—is valid, we need to examine a number of variables that could influence these results. Among the variables is the choice of a biological agent used for therapeutic purposes, the required pharmacokinetics, the responsiveness of the target tissue to growth factor stimulation, the genetic determinants of neovascularization, our ability to monitor and assess functional benefits of angiogenic therapy in clinical settings, and the correct selection of patients and the proper conduct of clinical trials. These are discussed in turn.

Choice of Biological Agent

Curiously, little physiological rationale has gone into the selection of biological agents for clinical trials. This seems to have been based largely on factors such as availability of the growth factor for a study (FGF-2 over more potent FGF-1), intellectual property rights (a choice of VEGF$_{121}$ or VEGF-C over much stronger cousins VEGF$_{165}$ or VEGF-D), or the desire to target only endothelial cells to prevent side effects (hence the choice of VEGFs over FGF, PDGFs, and many growth factors) among others. Although past selection is easily criticized in retrospect, it should be noted that many of these choices were made at a time when it seemed that anything worked; why complicate matters unnecessarily?

Because we are facing a different environment, the rationales for choosing biological agents should be examined closely. It can be argued that because arteriogenesis is far more potent than is angiogenesis in restoring blood flow to tissues compromised by a flow-limiting lesion in a large arterial trunk such as the epicardial coronary artery or the large lower leg arteries,[35] the primary ability of the agent for treatment of such conditions should be its ability to induce the development of large arterial trunks. Alternatively, treatment of critical limb ischemia resulting from diffuse compromise of the distal vascular bed may be best effected by stimulating angiogenesis and lymphogenesis, and therefore agents for this purpose should demonstrate these effects.

Furthermore, because blood vessel growth is a complex, multigene event,[36] it is possible that multiple growth factors acting at different times may be required. Thus, we can conceive of one agent that stimulates the growth of new vascular structures while another induces their maturation, thereby ensuring longevity. Such a sophisticated therapeutic strategy requires a detailed understanding of the kinetics of vessel growth and the ability to noninvasively assess the state of neovascular response. Neither of these abilities exists. It is argued frequently that all that needs to be done is to initiate

the process of vascular growth, and nature will take its course. Thus far, nature has perversely refused to cooperate. The other extreme point of view is that because this is too difficult to figure out, we should throw the whole kitchen sink at the problem, which can be achieved by injecting cells,[37] fibrin glue,[38] or another biological material chock-full of the "good stuff." The expectation here is that such injectables will release all of the factors they have and then these factors will figure out among themselves how to grow vessels effectively; however, this does not address the issue of proper timing of the activity and the pharmacokinetic issues discussed below.

One possible criterion for choosing a growth factor is to administer something that is missing at the site of ischemia. That is, perhaps it is the absence of a factor A in a given patient that is responsible for the lack of angiogenic compensation to advancing coronary stenosis. Extensive investigations have failed to conclusively demonstrate a reduced presence of most growth factors, including VEGFs and PlGF, in patients with reduced collateral development,[39] although the absence of collaterals was noted to correlate with increased angiogenesis-inhibitor endostatin levels in pericardial fluid.[40] Intriguingly, hepatocyte growth factor levels are reduced in the setting of ischemic disease,[41] but VEGF levels are markedly elevated in ischemic tissues and in the serum of patients with CAD.[42,43]

None of the techniques that have been used in VEGF therapy trials, including protein, plasmid, and adenoviral-based therapies, would have any significant impact on the amount of VEGF in circulating blood or in ischemic tissues. It can be argued that it is the relative lack of VEGF that is responsible for the lack of native biological adaptation—a situation that is similar to that of type 2 diabetes mellitus, in which elevated insulin levels are the consequence of poor tissue responsiveness to insulin. At the risk of extending this analogy, it can be noted that additional insulin therapy never cures type 2 diabetes mellitus. The same logic could be applied to other tested growth factors that demonstrate unchanged or increased expression in ischemic beds.

Thus, many of the observed angiogenic defects in advanced atherosclerosis could be secondary to impaired growth factor signaling at the receptor/postreceptor levels. Indeed, this hypothesis would explain why any number of the growth factors are effective in young, healthy animals and not in old, diseased patients studied in clinical trials. Some experimental evidence is in tune with this premise, including the lack of VEGF effectiveness in ApoE$^{-/-}$ mice and defective VEGF signaling in endothelial cells exposed to high glucose medium.[44] Furthermore, depressed chemotactic response to VEGF of monocyte isolated from patients with diabetes as compared with control patients could be one of the reasons for decreased collateral development in diabetes.[45]

In summary, these considerations suggest that to be effective, a therapeutic angiogenesis agent should predominantly induce arteriogenesis and it should be capable of doing so not in a normal but in a diseased vascular bed. Furthermore, the best means of inducing arteriogenesis may be via restoration of effective endothelial signaling rather than by supraphysiological administration of a growth factor. One caveat to be considered is that if vasculogenesis plays an important role in adult tissue neovascularization, then agents that promote this process may prove effective. The effectiveness of these agents may be limited, however, by the fact that stem cells numbers and release from bone marrow decline with age[46] and disease.[47,48]

Pharmacokinetics: Proteins, Genes, Gels
The next challenge, once a putatively effective agent has been identified, is to develop an administration strategy that provides a necessary concentration of the agent in a desired location for an amount of time sufficient not only to induce the new vessel growth but also to allow their maturation. We know remarkably little about any of these issues. In particular, we know little about arteriogenesis, a process we want to influence physiologically. It is likely that arterial growth, whether by way of remodeling preexisting vasculature or de novo, occurs during an extended period of time that can be measured in weeks. Similarly, maturation of newly formed vessels also may take a while. One hint that this is the case comes from a study that demonstrated the persistence of neovasculature after 32 but not 14 days of continuous VEGF expression in mice hearts.[49]

The chosen delivery modality likely must provide an extended (4 to 6 weeks or potentially longer) presence of the therapeutic agent at the site of desired vessel growth. Such a prolonged presence clearly cannot be achieved with single-dose administration of proteins or peptides. A slow-release gel formulation may allow this, but this is inconvenient to administer by noninvasive means.[50] A sustained benefit of heparin-alginate–based FGF-2 delivery provides a strong endorsement of this strategy.[51,52] Gene therapy has been touted as the answer to the short half-life of naked protein delivery; however, neither of the 2 vector systems most in use, plasmid or adenovirus based, provide more than a few weeks of high-level expression.[53] More long-lived gene transfer vectors such as AAV or lentiviruses have not yet been tested. The concern here is a long, unregulated expression of an angiogenic agent that may lead to substantial side effects.

The remaining options include the systemic administration of an agent that specifically acts only in the desired organ or tissue. For example, PlGF appears to induce vessel growth only in the setting of ischemia.[54] If this observation is correct, then a prolonged systemic administration of PlGF (eg, by means of a wearable subcutaneous-injection minipump) may be an option. Finally, the recent discovery of endocrine tissue–specific VEGF raises the possibility that tissue-specific growth factors exist that could then be used in a systemic fashion.[55]

Thus, given our understanding of the biology of arteriogenesis, a prolonged treatment modality appears necessary. At present, this can be achieved by either sustained-release delivery of growth factor proteins or systemic administration of organ- or tissue-specific agents.

Vasculature Responsiveness
As already suggested, the ability of vasculature to respond to growth factor stimulation may be the most important parameter of successful angiogenic therapy. Although we know

relatively little about what regulates vascular responsiveness, responsiveness clearly diminishes with age as well as with associated hyperglycemia and atherosclerosis. Genetics is another factor that may play a role. Decreased vascular responses in the setting of systemic diseases such as diabetes is probably related to postreceptor intracellular signaling defect because receptor expression for most growth factors appears to be unaltered, although once again, the data are sketchy. Thus, in individuals with diabetes, the ability of monocytes to migrate toward a gradient of VEGF-A is severely impaired, and this impaired response seems to be secondary to a signal transduction defect within the monocyte.[45] Elevated homocysteine levels likewise impair angiogenic responses.[56] Atherosclerosis is also associated with vascular dysfunction, manifested in part by reduced vasodilation to endothelium-dependent agents such as acetylcholine, and with severe impairment of angiogenesis and arteriogenesis.[57] Whether both of these defects result from impaired signaling at the cellular level or reduced production of growth factors (eg, VEGF) in response to ischemia has not been fully established.

Genetic Determinants of Neovascularization

Genetic differences also may play a role in an individual's ability to develop collateral vessels in response to occlusive arterial disease. Clinical observations have long noted a variable presence or absence of collateral circulation on coronary angiograms. Although some clinical parameters such as the anatomic extent of disease and duration of symptoms are somewhat predictive of the ability to develop angiographically visible collaterals, much of the difference remains unaccounted for.[58,59] One interesting study suggested that the ability of monocytes from different individuals to respond to hypoxia by increasing HIF-1α expression correlated with the extent of collateral development.[60] Another study demonstrated higher expression of monocytes CD44 antigen in patients with more compared with less extensive collateral development.[61] Reduced pericardial endostatin level[40] and a haptoglobin phenotype[62] also have been linked to collateral development. These observations suggest that genetic differences may play a significant role not just in the occurrence of spontaneous collateral response but also in patients' abilities to respond to angiogenic therapy.

Monitoring and Assessment of Angiogenesis

The ability to monitor the effect of angiogenic therapy has been a long-standing challenge. In principle, this can be accomplished by either directly monitoring blood vessel growth or observing the functional effects of such therapy. Molecular imaging of angiogenesis has received prominent attention recently, with a number of reports demonstrating the feasibility of observing blood vessel growth in tissues by targeting several "angiogenic" endothelial cell-specific antigens such as $\alpha v \beta 3$ integrin,[63] VEGF receptors,[64] or an NGR receptor.[65] Although such studies are clearly intriguing, none of the approaches has been applied in clinical trials. Another alternative is direct visualization of new vasculature. Large collaterals (<130 μm diameter) can be observed and perhaps even quantified with standard angiographic techniques; how-
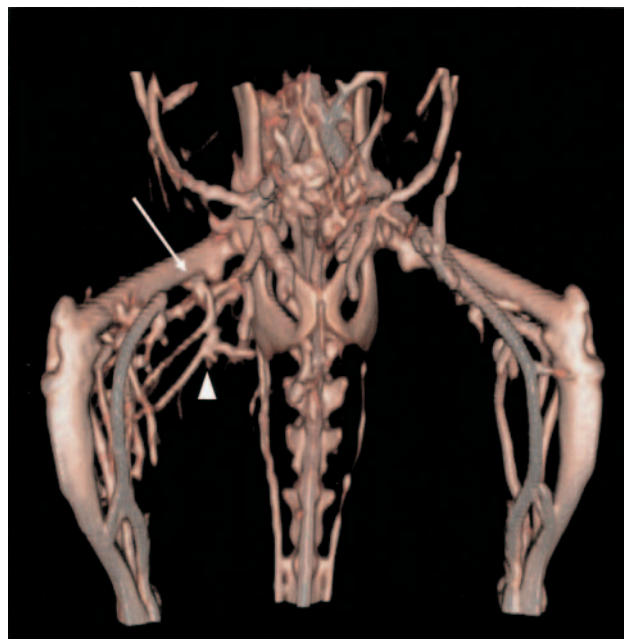

Figure 3. 3D CT reconstruction of the mouse peripheral vasculature. Note disrupted common femoral artery (arrow) and forming collaterals (arrowhead). Courtesy of Z. Zhang and E. DeMuinck, Dartmouth Medical School.

ever, a number of difficult-to-control factors influence the angiographic appearance of vessels, including vascular tone, amount of the injected contrast, force of injection, and medication. Furthermore, angiograms are notoriously difficult to quantify, although a number of approaches have been proposed.[66] CT, especially with the advent of multislice scanners, can be an appealing alternative, but the clinical experience with this technique has been rather limited.[67] A particularly interesting technology is the 3D reconstruction of tomographic images (Figure 3). Finally, magnetic resonance angiography can also provide visualization of collaterals.[68] Although relatively effective in the limb, the sensitivity of MRI coronary reconstruction is not yet sufficient.

The alternative to direct vasculature visualization is the assessment of the physiological consequences of vessel growth, such as improvement in tissue perfusion, oxygenation, or function. In the case of CAD trials, the most obvious alternative was the use of time-tested nuclear perfusion imaging. Remarkably little effect was observed with this imaging modality even when patients appeared to be improved symptomatically. This raised questions about the spatial resolution of single-photon emission computed tomography (SPECT) imaging, variability of SPECT findings over time in the same patient population,[69] or the wisdom of instituting exercise protocols in which patients may exercise longer before reaching the ischemic state that would be similar to the pretreatment study.[70] It is possible that SPECT imaging did not show the benefits of angiogenic therapy because the technique is simply not sensitive enough. It is also possible that it did not show these benefits because they were absent.

Positron-emission tomography (PET) and MRI are the main alternatives to SPECT imaging. PET boasts somewhat

higher spatial resolution, elimination of attenuation, and quantitative assessment of perfusion[68]; however, experience with PET in clinical CAD trials in the United States is limited, and no large angiogenesis trial to date has used PET as an end point. There is more experience with MRI for perfusion and cardiac function assessment,[71] but even here no agreement has been reached with regard to how it should be measured. One approach relies on assessing relative differences in perfusion between normal and ischemic zones, thereby providing an assessment of the ischemic zone size.[72] Alternative approaches attempt to measure perfusion by the first-pass technique.[73] The advantages of MRI are its high spatial resolution and sensitivity to even small changes in flow. Similar to PET, however, experience with the use of MRI perfusion in large clinical trials is limited.

Clinical Trials Issues: Population Selection, Placebo Effect

An important part of therapeutic angiogenesis trials is the selection of an appropriate patient population. As with all radically new therapies, there is a tendency to initially restrict the therapy to the no-option population. Indeed, most therapeutic angiogenesis trials have been carried out in symptomatic patients who have exhausted standard therapy modalities such as coronary artery bypass graft and percutaneous coronary intervention.[70] These patients tend be older, with more extensive disease and clinical evidence of not being responsive to standard therapies, thus suggesting defects in intrinsic neovascularization response. These characteristics could make these patients especially poor candidates for angiogenesis; however, the choice of a less severe population with available therapeutic options, although appealing on theoretical grounds, faces significant regulatory and recruitment hurdles because both the US Food and Drug Administration and local institutional review boards are reluctant to open enrollment of patients into experimental gene therapy protocols when standard options are available. A partial solution to this problem would be the availability of biomarkers predictive of neovascularization response or lack thereof. To date, no such markers have been found.

Another issue that has bedeviled early clinical trials is the occurrence of a significant placebo response.[74,75] Although placebo effects are well described in many fields of medicine, the sheer magnitude of the effect observed in these trials was surprising because few clinicians expected that the "no-option patients" could increase their exercise capacity by 45 to 60 seconds. Other measures of functional capacity and symptomatic improvement such as the Seattle Angina Questionnaire, Short Form-36, and pill counts also showed surprising changes in the placebo group. Regardless of the reason why the placebo response is so prominent and significant in this patient population, the importance of this phenomenon clearly mandates that no conclusions should be drawn from the open-label trials and that all trials should be conducted in a double-blind randomized manner.

Yet another complication to emerge from the angiogenesis trials was the demonstration of significant fluctuations in "hard" end points such as myocardial perfusion and function in patients treated with placebo. A recent study confirmed that patients with advanced CAD demonstrate high variability on their SPECT perfusion studies even in the absence of any changes in therapy, with the percentage of myocardial ischemia varying by an average of 50%.[76] Similarly large changes in ostensibly hard physiological end points such as ankle-brachial index and transcutaneous partial oxygen pressure have been observed in PAD trials. The occurrence of frequent and significant changes in physiological parameters in these patients suggests that we are dealing with a heterogeneous population.

Restenosis and Arterial Injury Repair

The role of angiogenic growth factors and angiogenesis has long been debated in relation to the repair of arterial injury and the promotion of atherosclerotic plaque growth. Early studies with the balloon injury model in normal rats suggested that VEGF and FGF may reduce neointima formation by promoting the reendothelialization of the injured arterial segment[77–79] and that VEGF may, in general, have a vascular protective effect.[80] Other studies, however, challenged these findings.[81–84] Still other studies suggested that circulating endothelial progenitor cells play an important role in postinjury reendothelialization[85,86] and that infusion of these cells or the stimulation of their release by angiogenic growth factors can promote a reduction in restenosis.[87–89]

On the other side of the ledger, systemic VEGF administration in atherosclerotic mice and rabbits was reported to promote atherosclerotic plaque growth, presumably by inducing vessel wall angiogenesis,[90] and the extent of vessel wall vascularization appears linked to plaque growth.[91] Clearly, in assessing the potential of angiogenic therapy to reduce neointima formation the effect of these factors on atherosclerosis needs to be taken into account.

The relationship between vessel wall angiogenesis and neointima growth was first suggested by studies that found that intraplaque microvessels were more commonly found in restenotic compared with primary atherosclerotic specimens.[92] Subsequently, ApoE$^{-/-}$ mice treated with angiogenesis inhibitors TNP-470 and endostatin were shown to have reduced neointima as compared with untreated animals,[93] an effect mediated in part by reduced macrophage accumulation,[94] as well as reduced arterial wall vascularity. Similar findings were reported with angiostatin.[95] Whether all of these effects are directly attributable to the antiangiogenic effects of these compounds is uncertain, however. Thus, TNP-470 has a direct antiproliferative effect on smooth muscle cells and this effect may contribute to its antirestenotic properties in rats.[96]

These data suggest that angiogenic growth factors may have different effects according to the state of the arterial tree, the presence or absence of atherosclerosis, the mode of administration, and systemic factors (eg, the presence of circulating endothelial progenitor cells). Intracoronary injection of FGF-2 after balloon angioplasty in a double injury model had only a minimal effect on the extent of neointimal development. When stents were used, the increase in neointima was more substantial, suggesting that the greater the extent of development, the higher the proliferative response to the growth factor.[97] When growth factors were delivered to
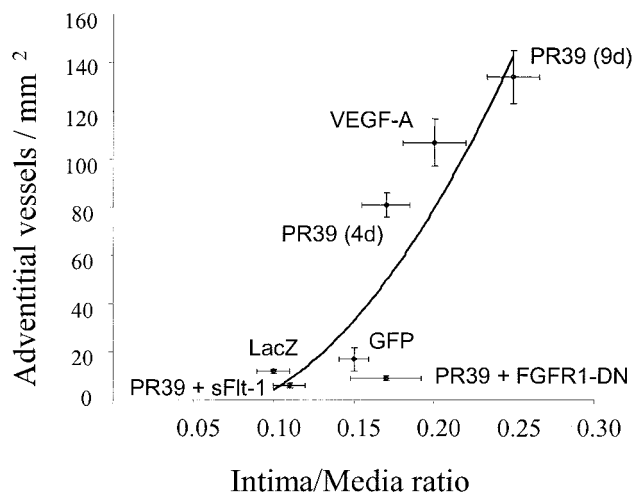
Figure 4. The relationship between periadventitial angiogenesis and neointima formation. A plot of the extent of periadventitial angiogenesis (adventitial vessels/mm²) vs morphometric extent of neointima formation (intima/media ratio) in rat and rabbit models of vascular injury. The individual plot points represent values after treatment with angiogenic growth factor PR39 (4 or 9 d) and VEGF-A, angiogenesis inhibitors sFlt-1 and FGFR1-DN, controls (Lac Z and GFP), or a combination of these treatments. Note that even full inhibition of adventitial angiogenesis does not inhibit neointima development. FGFR1-DN indicates fibroblast growth factor receptor-1 dominant negative; sFlt-1, soluble Flt-1 (VEGF receptor 1). Reprinted with permission from Khurana et al.[98] Copyright 2004, American Heart Association.

the adventitial surface of the artery after injury with or without endothelial damage, a marked increase in neointima size occurred, which was directly correlated with the extent of vessel wall angiogenesis (Figure 4). Inhibition of the angiogenic process with either FGF or VEGF pathway inhibitors fully blocked vessel wall neovascularization; however, the extent of neointimal growth was decreased to the level seen with mechanical injury alone.[98] The study of Khurana et al illustrates 2 important points: (1) The sustained presence of the growth factor in the setting of arterial injury will induce neointima growth, and (2) neointima growth had both angiogenesis-dependent and -independent phases (Figure 5). If correct, this means that antiangiogenic treatments can reduce but not eliminate postangioplasty restenosis.
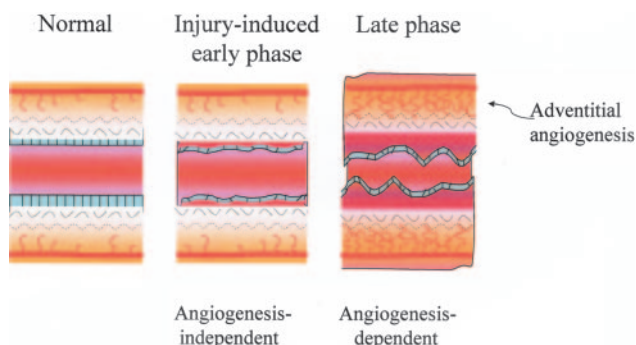

Figure 5. The role of periadventitial angiogenesis in neointima development. The drawing illustrates angiogenesis-independent and -dependent phases of neointima formation. Reprinted with permission from Khurana et al.[98] Copyright 2004, American Heart Association.

At present, the effect of angiogenic therapies on neointima and atherosclerotic plaque growth is difficult to assess. A recent clinical trial of balloon-catheter–delivered plasmid or adenoviral-encoded VEGF to the vessel wall in the setting of coronary angioplasty failed to show any beneficial or adverse effect of therapy on restenosis.[99] This result is consistent with the above-cited animal study results showing that transient exposure of blood vessels to a growth factor is unlikely to produce significant effects. Intracoronary infusion of granulocyte-colony stimulating factor–mobilized peripheral blood endothelial progenitor cells in the setting of acute myocardial infarction resulted in a marked increase in in-stent restenosis.[100]

Angiogenesis and Body/Organ Size

Although angiogenesis has long been associated with tumor growth, it is equally reasonable to ask whether endothelial cell mass determines organ or body size or whether it is determined by it.[101] That is, is one heart larger than another heart because it has more vessels or does it have more vessels because it is larger? Furthermore, if the former is true, then can organ size be increased by stimulating angiogenesis? Some available evidence suggests that this may be the case. In one set of studies, treatment of castrated rats with testosterone induced endothelial cell proliferation in the prostate, and then proliferation of the glandular epithelium and an increase in the prostate size.[102] In a related study, castration of mice bearing androgen-sensitive prostate carcinoma resulted in a dramatic decrease in the gland vascularity and then a reduction in its mass.[103] Additional evidence comes from studies of the ability of FGF-2 to promote and TNP-470 to inhibit hepatic regeneration after partial hepatectomy.[104] In both cases, changes in the extent of vascularization preceded changes in the size of the liver.

Similar results have been noted in the case of adipose tissue, in which stimulation or inhibition of angiogenesis directly affected the adipose tissue mass.[105,106] Yet more tangential evidence comes from the demonstration that genetic loci that control the response to FGF-2 coincide with a number of genetic loci that control body growth, body length, and adult weight.[107]

Although myocardial hypertrophy is known to be associated with increased capillary cross-sectional area maintaining constant capillary surface area/myocyte volume and an increase in arteriolar number, the cause-and-effect relationship has not been established.[108,109] Several elegant studies have demonstrated that myocardial stretch secondary to increased afterload or bradycardia induces the release and synthesis of a number of angiogenic growth factors including VEGF.[110–112] It is interesting to speculate whether in some cases myocardial hypertrophy is a consequence of angiogenesis. In this vein it can be noted that captopril, a drug known to reverse myocardial hypertrophy, is also an effective angiogenesis inhibitor.

Angiogenic Side Effects of Anti- and Proangiogenic Trials

The advent of drugs capable of stimulating or inhibiting angiogenesis led to the recognition of a new spectrum of side

effects that, in addition to their importance in terms of clinical patient treatment, provide important biological insights into the role of growth factors and native angiogenesis in human health.

On the proangiogenesis side, the most recognized side effect to date is hypotension that occurs with VEGF and, to a lesser extent, FGF-2 administration.[32] In both cases it is mediated by nitric oxide production and, in the case of VEGF, rapid tachyphylaxis can be induced with subtherapeutic doses.[113] A side effect specific to VEGF is edema. It is not clear whether edema is simply a reflection of increased vascular permeability, a hallmark of VEGF activity,[114] or related to the "immaturity" of VEGF-induced new vessel growth. If the latter is the case, then agents promoting vascular maturation such as Ang-1 can be expected to reduce edema. Much additional concern with regard to VEGF, and to a lesser extent FGF, was expressed about their ability to induce "off-target" angiogenesis, particularly in the retina, and to induce the growth of occult tumors. Although proliferative retinopathy is associated with high local levels of VEGF[115] and anti-VEGF therapies are being tested for treatment of diabetic retinopathy and macular degeneration,[116] to date there is no evidence that VEGF administration in therapeutic angiogenesis trials has worsened these processes. In part, this is a reflection of low levels of systemic VEGF after therapeutic administration and also may be the result of the poor penetration of systemic VEGF into eye tissues. A primary concern associated with FGF therapy is induction or promotion of membranous nephropathy. This is a real concern, and FGF clearly is capable of worsening renal protein loss in patients with preexisting disease.

A concern related to these as well as other growth factors is the possibility that systemic administration may promote tumor growth. To date, there is no evidence and no biological reason to suggest that an angiogenic growth factor may induce de novo malignancy. On several rare occasions when a preexisting malignancy was missed, administration of a growth factor resulted in an apparent acceleration of tumor growth. Thus, careful screening programs are necessary before therapeutic angiogenesis trials are initiated.

Several interesting side effects were recorded with the use of the anti-VEGF antibody bevacizumab. These included hypertension, thrombosis, proteinuria (with occasional nephrotic syndrome), and epistaxis. Serious tumor-related bleeding episodes (eg, hemoptysis and hematemesis) were a particular concern in patients with non-small cell lung cancer.[117] The occurrence of hypertension suggests that VEGF, likely via release of nitric oxide, regulates basal blood pressure. The occurrence of thrombosis is more difficult to explain, but this may also relate to diminished nitric oxide production. Epistaxis likely is the consequence of tumor necrosis, with the necrotic mass eroding into the bronchial tree.

A more recently recognized side effect is an increased risk of thromboembolic events including stroke, transient ischemic attack, angina, and myocardial infarction. Another, albeit still theoretical side effect, is the prevention of collateral development in the heart and other organs compromised by ischemia. In this regard, it is interesting to note that anti-

VEGF receptor-2 antibody blocks port-systemic collateral formation in portal hypertensive mice.[118]

Summary

Therapeutic modulation of angiogenesis is still in its infancy, but the baby is showing considerable potential. The ultimate success of this therapeutic modality will depend on careful translational research program that will incorporate the ever-evolving basic understanding of the biology of angiogenesis into an effective clinical trials program. The likely keys include the careful choice of a biological agent that may be different for different indications, effective delivery modality with pharmacokinetic properties matching biological needs, and the meticulous choice of patient population and end points for study.

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