Growing blood vessels to treat limb ischemia: studie in mice and man
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Review and Interpretation of
Pre-Clinical and Clinical Research
Chapter 1

Vascular Growth in Ischemic Limbs: A Review of Mechanisms and Possible Therapeutic Stimulation

V. van Weel, R.B. van Tongeren, V.W.M. van Hinsbergh, J.H. van Bockel, P.H.A. Quax
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 characterized by pain at rest, ulceration or gangrene of ischemic tissues, summarized as Critical Limb Ischemia.\(^1\) 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

Neovascularization 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. Furthermore, it contributes to the pathogenesis of many disorders, either by excessive vessel growth, for example in cancer, atherosclerosis, diabetic retinopathy, psoriasis, and arthritis, or by insufficient vessel growth, for example in 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 neovascularization 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-1\(\alpha\) (HIF-1\(\alpha\)). 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 stabilized 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 have tentatively been indicated as endothelial progenitor cells (EPCs).\(^{10}\) EPCs have been identified in peripheral blood\(^{11,12}\), and have been demonstrated to contribute to adult neovascularization.\(^{13,14}\) To date, the mechanism how these bone marrow-derived cells (BMCs) exactly contribute to neovascularization remains unclear. Substantial incorporation of EPCs in the vessel wall is rarely reported\(^{15,16}\), and often

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**Figure 1** Schematic representation of arteriogenesis, angiogenesis and vasculogenesis. EC, endothelial cell; BMC, bone marrow cell; SMC, smooth muscle cell.
there was only a minor contribution\textsuperscript{17-20}, leaving a paracrine function of cells with secretion of angiogenic factors more probable.\textsuperscript{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.\textsuperscript{23}

\textit{Adaptive arteriogenesis}, or shortly arteriogenesis, was defined by Wolfgang Schaper as the development of adult collateral arteries from a pre-existing arteriole network.\textsuperscript{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 induced by inflammatory factors, for instance, monocyte chemoattractant protein-1 (MCP-1), than by hypoxia inducible (growth) factors, such as HIF-1$\alpha$ and VEGF, and shows no temporal relation with ischemia.\textsuperscript{25,26} 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,\textsuperscript{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.\textsuperscript{28} In addition, degradation of connective tissue surrounding collateral arteries by for example metalloproteinases facilitates their remodeling.\textsuperscript{29,30}

The three above described concepts of vascular formation probably all play a role in adult neovascularization, and usually occur simultaneously at different levels. However, it should be realized that differences between angiogenesis, vasculogenesis and arteriogenesis are not as outspoken. 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. Unlike in the limb, in the heart the distances between arterial obstruction and ischemia are small to none, by which both arteriogenesis and angiogenesis, and their growth factors, are intertwined.

\textbf{Angiogenic and arteriogenic growth factors}

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 1$\alpha$ (HIF-1$\alpha$). HIF-1$\alpha$ 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 1). Moreover, most of these agents successfully promote vascular growth in models of hind limb ischemia.

<table>
<thead>
<tr>
<th>Growth factor</th>
<th>Angiogenesis/ Vasculogenesis</th>
<th>Model</th>
<th>Ref</th>
<th>Arteriogenesis</th>
<th>Model</th>
<th>Ref</th>
</tr>
</thead>
<tbody>
<tr>
<td>VEGF-A</td>
<td>++</td>
<td>Rabbit, murine hind limb</td>
<td>114, 115</td>
<td>+</td>
<td>Rabbit hind limb</td>
<td>116</td>
</tr>
<tr>
<td>VEGF-B</td>
<td>+/-</td>
<td>Matrigel implants, murine skin, rabbit hind limb</td>
<td>117, 118</td>
<td>?</td>
<td>Rabbit hind limb</td>
<td>119</td>
</tr>
<tr>
<td>VEGF-C</td>
<td>+</td>
<td>Rabbit hind limb</td>
<td>119</td>
<td>+</td>
<td>Rabbit hind limb</td>
<td>118</td>
</tr>
<tr>
<td>VEGF-D</td>
<td>+</td>
<td>Rabbit hind limb</td>
<td>118</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>118</td>
</tr>
<tr>
<td>PI GF</td>
<td>+</td>
<td>Rabbit, murine hind limb</td>
<td>116, 120</td>
<td>++</td>
<td>Rabbit, murine hind limb</td>
<td>116, 120</td>
</tr>
<tr>
<td>SDF-1</td>
<td>++</td>
<td>Murine hind limb</td>
<td>121</td>
<td>+</td>
<td>Rat hind limb</td>
<td>122</td>
</tr>
<tr>
<td>FGF-2</td>
<td>++</td>
<td>Murine, rabbit hind limb</td>
<td>36, 123</td>
<td>++</td>
<td>Murine, rabbit hind limb</td>
<td>36, 123</td>
</tr>
<tr>
<td>Angiopoietin-1</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>124</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>124</td>
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<tr>
<td>Angiopoietin-2</td>
<td>-</td>
<td>Rabbit, murine hind limb</td>
<td>124, 125</td>
<td>-</td>
<td>Rabbit, murine hind limb</td>
<td>124, 125</td>
</tr>
<tr>
<td>HGF</td>
<td>++</td>
<td>Rat, rabbit hind limb</td>
<td>126</td>
<td>++</td>
<td>Rat, rabbit hind limb</td>
<td>126</td>
</tr>
<tr>
<td>IGF</td>
<td>++</td>
<td>Murine hind limb</td>
<td>127</td>
<td>?</td>
<td>Rabbit hind limb</td>
<td>127</td>
</tr>
<tr>
<td>Tissue kallikrein</td>
<td>++</td>
<td>Murine hind limb</td>
<td>128</td>
<td>?</td>
<td>Rabbit hind limb</td>
<td>128</td>
</tr>
<tr>
<td>Erythropoietin</td>
<td>++</td>
<td>Murine hind limb</td>
<td>129</td>
<td>+/?</td>
<td>Murine hind limb</td>
<td>130</td>
</tr>
<tr>
<td>HIF-1α (masterswitch gene)</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>131</td>
<td>+/0</td>
<td>Rabbit hind limb</td>
<td>131, 25</td>
</tr>
<tr>
<td>EGR-1 (masterswitch gene)</td>
<td>++</td>
<td>Matrigel implants, tumor in mice, rat cornea</td>
<td>132</td>
<td>++</td>
<td>Murine hind limb</td>
<td>133</td>
</tr>
<tr>
<td>PR39 (masterswitch gene)</td>
<td>++</td>
<td>Murine myocardium</td>
<td>134</td>
<td>++</td>
<td>Pig myocardium, murine hind limb</td>
<td>135, 136</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>-</td>
<td>Murine melanoma</td>
<td>137</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>138</td>
</tr>
<tr>
<td>TNF-α</td>
<td>++</td>
<td>Rat cornea, chick chorioallantoic membrane</td>
<td>139</td>
<td>+</td>
<td>Murine hind limb</td>
<td>140</td>
</tr>
<tr>
<td>TGF-β</td>
<td>+/-</td>
<td>Developmental studies in mice</td>
<td>141</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>142</td>
</tr>
<tr>
<td>MCP-1</td>
<td>++</td>
<td>Chick chorioallantoic membrane</td>
<td>143</td>
<td>++</td>
<td>Rabbit hind limb</td>
<td>144</td>
</tr>
<tr>
<td>CD44</td>
<td>++</td>
<td>Murine matrigel, tumor, wound</td>
<td>145</td>
<td>++</td>
<td>Murine hind limb</td>
<td>146</td>
</tr>
</tbody>
</table>

++ (strongly stimulatory), + (mildly stimulatory), 0 (no effect), - (inhibitory), ? (unknown effect).

Table 1 Effect of some important growth factors on angiogenesis, vasculogenesis, and arteriogenesis in vivo

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. 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 PIGF; VEGFR2 and VEGFR3 for VEGF-C and VEGF-D. The latter ones contribute to lymphangiogenesis via VEGFR3. A variety of cells, such as endothelial cells, hematopoietic 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. With respect to arteriogenesis MCP-1 and GM-CSF received much attention. MCP-1 activates the C-C chemokine receptor-2 (CCR-2) on monocytic cells, thereby exerting its effect on collateral formation. GM-CSF receptor is expressed on a variety of cell types, e.g. hematopoietic 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. Similarly, over-expression of VEGF in tissues causes initially rapid outgrowth of immature endothelial tubes. However, these new micro-vessels lack a stabilizing mural cell layer around their endothelium, which must become stabilized by pericytes. The formation of such immature and leaky neovascularization 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). 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, such as monocytes, T-cells, natural killer cells, neutrophils, mast cells and dendritic cells have been demonstrated to play a role in angiogenesis in for instance cancer development, by production of angiogenic factors, cytokines and proteinases.

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 or CD133 markers, and non-endothelial progenitor cells, selected with CXCR4 in combination with VEGFR1 markers, have been proposed to be involved in adult neovascularization. Further research is needed to optimize specificity of cellular markers to define the role of progenitor cells in neovascularization.

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. The actual growth of collaterals is dominated by proliferation of SMCs, adventitial fibroblasts and endothelial cells. Arteriogenesis is
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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. Only recently, lymphocytes, such as CD4+ T-cells, CD8+ T-cells and natural killer cells (see Chapter 3), have been shown to be involved as well. Moreover, stem cells have come into play 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).

![Figure 2 Subpopulations of mononuclear cells in the bone marrow and their differentiation](image)

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 neovascularization by direct incorporating into the vessel wall. 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. Augmentation of arteriogenesis by
administration of bone marrow-derived cells was successful in pre-clinical studies\textsuperscript{47;50-52}, 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.\textsuperscript{53}

**Therapeutic stimulation of vascular growth**

**Concept**

Arteriogenesis or collateral artery growth is proposed to be more important for restoration of blood flow than capillary growth. As flow through a vessel mainly depends on the radius according to the well-established Poiseuille relationship\textsuperscript{54}, a few large vessels (collateral arteries) are hemodynamically much more advantageous over many small ones (capillaries). 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 $\mu$ 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.

**Modes of delivery: protein or gene therapy**

Stimulation of neovascularization 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.\textsuperscript{55} 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\textsuperscript{56}, or their transfection efficiency may be improved. The latter is achieved by for example developing cationic liposome complexes\textsuperscript{57} or intelligent polymers\textsuperscript{58} as vectors that allow efficient cellular uptake and endosomal escape. Other emerging methods to enhance non-viral gene transfer are ultrasound-mediated microbubbles destruction\textsuperscript{59} 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.\textsuperscript{60,61} 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.\textsuperscript{62}

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.\textsuperscript{36} 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.\textsuperscript{55} 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.\textsuperscript{63} 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\textsuperscript{64-70}, but also using hepatocyte growth factor\textsuperscript{71}, were promising. Similar beneficial results were obtained from early-phase trials in patients with coronary arterial disease using VEGF-A \textsuperscript{72-75}, VEGF-C \textsuperscript{76} or fibroblast growth factor (FGF) \textsuperscript{77-80}. However, of the larger randomized placebo-controlled trials of therapeutic angiogenesis that have been published\textsuperscript{81-85}, all but one, using recombinant FGF-2 protein\textsuperscript{85}, 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\textsuperscript{86}, whereas promising results for treatment of coronary artery disease\textsuperscript{87}. 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
hemodynamics as compared to placebo in diabetic patients with critical limb ischemia. 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 2.

<table>
<thead>
<tr>
<th>Study</th>
<th>Phase</th>
<th>Patients</th>
<th>N</th>
<th>Factor</th>
<th>Delivery</th>
<th>Beneficial</th>
<th>Improved parameter(s)</th>
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<tr>
<td><strong>Angiogenic factors</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Baumgartner, 1998</td>
<td>I</td>
<td>CLI</td>
<td>9</td>
<td>VEGF165 plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>ABI, angiography, flow, ulcer healing, limb salvage</td>
</tr>
<tr>
<td>Isner, 1998</td>
<td>I</td>
<td>TAO</td>
<td>6</td>
<td>VEGF165 plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>ABI, angiography, flow, ulcer healing, nocturnal rest pain</td>
</tr>
<tr>
<td>Isner, 1998</td>
<td>I</td>
<td>CLI</td>
<td>28</td>
<td>VEGF165 plasmid</td>
<td>Hydrogel-coated balloon</td>
<td>Yes</td>
<td>Angiography</td>
</tr>
<tr>
<td>Rajagopalan, 2001</td>
<td>I</td>
<td>IC or RP</td>
<td>6</td>
<td>VEGF121 adenovirus</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>Lower-extremity flow reserve, peak walking time</td>
</tr>
<tr>
<td>Makinen, 2002</td>
<td>II</td>
<td>Stenosis suitable for PTA, no DMI</td>
<td>54</td>
<td>VEGF165 plasmid</td>
<td>Intra-arterial</td>
<td>Yes</td>
<td>Angiography</td>
</tr>
<tr>
<td>Lederman, 2002</td>
<td>II</td>
<td>CI</td>
<td>190</td>
<td>bFGF protein</td>
<td>Intra-arterial</td>
<td>Yes</td>
<td>Peak walking time</td>
</tr>
<tr>
<td>Shyu, 2003</td>
<td>I</td>
<td>CLI</td>
<td>21</td>
<td>VEGF165 plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>ABI, flow, ulcer healing, rest pain</td>
</tr>
<tr>
<td>Rajagopalan, 2003</td>
<td>II</td>
<td>CI, stratified on diabetic status</td>
<td>105</td>
<td>VEGF121 adenovirus</td>
<td>Intra-muscular</td>
<td>No</td>
<td>None (primary end point was peak walking time)</td>
</tr>
<tr>
<td>Kipshidze, 2003</td>
<td>I/II</td>
<td>CLI, referred for amputation</td>
<td>23</td>
<td>Fibrin+/- VEGF165 plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>ABI, transcutaneous oxygen pressure, IC, rest pain, limb salvage</td>
</tr>
<tr>
<td>Morishita, 2004</td>
<td>I</td>
<td>CLI, incl TAO</td>
<td>6</td>
<td>HGF plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>Pain scale, ABI, ulcer healing</td>
</tr>
<tr>
<td>Kim, 2004</td>
<td>I</td>
<td>CLI, incl TAO</td>
<td>9</td>
<td>VEGF165 plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>Ischemic pain, ulcer healing, ABI, angiography</td>
</tr>
<tr>
<td>Kusumanto, 2006</td>
<td>II</td>
<td>CLI and DM</td>
<td>54</td>
<td>VEGF165 plasmid</td>
<td>Intra-muscular</td>
<td>Yes</td>
<td>ABI, ulcer healing</td>
</tr>
</tbody>
</table>

| **Arteriogenic factors** | | | | | | | |
| Van Royen (START)    | II    | CI       | 40  | GM-CSF protein  | Subcutaneously | No         | None (primary end point was change in walking time) |
| Matyas, 2005         | I/II  | CLI      | 13  | FGF-4 adenovirus | Intra-muscular | Unknown    | No conclusions regarding efficacy due to small patient cohort |

Table 2 Clinical trials for stimulation of neovascularization in patients with peripheral arterial disease

Numerous clinical trials 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. 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 down-stream VEGF and SDF-1, in chronic ischemia as opposed to acute-on-chronic ischemia.

**Clinical trials using cell-based therapy**

A cell-based therapeutical approach has evolved when it was suggested that administration of bone marrow-derived stem or endothelial progenitor cells might 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. 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.

The safety profile has been reassuring thus far, yet long-term results have recently been questioned. 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. End points included pain free walking distance and ankle brachial indices, which are at best indirect parameters for assessing peripheral blood flow.

An overview of the published clinical articles in English language with more then 5 patients is given in Table 3.

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 intra-coronary infusion of mobilized PBMCs.

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 primarily been designed or powered to evaluate clinical outcomes. Furthermore, long-term safety issues have also to be evaluated.

<table>
<thead>
<tr>
<th>Study</th>
<th>Phase</th>
<th>Patients</th>
<th>N</th>
<th>Factor</th>
<th>Delivery</th>
<th>Beneficial</th>
<th>Parameter(s)</th>
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<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Tateishi-Yuyama, 2002</td>
<td>III</td>
<td>CLI</td>
<td>45</td>
<td>BMC</td>
<td>IM</td>
<td>Yes</td>
<td>ABI, TcO2, pain-free walking time, angiography (in 27 of 45 patients)</td>
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<td>Huang, 2005</td>
<td>III</td>
<td>CLI</td>
<td>14+14</td>
<td>G-CSF mobilized PBMC</td>
<td>IM</td>
<td>Yes</td>
<td>Ulcer healing, limb salvage, ABI, laser Doppler flow, angiography</td>
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<td>Barc, 2006</td>
<td>III</td>
<td>CLI</td>
<td>14+15</td>
<td>BMC</td>
<td>IM</td>
<td>No</td>
<td>No improvement in ABI, TcO2, angiography. Marginal improved ulcer healing and limb salvage ABI, pain-free walking distance, capillary-venous oxygen saturation, venous plethysmography</td>
</tr>
<tr>
<td>Bartsch, 2006</td>
<td>III</td>
<td>IC</td>
<td>13+12</td>
<td>BMC</td>
<td>IM + IA</td>
<td>Yes</td>
<td>ABI, TcO2</td>
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<td>Esato, 2002</td>
<td>III</td>
<td>CLI and IC</td>
<td>8</td>
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<td>IM</td>
<td>Varying</td>
<td>Rest pain, ulcer healing, skin temperature, ABI, angiography</td>
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<td>Higashi, 2003</td>
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<td>7</td>
<td>BMC</td>
<td>IM</td>
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<td>BMC</td>
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<td>BMC</td>
<td>IM</td>
<td>Varying</td>
<td>ABI, TcO2</td>
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<tr>
<td>Lenk, 2005</td>
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<td>CLI</td>
<td>7</td>
<td>G-CSF mobilized PBMC</td>
<td>IA</td>
<td>Yes</td>
<td>ABI, TcO2, pain-free walking distance, pain score</td>
</tr>
<tr>
<td>Yang, 2006</td>
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<td>G-CSF mobilized PBMC</td>
<td>IM</td>
<td>Varying</td>
<td>Ulcer healing, limb salvage, ABI, TcO2</td>
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<tr>
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<td>CLI and IC</td>
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<td>IM</td>
<td>Varying</td>
<td>Ulcer healing, limb salvage, pain score, ABI, walking distance</td>
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<tr>
<td>Bartsch, 2006</td>
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<td>8</td>
<td>BMC</td>
<td>IM + IA</td>
<td>Yes</td>
<td>ABI, pain-free walking distance, capillary-venous oxygen saturation</td>
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<td>CLI</td>
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<td>Yes</td>
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<tr>
<td>Miyamoto, 2006</td>
<td>III</td>
<td>CLI</td>
<td>8</td>
<td>BMC</td>
<td>IM</td>
<td>Varying</td>
<td>Ulcer healing, ABI, VAS, angiography</td>
</tr>
<tr>
<td>Kawamura, 2006</td>
<td>III</td>
<td>CLI and IC</td>
<td>92</td>
<td>G-CSF mobilized PBMC</td>
<td>IM</td>
<td>Varying</td>
<td>Limb salvage, thermography, plethysmography, CT-angiography</td>
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<tr>
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<td>III</td>
<td>CLI</td>
<td>7</td>
<td>BMC (6) PBMC (1)</td>
<td>IM</td>
<td>Varying</td>
<td>ABI, TcO2, VAS</td>
</tr>
<tr>
<td>Saito, 2007</td>
<td>III</td>
<td>CLI</td>
<td>14</td>
<td>BMC</td>
<td>IM</td>
<td>Yes</td>
<td>Ulcer healing, pain score</td>
</tr>
</tbody>
</table>

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 analog scale.

Table 3 Overview of cell-based clinical trials in patients with peripheral arterial disease
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 \(^{93}\) or micro-infarction using mesenchymal stromal cells in a dog model.\(^{94}\) In line with this, a so-called Janus phenomenon has been proposed by Epstein and colleagues between arteriogenesis and atherosclerosis\(^{95}\), meaning that pro-arteriogenic factors, such as MCP-1, may also contribute to plaque progression and neointima formation, as reported.\(^{96,97}\) Moreover, there is evidence that development of atherosclerotic plaques is associated with proliferation of the vasa vasorum\(^{98-100}\), 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\(^{101}\) to adverse\(^{102}\). Other limitations of therapeutic neovascularization may consist of inappropriate blood vessel growth at unwanted sites\(^{103}\), 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.\(^{104;105}\) 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.\(^{88}\) 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 neovascularization 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 neovascularization, 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\(^{44,45}\), CD8+ T-cells\(^{46}\) and natural killer cells\(^{45}\), 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 mobilization of circulating BMCs, such as VEGF or GM-CSF, or factors to retain BMCs in ischemic tissues, such as SDF-1, 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 neovascularization 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 neovascularization, such as reduced migration of monocytes towards VEGF in diabetics, reduced endothelial cell proliferation and motility by disturbed lipid metabolism, and reduced neovascularization capacity of bone marrow mononuclear cells, 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 neovascularization in ischemic tissue is efficient or defective should be unraveled 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. 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 remodeling or pruning during development. 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 manufactured 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.\textsuperscript{113}
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Therapeutic neovascularization, a review


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Therapeutic neovascularization, a review


