Experimental therapeutic strategies in restenosis and critical limb ischemia

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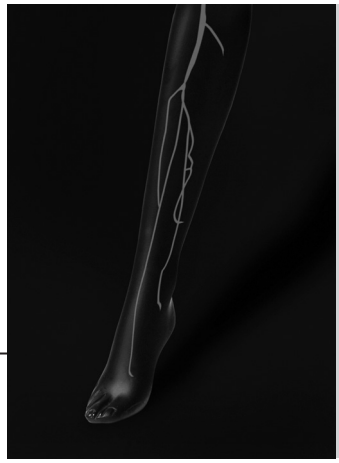

Chapter 05

Vascular growth in ischaemic limbs: a review of mechanisms and possible therapeutic stimulation

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Abstract

Stimulation of vascular growth to treat limb ischemia is promising, and early results obtained from uncontrolled clinical trials using angiogenic agents, for instance, vascular endothelial growth factor (VEGF), led to high expectations. However, negative results from recent placebo-controlled trials warrant further research. Here, current insights into mechanisms of vascular growth in the adult, in particular the role of angiogenic factors, the immune system, and bone marrow, were reviewed, together with modes of its therapeutic stimulation and results from recent clinical trials.

Three concepts of vascular growth have been described to date, being angiogenesis, vasculogenesis and arteriogenesis (collateral artery growth), which represent different aspects of an integrated process. Stimulation of arteriogenesis seems clinically most relevant, and has most recently been attempted using autologous bone marrow transplantation with some beneficial results, although the mechanism of action is not completely understood. Better understanding of the highly complex molecular and cellular mechanisms of vascular growth may yet lead to meaningful clinical applications.

Introduction

Peripheral arterial obstructive disease (PAOD), mainly caused by atherosclerosis, is a major problem, which is known to affect 10-15% of the aged adult population. PAOD may at first exist without symptoms, but with further progression it may lead to intermittent claudication. Advanced disease is characterised by pain at rest, ulceration or gangrene of ischemic tissues, summarised as Critical Limb Ischaemia.[1] Furthermore, in PAOD atherosclerosis is often not limited to the leg, leading to increased mortality due to cerebro-vascular events or myocardial infarction.[2] In case of progression of PAOD with vascular occlusions at multiple levels and particularly low quality run-off crural vessels with limited outflow, options for vascular interventions, such as percutaneous transluminal angioplasty, stenting or bypass surgery, become limited. Amputation of ischemic toes, foot or limb remain the only option in 50% of patients with critical limb ischemia within 1 year, because of insufficient response to the treatments.[3] Most of these amputees suffer from a poor collateral arterial network, as evidenced by angiography. The large unmet medical need of these "no-option" patients has propelled the development of biological revascularization. Clinical trials using angiogenic growth factors have been launched in the field of both PAOD and coronary artery disease. This review mainly focuses on the mechanisms of vascular adaptation to limb ischemia and its stimulation to treat PAOD.

Basic mechanisms of vascular growth

Three principles: angiogenesis, vasculogenesis and arteriogenesis

Neovascularisation plays a major role in both health and diseases. In physiology, it plays a role in embryogenesis and development, the female reproductive system and wound healing. On the other hand neovascularisation also attributes to a great variety of diseases. It has long been recognized that excessive vessel growth is a large contributing factor in the pathogenesis of cancer, atherosclerosis, diabetic retinopathy, psoriasis, and arthritis. Contrary, insufficient vessel growth is associated with ischemic disease of heart, limb or brain, neurodegeneration, pre-eclampsia, and osteoporosis.[4] Recently, major progress has been made in understanding the mechanisms underlying vascular formation both in the adult as in embryogenesis. To date, three concepts of neovascularisation have been described, being angiogenesis, vasculogenesis and arteriogenesis,[5] which represent different aspects of an integrated process (Figure 1).

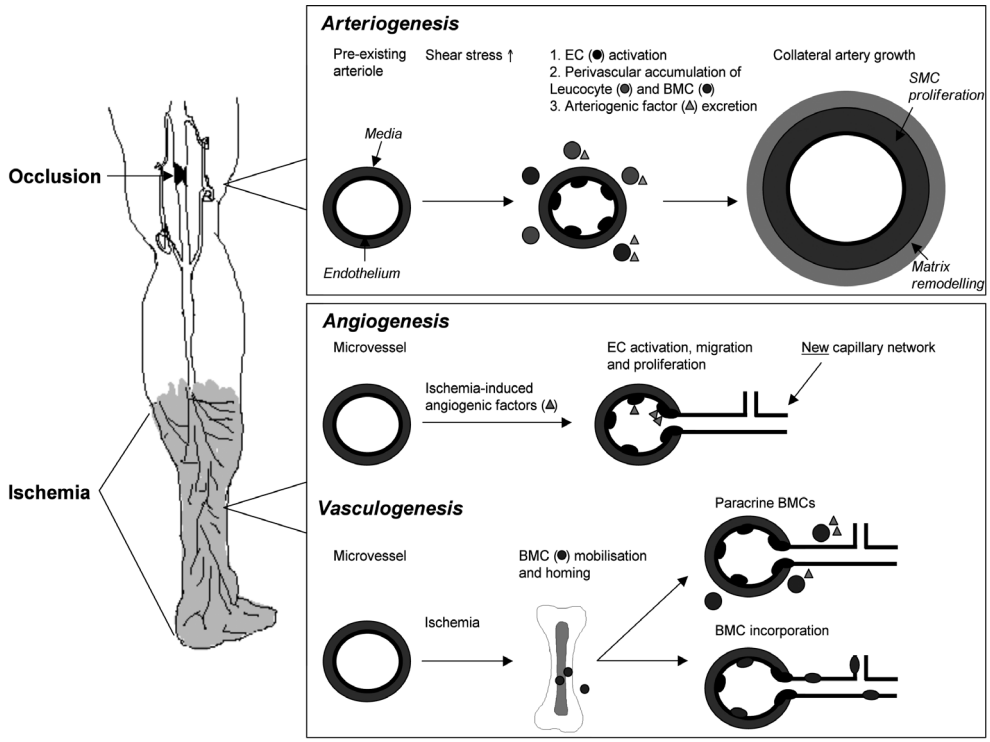
Angiogenesis involves the sprouting of new capillary-like structures from existing vasculature[4], and is regulated by pro- and anti-angiogenic factors.[6;7] Hypoxia is a strong stimulus, which induces pro-angiogenic factors, such as vascular endothelial growth factor A (VEGF) via activation of hypoxia-inducible factor-1α (HIF-1α). A series of sequential events can be distinguished during the formation of new microvessels, consisting of degradation of the vascular basement membrane and interstitial matrix by

endothelial cells, endothelial cell migration, endothelial proliferation, and the formation of new capillary tubes and a new basement membrane.[8] These newly formed tubes are subsequently stabilised by surrounding pericytes or smooth muscle cells (SMCs).

Vasculogenesis was originally defined by Risau[9] as the formation of a capillary plexus from blood islands, and is presently commonly used for the intussusception of bone marrow derived progenitors cells into the expanding vascular area.[4] These cells were primarily addressed as endothelial progenitor cells (EPCs)[10] and have been identified in peripheral blood[11;12] Moreover, they have been demonstrated to contribute to adult neovascularization.[13;14] To date, the mechanism how these bone marrow-derived cells (BMCs) exactly contribute to neovascularisation remains unclear. Substantial incorporation of EPCs in the vessel wall is rarely reported[15;16], and often there was only a minor contribution[17-20], leaving a paracrine function of cells with secretion of angiogenic factors more probable.[21;22] Furthermore, also non-endothelial bone marrow-derived progenitor cells have been described to contribute to ischemia-induced angiogenesis/vasculogenesis in a paracrine fashion.[23]

Adaptive arteriogenesis, or shortly arteriogenesis, was described by Wolfgang Schaper as the development of adult collateral arteries from a pre-existing arteriole network.[24] Via arteriogenesis a natural bypass is developed around an occluded main artery. This collateral artery growth mostly occurs proximal to ischemic tissues where angiogenesis and vasculogenesis occur (Figure 1). As compared to the two latter processes, arteriogenesis is more prominently stimulated by inflammation, and does not seem to be hypoxia-driven. In experiments in rabbits[25;26], angiographic collateral growth was not associated with production of metabolic intermediates indicative for ischemia or with expression of hypoxia-inducible genes, such as VEGF or HIF-1. Moreover, the time course of capillary growth and collateral growth was distinct: capillaries were formed 5 days after femoral artery removal and this was associated with increased lactate release in plasma and expression of VEGF in adductor muscle, whereas collateral growth occurred at 10 days, without the above mentioned signs of ischemia. Moreover, there is evidence that arteriogenesis is triggered by increased shear stress through specific pre-existing arterioles, by which the vessel wall is activated. This causes up-regulation of adhesion molecules for leucocytes, such as ICAM-1,[27] followed by attachment and transmigration of leucocytes. These leucocytes may secrete additional factors leading to growth of collateral arteries with media thickening and increase of SMC content of the vascular wall.[28] In addition, degradation of connective tissue surrounding collateral arteries by for example metalloproteinases facilitates their remodelling.[29;30]

Figure 1. *Schematic representation of arteriogenesis, angiogenesis and vasculogenesis. EC, endothelial cell; BMC, bone marrow cell; SMC, smooth muscle cell.*



The three above described concepts of vascular formation probably all play a role in adult neovascularisation, and usually occur simultaneously at different levels. However, it should be realized that distinction between angiogenesis, vasculogenesis and arteriogenesis is not unambiguous. They share common mechanisms, e.g. invasion of inflammatory cells, and expression of growth factors and cytokines. In the adult, vasculogenesis is merely a term for angiogenesis that involves progenitor cells intussuscepting in and around the new vascular structures. Moreover, arteriogenesis may not only be triggered by shear stress-induced arteriogenic factors, but also by circulating angiogenic factors that are produced in distant ischemic tissues. Contrary to the limb, arterial obstruction in the heart is situated near the ischemic regions in the vast majority of cases. Consequently, arteriogenesis and angiogenesis in the heart occur in close proximity of each other, possibly influencing each other via growth factor expression.

Angiogenic and arteriogenic growth factors: successful promotion of neovascularisation in animal models

Many vascular growth factors, but also inflammatory cytokines and chemokines, have been shown to promote angiogenesis, vasculogenesis and/or arteriogenesis, either in cell

cultures or in animal models. Angiogenesis and vasculogenesis are usually triggered by the induction of angiogenic factors, particularly by activation of hypoxia-inducible factor 1α (HIF-1α). HIF-1α is a transcription factor (master switch gene) that up-regulates a number of pro-angiogenic genes, such as VEGF, VEGF-receptor 2, stromal cell derived factor-1 (SDF-1) and its receptor CXCR4, angiopoietin-2 and erythropoietin (Epo), resulting in a coordinated angiogenic response. Numerous growth factors have been shown to play a role in angiogenesis, vasculogenesis and arteriogenesis *in vivo* (Table I). Moreover, most of these agents successfully promote vascular growth in animal models of hind limb ischemia. Nevertheless, results in placebo-controlled studies in patients were less beneficial to date. This may be explained by that current animal models suffer from considerable limitations: first, "healthy" animals are used that upon femoral artery occlusion demonstrate acute ischemia, whereas patients with arterial disease suffer from various metabolic disorders leading to chronic ischemia. Moreover, differences in expression patterns of endogenous VEGF in ischemic muscle were reported for muscle either derived from rabbits after femoral artery occlusion as compared to human amputation material.[65] Another limitation is that results from hind limb ischemia models are very much dependent upon the applied surgical technique. Many research groups have used a model of complete excision of the femoral artery and its side-branches leading to deep ischemia and mainly capillary formation. Other groups applied a short occlusion of the proximal femoral artery, which is more suitable to study collateral artery growth. The diversity of surgical techniques together with a large variety of applied end points measurements (clinical score, blood flow using laser-doppler imaging, microspheres, flow probes, or MRI, (post-mortem) angiography, CT, histology) merit carefully interpretation of results derived from these models.[66]Vascular growth factors may contribute in different ways to new vessel formation depending on which cell types their receptors are expressed. VEGF is the most extensively studied and crucial pro-angiogenic factor. Homozygous and even heterozygous VEGF-deficient murine embryos show a lethal phenotype by abnormal blood vessel formation.[67] Molecular targets for the VEGF gene family have been identified, being VEGF-receptor-1 and -2 (VEGFR1, VEGFR2) for VEGF-A; VEGFR1 for VEGF-B and PlGF; VEGFR2 and VEGFR3 for VEGF-C and VEGF-D[68;69]. The latter ones contribute to lymphangiogenesis via VEGFR3.[68;69] A variety of cells, such as endothelial cells, haematopoietic stem cells, and monocytes respond to VEGF-A either via VEGFR1 or VEGFR2. This indicates that VEGF-A (further indicated as VEGF) plays a role in angiogenesis, vasculogenesis, and arteriogenesis, respectively.

Table I. *Effect of some important growth factors on angiogenesis, vasculogenesis, and arteriogenesis in vivo.*

Growth factor	Angiogenesis/ Vasculogenesis	Model	Reference	Arteriogenesis	Model	Reference
VEGF-A	++	Rabbit, murine hind limb	31;32	+	Rabbit hind limb	33
VEGF-B	+/-	Matrigel implants, murine skin, rabbit hind limb	34;35	?		
VEGF-C	+ lymphangiogenesis +	Rabbit hind limb	36	+	Rabbit hind limb	36
VEGF-D	++ lymphangiogenesis ++	Rabbit hind limb	35	++	Rabbit hind limb	35
PlGF	+	Rabbit, murine hind limb	33;37	++	Rabbit, murine hind limb	33;37
SDF-1	++	Murine hind limb	38	+	Rat hind limb	39
FGF-2	++	Murine, rabbit hind limb	40;41	++	Murine, rabbit hind limb	40;41
Angiopoietin-1	++	Rabbit hind limb	42	++	Rabbit hind limb	42
Angiopoietin-2	-	Rabbit, murine hind limb	42;43	-	Rabbit, murine hind limb	42;43
HGF	++	Rat, rabbit hind limb	44	++	Rat, rabbit hind limb	44
IGF	++	Murine hind limb	45	?		
Tissue kallikrein	++	Murine hind limb	46	?		
Erythropoietin	++	Murine hind limb	47	+/?	Murine hind limb	48
HIF-1α (masterswitch gene)	++	Rabbit hind limb	49	+/0	Rabbit hind limb	25;49
EGR-1 (masterswitch gene)	++	Matrigel implants, tumor in mice, rat cornea	50	++	Murine hind limb	51
PR39 (masterswitch gene)	++	Murine myocardium	52	++	Pig myocardium, murine hind limb	53;54
GM-CSF	-	Murine melanoma	55	++	Rabbit hind limb	56
TNF-α	++	Rat cornea, chick chorioallantoic membrane	57	++	Murine hind limb	58
TGF-β	+/-	Developmental studies in mice	59	++	Rabbit hind limb	60
MCP-1	++	Chick chorioallantoic membrane	61	++	Rabbit hind limb	62
CD44	++	Murine matrigel, tumor, wound	63	++	Murine hind limb	64

++ (strongly stimulatory), + (mildly stimulatory), 0 (no effect), - (inhibitory), ? (unknown effect).
VEGF, vascular endothelial growth factor; PlGF, placental growth factor; SDF-1, stromal cell derived factor-1; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; IGF, insulin-like growth factor; HIF-1α, hypoxia-inducible factor 1α; EGR, early growth response protein; GM-CSF, granulocyte-macrophage colony-stimulating factor; TNF, tumor necrosis factor; TGF, transforming growth factor; MCP-1, monocyte chemoattractant protein.

Currently, MCP-1 and GM-CSF are a main focus in arteriogenesis research. MCP-1 activates the C-C chemokine receptor-2 (CCR-2) on monocytic cells, thereby exerting its effect on collateral formation.[70] GM-CSF receptor is expressed on a variety of cell types, e.g. haematopoietic cells, monocytes, endothelial cells and cardiomyocytes.

Role of cellular components: vascular cells, inflammatory cells, and stem cells

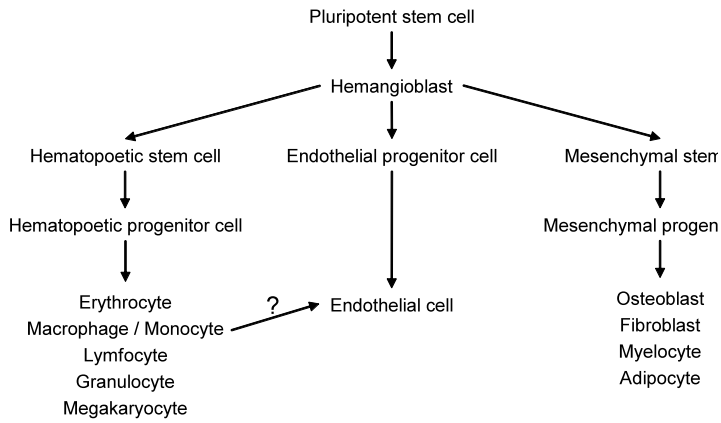
Endothelial cells are the vectors of *angiogenesis*. They are triggered by vascular growth factors, such as VEGF. Cultured (human) endothelial cells by themselves are capable of forming capillary-like tubes in three-dimensional matrices in the presence of VEGF.[71] Similarly, overexpression of VEGF in tissues causes initially rapid outgrowth of immature endothelial tubes.[23] However, these new micro-vessels lack a stabilising mural cell layer around their endothelium, which must become stabilised by pericytes. The formation of such immature and leaky neovascularisation *in vivo* may be an important limitation of therapeutic angiogenesis using a single endothelial cell-selective growth factor, such as proposed for gene therapy with VEGF (initially called vascular permeability factor).[40;72] This suggests the important contribution of additional growth factors, such as FGF-2, which has been shown to act on SMC proliferation. In addition, a variety of inflammatory cell types have been demonstrated to play a role in *angiogenesis* in e.g. cancer development. For example monocytes, T-cells, natural killer cells, neutrophils, mast cells and dentritic cells have been shown to produce angiogenic factors.[4]

It is problematic to determine whether and which (endothelial) progenitor cell types are involved in *vasculogenesis*. This is caused by a significant lack of appropriate cellular markers to identify these cells. Both endothelial progenitor cells, selected with CD34[73] or CD133[74] markers, and non-endothelial progenitor cells, selected with CXCR4 in combination with VEGFR1 markers[23], have been proposed to be involved in adult neovascularisation. Further research is needed to optimise specificity of cellular markers to define the role of progenitor cells in neovascularisation.

A variety of cell types have been shown to be involved in *arteriogenesis*, including endothelial cells, SMCs, fibroblasts, monocytes, lymphocytes, mast cells, platelets and bone-marrow-derived cells.[75] The actual growth of collaterals is dominated by proliferation of SMCs, adventitial fibroblasts and endothelial cells. Arteriogenesis is initiated by the activation of endothelial cells, followed by perivascular accumulation of various types of leucocytes and bone marrow-derived cells, which orchestrate collateral growth by producing cytokines, growth factors and proteases. Various studies have demonstrated a crucial role for monocytes in arteriogenesis.[76-78] Only recently, lymphocytes, such as CD4+ T-cells[79;80], CD8+ T-cells[81] and natural killer cells[80], have been shown to be involved as well. Recently, stem cells have become a main interest for stimulation of arteriogenesis. Stem cells can be obtained from different sources: among these are cells from bone marrow, peripheral blood or umbilical cord. Stem cells have clonogenic and self-renewing capabilities and may differentiate into multiple cell lineages, a phenomenon known as

plasticity. Apart from the cell-lineage for red blood cells, the bone marrow contains a collection of mononuclear cells (BMCs) (Figure 2). Hematopoetic stem cells represent a subpopulation of those BMCs. Given the amount of in vitro data on the plasticity of various bone marrow-derived cell populations, it is tempting to suggest that cell-based therapy enhances neovascularisation by direct incorporating into the vessel wall.[11;82] However, conflicting data on this transdifferentiation of BMCs / EPCs into new endothelial cells exist. Others challenged this theory with compelling evidence that BMCs do hardly, or not at all, incorporate and vascular growth is promoted by a paracrine effect of these cells. Bone marrow cell populations contain very small number of stem cells, <0.01% of total cells. Since many bone marrow subpopulations are a source of growth factors, cytokines and chemokines, a complementary hypothesis is that the cells act in a more supportive role.[20;83;84] Augmentation of arteriogenesis by administration of bone marrow-derived cells was successful in pre-clinical studies[82;85-87], and initial results from clinical trials are intriguing. Furthermore, implantation of peripheral blood mononuclear cells (PBMNCs) and platelets by injection into the ischemic thigh area in rats also induced collateral vessel formation by supplying angiogenic factors and cytokines.[88]

Figure 2. *Subpopulations of mononuclear cells in the bone marrow and their differentiation.*



Therapeutic stimulation of vascular growth

Concept

In aiming to restore sufficient blood flow towards the chronically ischemic limb, formation of mature collateral vessels is more essential than capillary growth. In this view, a few large conduits (collateral arteries) are hemodynamically much more advantageous

over many small high resistance capillaries, as flow through a vessel mainly depends on the radius according to the well-established Poiseuille relationship.[89] According to this mathematical model, the flow resistance, R, in mmHg/mL per minute, along each separate collateral parallel pathway, is estimated for laminar tube flow: $R=0.5 \cdot \mu \cdot L/d^4$, where μ is blood viscosity (0.03 g/cm per second), L is estimated length (mm), and d is diameter (mm). Therefore, therapeutic stimulation of vascular growth should primarily aim at large-diameter collateral vessels. Nevertheless, to improve oxygenation status of ischemic tissues, stimulation of both arteriogenic collaterals and angiogenic capillaries are crucial for sufficient blood inflow and gas exchange, respectively.

Increased fluid shear stress is thought to be responsible for initiating collateral artery growth, because a sudden decrease in peripheral blood pressure following an arterial occlusion increases the flow velocity through pre-existent collateral arterioles that interconnect the pre-occlusion high-pressure territories with the post-occlusion low-pressure regions. Shear stress is defined as the tangential force per unit area applied by the blood flow stream on endothelium. Many studies had previously implicated increased fluid shear stress as an arterial moulding force.[90-92] Shear stress levels are actively maintained in the arterial circulation as vascular tissues respond to shear stress changes with acute adjustments in vascular tone and with chronic structural remodelling, resulting in adjustments of vessel diameter. Only recently, evidence is accumulating that increased shear stress indeed plays a role in the induction of arteriogenesis.[93] Pipp and colleagues clearly showed that a primary change in shear stress is the dominant mechanical force in collateral artery growth in an AV shunt model in pigs and rabbits.

Modes of delivery: protein or gene therapy

Stimulation of neovascularisation can be achieved either by the use of growth factor proteins or by the introduction of genes encoding these proteins. The use of proteins is significantly restricted by their limited tissue half-life, which may require sustained-release preparation or repeated administration. Moreover, proteins in general require systemic administration with potentially more side effects as opposed to local delivery. Nevertheless, proteins are closer to clinical use than gene therapy.[94] Gene therapy is a very promising therapeutic tool in cardiovascular diseases that can overcome the inherent instability of angiogenic proteins by facilitating sustained, local production of these angiogenic factors. The use of viral vectors to carry angiogenic genes, for example adenovirus, adeno-associated virus or retrovirus, has the advantage of high transfection efficiency of target tissues. However, viruses disadvantageously trigger immunological responses or, in case of retrovirus, insertional mutagenesis is possible. Non-viral vectors (plasmids) are much safer and cheaper, can be produced easily in large quantities, and have higher genetic material carrying capacity. Plasmids are closer to clinical use than viral vectors due to less health issues. Yet, they are generally less efficient in delivering DNA and initiating gene expression, and duration of transgene expression is relatively

short as compared to viral vectors. Hence, plasmids can be delivered repeatedly[95], or their transfection efficiency may be improved. The latter is achieved by for example developing cationic liposome complexes[96] or intelligent polymers[97] as vectors that allow efficient cellular uptake and endosomal escape. Other emerging methods to enhance non-viral gene transfer are ultrasound-mediated microbubbles destruction[98] or electroporation. Electroporation is a physical method to deliver genes, drugs or other molecules to many different types of tissue (e.g. skeletal muscle, liver, lung and vasculature) by electrical pulses that result in cell electropermeabilization and DNA electrophoresis.[99;100] Recently, we showed that intra-muscular gene transfer by electroporation of plasmid DNA results in similar or even higher transfection efficacy and transgene expression duration as compared to adenoviral vectors.[101]

Although high transfection efficacy is the aim, one should be cautious that too high expressions of angiogenic factors may have deleterious effects, as shown for recombinant Sendai viral vector highly over-expressing VEGF, resulting in accelerated limb loss after administration in mice.[40] Moreover, the most optimal delivery strategy of angiogenic vectors or proteins is yet to be determined. There are multiple delivery modes, such as systemic (intra-venous, intra-arterial), intra-muscular, intra-vascular, peri-vascular, intra-pericardial or subcutaneous, which remain unproven in terms of clinical efficacy and superiority.[94] Finally, optimal dose schedules are largely unknown, and should be further explored.

Clinical trials using angiogenic growth factors

The therapeutic implications of angiogenic growth factors were identified by the pioneering work of Judah Folkman in the field of tumor biology and Jeffrey Isner in cardiovascular regeneration.[102] Subsequent beneficial effects of these growth factors in ischemia models in animals led to great expectations for the treatment of PAOD. Permission for subsequent clinical trials administering angiogenic factors, even by gene therapy, were relatively easy to obtain since patients with advanced ischemic disease did not have any other therapeutic options. Early results obtained from small phase I/II human trials using angiogenic growth factors, mainly using vascular endothelial growth factor A[103-109], but also using hepatocyte growth factor[110], were promising. Similar beneficial results were obtained from early-phase trials in patients with coronary arterial disease using VEGF-A [111-114], VEGF-C [115] or fibroblast growth factor (FGF)[116-119]. However, of the larger randomized placebo-controlled trials of therapeutic angiogenesis that have been published[120-124], all but one, using recombinant FGF-2 protein[124], were negative. In addition, small randomized trials that tested a more arteriogenic approach by using GM-CSF protein showed negative results in patients with intermittent claudication[125], whereas promising results for treatment of coronary artery disease[126]. Unfortunately, the mainly disappointing results of the larger clinical trials have now tempered the therapeutic angiogenesis hype. In contrast, we recently showed, for the first time in a double-blind randomized trial, that VEGF gene transfer may significantly

improve ulcer healing and haemodynamics as compared to placebo in diabetic patients with critical limb ischaemia.[127] Hopefully, the latter results may regenerate interest in treatment of peripheral arterial disease with angiogenic gene transfer approaches, especially using naked plasmid DNA as a vector. For an overview of clinical angiogenesis trials in patients with peripheral arterial disease from 1998 to present date please see Table II. Numerous reasons have been suggested to account for the negative results from clinical angiogenesis trials, such as the use of only a single factor, factor dose, duration of expression, mode of delivery, multiple splice-variants for agents, patient selection, pre-selected trial end-points, patient heterogeneity, angiogenesis inhibitors, and strong placebo effect.[130] Moreover, biological responses to growth factor therapy may be hampered in chronically ischemic muscle in which endogenous angiogenesis has become exhausted; we have recently observed in muscle samples of amputated limbs that there is an inability of hypoxic tissues to express sufficient hypoxia inducible factor-1α, and downstream VEGF and SDF-1, in chronic ischemia as opposed to acute-on-chronic ischemia.[131]

Clinical trials using cell-based therapy

A cell-based therapeutic approach has evolved when it was suggested that administration of bone marrow-derived stem or endothelial progenitor cells may improve blood flow recovery in various ischemic models. Despite the lack of understanding regarding the complex issues of cell origin and fate, quite some attention has been focused on demonstrating the clinical benefits of cell-based therapy. Tateishi-Yuyama and colleagues published their pioneering work in 2002 showing beneficial results with autologous transplantation of bone marrow cells in patients with limb ischemia.[132] Bone marrow and peripheral blood provide stem cells of autologous origin. Practical issues as immunologic rejection and possible teratoma formation, as well as ethical issues, have hampered the use of embryonic stem cells in a clinical setting. Most clinical trials made use of the mononuclear cell fraction from the bone marrow. Alternatively, PBMCs are administered after mobilization of these cells from the bone marrow with G-CSF application. Others administered more specifically EPCs.

Table II. *Clinical trials for stimulation of neovascularization in patients with peripheral arterial disease.*

Study	Phase	Patients	N	Factor	Delivery	Beneficial	Improved parameter(s)
Angiogenic factors							
Baumgartner, 1998[103]	I	CLI	9	$VEGF_{165}$ plasmid	Intra-muscular	Yes	ABI, angiography, flow, ulcer healing, limb salvage
Isner, 1998[104]	I	TAO	6	$VEGF_{165}$ plasmid	Intra-muscular	Yes	ABI, angiography, flow, ulcer healing, nocturnal rest pain
Isner, 1998[105]	I	CLI	28	$VEGF_{165}$ plasmid	Hydrogel-coated balloon	Yes	Angiography
Rajagopalan, 2001[106]	I	IC or RP	6	$VEGF_{121}$ adenovirus	Intra-muscular	Yes	Lower-extremity flow reserve, peak walking time
Makinen, 2002[107]	II	Stenosis suitable for PTA, no DMI	54	$VEGF_{165}$ plasmid +adenovirus	Intra-arterial	Yes	Angiography
Lederman, 2002 (TRAFFIC)[124]	II	CI	190	bFGF protein	Intra-arterial	Yes	Peak walking time
Shyu, 2003[108]	I	CLI	21	$VEGF_{165}$ plasmid	Intra-muscular	Yes	ABI, flow, ulcer healing, rest pain
Rajagopalan, 2003 (RAVE)[122]	II	CI, stratified on diabetic status	105	$VEGF_{121}$ adenovirus	Intra-muscular	No	None (primary end point was peak walking time)
Kipshidze, 2003[128]	I/II	CLI, referred for amputation	23	Fibrin+/- $VEGF_{165}$ plasmid	Intra-muscular	Yes	ABI, transcutaneous oxygen pressure, IC, rest pain, limb salvage
Morishita, 2004[110]	I	CLI, incl TAO	6	HGF plasmid	Intra-muscular	Yes	Pain scale, ABI, ulcer healing
Kim, 2004[109]	I	CLI, incl TAO	9	$VEGF_{165}$ plasmid	Intra-muscular	Yes	Ischemic pain, ulcer healing, ABI, angiography
Kusumanto, 2006[127]	II	CLI and DM	54	$VEGF_{165}$ plasmid	Intra-muscular	Yes	ABI, ulcer healing
Arteriogenic factors							
Van Royen (START)[125]	II	CI	40	GM-CSF protein	Subcutaneously	No	None (primary end point was change in walking time)
Matyas, 2005[129]	I/II	CLI	13	FGF-4 adenovirus	Intra-muscular	Unknown	No conclusions regarding efficacy due to small patient cohort

CLI, critical limb ischemia; TAO, thromboangiitis obliterans (Buerger's disease); IC, intermittent claudication; RP, rest pain; PTA, percutaneous transluminal angioplasty, DM, diabetes mellitus; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; G(M)-CSF, granulocyte-(macrophage) colony-stimulating factor; ABI, ankle-brachial index.

The safety profile has been reassuring thus far, yet long-term results have recently been questioned.[133] Unfortunately, these studies are also not easy to interpret. It is particularly difficult to state firm conclusions about treatment efficacy since most studies are lacking controls, have diverse treatment modalities, endpoints and inclusion/exclusion criteria. Furthermore, the emphasis has been on demonstrating recovery of clinical parameters, rather than the evaluation of new vessel formation.

Practically all studies use indirect parameters as ulcer healing, limb salvage, pain-free walking distance, ankle-brachial index, transcutaneous oxygen measurements and pain scores to assess the outcome. Although clinical effect is of pivotal importance, objective parameters for the evaluation of vascular growth seem essential, considering the suggested mechanism. Few studies include follow-up digital subtraction angiograms of which the assessment is based on qualitative visual comparison. This limitation of current clinical endpoints also holds for trials on angiogenic growth factors. An overview of published clinical studies in English language using cell-based therapy with more then 5 patients is given in Table III.

Regarding harvest procedure, a dichotomy exists between the origins of the cells. Initially, mononuclear cells were collected from the iliac crest; more recently also PBMCs are administered after G-CSF mobilization. Since a firm conclusion about efficacy of cell-based therapy in general cannot be drawn, one can only speculate about differences between BMCs and PBMCs. Collection from the iliac crest requires general or epidural anesthesia. Otherwise, some concern has raised that G-CSF therapy might be related to an unexpected high rate of in-stent restenosis at the culprit lesion after intracoronary infusion of mobilized PBMCs.[149]

In summery, cell-based therapy seems an encouraging strategy for patients with severe peripheral arterial disease who are not amenable for conventional treatment. Clinical studies performed to date however, have not primary been designed or powered to evaluate clinical outcomes. Furthermore, long-term safety issues have also to be evaluated.

Limitations of therapeutic angio-/arteriogenesis

Some adverse effects of therapeutic angiogenesis have been reported, such as aggravation of re-stenosis using peripheral blood stem cells in patients with myocardial infarction [149] or microinfarction using mesenchymal stromal cells in a dog model.[150] In line with this, a so-called Janus phenomenon has been proposed by Epstein and colleagues between arteriogenesis and atherosclerosis[151], meaning that pro-arteriogenic factors, such as MCP-1, may also contribute to plaque progression and neointima formation, as reported.[152;153] Moreover, there is evidence that development of atherosclerotic plaques is associated with proliferation of the vasa vasorum[154-156], which may thus be accelerated using angiogenic factors. Nevertheless, the effects of exogenous angiogenic factors, such as VEGF, on re-stenosis and atherosclerosis are still debated ranging from beneficial[157] to adverse[158]. Other limitations of therapeutic neovascularisation may consist of inappropriate blood

Table III. *Overview of cell-based clinical trials in patients with peripheral arterial disease.*

Study	Phase	Patients	N	Factor	Delivery	Beneficial	parameter(s)
With control group							
Tateishi-Yuyama, 2002[132]	I/II	CLI	45	BMC	IM	Yes	ABI, TcO2, pain-free walking time, angiography (in 27 of 45 patients)
Huang, 2005[134]	I/II	CLI	14+14	G-CSF mobilized PBMC	IM	Yes	ulcer healing, limb salvage, ABI, laser Doppler flow, angiography
Barc, 2006[135]	I/II	CLI	14+15	BMC	IM	No	No improvement in ABI, TcO2, angiography. Marginal improved ulcer healing and limb salvage
Bartsch, 2007[136]	I/II	IC	13+12	BMC	Combined IM + IA	Yes	ABI, pain-free walking distance, capillary-venous oxygen saturation, venous plethysmography
Without control group							
Esato, 2002[137]	I/II	CLI and IC	8	BMC	IM	Varying	Rest pain, ulcer healing, skin temperature, ABI, angiography
Higashi, 2003[138]	I/II	CLI	7	BMC	IM	Yes	ABI, TcO2, pain-free walking time
Miyamoto, 2004[133]	I/II	CLI	12	BMC	IM	Yes	ABI, pain-free walking time, VAS, ^{99m}Tc-TF perfusion scintigraphy
Saigawa, 2004[139]	I/II	CLI and IC	8	BMC	IM	Varying	ABI, TcO2
Lenk, 2005[140]	I/II	CLI	7	G-CSF mobilized PBMC	IA	Yes	ABI, TcO2, pain-free walking distance, pain score
Yang, 2006[141]	I/II	CLI and IC	152	G-CSF mobilized PBMC	IM	Varying	ulcer healing, limb salvage, ABI, TcO2
Tateno, 2006[142]	I/II	CLI and IC	29	G-CSF mobilized PBMC	IM	Varying	ulcer healing, limb salvage, pain score, ABI, walking distance
Bartsch, 2006[143]	I/II	CLI and IC	8	BMC	Combined IM + IA	Yes	ABI, pain-free walking distance, capillary-venous oxygen saturation
Durdu, 2006[144]	I/II	CLI	26	BMC	IM	Yes	ulcer healing, ABI, VAS, peak walking time, quality of life, angiography
Miyamoto, 2006[145]	I/II	CLI	8	BMC	IM	Varying	ulcer healing, ABI, VAS, angiography
Kawamura, 2006[146]	II	CLI and IC	92	G-CSF mobilized PBMC	IM	Varying	Limb salvage, thermography, plethysmography, CT-angiography
Kajiguchi, 2007[147]	I/II	CLI	7	BMC (6) PBMNC (1)	IM	Varying	ABI, TcO2, VAS
Saito, 2007[148]	I/II	CLI	14	BMC	IM	Yes	Ulcer healing, pain score

CLI, critical limb ischemia; IC, intermittent claudication; G-CSF, granulocyte-colony-stimulating factor; BMN, bone marrow mononuclear cells; PBMC, peripheral blood mononuclear cells; IM, intra-muscular; IA, intra-arterial; ABI, ankle-brachial index; VAS, visual analogue scale.

vessel growth at unwanted sites[159], which may theoretically lead to increased incidence of diabetic retinopathy or cancer. In line with this, inhibition of angiogenesis has developed into an important adjuvant treatment of neoplasms.[160;161] Nevertheless, to our knowledge, no case of *de novo* cancer or progression of cancer after angiogenic therapy has been described in patients to date. Furthermore, in our experience, there was no evidence of increased occurrence of malignancies after plasmid VEGF treatment.[127] Local delivery and specificity to target tissue of angiogenic proteins or genes may overcome these concerns.

Discussion

As can be concluded from the above, adult neovascularisation is a very complex phenomenon involving a large variety of cellular components, in turn excreting a large variety of vascular growth factors, cytokines and chemokines. All cellular components are tightly orchestrated concerning chronology of involvement, location and expression patterns. Many steps in this process remain to be elucidated. It is no coincidence that, parallel to ongoing basic research, autologous bone marrow cell transplantation has come into play, since bone marrow seems to potentially consist of almost all cell types involved. Nevertheless, results from clinical bone marrow trials are inconsistent. Refining our knowledge on which and how subsets of BMCs are involved in neovascularisation, and isolating these cells before administration, will probably improve efficacy of this treatment in the future. For instance, a significant subpopulation of bone marrow consists of inflammatory cells. Moreover, others and we recently found that lymphocytes, in particular CD4+ T-helper cells[79;80], CD8+ T-cells[81] and natural killer cells[80], play a modulating role in arteriogenesis. Administration of defined lymphocyte subsets or their specific activation/inhibition with ligands for activating or inhibitory receptors, respectively, may thus prove beneficial for stimulation of arteriogenesis in the future. Furthermore, the administration of factors for mobilisation of circulating BMCs, such as VEGF or GM-CSF, or factors to retain BMCs in ischemic tissues, such as SDF-1[162], holds promise as well.

As mentioned, most placebo-controlled trials using angiogenic factors were negative. One obvious explanation may be that the administration of a single factor is not sufficient to set the complex process of neovascularisation in motion. Therefore, future trials should be designed to use a combination of growth factors, preferably combining angiogenic and arteriogenic factors, or including "master-switch genes", such as HIF-1α, that trigger a coordinated expression of many other angiogenic factors. Another explanation for unsuccessful trials may be that patient selection occurs guided by ethical concerns, especially for gene therapy trials. In many trials, only patients that were no candidate for standard vascular intervention, e.g. due to advanced disease, were selected. Hopefully, with the development of safer vectors, patients may be included in earlier stages of the disease with more beneficial results. In our opinion, electroporation has most potential in delivering genes packed in these safer vectors due to high and prolonged gene

expression. Furthermore, patients with end-stage ischemic disease may be less susceptible to angiogenic therapies due to a diseased vessel wall with endothelial dysfunction and concomitant defective receptors, for instance, VEGF receptors, ICAM-1 or V-CAM. Other problems may lay in dysfunction of cells involved in neovascularisation, such as reduced migration of monocytes towards VEGF in diabetics[163], reduced endothelial cell proliferation and motility by disturbed lipid metabolism[164;165], and reduced neovascularisation capacity of bone marrow mononuclear cells[166], or lymphocytes. Future research should focus on better understanding these problems in order to improve susceptibility to either endogenous or exogenous growth factors.

Other issues that merit future investigation are, first, that arteriogenic factors may additionally accelerate atherosclerosis (the Janus phenomenon). Optimal arteriogenic factors, that are not atherogenic, may be identified by differential expression studies comparing models of arteriogenesis and atherosclerosis. Second, genetic profiles of patients determining whether neovascularisation in ischemic tissue is efficient or defective should be unravelled to identify new therapeutic targets and open possibility for disease prevention. Animal models may help in this by for instance comparing strains with different vessel-forming capacity.[80;167] In this respect, differences in pre-existing collateral networks may be genetically determined, which may explain why one patient forms an adequate collateral network or responds well to arteriogenic treatment, and the other patient does not. Interestingly, leucocytes were recently proposed to play a role in retinal vascular remodelling or pruning during development.[168] A role for the immune system in embryonic development of a collateral network is yet to be determined. Third, study of differential expression of angiogenic genes between acutely and chronically ischemic tissues may bring forward novel candidate-growth factors.

Finally, designer blood vessels fabricated by tissue engineering may ultimately prove to be the solution for patients with ischemic disease, however an artificial non-thrombogenic, immunocompatible, strong, yet biologically responsive blood vessel seems not in sight in the near future.[169]

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