REVIEW ARTICLE

Angiogenesis and arteriogenesis in limb ischemia

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Formation of true new blood vessels, or angiogenesis, and development of collateral vessels from preexisting blood vessels, or arteriogenesis, are important in the pathophysiology of vascular disease. By stimulating these processes we might be able to provide an alternative treatment strategy for patients with lower limb ischemia and coronary artery disease.

Use of therapeutic angiogenesis, eg, stimulating angiogensis to treat ischemia in the heart or leg, was begun almost a decade ago. Since then a number of studies have reported improved restoration of perfusion as a result of angiogenesis in animal models of limb ischemia after administration of vascular growth factors or after overexpression with gene therapy. Therapeutic angiogenesis has also been tried in patients. Trials showing beneficial effects of treatment with recombinant vascular growth factors in nonrandomized patient series are common, whereas data from controlled clinical trials in patients with limb ischemia are scarce. More trial data are available for coronary artery disease, but the results are not consistent, showing both no effect compared with placebo, as well as slight improvement of heart perfusion and function.

After the initial enthusiasm for therapeutic angiogenesis, lack of positive trial data has reduced the optimism somewhat. Negative study results have focused researchers and clinicians on the fact that the angiogenesis and arteriogenesis processes are complex and that simply supplying vascular growth factor to the leg is usually not sufficient for effective therapy. Therefore more interest and research efforts are now devoted to understanding the basic mechanisms of these processes. This review discusses the basic mechanisms underlying angiogenesis and arteriogenesis, and provides speculation as to how this knowledge influences our view of the pathophysiology and treatment of lower limb ischemia.

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BASIC MECHANISMS OF ANGIOGENESIS

Angiogenesis is a physiologic process required for the menstrual cycle and wound healing in adults. It also has a role in pathologic processes, eg, tumor growth, rheumatoid arthritis, diabetes, and cardiovascular disease. The angiogenic process is complex and not yet fully understood, but a number of growth-promoting factors regulate induction of angiogenesis (Table). Most stimulate proliferation and migration of cells in the vascular wall and inhibit apoptosis.

The angiogenic process originates from preexisting capillaries in the vicinity of the ischemic insult (Fig 1). The initial step is stimulation of endothelial cells by vascular growth factors. The vascular endothelial cell growth factor (VEGF) family of proteins is probably the most important for angiogenesis, but this has recently been questioned.[1] An ischemic environment in the tissue induces VEGF synthesis in many different cell types. Hypoxia is one of the stronger inducers, which act by binding hypoxia-inducible factor-1α (HIF-1α) to the hypoxia response area in the VEGF gene promoter region. VEGF transcription is also induced by hypoglycemia and acidosis and is further stimulated by other growth factors, eg, platelet-derived growth factor and fibroblast growth factor-2 (FGF-2).[2] The VEGF family consists of six members, where VEGF-A is the first identified (also called simply VEGF). Splicing of the VEGF-A gene results in five isoforms of VEGF-A, differing in total amino acid number. It is probable that these isoforms have different functions in the angiogenic process. For example, VEGF-A189 may exclusively stimulate endothelial cell proliferation, whereas VEGF-A165, the predominant form, also stimulates endothelial cells to coalesce and form larger vessels.

The next step in the angiogenic process is a VEGF-mediated increase in vascular permeability. This is due to alterations in cell membrane structure and redistribution of intracellular adhesion molecules PECAM-1 and vascular endothelial-cadherin. Vascular permeability is controlled and downregulated by angiopoetin-1. Extravasation of plasma proteins, stimulated by VEGF, follows, creating an environment to support endothelial cell migration. Degradation of extracellular matrix, by angiopoetin-2 and a number of proteinases, mostly matrix metalloproteinases (MMP), is also a part of this preparation.

Table 1. Examples of cytokines believed to be involved in angiogenesis and arteriogenesis

Growth factor	*Cellular function*			*Upregulates expression or function*	*Effect on angiogenesis/arteriogenesis*
	EC proliferation	*SMC proliferation*	*Matrix production*		
VEGF	++	+	0	eNOS, MCP-1	++/+
FGF-2 (bFGF)	+	+	0	VEGF, eNOS, MCP-1, HGF	+(?)/++
PDGF	+(?)	++	0	TGF-beta, FGF-2, VEGF	0/+
TGF-beta	+ and −	+ and −	+	PDGF, FGF-2, VEGF	+/+
Angiopoetin-1	+	0	+(?)		+/0(?)
Angiopoetin-2	− and +	−	−		−/−
MCP-1	+	+(?)	0		0/++
GM-CSF				IL-8	0/+
TNF-alfa	+ and −	0(?)		VEGF (?)	+/+
HGF				FGF-2	++/+(?)
Placental growth factor	(+)			VEGF	+/+

EC, Endothelial cell; *SMC*, smooth muscle cell; *VEGF*, vascular endothelial cell growth factor; *FGF*, fibroblast growth factor; *PDGF*, platelet-device growth factor; *TGF*, transforming growth factor; *MCP*, monocyte chemoattractant protein; *GM-CSF*, granulocyte-macrophage colony-stimulating factor; *TNF*, tumor necrosis factor; *eNOS*, endothelial nitric acid synthase; *HGF*, hepatocyte growth factor; *IL*, interleukin; +, stimulatory; − inhibitory; 0, no effect; ?, unclear.

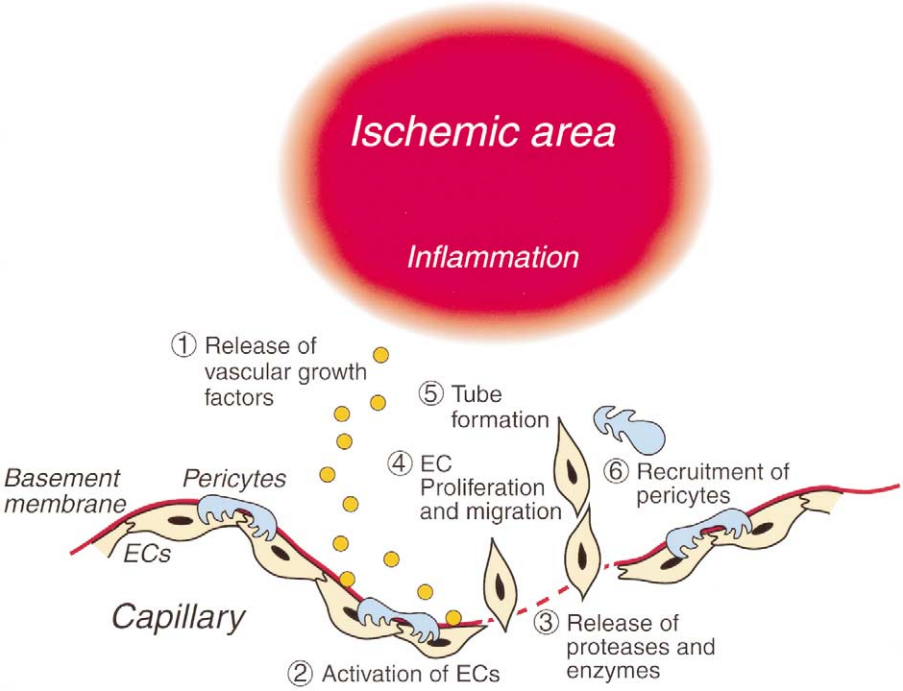

Fig 1. Schema of possible events in the angiogenic process. *EC,* Endothelial cell.

Endothelial cell proliferation and migration, two important effects of VEGF, are mediated through three cell surface VEGF receptors. VEGFR-2 (KDR/Flk-1) mediates most of the mitogenic, survival, and permeability effects of VEGF and is mainly expressed on endothelial cells. VEGFR-1 (Flt-1) is expressed on inflammatory cells, and VEGFR-3 is found in lymphatic endothelium. There are also a number of co-receptors, eg, neurophilins, proteogly-

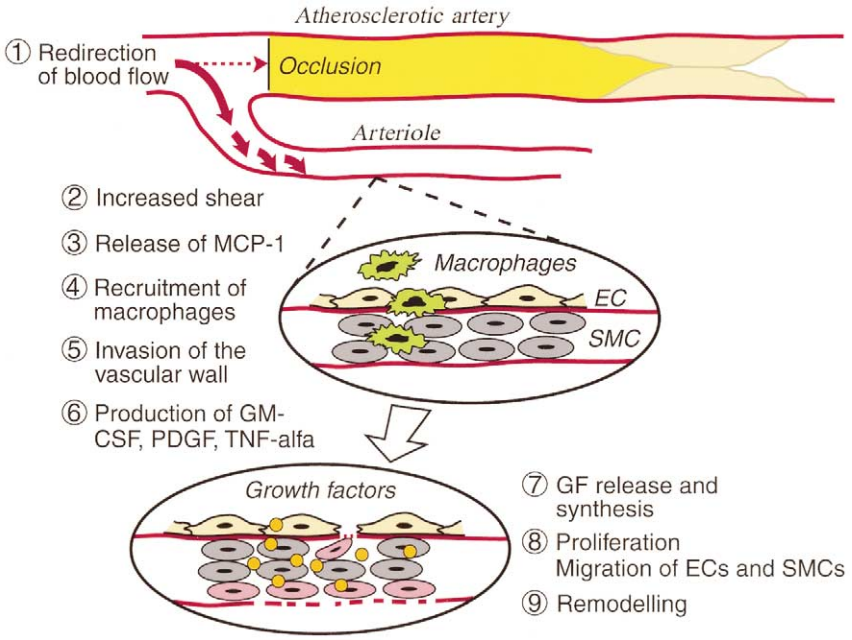

Fig 2. Schema of possible events in the arteriogenic process. *EC,* Endothelial cell; *SMC,* smooth muscle cells; *MCP-1,* monocyte chemoattractant protein-1; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *PDGF,* platelet-derived growth factor; *TNF-alfa,* tumor necrosis factor-α.

cans, cadherins, and integrins. VEGF receptor signal transduction is not entirely clear. For example, the purpose of VEGFR-1 binding may be only as a negative regulator of VEGFR-2. In common with the VEGF protein, receptor expression is upregulated by hypoxia.

When the endothelial cells reach the extracellular matrix, they form cords and subsequently a lumen. This is accomplished by thinning of the endothelial cells and fusion with existing vessels. Various VEGF isoforms, angiopoetins, and integrins regulate lumen diameter. Transforming growth factor–β and platelet-derived growth factor stimulate extracellular matrix production and recruit pericytes to stabilize the new vessel structure.

BASIC MECHANISMS OF ARTERIOGENESIS

Arteriogenesis, when the lumen of preexisting vessels increases to form collateral arteries, is a process that can ameliorate the harmful effects of vessel obstruction. Usually arterioles become large conductance vessels that maintain blood flow after myocardial infarction or limb ischemia. Not all vessels can become collateral vessels, and there is a large difference in this capacity between species, vascular beds, and probably also individuals.

Once stenosis in a large main artery becomes hemodynamically significant, blood flow is directed toward the lowest resistance into the periphery via preexisting arterioles (Fig 2). The initial step in the arteriogenesis process is elevation of shear stress against the wall of these arterioles. Shear stress can be considered frictional wall pressure at the cell surface, caused by blood flow and the compensatory forces striving to counteract this pressure. It is not known how endothelial cells become activated in response to increased shear stress. There may be specific shear stress receptors on the cell surface, and multiple intracellular signaling pathways probably mediate shear stress regulation of gene expression.[3] The latter converge to activate transcription factors, shear stress response elements. Candidates for shear stress receptors are caveole, integrins, and tyrosine kinase receptors (one is VEGFR-1).

Endothelial cells react by activating endothelial nitric oxide synthetase and genes for cytokines (probably through shear stress response elements). the most important being monocyte chemoattractant protein-1 (MCP-1), granulocyte-macrophage colony-stimulating factor (GM-CSF), tumor necrosis factor-α, and cell adhesion molecules.[4] Attracted by MCP-1 and aided by adhesion molecules, circulating monocytes adhere to and invade the vascular wall. Platelets also adhere and produce interleukin-4, further stimulating adhesion molecule synthesis. The main role of GM-CSF is to provide an environment for stable monocyte function. Accordingly, this stage can be regarded as local inflammation in the vessel wall.

After transformation to macrophages, monocytes produce fibronectin, proteoglycans, and proteases, which remodel the extracellular matrix. These inflammatory cells also produce large amounts of vascular growth factors (Table), essentially from the FGF family, and particularly FGF-2, basic FGF. Many FGF isoforms affect cells in the vascular wall. FGF use four cell surface receptors, FGFR-1 to FGFR-4.[5] One of the effects of FGF-2 binding to its

receptor is stimulation of endothelial cells and smooth muscle cell (SMC) proliferation. Cell proliferation is followed by disruption of lamina elastica interna and migration of SMC to form a neointima. Remodeling of the vessel is the final step that, in large part, occurs in the adventitial layer. SMCs and fibroblasts are important during this stage.[6] The external elastic lamina and elastin in the adventitia and surrounding tissue are broken down by protelytic enzymes, eg, MMP and plasmin. This provides space for the growing vessel. At this stage the vessel's old structure is more or less taken apart. FGF-2 then stimulates fibroblast maturity, and additional cell layers are added to the vessel wall.

Many collateral vessels contribute to counteract the increase in shear, but some vessels can enlarge more than 20 times. The newly formed collateral vessel is initially tortuous, to compensate for still increased shear forces, but eventually it becomes indistinguishable from a normal artery. It has then both a medial layer and normal reactivity.

EMBRYONAL DEVELOPMENT OF BLOOD VESSELS: EQUIVALENT TO VESSEL GROWTH IN THE ADULT?

There is reason to believe that the basic mechanisms of angiogenesis and arteriogenesis are similar in the adult, in normal states as well as in disease, as in vascular development in the embryo and during the postnatal period. The stages involved appear to be the same, perhaps with the exception of vasculogenesis. This is the initial process during vascular development of the major vessels and the heart, when endothelial cells evolve from mesoderm-derived progenitors in areas that lack blood vessels. These endothelial cells then adhere to one another and form tubes and networks. Development of leg arteries, however, occurs later during gestation, and vasculogenesis is probably not a major feature. Limb development starts around week 7 with formation of buds from the trunk. The processes responsible for leg artery development and growth in these buds are primarily angiogenesis and remodeling. Remodeling has many similarities to arteriogenesis in the adult, and is most prominent during the early postnatal period.

It has been suggested that primitive undifferentiated vascular cells are present systemically also in the adult.[7] Such endothelial precursor cells originate from bone marrow. These cells may be able to start a process similar to vasculogenesis in the adult. Of interest, the promising therapeutic angiogenesis concept of injecting bone marrow cells into ischemic limbs to form new vessels can be regarded as "vasculogenesis."[8]

Growth factors that control angiogenesis and vascular remodeling are most likely also identical in the embryo and the adult. As in the adult, the interplay between various growth factors is complex throughout vascular development and has not yet been completely explained. Various VEGF family members and receptors also appear to have specific functions during development. For example, VEGF-B is most prominently expressed during tube formation. Angiogenic growth factors may nonetheless be more important during certain stages than others. For example, recruitment of mesodermal cells seems to be controlled primarily by FGF-2, whereas differentiation of angioblasts to endothelial cells and formation of primary networks are largely regulated by VEGF. Angiopoetin and its receptor Tie-2 are probably important, in addition to VEGF, for vascular branching.[9] However, it must be remembered that optimal vessel development and growth require several growth factors and timing of events.

Considering the similarities between embryonal and adult vessel formation, much of the information gained by studying embryonal vessel formation can be applied to adult vessels and as mechanistic background for therapeutic angiogenesis purposes. For example, conditions and peptides involved in vascular branching during vascular development can be used to stimulate development of new arteries from atherosclerotic main branches.

IMPLICATIONS FOR PATHOPHYSIOLOGY AND TREATMENT OF LIMB ISCHEMIA

Angiogenesis. The angiogenesis mechanisms described are based on experimental data not focused on limb ischemia. While not yet established in patients, it is probable that angiogenesis occurs in ischemic tissue in acute limb ischemia. In animal models a burst of VEGF appears to be released directly after ischemic induction in ischemic skeletal muscle. Upregulated transcription and production of VEGF, VEGFR-2, and HIF-α have also been observed. Most VEGF comes from myocytes and macrophages, and expression appears to be greater in oxidative muscles than in glycolytic muscles. The increase in VEGF production lasts at least 3 weeks and seems to be accompanied by increased capillarity and increased uptake of bromodeoxyuridine by endothelial cells in these animal models. FGF-2 is also present in large amounts in ischemic tissue directly after ischemic induction, but not after 1 week.[10] The main producers of FGF-2 are inflammatory cells. Anecdotal data in patients with acute limb ischemia supports these observations. VEGF and its receptors are also upregulated after rather short periods of arterial occlusion in healthy subjects. Little is known about expression of other angiogenic proteins in acute limb ischemia.

Information on angiogenesis is even more limited in chronic disease, because of lack of appropriate animal models. Patients with chronic ischemia are reported to have higher systemic levels of FGF-2 compared with control subjects. A probable source of this is inflamed skin rather than ischemic muscle. VEGF levels do not seem to be extensively elevated systemically or locally in the chronically ischemic leg. There are, however, conflicting data regarding this. In tissue samples obtained during reconstructive vascular surgery, very low levels of VEGF are found in muscle tissue at distal sites (Palmer-Kazen, unpublished data), whereas high concentrations have been measured in certain parts of amputated legs.[11] In the study by Rissanen et al,[11] VEGF was co-localized with HIF-α and macrophages in atrophic and regenerating muscle tissue. Increased levels of VEGF in that study correlated with

VEGFR-2 upregulation. In the literature there is little evidence that increased receptor expression compensates for attenuated growth factor production. Presently there appear to be few reports that show active angiogenesis in chronic limb ischemia. This may be because of heterogeneous distribution of angiogenesis in the ischemic leg or lack of methods to assess angiogenesis, or because this process is not central in chronic disease.

Almost 50% of patients with limb ischemia have diabetes. It has been suggested that increased risk for ischemia and the relatively poor prognosis for patients with diabetes compared with patients without diabetes may be due to defective angiogenesis. This may appear contradictory, considering diabetic retinopathy, a complication largely caused by increased neovascularization and VEGF upregulation. The evidence supporting impairment in diabetes consists of a reduced number of capillaries compared with control in biopsy specimens from patients with coronary artery disease and low levels of vascular growth factors, receptors, and messenger RNA around ischemic ulcers and in ischemic muscle from animal models of diabetes.[12] The mechanism of such presumed impaired angiogenesis in limb ischemia is probably complex and may be due to disturbances in one of the steps in the angiogenesis process.

Smoking may also affect ability to form new blood vessels. While not proved in patients with peripheral artery disease, there is indirect evidence that the angiogenesis process is negatively influenced by smoking. Healthy smokers have decreased levels of VEGF systemically, and patients with squamous cell carcinoma who smoke have lower microvessel density and VEGF concentration in the tumor compared with matched nonsmokers. The substance in cigarette smoke that exerts this effect is unknown. Of interest, nicotine appears to have a dose-dependent effect, stimulating endothelial cell proliferation in vitro at low levels while being directly cytotoxic at higher concentrations.[13]

Arteriogenesis. The arteriogenic mechanisms described have, to a large extent, been elucidated in animal models of acute limb ischemia. Large animal models developed to create gradually constricting stenosis in one coronary artery have also been helpful. The location, timing, and extent of growth factor, receptor, and enzyme production have largely been demonstrated by Schaper et al in Germany, using a combination of in vitro and in vivo approaches. Overall, there is ample evidence that arteriogenesis occurs and is essential for restoring perfusion in animal models of limb ischemia.

There is little information, however, about arteriogenesis in patients. The presence of collateral vessels is obvious to anyone that has seen angiograms, but not much is known about the development of such angiographically evident collateral vessels or their capacity to ease symptoms. For example, the effect of exercise training programs in patients with claudication seems to improve muscle metabolism and oxygen uptake rather than collateral vessel capability. There is a weak relationship between increased lower limb blood flow and walking distance after exercise treatment, indicating that the increased flow accounts for less than 30% of the improvement.[14] The main benefit of exercise is less reliance on anaerobic metabolism and better muscle energy.[15]

It is also unknown what risk factors affect collateral vessel development in limb ischemia. We found that having few profunda collateral vessels when the superficial femoral artery was occluded was related to diabetes, short duration of symptoms, and smoking in patients with claudication (De Vivo S, unpublished data). Patients with diabetes and coronary artery disease also have a reduced number of collateral vessels compared with patients with coronary artery disease without diabetes.[16] As in animal models, there does not seem to be upregulation of vascular growth factors or extensive inflammation in the tissue surrounding the developing collateral artery in patients with limb ischemia.

Pathophysiology. Arteriogenesis appears to be an important part of the pathophysiology of limb ischemia, continuing for a long time after onset of disease, whereas angiogenesis may function mainly in acute situations. One may speculate, however, that angiogenesis may occur in certain parts of the leg with chronic disease that experience abrupt inhibition of blood supply, as in even subclinical acute thrombosis. Moreover, border zones between necrotic metabolically inactive tissue may become ischemic during increased demand, creating a milieu suitable for new vessel formation.[17] This explanation would also be beneficial for synergetic effects of angiogenesis and arteriogenesis. Reaching the absolute claudication distance may also stimulate both processes.

Implications for treatment. Increased knowledge of the effects of endogenous angiogenesis and arteriogenesis in limb ischemia may facilitate optimization of exercise therapy and pharmacologic treatment. With identification of patients with a poor prognosis, active measures could be started and selection to vascular surgical procedures would be easier. To achieve such effects and to make therapeutic angiogenesis more successful, research efforts focusing on patients and the physiology of endogenous angiogenesis and arteriogenesis are needed.

The TRAFFIC trial[18] is the first randomized clinical trial to show a positive effect of growth factor administration in patients with ischemic limbs. Recently bone marrow mononuclear cells injected into muscle have also been shown to stimulate angiogenesis in patients with chronic ischemia in a small randomized trial.[8] Other than these examples, there are no studies proving the concept of therapeutic angiogenesis. Considering the basic mechanisms of angiogenesis and arteriogenesis described, attempts to treat limb ischemia should probably be directed toward arteriogenesis. The ideal therapeutic concept would probably involve efforts to make vessels grow further beyond harmonization of shear forces so that collateral capacity could sufficiently ameliorate all symptoms. Treatment to keep collateral vessels "active" as long as possible by prolonging the proliferation and remodeling phases of arteriogenesis may be a way to achieve this. This concept is being

tried in clinical trials presently starting up using MCP-1 and GM-CSF supplementation. Another possibility is combination treatment, eg, FGF-2 and platelet-derived growth factor-BB, which creates long- lasting functional collateral vessels in animal models of limb ischemia.[19]

SUMMARY

When the concept of therapeutic angiogenesis was launched, the complexity of angiogenesis and arteriogenesis were underestimated. Today most researchers believe that simply adding vascular growth factors to an ischemic area is not sufficient for effective therapeutic angiogenesis. While the mechanisms involved in these processes are starting to be revealed, their effect on the pathophysiology of limb ischemia needs to be further clarified. Such information will make future treatment efforts more likely to succeed.

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