Eur J Vasc Endovasc Surg **28**, 9–23 (2004)
doi: 10.1016/j.ejvs.2004.03.021, available online at http://www.sciencedirect.com on SCIENCE DIRECT®

REVIEW

Therapeutic Angiogenesis in Peripheral Arterial Disease: Can Biotechnology Produce an Effective Collateral Circulation?

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The physiological processes of angiogenesis, vasculogenesis and arteriogenesis contribute to the growth of collateral vessels in response to obstructive arterial disease causing lower limb or myocardial ischaemia, but in clinical practice the endogenous angiogenic response is often suboptimal or impaired, e.g. by factors such as ageing, diabetes or drug therapies. Therapeutic angiogenesis is an application of biotechnology to stimulate new vessel formation via local administration of pro-angiogenic growth factors in the form of recombinant protein or gene therapy, or by implantation of endothelial progenitor cells that will synthesize multiple angiogenic cytokines. Numerous experimental and clinical studies have sought to establish 'proof of concept' for therapeutic angiogenesis in PAD and myocardial ischaemia using different treatment modalities, but the results have been inconsistent. This review summarises the mechanisms of angiogenesis and the results of recent trials evaluating the efficacy and safety of different gene therapy, recombinant protein and cellular-based treatment approaches to enhance collateral vessel formation.

Key Words: Therapeutic angiogenesis; Basic fibroblast growth factor; Peripheral arterial disease; Endothelial growth factor; Gene transfer; Critical ischaemia; Intermittent claudication.

Introduction

The strengths and limitations of surgical revascularization in peripheral arterial disease (PAD) are well recognised. In general, by-pass grafting and percutaneous interventions are reserved for patients with critical limb-threatening ischaemia and those with disabling claudication due to discrete, proximal disease.[1,2] This leaves a significant number of patients with moderate-to-severe PAD with few, if any, effective treatment options to improve symptoms, restore distal perfusion and preserve tissue viability. This particularly applies to patients with diffuse and distal disease, who are often diabetic, and those who, despite successful revascularization, present with recurrent symptoms that are not amenable to further surgical intervention.

There is good evidence that the development of collateral vessels has a favourable effect on the symptoms and outcomes of atherosclerotic disease. For example, in acute myocardial infarction the presence of a collateral circulation decreases infarct size and improves left ventricular function and patient survival.[3] Over the last 10 years, there has been considerable interest in the physiological mechanisms that regulate new vessel formation.[4] The main factors that stimulate growth of collateral vessels are, firstly, the duration and severity of ischaemia, as well as shear stress and inflammation, but numerous local and systemic cytokines also have pro- or anti-angiogenic effects. Cancer researchers have been particularly interested in blocking angiogenesis as a method of inhibiting tumour growth; meanwhile, cardiovascular researchers have explored the feasibility of enhancing collateral vessel formation in patients with chronic ischaemia of the myocardium or lower limb. This review describes the underlying mechanisms and principles of therapeutic angiogenesis, and summarises important results from recent clinical and experimental studies in PAD.

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1078–5884/010009 + 15 $35.00/0

Terminology Relating to New Vessel Formation

Angiogenesis is the sprouting of new capillaries from existing vascular structures, a process that is triggered by endothelial cell migration and proliferation. Remodelling of the extracellular matrix (ECM), tubule formation and expansion of the surrounding vascular tissues are key elements of angiogenesis (Fig. 1). Vasculogenesis, however, is quite different: the *in situ* formation of new blood vessels from circulating bone marrow-derived endothelial progenitor cells (EPCs) which differentiate into endothelial cells and fuse into luminal structures. It was previously assumed that vasculogenesis only occurred during embryological development, but there is increasing evidence that neovascularization in adult tissues involves both processes of angiogenesis and vasculogenesis.[5,6] Under normal conditions, the number of circulating EPCs is relatively small but vascular trauma or ischaemia results in mobilization and proliferation of EPCs from the bone marrow.[7] EPCs may contribute up to 25% of endothelial cells in newly formed vessels,[8] and there is evidence that the biological activity of EPCs and vasculogenesis are impaired in conditions such as diabetes.[9]

A more recently defined term, arteriogenesis, refers to an increase in the calibre of pre-existing arteriolar collateral connections by recruitment of perivascular cells and expansion and remodelling of the extracellular matrix.[10] Arteriogenesis increases the size and wall thickness of collateral vessels, and shear stress (rather than hypoxia) seems to be the main factor that stimulates arteriogenesis.[11] Thus, in response to occlusion or stenosis of a major artery the haemodynamic changes in proximal vessels lead to increased blood flow through preformed collateral arterioles. The associated increases in shear stress promote arteriogenesis involving monocyte invasion of the wall of the growing collateral arteriole.[12] Migration of circulating monocytes and their differentiation into macrophages within the blood vessel wall is a key element of arteriogenesis, together with recruitment and expansion of smooth muscle cell and matrix components of the vessel wall.

Thus, the initiation and development of new vessels to provide an effective collateral circulation in occlusive arterial disease involves all three processes of angiogenesis, vasculogenesis and arteriogenesis.

Regulation of Angiogenesis

Initiation of angiogenesis requires the normally quiescent vascular endothelium to become activated, e.g. by ischaemia or circulating growth factors, which trigger individual cells to break their intercellular adhesions with neighbouring endothelial cells. One 'leader cell' then begins to migrate, followed by other cells, in a process of capillary budding and endothelial cell proliferation (Fig. 1). Local release of matrix metalloproteinases (MMPs) and plasminogen degrades the surrounding ECM while the migrating endothelial cells form bands which develop into loops and eventually canalize to allow blood to flow. The new endothelial cells re-establish intercellular connections, and in the later stages of angiogenesis recruitment of smooth muscle cells and fibroblasts creates mature thicker-walled vessels (Fig. 1).

The key steps in angiogenesis—namely endothelial cell activation, migration, proliferation and reorganisation—are tightly regulated in a complex balance between pro- and anti-angiogenic mechanisms. Over the last 20–30 years, a large number of molecules have been identified which either stimulate or inhibit angiogenesis (Table 1). The most important pro-angiogenic growth factors are vascular endothelial growth factor (VEGF) (there are four spliced variants of VEGF containing 121, 165, 189 and 206 amino acids),[13] and basic fibroblast growth factor (bFGF), also known as FGF-2 (one of nine members of the FGF family). VEGF is an endothelial cell specific mitogen that is markedly upregulated by hypoxia,[14] and plays an important role in endothelial cell proliferation, differentiation and survival. Platelets are a major source of circulating VEGF,[15] which interacts with three tyrosine-kinase receptors (flt-1, flk-1 and flt-4) to promote neovascularization and increased vascular permeability. Production of a soluble form of the flt-1 receptor, s.flt-1, may be important in determining

Table 1. Factors that stimulate and inhibit angiogenesis

Stimulate	Inhibit
VEGF	Angiostatin
aFGF	Thrombospondin
bFGF	Endostatin
PDGF	Troponin-I
TGFα/β	TIMPs
TNFα	Suramin
HGF	
Angiogenin	
PlGF	
Angiopoetin	
Oestrogen	
Nitric oxide	

VEGF, vascular endothelial growth factor; aFGF and bFGF, acid and basic fibroblast growth factor (FGF-1 and FGF-2, respectively); PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF, tumour necrosis factor; HGF, hepatocyte growth factor; PlGF, placental growth factor; TIMPs, tissue inhibitors of matrix metalloproteinases.

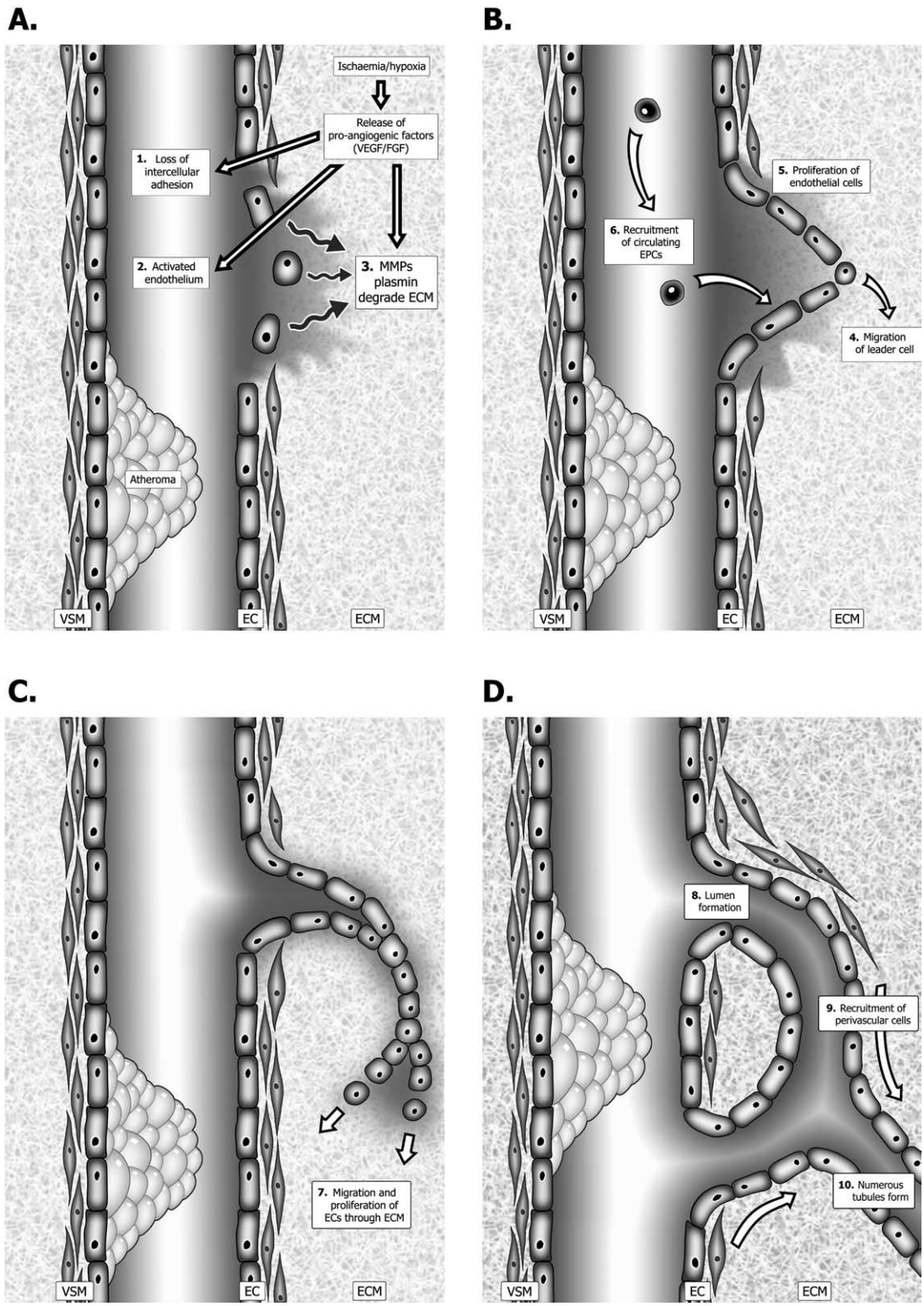

Fig. 1. Schematic showing the various stages in new vessel formation (including angiogenesis, vasculogenesis and arteriogenesis). Ten individual steps are described in panels A to D showing endothelial cell (EC) proliferation and migration (angiogenesis) followed by extracellular matrix (ECM) remodelling and expansion of vascular smooth muscle (VSM) cells (arteriogenesis).

VEGF responses, and there is evidence that s.flt-1 production may be impaired in PAD.

FGFs, unlike VEGF, are non-secreted growth factors that are released only during cell death or ischaemic cell injury. FGFs are also powerful endothelial cell mitogens, but the FGF family of cytokines is not endothelial cell specific. At least four high-affinity FGF receptors have been cloned and characterised. *In vitro* studies have shown that FGF and VEGF have different effects on the angiogenic process, e.g. FGF stimulates vascular cells other than endothelial cells. *In vivo* the inter-play between positive and negative regulators of new vessel formation creates a complex microenvironment that is often called the 'angiogenic switch'.[16,17]

Cellular interactions are also very important in determining the response of endothelial cells to proliferate and migrate. There is evidence, for example, that leucocytes and platelets exert important effects on endothelial cells and angiogenesis either by direct intercellular contact or via local release of cytokines.[4,18] Clinical and biochemical factors also influence the formation of, and biological response to, different angiogenic growth factors. For example, hypoxia is one of the most potent inducers of angiogenesis,[17] principally via up-regulation of VEGF,[14] whereas diabetes and raised levels of cholesterol and lipoprotein (a) are associated with a reduced angiogenic response.[19–21] Glycation of angiogenic growth factors, e.g. bFGF, reduces their activity,[22,23] while hypercholesterolaemia seems to affect angiogenesis indirectly via reduced nitric oxide (NO) availability.[20]

Although ischaemia activates physiological mechanisms leading to collateral vessel formation, there is evidence that in clinical practice the endogenous angiogenic response in patients with PAD is often impaired or insufficient. For example, it has been shown that angiogenesis is reduced in the elderly,[24] and in patients with diabetes[9,19,25] or dyslipidaemia.[20] In addition, many of the commonly prescribed cardiovascular drugs may also impair angiogenesis, e.g. ACE inhibitors,[26] statins[27] and non-steroidal anti-inflammatory drugs (NSAIDs).[28] Conversely, physical exercise enhances angiogenesis.[29]

Therapeutic angiogenesis aims to overcome any limitations of the natural angiogenic response by increasing substantially the local concentrations of angiogenic growth factor(s) in the lower limb or myocardium, either by administering recombinant protein or the gene that codes for an angiogenic growth factor, or by administering EPCs that will synthesize a cocktail of growth factors in the vicinity of new vessel formation.

Experimental Studies of Therapeutic Angiogenesis in Animal Models of Lower Limb and Myocardial Ischaemia

There has been considerable research into the pharmacodynamics and pharmacokinetics of different therapeutic interventions to increase levels of angiogenic growth factors in animal models of lower limb and myocardial ischaemia. These studies can broadly be divided into three groups: (1) evaluation of different angiogenic growth factors;[30–38] (2) evaluation of different treatment modalities and different routes of administration, e.g. recombinant protein versus gene transfer;[39,40] and (3) studies evaluating cellular techniques, including chemotactic methods to attract monocytes to ischaemic tissues,[41] local administration of EPCs harvested from the peripheral circulation,[42] and even autologous bone marrow transplantation to provide EPCs capable of augmenting vasculogenesis and synthesizing multiple angiogenic growth factors.[43]

Administration of recombinant protein

Several growth factors administered in the form of recombinant proteins have been shown to augment collateral vessel formation *in vivo*, e.g. VEGF is effective in models of hindlimb and myocardial ischaemia even after single dose administration.[32–34] Similarly, there is good evidence that recombinant bFGF produces dose-dependent increases in collateral vessel formation, capillary density and blood flow in animal models of intermittent claudication and stable angina.[35–37] Other recombinant angiogenic growth factors also have therapeutic activity in experimental models of PAD, e.g. hepatocyte growth factor (HGF)[38] and placental growth factor.[44]

Gene transfer

Gene transfer is the introduction of foreign DNA into target cells in order to achieve a localised, sustained therapeutic over-expression of the chosen gene. Several different approaches have been evaluated to transfer an angiogenic growth factor gene into vascular endothelial cells, but the success of any technique depends upon the efficiency with which the transgene is introduced and expressed within the target cell population.[45] Naked DNA is poorly taken-up, but different types of DNA vectors have been successfully used to increase the efficiency of gene transfer. In cardiovascular research, the most commonly used vectors are either adenoviruses or

plasmids, often formulated with liposomes to facilitate the transfer of DNA across the cell membrane. However, even the best methods of gene transfer still encounter significant problems in achieving high enough rates of transfection to result in clinically significant levels of protein production.

Adenoviral vectors produce a higher efficiency of gene transfer, but there is a risk of triggering an immune response to the viral DNA. In addition, none of the methods of gene transfer ensure that only the target cells are transfected; introducing foreign DNA into non-target cells may cause adverse effects. Thus, more recently there has been considerable interest in '*ex vivo* gene transfer'—i.e. harvesting cells which are then transfected *in vitro* before being replaced.[46,47] This method increases the transfection efficiency and ensures that foreign DNA is only introduced into target cells.

Several angiogenic genes have been evaluated in experimental models of lower limb and myocardial ischaemia, e.g. VEGF, bFGF, HGF and hypoxia inducible factor (HIF)-1α. VEGF has been the most intensively studied over the past 10 years, and several studies have shown that gene transfer using naked DNA or adenoviral vectors augments collateral formation and tissue perfusion in models of myocardial and hindlimb ischaemia.[48–51] Unlike VEGF, bFGF lacks a secretory signal sequence and is therefore not actively secreted from cells following gene transfer.[52] Thus, in order to achieve a clinical response the bFGF gene would need to be modified to add a signal sequence prior to transfection. This added difficulty has resulted in less interest in developing gene transfer methods for bFGF compared with VEGF, but limited success has been reported with adenoviral and *ex vivo* gene transfer of FGFs in animal models of myocardial and hindlimb ischaemia.[46,47,53]

HGF has also shown some potential in gene transfer studies in experimental animals, e.g. transfection of naked DNA augmented collateral vessel growth in a model of hindlimb ischaemia.[54] HIF-1α is a transcription factor that regulates the expression of several genes encoding angiogenic proteins including VEGF and its receptors. An active form of the transcription factor has been synthesized and transferred effectively in animal studies to induce collateral growth.[55,56]

Delivery of cells that express multiple angiogenic cytokines

There has always been a concern that angiogenesis may be too complex a process to be stimulated effectively by administration of a single angiogenic cytokine, and therefore a separate line of research has explored ways of increasing cellular recruitment with a view to increasing local production of a cocktail of growth factors. For example, there is evidence that circulating monocytes play a crucial role in arteriogenesis,[41] and that differentiation of monocytes into tissue macrophages leads to local secretion of VEGF, nitric oxide and angiogenic cytokines. Thus, *in vitro* studies have shown that administration of monocyte chemoattractant protein-1 (MCP-1), which increases the recruitment of monocytes to ischaemic tissue, improves collateral flow in a rabbit model of hindlimb ischaemia.[41,57]

EPCs derived from bone marrow are present in the peripheral circulation, and are mobilized and incorporated into sites of neovascularization in response to tissue ischaemia.[5] This has led to the investigation of 'supply-side angiogenesis' whereby EPCs are harvested from the bone marrow or peripheral blood, expanded and concentrated *ex vivo* and then re-administered into the lower limb of the animal.[58,59] A further development of this technique involves treating the EPCs with adenovirus-containing VEGF or bFGF prior to re-implantation.[60] The initial results have shown augmented neovascularization and increased perfusion in animal models of myocardial and hindlimb ischaemia.[60,61] Other cellular based angiogenic techniques have evaluated bone marrow derived mononuclear cells and embryonic stem cells.[62,63]

Pharmacokinetic and pharmaceutical aspects of therapeutic angiogenesis

There is uncertainty about the optimum method and frequency of delivery of angiogenic growth factors, whether in the form of recombinant protein or as gene therapy. Ideally, the protein or gene therapy should be easy to administer and produce a sustained, high local concentration of the angiogenic cytokine with low systemic availability. In practice, the i.v. or i.a. routes of administration require massive doses in order to achieve a localised therapeutic effect. This often results in high systemic plasma concentrations and potentially serious side effects.[64] It has been shown that administration of recombinant bFGF into a peripheral vein is ineffective, whereas local i.a. injection achieves higher local concentrations and a clear angiogenic effect.[65]

In the case of gene transfer, transfection efficiency is particularly poor when naked DNA is injected into the circulation, probably because of degradation by circulating nucleases. Nevertheless, animal studies have shown a therapeutic effect following i.v. or i.a.

injection of gene transfer vectors.[34,66] Intramyocardial injection is more effective in the heart,[67] and gene transfer by intramuscular injection has been successful in increasing collateral growth in animal models of PAD.[50,55,68]

Clinical Trials of Therapeutic Angiogenesis: Theoretical Risks and Current Information on Safety and Tolerability

A fairly large number of clinical studies have been reported in the literature, ranging from uncontrolled case reports to larger randomized controlled trials, using several treatment modalities (e.g. recombinant protein, gene transfer or cellular implantation) in patients with PAD (Fontaine stages II and III) and those with endstage myocardial ischaemia. These studies have used a variety of treatments, patient selection criteria and endpoints, as well as different study designs and duration of therapy, therefore meaningful comparisons are difficult and there is very little scope to undertake combined analyses of treatment efficacy. Establishing the safety of novel treatments has been an early priority for clinical studies.

Pharmacological stimulation of angiogenesis in humans raises a number of theoretical concerns, especially in relation to side effects in non-target tissues, e.g. unwanted neovascularization, tumour growth, haemorrhage from fragile new vessels, and even an adverse effect on atherosclerotic plaques. In addition, gene transfer techniques raise uncertainty about the hazards of introducing foreign DNA which, following intramuscular or intramyocardial injection, may disturb muscle cell growth and turnover.[69] The possibility of causing vascular malformations was illustrated in the first-ever report of VEGF gene transfer for PAD; the patient concerned developed three spider angiomas on the treated leg several weeks after DNA administration.[70]

Triggering neovascularization in patients with diabetic retinopathy has been another major concern, particularly since VEGF plays an important role in new vessel formation around the optic disc and in sight-threatening macular oedema.[71] Most clinical studies of therapeutic angiogenesis have included fundoscopic surveillance and so far no significant adverse effects have been reported in the eye[72,73] but several studies have excluded patients with pre-existing diabetic retinopathy (even the common form of background retinopathy). Given that patients with diabetes are an important subgroup that may benefit from therapeutic angiogenesis more safety data are required in this population.

A number of growth factors are important in tumourigenic angiogenesis, which raises concerns that underlying polyps, tumours and various benign abnormalities might be activated, or develop complications, as a result of systemic exposure to high pharmacological doses of angiogenic cytokines. These concerns are plausible and difficult to exclude, although no serious tumour-related side effects have emerged in clinical trials to date. In fact, in the recent VIVA trial, there were more new diagnoses of malignant disease in the control group.[74] Angiogenic growth factors may also stimulate neovascularization within the intima of the arterial wall and at sites of atherosclerotic disease, potentially causing plaque instability and plaque rupture due to intimal neovascularization.[75] The limited results of clinical trials, however, seem to refute this possibility, and indeed, by contrast, suggest that angiogenic factors inhibit neointimal thickening.[76]

Administration of high systemic concentrations of VEGF (also known as vascular permeability factor) has the potential to cause hypotension and oedema. These side effects have been reported in trials of recombinant VEGF, but seem to be relatively mild, transient and reversible.[74,77] In the case of recombinant bFGF, the main adverse effects are on the kidney, especially proteinuria.[78] Modest increases in urinary albumin excretion rate have been reported in several studies following single or double doses of bFGF, but one trial of repeated intravenous infusions of bFGF was terminated because of severe proteinuria in five out of 24 patients.[79]

Notwithstanding the potential hazards of gene transfer,[69] one major advantage of this technique is that systemic plasma concentrations of the gene product do not increase,[80,81] i.e. the therapeutic effect is very well contained locally. Gene therapy, however, may provoke an unwanted inflammatory response. Several studies have reported transient fevers following the procedure, especially with adenoviral vectors.[81,82] In one randomized controlled trial, 61% of patients developed adenoviral antibodies which may have important limitations for the feasibility of giving repeated treatments.[82]

Most clinical studies to-date have been relatively short (2 weeks to 1 year follow-up), but two recent trials have provided longer-term safety data. Firstly, outcomes up to 3 years after administration of bFGF via perivascular beads at the time of coronary artery bypass grafting showed two late deaths in the highest dose group, one from pancreatic carcinoma and one sudden death of unknown cause.[83] There were no

differences in overall mortality between the actively treated and control groups, and no other longer term serious adverse events.[83] Secondly, a trial of gene transfer of VEGF using plasmid-liposome and adenoviral vectors for critical limb ischaemia and intermittent claudication has reported safety data up to 2 years. There have been no differences in mortality, and no new tumours detected.[82]

Clinical Trials of Therapeutic Angiogenesis in Lower Limb Ischaemia: Information About Treatment Efficacy

Following the first case report of therapeutic angiogenesis in 1996, VEGF gene transfer in a patient with critical limb ischaemia,[70] there have been numerous clinical studies of different angiogenic agents in both PAD and inoperable myocardial ischaemia. The results have been inconsistent, and several studies were not adequately controlled or powered to make firm conclusions about treatment efficacy. Furthermore, it has been particularly difficult to undertake a combined meta-analysis because the various studies have used different treatment modalities, endpoints and inclusion/exclusion criteria. The placebo effect in cardiovascular interventions should not be underestimated, yet several clinical studies, for obvious practical reasons, were not fully blinded or did not include a properly matched control group. More recently, however, some larger multicentre randomized controlled trials have been published.

Clinical trials in patients with ischaemic heart disease: lessons about safety and trial design that are relevant to future prospects in PAD

There have been several (mostly small and uncontrolled) clinical studies of therapeutic angiogenesis in patients with endstage myocardial ischaemia (Table 2). The early results using intramyocardial VEGF gene transfer and intra-coronary injection of bFGF protein showed encouraging improvements in anginal frequency and angiographic scores of collateral growth,[72,76,77,84–87] but less impressive results have appeared from larger multicentre randomized controlled trials[74,80,81,88] (Table 2). Changes in surrogate endpoints, such as myocardial perfusion imaging or angiography, are much less clinically relevant than differences in anginal frequency or exercise tolerance. Thus, recent larger clinical trials have included the Seattle Angina Questionnaire (SAQ) and other quality of life indices.[74,88]

The three largest muticentre randomized controlled trials, i.e. the FIRST,[88] VIVA[74] and KAT II[81] studies, have been fairly disappointing. The FIRST trial, for example, evaluated the effects of intracoronary recombinant bFGF in over 330 patients. Although angina symptom scores were significantly improved after 90 days, treadmill exercise tolerance and myocardial perfusion were no different between the two groups.[88] Similarly, the VIVA and KAT II trials used recombinant VEGF and VEGF gene transfer, respectively, and there were only modest, inconsistent improvements in isolated endpoints.[74,81]

Clinical trials in patients with critical limb ischaemia

The initial studies of therapeutic angiogenesis in PAD were conducted in patients with critical limb ischaemia (Table 3). These were patients who were either unsuitable for surgical revascularization, or those who had failed other treatment options and were at high risk of distal amputation. Case reports and small uncontrolled studies appeared to show dramatic benefits. For example, VEGF gene transfer was successful in achieving clinical improvement (e.g. resolution of rest pain), increased density of collateral vessels and increased ABPI[69,73,89,90] (Fig. 2). However, in a larger randomized controlled trial of VEGF gene therapy, 87% of patients showed improved vascularity on digital subtraction angiography but there were no significant differences between the treatment and placebo groups in terms of restenosis after angioplasty, amputation rates, ulcer healing or severity of rest pain.[82] A further uncontrolled study using a plasmid vector for VEGF gene transfer has shown an 83% improvement in rest pain and 75% improvement in ulcer healing, but the number of patients is too small to draw firm conclusions.[73]

There is considerable interest in cellular based therapy for improving lower limb outcomes in critical ischaemia. For example, in the TACT study, a randomized controlled trial, autologous implantation of bone marrow mononuclear cells, including EPCs, into critically ischaemic limbs produced clinical improvements in rest pain in 39 out of 45 patients. In addition, limbs injected with bone marrow mononuclear cells showed a significant increase in ABPI (>0.1) in 31 out of 45 patients, from 0.35 at baseline to 0.47 after 4 weeks ($p < 0.001$).[91] Treadmill walking time also improved (1.6–5.0 min, $p < 0.001$), and ischaemic ulcers were healed in 21 out of 28 patients.[91] The investigators in the TACT study used peripheral blood mononuclear cells with 500-fold fewer EPCs as the placebo control.

Table 2. Summary of all published clinical studies of therapeutic angiogenesis in patients with myocardial ischaemia

Ref.	Treatment	No. of Subjects		Follow up	Outcomes		
		Active	Control		Safety	Endpoints	Results
84	Intra-myocardial VEGF gene	5	—	60 days	No SAEs	Angina frequency	Improved
						Myocardial perfusion	Improved ($p < 0.05$)
						Angiography	Improved collateral flow
85	Perivascular beads bFGF protein	16	8	3 years	2 deaths: 1 in control 1 in active	Angina frequency	Treatment group angina free
						Myocardial perfusion	Improved with high dose ($p = 0.01$)
86	Intra-myocardial VEGF gene	21	—	2 months	3 deaths	Angina class	Improved class
						Angiography	Improved collateral scores
						Treadmill exercise	Improved in 50%
76	Intra-coronary VEGF gene	10	5	6 months	No SAEs	Angiography	No improvement at 6 months
72	Intra-coronary bFGF protein	52	—	6 months	Mortality 8% hypotension proteinuria	Angina questionnaire	Improvement ($p < 0.001$)
						LV ejection fraction	Small improvement
						MR imaging	Improved ($p < 0.001$)
77	Intra-Coronary VEGF protein	15	—	60 days	Hypotension flushing	Angina class	Improvement ($p = 0.002$)
						Myocardial perfusion	Improved in 50%
87	Intra-myocardial VEGF gene	12	7	12 weeks	No SAEs	Angina class	Improved ($p = 0.04$)
						Treadmill exercise	Improved ($p = 0.02$)
FIRST 88	Intra-coronary bFGF protein	251	86	180 days	No difference in mortality/SAEs	Angina questionnaire	Improved at 90 days ($p = 0.035$)
						Myocardial perfusion	No difference
						Treadmill exercise	No difference
AGENT 80	Intra-coronary FGF-4 gene	60	19	10 months	No difference in mortality/SAEs	Treadmill exercise	Improved compared to placebo
						Stress echo	No difference from baseline
VIVA 74	IV + Intra-coronary VEGF protein	115	63	120 days	Hypotension flushing	Angina questionnaire	Improvement at 120 days ($p = 0.09$)
						Myocardial perfusion	No improvement
						Treadmill exercise	Improved at 120 days ($p = 0.15$)
KAT II 81	Intra-coronary VEGF gene	65	38	6 months	No difference in mortality/SAEs	Re-stenosis rate	No difference from placebo
						Myocardial perfusion	Improvement at 6 months ($p < 0.05$)

Table 3. Summary of all published clinical studies of therapeutic angiogenesis in patients with PAD

Ref.	Treatment	No. of subjects		Follow up	Outcomes		
		Active	Control		Safety	Endpoints	Results
70	Intra-arterial $VEGF_{165}$ Gene	1	—	12 weeks	3 angiomas	Angiography	Increased collaterals
89	Intra-Muscular $VEGF_{165}$ Gene	6	—	14 months	No SAEs	ABPI	Increased in 4 limbs
						Angiography	New collaterals
90	Intra-Muscular $VEGF_{165}$ Gene	9	—	6 months	Transient oedema	ABPI	Increased ($p = 0.028$)
						Angiography	Increased collaterals
						Symptoms	Reduced rest pain ($p = 0.043$)
78	Intra-arterial bFGF Protein	13	6	1 year	Mild proteinuria	Calf blood flow	Improved ($p < 0.05$)
						Symptoms	Some improvement
79	IV bFGF Protein	16	8	—	Severe proteinuria	None	Study stopped prematurely. No positive results at cessation
92 TRAFFIC	Intra-arterial bFGF Protein	127	63	6 months	Proteinuria	Peak walking time	Increased at 90 days ($p = 0.034$)
						ABPI	Increased at 90 days ($p = 0.037$)
82	Intra-arterial VEGF Gene	35	19	2 years	No SAEs	Angiography	Improved vascularity ($p = 0.03$)
						ABPI	No difference
						Symptoms	No difference
						Re-stenosis rate	No difference
73	Intra-Muscular $VEGF_{165}$ Gene	24	—	6 months	Transient oedema	ABPI	Improved ($p < 0.001$)
						Angiography	Increased collaterals ($p < 0.01$)
						Symptoms	Reduced rest pain and ulcer healing
93 RAVE	Intra-Muscular $VEGF_{121}$ Gene	33	72	26 weeks	Oedema	Peak walking time	No difference
						ABPI	No difference
						Symptoms	No difference

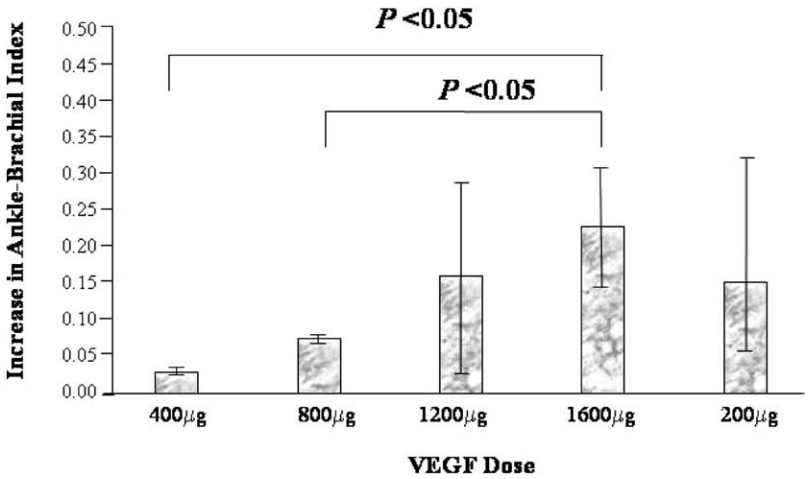

Fig. 2. Ankle-brachial pressure index (ABPI) before and after plasmid-mediated $VEGF_{165}$ gene transfer in 24 limbs of 21 patients with rest pain and chronic critical limb ischaemia. Doses varying between 400 and 2000 μg of ph $VEGF_{165}$ were injected intra-muscularly into the affected limbs, and a repeat dose administered 4 weeks later. Results show the change in ABPI following VEGF. Reproduced from an uncontrolled study, with permission.[73]

Clinical trials in patients with intermittent claudication

Two randomized, placebo-controlled trials have recently been reported in patients with intermittent claudication.[92,93] Both were phase II 'proof of concept' studies, one using intra-arterial recombinant bFGF[92] and the other using intra-muscular adenoviral gene transfer of $VEGF_{121}$.[93]

The TRAFFIC study was a randomized, double-blind placebo-controlled trial of single or repeat-dose i.a. recombinant bFGF 30 μg/kg.[92] The formulation of bFGF used in this study was a 146-amino acid, non-glycosylated, monomeric 16.5 kDa protein which is produced in genetically engineered yeast. The dose (30 μg/kg) was the maximum-tolerated dose of bFGF (limited by acute hypotension) in a phase 1 study of intracoronary perfusion.[72]

Patients with intermittent claudication and reproducible exercise tolerance on a treadmill received half the dose of bFGF down each femoral artery via a single arterial puncture and crossover catheter. Patients needed two reproducible (within 20%) Gardner treadmill tests (with peak walking time between 1 and 12 min) during a 30-day screening period in order to be eligible for inclusion. Other inclusion criteria included evidence of infra-inguinal obstructive arterial disease (>70% stenosis of femoral, popliteal, or tibial arteries on angiography) and a resting APBI <0.8 on the most affected limb. All patients received appropriate medical management and risk factor modification. Anyone with a history of malignancy within the past 10 years was excluded, as were those with other exercise limiting symptoms, e.g. arthritis or angina. A total of 377 patients were screened for the TRAFFIC study, and 190 were deemed eligible for randomization.

Patients were randomized to placebo, single-dose bFGF or two doses of bFGF (a second dose 30 days later). Clinical and demographic details were similar in the three groups (e.g. mean ages 65–69 years; 24–38% were current smokers; 33% were diabetic; and half had undergone previous revascularization for PAD). Most patients (85%) had femoropopliteal disease; 30% had isolated femoropopliteal disease and 45–55% had multiple sites of disease in the femoropopliteal region. Two-thirds of patients had evidence of bilateral disease.

The maximum walking distance at 90 days was the primary endpoint for the TRAFFIC study, and there were results for over 60 patients in each of the three treatment groups. The trial reported a statistically significant improvement in peak walking time, but there was no difference between single and repeated doses of bFGF: for example, compared with baseline patients in the placebo group increased peak walking time by 0.60 min (14%), patients in the single-dose group increased this time by 1.77 min (34%), and patients in the double-dose group increased by 1.54 min (20%) (Fig. 3). Results of the intention-to-treat analysis ($n = 190$) showed a significant difference between the three groups ($p = 0.034$). Active therapy also produced a small but significant increase in ABPI in the more affected limb ($p < 0.04$).[92] However, patients with non-compressible vessels ($n = 10$) and those who withdrew from the study early ($n = 14$) or who were revascularized ($n = 4$) were excluded from this analysis.

Three subgroups (smoking, diabetes and older age)

were prespecified in the TRAFFIC study because of their potential to influence the primary endpoint. Diabetes, age greater than median (68 years), and non-current smoking status were all associated with lower improvement in peak walking time in response to bFGF; however, only smoking status had an independent effect on peak walking time. Current smokers showed a greater increase in peak walking time (1.25, 2.10, and 2.26 min for placebo, single-dose and double-dose, respectively).[92]

One or two doses of bFGF was generally well tolerated in the TRAFFIC study. Transient acute hypotension was uncommon: two patients in the placebo group (3%), four in the single-dose group (6%) and five in the double-dose group (8%). Corresponding frequencies for development of proteinuria were 3% (placebo), 9% (single-dose) and 11% (double-dose). Seven out of nine patients who developed proteinuria also had diabetes. There were two deaths (one in placebo group and one in double-dose group) during the study. There was no evidence of tumourigenesis or adverse effects on the retina with bFGF administration.

The RAVE trial was also a phase II multicentre, randomized, placebo-controlled, double-blind study to evaluate the safety and efficacy of $AdVEGF_{121}$, a replication-deficient adenovirus encoding the 121-amino-acid isoform of VEGF.[93] In total 105 patients with unilateral exercise-limiting intermittent claudication were randomized after a run-in phase to establish that they had reproducible exercise performance on a treadmill (peak walking time 1–10 min). Patients were stratified by diabetes status and randomized to low-dose $AdVEGF_{121}$, high-dose $AdVEGF_{121}$ or placebo, administered as 20 intramuscular injections to the index leg in a single session. Over 105 patients were entered in the study, which gave >80% power to detect a difference of 1.5 min in peak walking time.[94] The results showed no significant difference in the primary endpoint, change in peak walking time after 12 weeks: e.g. mean values of 1.8 ± 3.2 min (placebo) vs. 1.6 ± 1.9 min (low-dose $AdVEGF_{121}$) vs. 1.5 + 3.1 min (high-dose $AdVEGF_{121}$).[93] Secondary endpoints, including ABPI and quality of life measures, were also unchanged after 12 and 26 weeks. The adenoviral therapy was associated with peripheral oedema.[93]

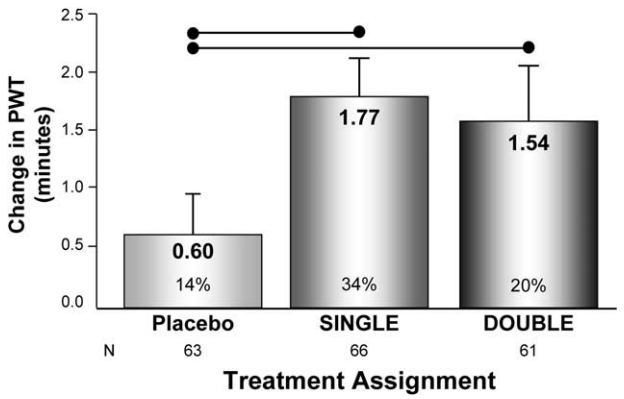

Fig. 3. Results from the TRAFFIC study. Improvement in peak walking time (PWT) 90 days after treatment with intra-arterial placebo ($n = 58$), single-dose bFGF ($n = 62$) or double-dose bFGF ($n = 54$) in patients with intermittent claudication. Single dose vs. placebo, $p = 0.026$; double-dose vs. placebo, $p = 0.45$. Reproduced with permission.[92]

Possible Explanations for Clinical Trial Results Being Inconsistent and Inconclusive

The randomized controlled clinical trials in PAD have produced results that are less consistent than those undertaken in animals and less impressive in terms of the absolute treatment effect. This discrepancy raises the possibility that, in the *in vivo* clinical situation of patients with vascular disease, it is the responsiveness to angiogenic stimuli that is impaired rather than a problem with the availability of angiogenic growth factors. For example, increased (not decreased) levels of VEGF have been reported in PAD,[95] which could be interpreted as evidence of a potential defect in VEGF responsiveness. Differences in the production of soluble flt-1 may account for inter-subject differences in VEGF effects. In addition, it is possible that angiogenesis is abnormal in some way in patients with PAD. Both of these possibilities might explain why supplementing the availability of angiogenic growth factors does not necessarily augment angiogenesis in patients with arterial disease. The animal models used in various experimental studies often do not have on-going vascular disease and therefore may not mimic the clinical problem of reduced angiogenic responsiveness.

Conclusion

The formation of new blood vessels, including collaterals, is a complex physiological process that occurs in adults in response to tissue injury or ischaemia. Neovascularization involves angiogenesis, vasculogenesis and arteriogenesis, and there are several pro- and anti-angiogenic cytokines that regulate endothelial cell migration and proliferation. The endogenous angiogenic response seems to be impaired and/or insufficient in patients with PAD or

myocardial ischaemia, and therefore therapeutic angiogenesis seeks to augment collateral vessel formation using local administration of recombinant proteins or genes for angiogenic growth factors, or by re-implantation of EPCs harvested from the bone marrow or peripheral circulation.

VEGF is a secreted, endothelial cell specific mitogen, whereas the family of FGFs, especially bFGF, are not secreted but stimulate non-endothelial cell types in the angiogenic process. Experimental and clinical studies have evaluated the effects of VEGF gene transfer and recombinant bFGF in animals and humans with critical limb ischaemia, intermittent claudication and endstage myocardial ischaemia. Although the early uncontrolled reports were highly encouraging, more recent results from multicentre randomized controlled trials have been far less convincing. In intermittent claudication, intra-arterial recombinant bFGF improved peak walking time at 90 days in the TRAFFIC study[92] but $VEGF_{121}$ gene transfer was ineffective in the RAVE trial.[93] In critical limb ischaemia, autologous bone marrow transplantation was effective in a randomized controlled trial.[91]

There is still much to learn about the optimum treatment modality, dosing frequency and route of administration, but intra-arterial recombinant protein therapy is closer to being available for routine use than gene therapy. It is becoming clear that trials of single angiogenic growth factors are not achieving the results anticipated from experimental studies, and therefore administration of multiple agents may be necessary to optimize the angiogenic response.[96] For example, the combination of VEGF and bFGF has synergistic effects.[97] If regulatory approval is ever granted for these novel (and no doubt expensive) technologies, therapeutic angiogenesis is likely to be reserved for those patients with severe limb-threatening PAD that is not suitable or has failed with conventional revascularization. Whether a one-off intervention at such an advanced stage can achieve sufficient reperfusion, in a short space of time, to avert amputation is uncertain.

Thus, can biotechnology produce an effective collateral circulation? At present, this seems more remote than it did perhaps 3 years ago when experimental studies were so encouraging. Angiogenesis is clearly complex, and it may be necessary to adopt a therapeutic strategy that has several components to improve angiogenic responsiveness as well as increasing the availability of angiogenic growth factors.

Acknowledgements

We are grateful to Samuel Gray for assistance with the artwork.

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Accepted 30 March 2004
Available online 6 May 2004