COLLATERAL DEVELOPMENT IN LIMB ISCHEMIA

Aspects of endogenous and stimulated arteriogenesis

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To Dan, Isa and Elias
Peripheral arterial disease (PAD) is common among elderly and affects about 20% of the population above the age of 60 years. PAD is a strong marker for future cardiovascular events and increased mortality due to widespread atherosclerotic lesions in the coronary and cerebral arteries. Intermittent claudication (IC) with lower extremity pain on walking has a prevalence of 3-6% in men at the age of 60-70 years according to large population studies. Among these patients the majority remain stable without progression of symptoms. The symptoms progress in about 25% of the patients and distal ulceration or gangrene and ischemic rest pain, i.e critical limb ischemia (CLI) develops. Despite liberal indications for surgical or endovascular revascularizations, major amputation is required in as much as one third of the patients with CLI and for these patients there is a great need for alternative treatment strategies.

Collateral development, bridging stenoses or occlusions, is one of the reparative processes that reduce the consequences of coronary artery disease and PAD. The growth of these collaterals from pre-existing arteriole is called arteriogenesis. Angiogenesis, the sprouting of new capillaries, may also be of importance in the pathophysiology of PAD. There is little data establishing any impact of angiogenesis on PAD but it is likely that an angiogenic process occurs in ischemic tissues as a response to acute ischemia. Accordingly, hemodynamic forces, cytokines and inflammatory processes appear to be of importance in arteriogenesis, but the exact role of these processes is not firmly established. The arteriogenic mechanisms studied in animal models of limb ischemia and in vitro models aiming to stimulate arteriogenesis by supplying vascular growth factors have shown promising results.

The aim of this thesis was to assess the role of vascular growth factors in the arteriogenic process in PAD patients, and furthermore to evaluate gene therapy of FGF-4 in an animal model and humans as a method for therapeutic arteriogenesis.

First we explored the expression of growth factors in skin and muscle samples from 25 patients with CLI. There were increased levels of FGF-2 but not VEGF in distal muscle compared to proximal. Furthermore the expression of growth factors and inflammatory cytokines was investigated in 20 patients with IC after walking provocation. The pro-inflammatory cytokine IL-6 was significantly elevated one hour after exercise. VEGF mRNA also increased significantly in ischemic muscle samples after exercise. Taken together these results indicate that VEGF expression rises locally in response to maximal exercise to the absolute limit of pain in patients with IC but not in patients with CLI at rest. FGF-2 seems to be upregulated in distal muscle in CLI patients which could be due to local inflammatory processes. Gene therapy with adenovirus mediated FGF-4 was assessed in a rat model of severe limb ischemia and in a phase I/II multicenter clinical trial in CLI patients. No signs of stimulated arteriogenesis were detected in the animal model 4 weeks after gene transfer. Gene therapy in 13 patients seemed safe, but no sign of stimulated arteriogenesis was able to be detected in this small patient cohort.

Keywords: PAD, CLI, intermittent claudication, VEGF, FGF-2, angiogenesis, arteriogenesis, gene therapy, adenovirus, AdFGF-4, rat hindlimb ischemia model, randomized clinical trial
VEGF and FGF-2 expression in patients with critical limb ischemia  

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<tr>
<td>AAV</td>
<td>Adeno associated viral vector</td>
</tr>
<tr>
<td>ABI</td>
<td>Ankle–brachial index</td>
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<tr>
<td>ACD</td>
<td>Absolute claudication distance</td>
</tr>
<tr>
<td>Ad</td>
<td>Adenoviral vector</td>
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<tr>
<td>BOLD</td>
<td>Blood oxygen level dependent</td>
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<tr>
<td>CAD</td>
<td>Coronary artery disease</td>
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<tr>
<td>CLI</td>
<td>Critical limb ischemia</td>
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<tr>
<td>CRP</td>
<td>C-reactive protein</td>
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<tr>
<td>Ct</td>
<td>Threshold cycle</td>
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<tr>
<td>DSA</td>
<td>Digital subtraction angiography</td>
</tr>
<tr>
<td>EC</td>
<td>Endothelial cell</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunoassay</td>
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<tr>
<td>EPC</td>
<td>Endothelial progenitor cell</td>
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<tr>
<td>FGF</td>
<td>Fibroblast growth factor</td>
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<tr>
<td>GM-CSF</td>
<td>Granulocyte/macrophage colony stimulating factor</td>
</tr>
<tr>
<td>Gd-DTPA</td>
<td>Gadolinium diethyl triamine pentaacetic acid</td>
</tr>
<tr>
<td>hsCRP</td>
<td>High sensitivity CRP</td>
</tr>
<tr>
<td>HS</td>
<td>Heparan sulphate</td>
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<tr>
<td>IC</td>
<td>Intermittent claudication</td>
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<tr>
<td>ICAM-1</td>
<td>Intracellular adhesion molecule 1</td>
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<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<td>LDI</td>
<td>Laser Doppler imaging</td>
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<tr>
<td>MCP-1</td>
<td>Monocyte chemoattractant factor 1</td>
</tr>
<tr>
<td>MMP</td>
<td>Matrix metalloproteinase</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>NO</td>
<td>Nitrix oxide</td>
</tr>
<tr>
<td>PAD</td>
<td>Peripheral artery disease</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
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<tr>
<td>PDGF</td>
<td>Platelet derived growth factor</td>
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<tr>
<td>SMC</td>
<td>Smooth muscle cell</td>
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<tr>
<td>TC</td>
<td>Technetium</td>
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<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor alpha</td>
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<tr>
<td>TNF-β</td>
<td>Tumor necrosis factor beta</td>
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<tr>
<td>VCAM-1</td>
<td>Vascular cell adhesion molecule 1</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
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<tr>
<td>vWF</td>
<td>von Willebrand factor</td>
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INTRODUCTION

ATHEROSCLEROSIS

Atherosclerosis is a disease in the wall of large and medium sized arteries that accounts for the manifestation of arterial occlusive disease with symptoms of ischemia in the heart, brain or extremities. It is a complex disease involving endothelial cell activation, invasion of inflammatory cells, accumulation of lipoproteins in the vessel wall, proliferation and migration of smooth muscle cells and formation of a fibrous cap with a central part of necrotic and calcified cells (Ross, 1993). This lesion can cause symptoms by stenosis of the lumen or by plaque rupture with subsequent acute thrombosis of the artery. Both genetic and environmental factors can contribute to the atherosclerosis process. Known risk factors are diabetes, smoking, hypercholesterolemia, hypertension and obesity.

Atherosclerotic plaque development begins with endothelial cell (EC) activation with expression of adhesion molecules such as VCAM-1 on their surface and secretion of monocyte chemoattractant factors (MCP-1 and GM-CSF), attracting leukocytes to the EC surface. Once adherent to the endothelium, the monocytes transmigrate into the intima and turn into macrophages that take up entrapped modified LDL and transform into lipid-rich foam cells. T-lymphocytes also transmigrate into the intima and secrete inflammatory cytokines such as IFN-γ and TNF-β that can stimulate macrophages as well as ECs and SMCs. SMCs proliferate and migrate into the intima and produce extracellular matrix forming a fibrous cap that covers the inflammatory process. As the inflammatory process continues, the plaque grows and can give rise to symptoms depending on the impact of the stenosis that is formed. This stenosis can eventually lead to total lumen obstruction or sudden rupture of the plaque with subsequent thrombosis or embolization. Activated macrophages produce proteolytic enzymes degrading the fibrous cap which makes it prone to rupture. This rupture exposes the underlying necrotic core thus leading to thrombus formation. The inflammatory process of atherosclerosis has many similarities to the process presumed to initiate arteriogenesis, the growth of collateral arteries (Arras et al., 1998; Scholz et al., 2000).

Inflammatory markers of atherosclerosis

The realization that atherosclerosis is a chronic inflammatory condition based on the response to injury hypothesis (Ross, 1999), has led to the evaluation of circulating inflammatory markers to predict the risk of clinical events. These markers of systemic inflammation are CRP, proinflammatory cytokines such as IL-6 and TNF-α and soluble adhesion molecules ICAM-1 and VCAM-1. CRP is an inflammatory marker synthesized by hepatocytes in response to cytokine stimulation. IL-6 is one of the most studied cytokines with proinflammatory activity and is expressed by a large number of cell types e.g. monocytes, lymphocytes, SMCs and ECs. It is the main stimulant for CRP production in the liver but it also stimulates upregulation of adhesion molecules and cytokine secretion by ECs. IL-6 is also known to stimulate the expression of several hemostatic factors such as tissue factor, vWF and fibrinogen (Kerr et al., 2001). TNF-α is a pleiotropic cytokine derived mainly from macrophages. It is responsible for adhesion and activation of additional monocytes via upregulation of cell adhesion molecules and by stimulation of other cytokines such as IL-8 and MCP-1 (Nilsen et al., 1998). Adhesion molecules are thought to contribute to atherosclerotic lesion formation and
plaque rupture. ICAM-1 and VCAM-1 attributes to the firm adhesion of leucocytes to the endothelium and thereby facilitates migration into the arterial wall (Blankenberg et al., 2003).

Studies of inflammatory cytokines have revealed a strong correlation of the CRP level in plasma to future risk of myocardial infarction and stroke (Ridker and Morrow, 2003) as well as for the development of peripheral arterial disease (PAD) (Ridker et al., 2001). Also more specific markers as VCAM-1 and ICAM-1 have been shown to correlate with the risk for cardiovascular events (Blake and Ridker, 2002) and PAD development (Pradhan et al., 2002). Recently, a publication from the Edinburgh Artery Study demonstrated an independent association between PAD patients’ baseline levels of CRP, IL-6 and ICAM-1 with progression of disease measured by ABI at 12 years (Tzoulaki et al., 2005).

Exercise training in general leads to a variety of changes in cardiovascular function including reduced blood pressure, reduced heart rate, increased maximal myocardial oxygen uptake and various metabolic modifications (Kojda and Hambrecht, 2005). Exercise therapy in patients with intermittent claudication (IC) is known to increase the pain-free walking distance (Leng et al., 2000) but without affecting the ankle-brachial index (ABI) (Tan et al., 2000; Gardner et al., 2005). The molecular mechanisms underlying the adaptations to exercise training are largely unknown in these patients and there is little evidence showing that major conduit arteries increase in size. In animal models it has been shown that increasing blood flow by introducing an arteriovenous shunt increases vessel diameter significantly due to elevation of shear stress on the endothelial surface (Tronc et al., 1996). The same process may take place during exercise. In a rat limb ischemia model an increase in blood flow to the calf occurs when the animals are subjected to exercise (Yang et al., 1998). Exercise training appears to stimulate angiogenesis and result in an increased capillary density in the muscle which could be of importance in improving exchange properties between blood and muscle tissue. It has been suggested that exercise training causes transient hypoxia in the legs that upregulates VEGF as well as their receptors.
contributing to exercise-dependent angiogenesis (Gustafsson and Kraus, 2001; Gustafsson et al., 2005). Overall, the evidence for exercise-induced increases in collateral blood flow in PAD patients is limited and inconsistent (Tan et al., 2000; Gardner et al., 2005). Hence, other mechanisms may account for the evident exercise-induced improvements in walking capacity in these patients. Animal experiments as well as human trials have shown that exercise results in an increased expression of endothelial cell NO synthase (eNOS) and an increased endothelium-dependent vasodilatation. The regulation of eNOS is highly complex and closely related to changes in physical forces in the vasculature, in particular shear stress (Davis et al., 2003). This upregulation however appears to be a transient effect that diminishes as the vessel diameter increases and the shear stress normalizes (Green et al., 2004). Chronic ischemia leads to an accumulation of lactate and other metabolites of oxidative metabolism in the muscle (Hiatt et al., 1992). An increase in the oxidative capacity in the muscle with exercise training appears to be an important contribution to the training effect seen in patients with IC (Stewart et al., 2002).

**Incidence and prevalence**

The prevalence of PAD is strongly correlated to age. The prevalence of PAD defined as an ABI < 0.9 is about 18% in men and women older than 60 years in a recent population based Swedish study of 5080 subjects (Sigvant et al. unpublished data). This is in the same range as in previous studies (Fowkes et al., 1991; Meijer et al., 1998). IC has a prevalence of about 6 to 7% and CLI about 1% in the age range 60 to 90 years (Sigvant et al.). Primary risk factors for development of PAD are similar to those of atherosclerotic disease in general: age, diabetes, smoking, hypertension, hyperlipidemia and hereditary factors (Kannel and McGee, 1985). However, smoking and diabetes are considered the most important predictors for progression of the disease to CLI (Dormandy et al., 1999).

**Clinical presentation**

Asymptomatic lower extremity PAD is usually defined as a finding of an ABI of < 0.9. The earliest and most frequent presenting symptom of PAD is pain in the muscle of the leg when walking, referred to as IC. As the disease progresses with manifest impairment of blood flow, the patient might suffer from rest pain and/or ischemic ulceration and gangrene, defined as CLI. Although the prognosis of IC is fairly benign with regard to the local disease in the leg with a 5-year risk of progression to CLI of about 5% and a risk for amputation of only 2%, the morbidity and mortality is considerably higher than in the normal population due to cardiovascular co-morbidity (Dormandy and Murray, 1991; Criqui et al., 1992). Not all patients with CLI have deteriorated gradually from IC. Dormandy et al found that 55% of patients amputated for CLI never had experienced claudication when referred for CLI (Dormandy et al., 1994). The fate of patients with CLI is less benign both locally and systemically. In one year after diagnosis about one third of CLI patients required amputation and the in one year mortality is as high as 20% (Dormandy et al., 1999).

**Treatment**

Treatment of PAD is aimed to prevent general consequences of atherosclerosis, but also to reduce the clinical symptoms and prevent local tissue loss. The management consists of lifestyle modifications and pharmacotherapy addressing the risk factors to minimize the risk for disease progression and mortality in myocardial infarction or stroke. Lifestyle modification includes smoking cessation and exercise training. These measures both decrease the progression of PAD symptoms and have a beneficial effect on cardiovascular risk (Jonason and Bergstrom, 1987; Jonason and Ringqvist, 1987; Tan et al., 2000). Regular exercise training improves walking capacity, peak oxygen uptake and health related quality of life in patients with IC (Gardner et al., 2005). As previously discussed the exercise-induced impact on vascular growth in PAD is however not known.
Pharmacological therapy aims to regulate hypertension, hypercholesterolemia and hyperglycemia (Stratton et al., 2000; Pedersen et al., 2004). The use of statins and angiotensin converting enzyme (ACE) inhibitors is also associated to an increase in pain free walking distance for PAD patients (Novo et al., 1996; Aronow et al., 2003; Mohler et al., 2003). Another important risk factor modification is antiplatelet therapy. Treating PAD patients with aspirin reduce the risk for major cardiovascular events as well as the need for surgical revascularization (Aronow, 2005). Clopidogrel, a thienopyridine antiplatelet drug, is recommended when aspirin is not tolerated. In PAD patients, clopidogrel has shown a 23.8% relative risk reduction of ischemic stroke, myocardial infarction or vascular death compared to aspirin (1996). Prostanoids are believed to exert beneficial effects on the microcirculation in CLI, and intravenous treatment with the prostacyclin analogue iloprost has shown effect as well in some studies (Dormandy, 1991; Danielsson et al., 2004). Symptomatic treatment consists of endovascular or surgical revascularization. Unfortunately, 20% to 30% of the CLI patients can not be treated by any of these interventional methods and the only option for them is often amputation. For this group of patients there is a lack of good pharmacological symptomatic treatment and a great need for alternative treatment strategies.

**VASCULAR GROWTH IN ARTERIAL DISEASE**

**Normal artery**

Arteries are divided into three types: large elastic, medium sized muscular and small arteries. The normal artery wall consists of three structural layers, the intima, media and adventitia. The intima, the innermost layer is composed of a single layer of endothelial cells (ECs) resting on the basement membrane, a subendothelial space comprised of extracellular matrix (ECM) and finally an elastic membrane (internal elastic lamina) separating the intima from the media. The media is a multicellular layer of smooth muscle cells (SMCs) and ECM which gives the artery the ability to contract and dilate to regulate blood flow. The external elastic lamina separates the media from the adventitia in medium sized muscular arteries. The adventitia is the outermost layer and consists mostly of connective tissue and fibroblasts. It also contains nerves and capillaries (vasa vasorum) which supply the outer vessel wall. Smaller arteries are called arterioles and consist of a single endothelial layer surrounded with 1-2 layers of SMCs. The capillaries are the smallest vessels, only 5-10 µm in diameter, composed only by a single layer of ECs sparsely coated by pericytes.

**Molecular basis of vascular growth**

Postnatal vascular growth occurs normally in the female reproductive system and in pathological conditions such as wound healing, tumor growth and cardiovascular disease. Vascular growth is mediated by three different processes: vasculogenesis, angiogenesis and arteriogenesis.

**Vasculogenesis**

Vasculogenesis is defined as the development of the circulatory system from endothelial progenitor cells (EPCs) during embryogenesis (Risau, 1997). EPCs have been observed also in adult human bone marrow and peripheral blood (Asahara et al., 1997; Rafii, 2000). However, the role of EPCs in supporting postnatal vasculogenesis is under intensive investigation and the importance of these cells is still under debate (Rehman et al., 2003; Hristov and Weber, 2004).

**Angiogenesis**

Angiogenesis is characterized by a combination of sprouting of new vessels from pre-existing ones, or by longitudinal division or intussusception (Risau, 1997; Carmeliet, 2000), a process required for physiological and pathological conditions such as cardiovascular disease, diabetes mellitus, rheumatoid arthritis and tumor growth. Understanding the importance of angiogenesis in tumor growth led to intensive research of the basic mechanisms (Folkman et al., 1971). One of the most important stimulus for angiogenesis is...
tissue hypoxia which stimulates upregulation of angiogenic growth factors such as VEGF through hypoxia-inducible transcription factor 1 alpha (HIF-1α) (Ferrara et al., 2003). VEGF stimulation of ECs leads to increased vascular permeability accomplished through redistribution of intercellular adhesion molecules and alterations in cell membrane structure with extravasation of plasma proteins (Gale and Yancopoulos, 1999). Endothelial sprouting is enhanced by proteinases that degrade extracellular matrix, allowing ECs to migrate, and releases growth factors (FGF-2 and VEGF) sequestered in the matrix. Growth factor stimulation (e.g. VEGF, FGF, PDGF, Ang-1) leads to proliferation and migration of ECs, directed by signaling through integrins such as αvβ3 (Eliceiri and Cheresh, 1999) and intracellular adhesion molecules as PECAM-1 (CD-31) (Ilan and Madri, 2003). At this step, the ECs assemble into tubular forms elongating the original vessel. TGF-β and PDGF stimulate extracellular matrix production and recruit pericytes to stabilize the new vessel structure (Lindahl et al., 1998).

Arteriogenesis

Arteriogenesis, the growth of arterioles into collateral arteries, may be a process that can ameliorate the harmful effect of arterial obstruction. The process behind it is likely to be complex involving several steps. Upon sudden arterial occlusion or a slowly progressing stenosis in a large artery, blood flow is directed to interconnecting networks of arterioles. This causes augmented shear stress against the wall of these vessels leading to activation of ECs. The shear stress experienced by the EC is a function of the frictional force which occurs as blood flows through the vessel and is dependant on blood viscosity and the radius of the vessel (Malek and Izumo, 1994). It is not known how ECs become activated by shear stress. Evidence points to an important role for transduction of the frictional force through the cytoskeleton to focal adhesion sites on the basal side of the cell, implying that cell-matrix interactions may be of importance (Helmke et al., 2000; Gloe and Pohl, 2002; Ingber, 2002). These interactions and probably mechanosensitive structures at the cell surface can activate mechanosensitive tyrosine kinase and mitogen-associated protein kinase (MAP kinase) pathways, leading to activation of a number of transcription factors (Chien et al., 1998). These are responsible for the subsequent upregulation of many EC genes, probably achieved by binding to shear stress response elements on the promoter region of the gene (Khachigian et al., 1995). Most genes that become upregulated code for growth factors (MCP-1, GM-CSF, FGF-2) and adhesion molecules (ICAM-1, VCAM-1) (Scholz et al., 2000; Gloe et al., 2002; Hoefer et al., 2004). The result is attraction, activation and adhesion of circulating white blood cells. The monocytes will transmigrate into the vessel wall and produce various proteases, cytokines and growth factors. The proteases cause degradation of lamina elastica interna which opens the barriers to signal transmission to the SMCs. Proliferation of ECs and SMCs, stimulated principally by FGFs, is initiated as early as 24 hours after experimental occlusion of the femoral artery (Scholz et al., 2000). During this phase, a neointima forms composed of SMCs and finally remodelling of these vessels takes place with expression of proteinases in the perivascular space creating additional space for the growing collateral artery (Wolf et al., 1998; Cai et al., 2003).

The increase in vessel diameter reduces shear stress. The increased circumferential wall stress for the SMC, also stimulating proliferation, normalizes. The transformation of a small resistance vessel into a large conductance artery is completed.

The local inflammatory response to altered physical forces in the vessel wall closely resembles the inflammatory atherosclerosis process. The focal pattern of atherosclerotic lesions, mainly distributed in the arterial branches and curved regions, suggests that turbulent flow patterns and the mechanotransduction by shear stress alteration are important factors for atherosclerosis. There is obviously a relation between atherosclerosis and arteriogenesis where both reactions starts out as a way to compensate for altered flow conditions.
**VASCULAR GROWTH FACTORS**

**Vascular Endothelial Growth Factor**

Vascular endothelial growth factor (VEGF), also referred to as VEGF-A, is a key regulator of physiological angiogenesis. VEGF belongs to a gene family that include placental growth factor (PIGF), VEGF-B, VEGF-C, VEGF-D and VEGF-E. VEGF-C and VEGF-D exert their effect on lymphatic endothelial cells whereas the other mainly act on vascular endothelial cells. At least six different VEGF-A isoforms have been identified through alternative splicing of exons (VEGF_{121}, VEGF_{165}, VEGF_{189}, VEGF_{206}, VEGF_{145} and VEGF_{183}) (Ferrara et al., 2003). They are all actively secreted from intact cells. Different binding ability to heparin and heparan-sulfate chains distinguishes the different isoforms. VEGF_{165}, the predominant one, is a heparin-binding homodimeric glycoprotein of 45 kDa intermediary bound to the cell surface and extracellular matrix (Ferrara and Henzel, 1989). VEGF_{121} does not bind heparin and is freely soluble whereas VEGF_{189} and VEGF_{206} bind to heparin with high affinity and are mostly sequestered in the extracellular matrix. VEGF_{145} and VEGF_{183} appear in very low concentrations (Neufeld et al., 1999). The sequestered forms can be released by proteolytic cleavage of the heparin-binding domain, although with a significant loss of mitogenic activity (Keyt et al., 1996).

VEGF mRNA expression is induced by exposure to low oxygen tension mainly through the activation of hypoxia-inducible factor 1 alpha (HIF-1α). Several growth factors, including TGF-β, FGF and PDGF as well as inflammatory cytokines such as IL-6 upregulate VEGF mRNA expression (Neufeld et al., 1999).

**VEGF- receptors**

VEGF binds two related receptor tyrosine kinases, VEGFR-1 (flt-1) and VEGFR-2 (KDR/flk-1). Both receptors have seven immunoglobulin-like domains in the extracellular part, a single transmembrane region and an intracellular tyrosine kinase domain (Ferrara et al., 2003). VEGFR-1 and VEGFR-2 are almost exclusively expressed on ECs but are also present on bone marrow derived cells (Rabbany et al., 2003) and SMCs (Ishida et al., 2001). VEGFR-1 but not VEGFR-2 is upregulated by hypoxia in a similar manner to VEGF. In addition to these receptors, VEGF also interacts with a family of co-receptors, the neuropilins, which facilitates binding of VEGF\textsubscript{165} to VEGFR-2 (Neufeld et al., 1999). Ligand binding induces dimerization of the receptor tyrosine kinases. Thereby the receptors become tyrosine phosphorylated which allows binding of signal transduction molecules (Heldin, 1995). This leads to activation of intrinsic or associated enzymatic activities, initiating signalling cascades leading to regulation of transcription of specific genes (Pawson, 1995).

It is believed that VEGFR-2 is the major mediator of the angiogenic effects of VEGF and that the function of VEGFR-1 in the regulation of angiogenesis is that it acts as a decoy receptor for VEGF. The importance of the VEGF receptors and proteins for arteriogenesis is under debate. The most apparent arteriogenic role for VEGFR-1 is induction of monocyte migration (Clauss et al., 1996) which could be significant as monocytes are central for arteriogenesis (Arras et al., 1998). A third receptor tyrosine kinase, VEGF-3, is mainly expressed on lymphatic endothelium and involved in lymphangiogenesis, stimulated by VEGF-C and VEGF-D (Kaipainen et al., 1995).

**Fibroblast growth factors**

Human FGFs are 18-25 kDa polypeptides and have a 120 amino acid residue core, with 30% - 60% homology. Currently, there are twenty-two members of the FGF family identified (Itoh and Ornitz, 2004). FGFs are multifunctional proteins that play critical roles in cellular processes such as embryogenesis, angiogenesis, arteriogenesis, wound healing and tissue repair (Gerwins et al., 2000; Deindl et al., 2003). The wide array of biological activities are mediated through four receptor types. Most FGFs have N-terminal signal peptides and are readily secreted from cells. FGF-1 and FGF-2, the two prototype FGFs, lack signal sequences for export through the classical secretory pathway but can be released from dam-
aged cells or by an exocytotic mechanism independent of the endoplasmatic-recticulum-Golgi pathway (Mignatti et al., 1992). FGFs interact with heparin or heparan sulphate proteoglycans which stabilizes the FGFs and is required for FGF receptor activation (Ornitz, 2000).

The members of the FGF family stimulate EC proliferation, migration, protease production, integrin and cadherin receptor expression differently. Stimulation by FGF-1, FGF-2 and FGF-4 leads to EC proliferation (Cross and Claesson-Welsh, 2001), an upregulation of uPA and MMP production in ECs (Mignatti et al., 1992) leading to extracellular matrix degradation which is an important step in both angiogenesis and arteriogenesis and EC migration. FGF-2 regulates the expression of different integrins and cadherins in a complex fashion promoting cell-cell and cell-ECM interactions enhancing migration and proliferation (Klein et al., 1993; Underwood et al., 2002). FGF-2 is also known to stimulate SMC proliferation (Jackson and Reidy, 1993; Lindner and Reidy, 1993) and platelet derived growth factor (PDGF) receptor expression which may be important for creating a stable arteriogenic process (Cao et al., 2003).

FGF-2 is expressed by inflammatory cells, including monocytes and T-lymphocytes (Blotnick et al., 1994). FGF-2 is also released from ECs in response to cell damage (Gajdusek and Carbon, 1989), hypoxia (Kuwabara et al., 1995), inflammatory cytokines and nitric oxide (NO) (Walford and Loscalzo, 2003; Lee et al., 2004). Moreover can shear stress induce the release of FGF-2 from ECs (Gloe et al., 2002).

**FGF-receptors**

FGF use four cell surface receptors, FGFR-1 to FGFR-4 (Goldfarb, 2001). FGF-receptors are tyrosine kinases that contain an extracellular ligand-binding domain, a transmembrane domain and an intracellular tyrosine kinase domain. The extracellular region has two or three immunoglobulin-like domains and a heparin-binding domain (Goldfarb, 2001). Ligand binding promotes receptor dimerization and tyrosine kinase phosphorylation, which in turn induces the activation of the MAP kinase pathway (Auguste et al., 2003).

**Other vascular growth factors**

Many other factors are also involved in vascular growth. PDGF was originally purified from platelets, as its name suggests, but has been found in many other cell types including fibroblasts, keratinocytes, myoblasts, epithelial cells, ECs and macrophages (Heldin, 1995; Heldin and Westermark, 1999). PDGFs exists as homodimers or heterodimers composed of PDGF chains A and B. PDGF stimulates the proliferation of SMCs and pericytes thus helping to stabilize the new vessel structure (Abramsson et al., 2003). Transforming growth factor-β (TGF-β) as well as its receptor are also expressed by a wide variety of cells including ECs and pericytes (Massague, 1990). TGF-β are chemotactic for many cells including monocytes (Wahl et al., 1987) and establish a structural integrity of newly formed vessels during angiogenesis through modulation of the synthesis of ECM components and proteases. Angiopoetins, a family of four secreted proteins, and their Tie-receptors have been reported to play a major role in angiogenesis. Angiopoetin-1 (Ang-1), binding to Tie2 receptor on ECs, is a natural anti-permeability factor but also act as chemoattractant for ECs. Angiopoetin-2 is an inhibitor of Tie2 and antagonizes Ang-1 but induces angiogenesis in concert with VEGF (Maisonpierre et al., 1997; Koblizek et al., 1998). TNF-α, a pro-inflammatory cytokine is secreted mainly by activated macrophages and stimulates angiogenesis in vivo (Frater-Schroder et al., 1987) and has been shown to be required for adaptive arteriogenesis in animal models of limb ischemia (Hoefer et al., 2002; Grundmann et al., 2005). Granulocyte/macrophage colony stimulating factor (GM-CSF), proteins required for growth and differentiation of hematopoetic stem cells, also stimulates migration and proliferation of ECs (Bussolino et al., 1989).

**STIMULATION OF VASCULAR GROWTH**

Stimulation of arteriogenesis and angiogenesis have been suggested and tried as alternative treatments for patients with coronary artery disease (CAD) and PAD. There are a wide variety
of possible therapeutic targets and strategies available when induction of vascular growth is attempted. The complexity of the angiogenesis and arteriogenesis process is easy to underestimate and must be taken under consideration. Assuming that supplementation of vascular growth factors to tissue is the main treatment modality possible at present, two basic strategies can be applied. One is to deliver the recombinant protein and the other is to administer the gene encoding the protein. Each approach has its advantages and disadvantages. The advantage with protein delivery is that a more precise dose-response relationship can be attained. The major disadvantage with this method is the short half-life of proteins in most tissues which may be insufficient for stimulation of vascular growth and remodelling (Post and Simons, 2001). The advantage of gene therapy is that a sustained local production of the protein is achieved after a single administration.

**Gene Therapy**

Gene therapy techniques utilized generally involves plasmid DNA or replication deficient adenovirus containing the gene of interest. Recombinant genes (cDNA) contain only the protein coding sequences and lack the ability to invade cells. They are thus linked to viral or non-viral vectors which enable transfection of the host cell and expression of the gene with subsequent translation to the protein. Non-viral methods generally involve delivery of a naked plasmid or delivery of the DNA material in a liposomal vehicle.

**Plasmid DNA**

Plasmids occur naturally in bacteria as extrachromosomal circular double-stranded DNA. Human genomic DNA can be inserted together with a strong promoter, most commonly cytomegalovirus (CMV), to drive cDNA expression efficiently (Kelly, 2003). The limitation with plasmid gene transfer is low transfection efficacy largely because of the difficulties of these vectors to overcome the numerous barriers to reach the cell nucleus (Wiethoff and Middaugh, 2003; Wolff and Budker, 2005).

**Viral vectors**

Viruses are widely used as vectors as they by nature target the host cell, penetrate the plasma membrane, pass through the cytoplasm and enter the nucleus. Also for viral based systems, there are limitations. There is a size limitation of the inserted gene due to packaging constrains. Furthermore the potential insertional mutagenesis is a risk and the potential immunogenicity of the virus which may limit the ability for repeated administration.

In the first human gene transfer trial 1989 using a retroviral vector “ex-vivo” gene therapy to lymphocytes was employed (Rosenberg et al., 1990). Since then retroviral vectors are rarely used. While this is a type of RNA virus which is integrated in the host’s genome, resulting in permanent expression of the inserted gene, it also makes insertional mutagenesis possible. Retroviruses are also small, limiting the size of the gene that can be incorporated. A major disadvantage is that they do not infect non-dividing cells. Adeno-assiciated virus (AAV) or lentiviral vectors are presently considered more promising for gene therapy based on their excellent ability to transduce non-dividing cells. These viruses also integrate into the host’s genome with a subsequent risk for insertional mutagenesis. AAV predominantly transduce SMCs but not ECs (Dishart et al., 2003).

The adenovirus (Ad) is a pathogen in humans causing mild common cold symptoms and the majority of adults have serologic evidence of prior infection (Schulick et al., 1997). Ad vectors have become widely used for gene transfer and is the most commonly used for cardiovascular gene therapy. They infect non-dividing cells and do not integrate into the host cell genome. Adenoviruses are nonenveloped DNA virons with a capsid consisting of three main exposed structural proteins; the hexon, fiber and penton base (Nicklin et al., 2005). The Ad genome is organized into four early and one major late transcriptional unit (Horwitz, 2001). The early genes (E1 to E4) principally alter host cell biology to favour virus production and the major late region encodes most of the structural proteins. The E2, E3 and E4 regions are directly activated as a con-
sequence of E1 protein expression, the rate-limiting transactivator. By deletion of the E1 region a replication-deficient vector is accomplished. This results in a reduced immune response and prevents replication of the virus in the host cell. The E3 region is also frequently deleted in most first-generation Ad vectors to provide room for insertion of the gene of interest. The Ad knob domain binds to coxsackie-adenovirus receptors (CAR) on the surface of a broad range of cell types (Bergelson et al., 1997). Upon CAR binding, an interaction between $\alpha_v\beta_3$ or $\alpha_v\beta_5$ integrins and the Ad penton base occurs and the virus gets internalized into endosomes (Wickham et al., 1993) and translocates into the nucleus where it assumes an episomal location. The viral genome unfolds immediately and the virus gains complete control of the cellular machinery resulting in transcription and translation of the gene of interest. First-generation Ad vectors evoke a strong host immune response and newer second- and third-generation Ad vectors have demonstrated significant improvements (Baker, 2004).

In general, currently available vectors have higher tropism for non-vascular tissue than vascular cells. This has suggested that modification of existing vectors is required in order to generate more efficient and selective vectors for cardiovascular gene therapy.

**Cellular based therapy**

Accumulating evidence in the past years indicate that adult peripheral blood contains bone marrow derived endothelial progenitor cells (EPCs) (Luttun et al., 2002). These cells have been shown to participate in neo-vascularization of ischemic tissue, but the exact mechanism regulating the differentiation and homing still remain unclear (Hristov and Weber, 2004). Transplantation of EPCs has been suggested to increase blood perfusion in animal models of myocardial and peripheral ischemia by integration of EPCs into growing blood vessels (Kalka et al., 2000; Kawamoto et al., 2001). Others doubt that EPCs have any potential to differentiate into organ-specific cells in adults (Wagers et al., 2002; Ziegelhoeffer et al., 2004). EPCs have however been suggested to contribute to vascular growth by releasing angiogenic growth factors (Rehman et al., 2003).
The principal objective of this thesis was to assess the role of endogenous vascular growth factors in the arteriogenic process in PAD and to evaluate AdFGF-4 gene therapy in an animal model and in humans as a strategy for therapeutic arteriogenesis.

The specific aims were:

I. To evaluate the importance of VEGF and FGF-2 expression in CLI pathogenesis in humans

II. To assess whether exercise-induced ischemia elicits a systemic inflammatory response and increase local vascular growth factor expression in patients with IC

III. To evaluate the effects of adenovirus mediated FGF-4 therapy on limb perfusion and collateral development in a rat model of severe limb ischemia

IV. To assess safety and potential clinical efficacy of adenovirus mediated FGF-4 therapy by intramuscular injection to patients with CLI
PATIENTS AND CONTROL SUBJECTS

Patients

Twenty-five patients with CLI defined according to the Trans Atlantic Inter Society Consensus (Dormandy and Rutherford, 2000) with stable symptoms of rest pain or ischemic distal ulcers with ABI <0.5, ankle pressure <60 mmHg or toe pressure <40 mmHg who were candidates for femoropopliteal bypass below knee or femorodistal bypass were recruited to Study I. Fourteen men and eleven women aged 57 to 91 years were included. None of the patients had a medically documented history of malignant disease or rheumatoid arthritis.

Thirty non-diabetic patients with IC were recruited to Study II. Patients were asked to participate if they had an ABI between 0.5 and 0.8 and a reproducible absolute claudicant distance (ACD) on treadmill walking, defined as <20% difference in ACD on two consecutive tests. Patients with severe heart failure, myocardial infarction within 6 months, unstable angina or neoplastic, immunologic or chronic inflammatory diseases were excluded. Twenty-four patients, 13 women and 11 men aged 53 to 85 years were finally included.

Twelve patients with unreconstructable CLI and one patient with reconstructable CLI were included in Study IV. Patients were asked to participate if they were above 40 years of age with CLI according to Fontaine stage III or IV with rest pain and/or ischemic ulceration.

Control subjects

Fifteen subjects took part in Study I serving as controls. The control subjects were screened to exclude persons with a medical history of PAD or those who had objective signs of distal ischemia (ABI <0.9). Ten orthopaedic patients with arthritis undergoing hip or knee arthroplasty served as control subjects in Study I. The hip arthroplasty patients were women aged 56 to 74 years. Among the knee arthroplasty patients three were women and two were men. Their ages ranged from 70 to 89 years. Five additional patients who underwent coronary artery bypass surgery (CABG) also served as control subjects for this study. The biopsies were then obtained from skin in the distal leg during vein harvest. These five CABG patients were men, aged 58 to 83 years.

All studies were approved by the local Ethics Committee. The subjects were given oral and written information and their consent was obtained before inclusion.

In Study IV all study sites had the study protocol approved by local institutional review boards and biosafety committees. The study was conducted in accordance with the Declaration of Helsinki and International Conference on Harmonization for GCP Guidelines. The study also followed country-specific requirements for the use of genetically modified micro-organisms (GMOs).

ANIMALS

Twenty male Sprague-Dawley rats with a mean weight of 275 g (range 250-335 g) were used in study III and housed 5/cage at 21 °C in a 12-h light/dark cycle with water and pellets ad libitum according to the Karolinska Institute protocol. The experimental model used in this study was a rat hindlimb ischemia model (Seifert et al., 1985) modified by Lundberg et al that produces
a severe unilateral resting limb ischemia with reduced skin perfusion in the ischemic paw up to eight weeks (Lundberg et al., 2003). The animals were anesthetized with a combination of Hypnorm (fentanyl citrate 0.315 mg/ml and fluanisone 10 mg/ml, 0.05 ml/100 g BW) and Pentobarbital (60 mg/ml, 0.05 ml/100 g BW) intraperitoneally (i.p.). Through a midline laparotomy all arterial branches on the left side of the aorta distal to the renal artery and all branches from the left iliac artery down to the inguinal ligament were ligated and divided. A second operation using similar anesthesia was performed after one week where the femoral and superficial epigastric arteries on the left side were ligated and divided through an inguinal incision. An analgesic (buprenorphin 0.05 mg/kg) was injected subcutaneously after each operation twice a day for two days after the first operation and for seven days after the second operation. After surgery and treatment the animals were kept single-housed. The protocol was approved by the Institutional Animal Care and by the Ethics Committee in Stockholm.

**EXPERIMENTAL DESIGN AND PROCEDURES**

**Study I**

Twenty-five patients with CLI were recruited to this prospective observational clinical study at the Vascular Surgical Department at Karolinska Hospital, Stockholm, Sweden. The patients were asked to participate if they met the inclusion criteria and lacked exclusion criteria. Skin and muscle biopsies were gathered perioperatively from the groin and calf regions. Samples from the distal part of the leg were then compared to proximal samples and to biopsies from healthy control subjects. Fifteen patients without signs of PAD served as controls.

**Study II**

Twenty-four non-diabetic patients with IC were included in this experimental clinical study at the Vascular Surgical Department at Karolinska Hospital, Stockholm, Sweden. Patients were seen by a study nurse on a separate day for measurement of ABI and to perform qualifying treadmill tests. On the day of the test, resting ABI was measured, blood samples drawn and a urine sample obtained prior to exercise. After walking on treadmill to ACD twice, patients’ ABI was measured again and blood samples were drawn 15, 60 and 120 minutes after the end of the exercise. A post exercise urine sample was also gathered after 60 minutes. In ten of the patients a percutaneous muscle biopsy from the lateral part of the gastrocnemius muscle was obtained before and 120 minutes after exercise.

Five IC patients who initially did a treadmill test with less slope did not experience any calf pain despite walking for 10 to 16 minutes. These patients were included as controls. One of them was included again 2 years later this time experiencing claudication symptoms after walking on treadmill.

**Study III**

The rats were randomly divided into two groups receiving active treatment (n=10) or placebo (n=10) one day after the second operation. They were given i.m. injections of 0.4 ml of Adenovirus serotype 5 mediated FGF-4 (AdFGF-4) (10^{11} virus particles) or placebo (PBS) at four sites close to the ligated femoral artery. After 4 weeks the rats were again anesthetized to undergo perfusion measurements and muscle biopsy harvest.

**Study IV**

The study was a prospective, double-blind, randomized, placebo-controlled study of AdFGF-4 gene therapy with escalating dose groups of 2.87 x 10^{8}, 2.87 x 10^{9}, and 2.87 x 10^{10} viral particles injected intramuscularly in CLI patients. At each dose step, patients were assigned to either active drug or placebo in a ratio of 3:1. Patients were included at six centers in the UK, Sweden, Germany, Finland and Hungary.

Eligible patients underwent screening examinations including blood tests, eye examinations, a
pregnancy test for females and examinations to screen for malignant tumors including pelvic examination, rectal examination, mammography, prostate-specific antigen, sigmoidoscopy and chest x-ray. Thirteen patients fulfilling entry criteria were randomized and underwent baseline examinations. The treatment started within four days of completing the baseline examinations. The study medication was administered in addition to the patient’s ordinary background therapy except for oral anticoagulants or heparin which was replaced by low molecular weight heparin 7-10 days before administering the study medication. Patients received eight intramuscular injections of active drug or placebo into the study leg on a single day. The injections were given pairwise. The first three injection pairs were given close to the first angiographically identified significant stenosis, and the fourth injection pair was given in the upper third of the calf muscle. Study drug was given once according to the assigned dose cohort, and dose variations were not permitted. Patients were followed up for 12 weeks and about 6 months after the injections the treating physician was required to submit records for assessment of the post-study outcome. Standard biosafety techniques were applied for handling and administration of the product. Patients and investigators remained double blinded throughout the trial. The sponsor was unblinded after each cohort for safety assessment and safety data was reviewed by an independent safety review committee. Figure 1 depicts the study flowchart.

PARAMETERS TO ASSESS PAD

Ankle-brachial Index

Brachial blood pressure was measured with a standard 12 cm cuff and a stethoscope. Ankle blood pressure was measured with the same cuff and a continuous wave pen-Doppler (Minidop ES-100VX Hadeco). The Doppler signal was measured over the dorsal pedal artery and the posterior tibial artery, and the highest value of these was registered. Toe blood pressure was measured when there was no possibility to measure the ankle pressure because of incompressive vessels or ulcers in the ankle region preventing assessment. The toe pressure was measured with an automated instrument (PressToe, Moor Instruments) using a 2.5 cm cuff on the first toe. The median of three pressure readings was registered. The ankle-brachial index was calculated as ankle pressure in mmHg/brachial systolic pressure in mmHg.
Treadmill test

Patients were screened by performing two qualifying tests and ABI measurements. The treadmill was set at 3.2 km/h and a slope of 14% was used. Included patients returned another day for the study procedure. On that day resting ABI was measured and blood samples and a urine sample were obtained within one hour prior to exercise. The patients then walked on the treadmill until reaching ACD twice, each walk separated by 5 min rest period. After walking, ABI was again measured within 2 minutes and blood samples were drawn 15, 60 and 120 minutes after the end of treadmill exercise. A post exercise urine sample was gathered 60 minutes after the end of exercise.

Muscle and skin biopsies

In 25 patients with CLI and 15 control subjects muscle and/or skin samples of 5x5 mm were collected during surgery. The biopsies were obtained from the distal part of the calf incision and from the groin. The availability of samples from different muscles depended on the anatomical prerequisites of the surgical procedure. In the orthopedic control subjects, the biopsies were collected from the proximal quadriceps muscle and from the skin in the proximal lateral thigh or from the distal medial part of the quadriceps muscle and the skin medial to the knee. The skin biopsies gathered from the patients undergoing CABG were gathered in the mid calf.

In 10 IC patients a percutaneous muscle biopsy was obtained before the walking test and 120 minutes after the end of exercise. An area on the lateral part of the mid calf was prepared sterile. The microbiopsy was performed with a spring-loaded, reusable instrument, Bard® Magnum® Biopsy Instrument. The device has been proven effective for fine needle percutaneous biopsies of various tissues and has recently been shown to be effective also for skeletal muscle biopsies in a validation study comparing it to needle biopsy according to Bergström. The needles used were sterile packed 12-gauge core tissue biopsy needles (Bard® Magnum®, MN1210, BARD) with a length of the sampling notch of 19 mm and a penetration depth set to 15 mm. After local anaesthesia with 2 ml of lidocaine 10 mg/ml, a 3 mm long skin incision was made by a scalpel and the biopsy needle was inserted until the fascia was pierced. The muscle sample was obtained by activation of a trigger which protrudes the needle. The biopsy needle was then pulled out and the sample immediately frozen in dry ice. The needle was inserted one to two additional times through the same skin incision but in other directions to obtain sufficient muscle tissue (10-15 mg). The whole procedure was repeated 120 minutes after the test situation with a new puncture at a distance of 1 cm from the first one.

All biopsies were immediately frozen on dry ice.

Laser Doppler Imaging

A moorLDI-VR (Visible Red Laser Doppler Imager, Moor Instruments Ltd, Axminster, UK) was used to estimate dermal perfusion of the foot of the rat. The laser Doppler source is mounted on a stand over the animal on the bench and a laser beam scans the tissue. The laser beam reflected from moving red blood cells is processed to provide a flux value and a map of perfusion of the tissue is provided through colour coding of the data. The regions of interest were marked manually using the Moor LDI Image Processing V 3.01 software. Mean flux values of these regions were calculated by the software.

Measurements were performed under anaesthesia as described above. The rats were kept on a 37°C heating pad to reduce heat loss and supplementary oxygen was delivered maintaining the saturation above 90%.

Digital Subtraction Angiography

For Study IV, the angiography equipment available at each participating hospital was used. To maintain the reliability and assist in the analysis of the results a rigorous Quality Control program was used. This program included collecting sys-
tem information and performing angiography of four Quality Assessment test objects sent to all participating centres. Test object images were used to assess image quality and to create specific image distortion correction maps. Test object images were converted to DICOM (digital imaging and communications in medicine) formats and assessed for uniformity, dynamic range, distortion and artefacts. The different centres had a wide range of equipment (8 Siemens, 2 Philips, 1 GE and 1 Toshiba) but all systems were considered acceptable. Sets of images were obtained from the hip joint to the lateral foot. Digitalized data were then submitted to a core lab for blinded reading and analyses. The effects of treatment were evaluated by measuring changes in vessel size and by counting the number of vessels as well as by assessing the image appearance. A special vessel sizing software program was designed. The program and methods were coded in IDL (Interactive Development Language). Vessel counting was carried out by a consultant radiologist who counted all vessels crossing a grid of horizontal lines. The grid was placed so it covered the same area of the corresponding pre- and post-therapy images. An IDL tool was also created to annotate the images and overlay them on any other image, used for the visual comparison of pre- and post-therapy images. An IDL tool was also created to annotate the images and overlay them on any other image, used for the visual comparison of pre- and post-therapy images. DSA images were obtained from the hip joint down to the foot on the affected limb. A reference “ruler” was provided. Radio opaque skin markers were used for marking the sites of i.m. injections. The diameter of the main collateral originating from the occluded artery was measured approximately 5 mm from its origin. The diameter and length of any new identified vessel was also measured to identify changes in the number of vessels. Image data from all centres was assessed centrally, blinded to image sequence.

Magnetic Resonance Imaging

MRI was used in Study IV to visualize changes in tissue signal intensity in the calf, 10 cm distal to the knee joint. Parameters evaluated included:

a) time to peak signal intensity after a contrast bolus (0.1 mmol Gd-DTPA/kg BW, 0.2 ml gadopentetate dimeglumine, Magnevist®/kg BW) and c) time to peak blood flow velocity within the normal pulse cycle. The images of the legs were acquired in a single-slice mode in the axial plane with a slice thickness of 10 mm, a field view of 30 x 30 cm, a data matrix of 128 x 128 points, and a flip angle of 40°. All subjects were placed in a supine position and a suitable blood pressure cuff was placed above the knee. The cuff was inflated 30 – 50 mmHg above systolic blood pressure for 3 minutes followed by a post ischemic recovery period of 3 minutes. For classifying the drug effect on the variables a change was regarded as significant if it exceeded 15% of the basal values, based on the expected variability of the MRI data of 10%. Analyses of data from all patients included in Study IV were done by a core lab (Charité Hospital, Berlin, Germany).

Blood oxygen level dependent (BOLD) imaging was a concept introduced 1990 for evaluating brain activation (Ogawa et al., 1990). This method relies on MRI contrast resulting from changes in the ratio of oxyhemoglobin to deoxyhemoglobin and can demonstrate the impact of improved perfusion on oxygenation as visualized by signal intensity changes also in skeletal muscle (Wigmore et al., 2004). The BOLD technique utilizes the difference in effect on T2 relaxation time between oxyhemoglobin and deoxyhemoglobin. Deoxyhemoglobin causes a decrease in T2 relaxation time while oxyhemoglobin has little direct effect on T2 (Young and Bydder, 2003).

Injection of paramagnetic tracers has been used to measure perfusion in myocardium, tumors and skeletal muscle (Atkinson et al., 1990; Griebel et al., 1997; Luo et al., 2002). Following an i.v. injection of a contrast agent e.g. Gd-DTPA, the distribution of the agent can be followed with T1-weighted fast MRI sequences.

Scintigraphy

Three phase blood pool scintigraphy using technetium99m (Tc99m) labelled red blood cells was performed to evaluate blood flow (ml/min)
below the knee of the affected leg in Study IV. Blood flow was measured during reactive hyperemia induced by 3 minutes of arterial occlusion of the affected leg by a pneumatic cuff placed around the thigh. For labelling red blood cells cold stannous pyrophosphate was injected into the anterocubital vein with the patient in supine position. The cuff was inflated 50 mmHg above systolic blood pressure and during this time Te⁉⁹m was given intravenously. The cuff was released after three minutes and image data was acquired at one frame per second for 100 seconds and a time-activity curve was created from regions of interest. Normally, during the perfusion phase, the curve displays three distinct phases; a linear inflow phase, a mixing phase and an equilibrium phase. In PAD, the curve is expected to be linear, indicating that the turnover circulation of the affected limb is impaired. Blood flow was determined from the initial gradient of the time-activity curve and from equilibrium blood-pool sampling. All analyses were done blinded to treatment.

**LABORATORY METHODS**

**Blood sample analysis**

Blood samples were drawn from the antecubital vein and analyzed for high sensitivity CRP (hs-CRP), von Willebrand factor (vWF), erythrocyte volume fraction (EVF), interleukin 6 (IL-6), tumor necrosis factor alpha (TNF-α) and the vascular growth factors VEGF and FGF-2 in Study II. Blood samples for analysis of vWF were centrifuged within 30 minutes, frozen and analysed (Liatest vWF kit) by the Karolinska University Laboratory. Hs-CRP was analyzed within 24 h by means of particle enhanced immunonephelometry and IL-6 and TNF-α were centrifuged, frozen and analysed once by ELISA technique by the same laboratory. Blood samples for analysis of growth factors were centrifuged within 2 hours at 2000 g for 20 minutes and the plasma was separated and stored at -80°C for subsequent analysis. The levels of total human VEGF and FGF-2 in plasma were determined with ELISA. In Study IV, venous blood samples (Study IV, Appendix 2 and 3) were gathered for safety evaluation at different time points and analyzed by a single core lab (Fig.1).

**Total protein quantification**

In Study I, muscle and skin biopsy samples were homogenized in ice-cold buffer (25 µl buffer/mg muscle) containing 1000 µL of 0.5 mol/L Tris, 300 µL of 5.0 mol/L NaCl, 0.02 g of sodium vanadate, 0.05 g of NaDeoxycolic acid, 50 µL of Triton X-100, 6500 µL of distilled water, 2000 µL of 0.5 mol/L NaF, 20 µL of 0.1 mol/L Na vanadate, 10 µL (10 mg/mL) of aprotinin, 10 µL (10 mg/mL) of leupeptin, 20 µL of 0.5 mol/L ethylenediaminetetraacetic acid, and 100 µL of 0.1 mol/L phenylmethylsulfonyl fluoride in 10 ml of distilled water. After homogenization, the suspension was processed by centrifuge at 3000 g for 10 minutes, and the supernatant was centrifuged at 12000 g for 10 minutes at 4°C and stored at -70°C. The total protein content was determined by BCA Protein Assay Kit (Pierce 23225) according to the manufacturer’s protocol.

**ELISA**

Enzyme-linked immunoassay (ELISA) employs the quantitative sandwich enzyme immunoassay technique. A monoclonal antibody specific for the antigen of interest is pre-coated onto a microplate. Any antigen in the sample or standard binds to the immobilized antibody in each well. After washing away any unbound antigen, an enzyme-linked polyclonal antibody specific for the antigen is added which binds to a substrate solution added after washing away the abundant antibodies. This evokes a color reaction in proportion to the amount of antigen bound in the initial step. The intensity of the color is determined spectrophotometrically.

The concentrations of total human VEGF (isoform 121 and 165 combined) and FGF-2 were determined in human tissues and plasma using ELISA technique (Quantikine, human VEGF, human FGF basic, high sensitivity human FGF basic R & D Systems). The concentrations of
VEGF and FGF-2 in muscle and skin samples were analyzed in relation to the total protein content in the tissues and presented as pg/mg total protein.

Real-time PCR

Real-time PCR (real-time reverse transcription polymerase chain reaction) was used for quantification of gene expression for vascular growth factors in muscle biopsies. Real-time PCR reflects the initial amount of template and is the most specific, sensitive and reproducible methodology available today for detection and quantification of genetic material (Giulietti et al., 2001). It is based on the detection and quantification of a fluorescent marker present on special dual-labeled probes that emit a fluorescence signal only on nuclease degradation of the probe during the PCR extension cycle. The signal increases in direct proportion to the amount of PCR product in the reaction. By recording the amount of fluorescence emitted in each cycle the method allows for the detection of PCR product during the exponential phase of the reaction, combining amplification and detection in one step. To quantify the results two different methods are commonly used: the standard curve of a dilution series and the comparative threshold method. A computer software program calculates ΔRn expressing the probe degradation during the PCR process. The ΔRn values are plotted against the cycle number and an arbitrary threshold based on the variability of the baseline is chosen. The threshold cycle \( C_t \) value is the cycle number where the fluorescence emission exceeds this chosen threshold. Thus, \( C_t \) values decrease linearly with increasing target quantity. The \( C_t \) value for the target gene is normalized to the \( C_t \) value of a housekeeping gene that is expressed at a constant level, to correct for minor variations.

RNA extraction

For Study III, 30 mg of tissue was taken from each biopsy while still frozen. Equal volumes of lysis buffer RLT (containing β-mercaptoethanol 10µl/ml) and Phenol (Sigma) was added to Lysing matrix D tubes (Qbiogen) together with the tissue. Disruption of tissue was made 3 times at 20 sec, speed setting 6.5 in the FastPrep FP 120 Instrument (Qbiogene, Inc., CA, USA). The samples were cooled on wet ice between each run for 2 minutes. Again equal volumes of RLT and Phenol was added to the sample and centrifuged for 10 minutes at 9800 G. The supernatant was removed to an Eppendorf tube and homogenised through a thin cannula 6 times. Equal volume of ethanol 70% was added to each tube. The homogenate was transferred to a mini column and total RNA was isolated using RNeasy Mini Kit (Qiagen) including a DNase step according to the manufacturer. RNA quantity and quality was analysed by a Bioanalyzer 2100 (Agilent Technologies) using RNA 6000 Nano chips (Agilent Technologies) and samples were frozen in -70ºC. RNA showing degradation was excluded from the study. For Study II, 10 mg of tissue was analyzed. Total RNA was isolated using RNeasy Mini Kit (Qiagen) as previously described.

cDNA synthesis

To generate cDNA in Study III, 2 µg of total RNA was used for reverse transcription with Superscript II (Gibco) using pdN6 primer (Amersham) as recommended by the manufacturer (Applied Biosystems). In Study II cDNA was obtained using MMLV reverse transcriptase (Invitrogen, Carlsbad, CA) according to protocols of the kits.

PCR amplification

Real-time PCR was performed in a TaqMan 7700 according to the manufacturer’s instructions (Applied Biosystems). Oligonucleotide primers and TaqMan probes were ordered as gene assay on demand (Applied Biosystems). In study III, cDNA were amplified by real-time PCR with primer and probe pairs to human FGF-4 (forward primer: TACTGCAACGTGGGCA TC, reverse primer: GTGGGTTACCTTCA TTGGTAGG) and TaqMan universal PCR master mix (Applied Biosystems). Beta 2 microglobulin (B2m), glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) and glucoronidase beta (Gusb)
were used as housekeeping genes to normalize for RNA loading (Applied Biosystems). In Study II, cDNA were amplified with primer and probe pairs to human VEGF-A and FGF-2. GCCCACTGAGGAGTCCAACA and TCCTATGTGCTGGCCTTGGT were used as primers for VEGF-A and CACCATGCA-GATTATCGGATCAAACC as probe, and TGTGTCTATCAAGGAGTGTGCTA and TCCGTAACACATTGAGCCAGTA were used as primers for FGF-2 and CCGTCTACCTGGCTATGAAGGAAGATGGAAG as probe. Predeveloped human GAPDH was used to normalize for RNA loading (Applied Biosystems). The reactions were performed using TaqMan Universal PCR, Master Mix (Applied Biosystems, Foster City, CA) in multiplex using an ABI 7700 Prism Sequence Detection System (Perkin Elmer). The cycle was configured as follows: incubation (95°C, 10 min), 50 cycles of denaturation (95°C, 15 s), and annealing/extension (60°C, 60 s). Relative standard curves were prepared by serial dilution of positive RNA. The expression level was normalized to the endogenous control gene.

Immunohistochemistry

The principle of immunohistochemistry is to attach a primary antibody against a specific antigen in the tissue section. The primary antibody can be detected by a biotinylated secondary antibody.

In these studies, three 5µm thick sections were cut transversely using a cryostat and placed on glass slides. The frozen sections were stained for immunohistochemistry in an attempt to localize VEGF and FGF-2 in Study I, and ECs and SMCs in study III. Anterior tibial muscle sections were stained with a monoclonal antibody against CD-31 (Platelet Endothelial Cell Adhesion Molecule-1) to identify and count capillaries and the quadriceps muscle was stained with a monoclonal antibody against smooth muscle α-actin to detect collaterals. Tissue sections prepared with Acetone fixation were rinsed in PBS. 3% Hydrogen peroxidase (diluted in distilled water) was added for 5 minutes, followed by three washes in PBS for a total of 5 minutes. Blocking solution (2% rabbit serum in PBS in Study I and 5% goat serum in PBS in Study III) was applied for one hour at room temperature (RT) and the slides were incubated for one hour at RT (Study I) or overnight at 4°C (Study III) with a goat anti-FGF basic antibody or a goat polyclonal anti-human VEGF antibody (both R & D Systems) at a concentration of 2 µg/mL or with a mouse monoclonal antibody against rat smooth muscle α-actin (NeoMarkers) or mouse monoclonal antibody against rat CD 31 (serotec) both at 1:100, all diluted in 0.1% bovine serum albumin (BSA) in PBS. Incubation was followed by three 5-minute washes in PBS. A secondary biotinylated rabbit anti goat antibody (Vector) was added at 1:200 for 1 hour at RT for primary antibodies in goat, and a goat anti mouse antibody was added at 1:200 in for 30 minutes at RT for primary antibodies in mouse, followed by three washes in PBS for a total of 5 minutes. Labeled avidin biotin peroxidase complex (Vectastatin ABC kit; Vector Laboratories) was used according to the manufacturer’s recommendations for 30 minutes at RT to visualize biotinylated antibodies. After rinsing in PBS, DAB (3,3'-diaminobenzidine) peroxidase substrate kit was added for 7 minutes to localize the immune complexes. The sections were counterstained with Mayers hematoxylin for 3 minutes and mounted with Mountex. Negative controls were obtained by substituting the primary antibody with PBS. In this method, the immune complexes appear brown, and nuclei are blue. A Nikon epifluorescence microscope (Eclipse E800 Yokohama, Japan) was used to analyze the sections.

Capillary and collateral vessel estimation

Capillary ratio (CD-31 stained cells/muscle fiber) was calculated from positively stained cells at the border of the muscle fibres at 20 x magnification. Collateral number and size were measured from α-actin positively stained vessels, excluding the ones with a venous shape, at 10 x magnification. Collaterals with a diameter greater than 10 µm were counted and measured. A to
tal number of capillaries, collaterals and muscle fibres were counted manually from ten different fields selected from two different sections from each sample in a blinded fashion. All counting was done blinded to the samples origin.

**STATISTICAL ANALYSIS**

**Study I**

For statistical analysis paired t-test was used to compare dependent proximal and distal samples in the patient group. Mann Whitney U-test was used to compare patients to control subjects.

**Study II**

Analysis of variance (ANOVA) was used to compare data over time for IL-6 and TNF-α. For post-hoc comparisons, Scheffé’s test was applied. Paired t-test was calculated for comparisons of ABI and vWF before and after exercise. Non-parametric Wilcoxon signed-rank test (ACR, VEGF and FGF-2 mRNA) or Friedman ANOVA by ranks (hs-CRP, VEGF and FGF-2 in plasma) were used.

**Study III**

Mann Whitney U-test was used to compare results of capillary and collateral counting between the active group and placebo. Wilcoxon signed-rank test was used to compare perfusion results as well as capillary and collateral counting between the ischemic and the non-ischemic side of each animal. The mean flux values of regions of interest were calculated and the ratio of the ischemic versus non-ischemic limb was compared as well as comparisons between legs receiving active treatment and placebo using Mann Whitney U-test.

**Study IV**

This phase I/II clinical trial was powered to detect safety concerns and potential beneficial efficacy in 28 patients. The study only included 13 patients and therefore no statistical analysis was calculated.

For all analyses, p<0.05 was considered statistically significant.
RESULTS

EXPRESSION OF VASCULAR GROWTH FACTORS AND INFLAMMATORY PROTEINS

VEGF and FGF-2 expression in ischemic tissue

In Study I the main finding was that FGF-2 was elevated in distal muscle samples compared to proximal samples in CLI patients (p=.006) but without any difference compared to control subjects (p=.391) (Fig.2). VEGF levels were higher in distal muscle in patients than in control subjects (p=.028) but were not elevated compared to patients’ proximal muscle. VEGF was detected in lower levels in distal skin than in proximal (p=.038) (Fig.3). Patients with diagnosed diabetes mellitus (n=14) had lower levels of FGF-2 in distal muscle compared to patients without diabetes (p=.009). There was a tendency to higher levels of VEGF in distal skin samples of patients with distal ulcers or gangrene (p=.053).

In Study II evaluating patients with IC, exercise caused a marked decrease in ABI (p=.0001) which together with the patients’ experience of pain in the calf was interpreted as that the test did achieve distal muscle ischemia. Expression of VEGF mRNA (Fig.4) but not FGF-2 mRNA in the calf muscle biopsies was increased (p=.043) 120 minutes after exercise to ACD. Plasma levels of VEGF and FGF-2 were not influenced by exercise.

Figure 2. FGF-2 protein (mean ± SD) in CLI patients and control subjects (Study I). Levels were elevated in distal muscle samples compared to proximal samples in CLI patients (p=.006) but without any difference compared to control subjects (p=.391).

Figure 3. VEGF protein (mean ± SD) in CLI patients and control subjects (Study I). Levels were higher in distal muscle in patients than in control subjects (p=.028) but not compared to patients’ proximal muscle. VEGF was detected in lower levels in distal skin than in proximal (p=.038).
**Systemic expression of inflammatory proteins**

In Study II the patients with IC increased plasma levels of IL-6 after exercise (p=.004). TNF-α and hs-CRP were not changed at any of the time-points (p=.191, p=.709 respectively) (Fig.5 and 6). Plasma vWF showed a small but significant decrease after exercise (p=.034) (Fig.7). The ACR increased after the treadmill test (p=.039).

We analyzed additionally 5 IC patients as a control group. For this group the treadmill was set at the same speed but without any slope. The patients performed the test situation for 12 minutes (range 10–16 min) twice without experiencing any claudication symptoms. The blood and urine sampling was the same as previously described. In this group, exercise caused a decrease in mean ABI from 0.6 to 0.4 similar to the group experiencing calf pain where ABI decreased from 0.6 to 0.3. The ACR, however, did not increase at all after exercise (Fig.8). Furthermore, plasma levels of IL-6 were increased after exercise in the same manner as in the patients experiencing calf pain (Fig.9). The other parameters (hs-CRP, TNF-α and vWF) did not differ from the group experiencing calf pain.
ADFGF-4 GENE THERAPY STIMULATION OF VASCULAR GROWTH

ADFGF-4 in an animal model of limb ischemia

Perfusion measured by LDI was significantly decreased in both the active treatment group and in the placebo group 4 weeks after ischemic induction and treatment (Fig.10). The ratio of flow in the ischemic foot compared to the non-ischemic was less in the active treatment group (0.51) than the placebo group (0.62), p=.051. Figure 11 shows an example of LDI appearance.

No difference in capillary to muscle fiber ratio could be detected when comparing the ischemic limb of the active treatment group to placebo (p=.178) or in the non-ischemic limb of the two groups (p=.275), (Fig.12). Collaterals from the quadriceps muscle of the ischemic and the non-ischemic side had equal diameters (p=.593), and no difference in size could be detected between the active treatment group and the placebo group (p=.205) (Fig.13a). Similar number of collaterals per view, with a diameter greater than 10 µm, could be detected in the active treatment group compared to placebo (p=.807) (Fig.13b). Figure 14 shows an example of immunohistochemical staining.

No expression of mRNA for FGF-4 was detected 4 weeks after treatment.

Figure 8. Albumin/Creatinine Ratio (mean ± SEM) in IC patients before and 60 minutes after treadmill exercise showed an increase after the test (p=.039). In IC patients exposed to a treadmill test, not generating calf pain (controls), the ratio did not change after exercise (p=.144).

Figure 9. Plasma levels of IL-6 (mean ± SEM) in IC patients before exercise and 15, 60 and 120 minutes after treadmill exercise. In IC patients exposed to a treadmill test, not generating calf pain (controls), IL-6 increased even further.

Figure 10. LDI (perfusion units) was significantly decreased in both the active treatment group (p=.005) and in the placebo group (p=.028) 4 weeks after ischemic induction and treatment. The ratio of flow in the ischemic foot compared to the non-ischemic was less in the active treatment group (0.51) than the placebo group (0.62), p=.051 (Study III).
**Figure 11.** Example of LDI of the plantar aspects of the foot of the rat. Red and white color corresponds to high perfusion. Poorly perfused tissue is blue.

**Figure 12.** Capillary to muscle fiber ratio (Study III). No difference in capillary to muscle fiber ratio (number of CD-31 positive stained cells / muscle fiber) could be detected when comparing the ischemic limb of the active treatment group to placebo (p=.178) or in the non-ischemic limb of the two groups (p=.275).

**Figure 13.a)** Collateral diameter (Study III). The size of collaterals (smooth muscle α-actin positive stained vessels) in the quadriceps muscle of the ischemic leg in the active group and the placebo group were similar (p=.205).

**Figure 13.b)** Collateral number (Study III). Similar number of collaterals per view, with a diameter greater than 10 µm, could be detected in the active treatment group compared to placebo (p=.807).
Figure 14. Immunohistochemical staining with anti-CD-31 of anterior tibial muscle (a, b, c) and anti-smooth muscle α-actin of quadriceps muscle (d, e, f). Active treatment with AdFGF-4 (a and d), placebo group (b and e) and negative control without primary antibody (c and f). Brown colour (DAB) represents positive staining. Counterstaining with Hematoxylin.

AdFGF-4 in a Randomized Clinical Trial

Enrolment was prematurely terminated because of slow recruitment. Eventually 13 patients, 5 female and 8 male, with a mean age of 64 years (range 45-90) were included. Patient characteristics are displayed in Table 1. Ten patients received active treatment (AdFGF-4) and three received placebo (PBS).

Safety
There were no changes in vital signs, ECG, physical examination, eye examination or tumor screening results after treatment. The laboratory values were mostly associated to the underlying or concomitant disease and no differences were detected between the groups. The most frequently reported change in laboratory values involved glucose, CRP, differential blood count and gamma glutamyl transpeptidase (γ-GT). There was a tendency towards higher levels of CRP (Fig.15) in the active treatment group compared to placebo and γ-GT (Fig.16) also seemed to be higher at all time points in the active treatment group. The levels were however constantly higher already at baseline and were not related to study therapy. Adverse events were reported 35 times during the study period and of these 14 were considered as serious adverse events (SAE). Three of them were considered possibly related to study therapy (myalgia, toe pain and toe amputation) (Table 2). One patient, a 90 year old woman died of myocardial infarction six months after treatment. She had medical treatment for hypertension but no medical history of ischemic heart disease. This was not considered related to study therapy.
**TABLE 1. PATIENT CHARACTERISTICS**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Group</th>
<th>Age</th>
<th>Sex</th>
<th>PAD (III/IV), duration months</th>
<th>Concomitant disease</th>
<th>Previous interventional therapy</th>
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<tbody>
<tr>
<td>1(^a)</td>
<td>dose 1</td>
<td>62</td>
<td>M</td>
<td>III, 3</td>
<td>HT, IHD</td>
<td>1 (bilat), 2 (study leg)</td>
</tr>
<tr>
<td>2(^b)</td>
<td>dose 1</td>
<td>65</td>
<td>M</td>
<td>IV</td>
<td>HT, IDDM</td>
<td>4 (contralat)</td>
</tr>
<tr>
<td>3</td>
<td>dose 1</td>
<td>90</td>
<td>F</td>
<td>IV</td>
<td>HT</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>dose 1</td>
<td>45</td>
<td>M</td>
<td>IV, 6</td>
<td>IHD</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>dose 1</td>
<td>76</td>
<td>F</td>
<td>III, 4</td>
<td>HT</td>
<td>1, 2 (study leg)</td>
</tr>
<tr>
<td>6</td>
<td>dose 2</td>
<td>61</td>
<td>F</td>
<td>IV, 8</td>
<td>HT, IHD, DM, hyperchol.</td>
<td>1, 2 (study leg)</td>
</tr>
<tr>
<td>7</td>
<td>dose 2</td>
<td>63</td>
<td>M</td>
<td>IV, 10</td>
<td>IDDM, HT</td>
<td>1, 2, 3 (study leg)</td>
</tr>
<tr>
<td>8</td>
<td>dose 2</td>
<td>53</td>
<td>M</td>
<td>III, 6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>dose 3</td>
<td>68</td>
<td>M</td>
<td>IV, 1</td>
<td>HT</td>
<td>1, 3 (study leg)</td>
</tr>
<tr>
<td>10</td>
<td>dose 3</td>
<td>68</td>
<td>F</td>
<td>III, 3</td>
<td>IHD, HT, hyperchol.</td>
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</tr>
<tr>
<td>11</td>
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<td>49</td>
<td>M</td>
<td>IV, 2</td>
<td>Minor stroke, HT, left carotid artery occlusion, hyperchol.</td>
<td>1 (bilat)</td>
</tr>
<tr>
<td>12</td>
<td>placebo</td>
<td>76</td>
<td>M</td>
<td>III, 6</td>
<td>CABG, IDDM</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>placebo</td>
<td>55</td>
<td>F</td>
<td>III, 1</td>
<td>HT, CVL, Op ICA stenosis</td>
<td>1 (study leg)</td>
</tr>
</tbody>
</table>

\(^a\) study 1: PAD stage III  
\(^b\) study 2: PAD stage IV with diabetes mellitus  
\(^c\) (1) Reconstructive arterial surgery, (2) angioplasty, (3) toe amputation, (4) below knee amputation

Abbreviations: hypertension (HT), ischemic heart disease (IHD), insulin dependant diabetes mellitus (IDDM), non-insulin dependant diabetes mellitus (DM), coronary artery bypass surgery (CABG), cerebral vascular lesion (CVL), internal carotid artery (ICA) stenosis

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**Figure 15.** There was a tendency towards higher levels of CRP (mean ± SEM) in the three active treatment groups compared to placebo at all time points except for the last two timepoints. The levels were however higher in the active treatment group already at baseline.

**Figure 16.** The levels of γ-GT (mean ± SEM) were similar at all time points, also when comparing treatment groups to placebo.
TABLE 2: ADVERSE EVENTS

<table>
<thead>
<tr>
<th>Adverse Events</th>
<th>Placebo n=3</th>
<th>dose 1 n=5</th>
<th>dose 2 n=3</th>
<th>dose 3 n=2</th>
<th>Intensity</th>
<th>Relation to study drug</th>
</tr>
</thead>
<tbody>
<tr>
<td>Angina pectoris</td>
<td>1</td>
<td>mild</td>
<td></td>
<td>mild</td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Retinopathy</td>
<td>1</td>
<td>mild</td>
<td></td>
<td>mild</td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Back pain</td>
<td>1</td>
<td>mild</td>
<td></td>
<td>mild</td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Leg pain</td>
<td>2</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Headache</td>
<td>1</td>
<td>mild</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Peripheral edema</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Leg infection</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Urinary tract infection</td>
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<td>mild</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Joint swelling</td>
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<td>mild</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Claudication</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Spreading gangrene</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>possible</td>
<td></td>
</tr>
<tr>
<td>Below knee amputation</td>
<td>1</td>
<td>severe</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Phantome pain</td>
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<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
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</tr>
<tr>
<td>Fall on head</td>
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<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Femoro-popliteal bypass</td>
<td>1</td>
<td>severe</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Exclusion of neoplasm</td>
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<td>mild</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Anaemia</td>
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<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Nausea</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
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<tr>
<td>Appendicitis</td>
<td>1</td>
<td>severe</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Impaired healing postop</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Sepsis</td>
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<td>unlikely</td>
<td></td>
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<td>Hypoproteinemia</td>
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<td></td>
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<tr>
<td>Myalgia</td>
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<td>severe</td>
<td></td>
<td></td>
<td>possible</td>
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</tr>
<tr>
<td>Superficial phlebitis</td>
<td>1</td>
<td>moderate</td>
<td></td>
<td></td>
<td>unlikely</td>
<td></td>
</tr>
<tr>
<td>Toe amputation</td>
<td>1</td>
<td>severe</td>
<td></td>
<td></td>
<td>possible</td>
<td></td>
</tr>
</tbody>
</table>

Transfection and environmental risk

No AdFGF-4 was detected in any samples (blood, throat swab, urine or faeces), nor was FGF-4 protein detected in blood. Ad5 neutralizing antibodies were detected in 54% of patients before treatment and non specific IgG was slightly elevated in 39%. Antibody titres appeared to increase in a dose dependent manner and were not detected in the placebo group (Fig.17 a, b). No FGF-4 protein could be detected by immunohistochemistry in a muscle sample gathered eight weeks after gene transfer.

Efficacy evaluation

One patient experienced worsening of symptoms seven days after receiving active treatment and bypass surgery was performed three weeks later. Two patients were amputated during the 12 weeks follow up and another three within 11 months, all in the active treatment group. The clinical outcome for each patient is displayed in Table 3. The number or size of lesions or need for amputation as well as the analysis of all efficacy parameters combined did not indicate any differences between active treatment and placebo during the 12 week study period. Data resulting from the employment of methods intended to assess efficacy were difficult to interpret because of the small sample size. Figure 18 to 20 depicts the results of DSA (n=11), MRI (n=4) and Scintigraphy (n=5).
### TABLE 3. CLINICAL OUTCOME

<table>
<thead>
<tr>
<th>Group</th>
<th>ABI</th>
<th>VAS</th>
<th>Trophic lesions</th>
<th>Clinical outcome 6 months</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>bl w8</td>
<td>bl w8</td>
<td>bl w8</td>
<td></td>
</tr>
<tr>
<td>1*</td>
<td>dose 1</td>
<td>0.18</td>
<td>48</td>
<td>1</td>
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<td>2*</td>
<td>dose 1</td>
<td>0.38</td>
<td>71</td>
<td>80</td>
</tr>
<tr>
<td>3</td>
<td>dose 1</td>
<td>0.57</td>
<td>30</td>
<td>95</td>
</tr>
<tr>
<td>4</td>
<td>dose 1</td>
<td>0.92</td>
<td>56</td>
<td>62</td>
</tr>
<tr>
<td>5</td>
<td>dose 1</td>
<td>0.31</td>
<td>41</td>
<td>3</td>
</tr>
<tr>
<td>6</td>
<td>dose 2</td>
<td>1.47</td>
<td>72</td>
<td>81</td>
</tr>
<tr>
<td>7</td>
<td>dose 2</td>
<td>0.43</td>
<td>87</td>
<td>73</td>
</tr>
<tr>
<td>8</td>
<td>dose 2</td>
<td>0.58</td>
<td>64</td>
<td>22</td>
</tr>
<tr>
<td>9</td>
<td>dose 3</td>
<td>0.19</td>
<td>82</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>dose 3</td>
<td>0.60</td>
<td>27</td>
<td>13</td>
</tr>
<tr>
<td>11</td>
<td>placebo</td>
<td>0.27</td>
<td>80</td>
<td>60</td>
</tr>
<tr>
<td>12</td>
<td>placebo</td>
<td>0.61</td>
<td>78</td>
<td>40</td>
</tr>
<tr>
<td>13</td>
<td>placebo</td>
<td>0.38</td>
<td>76</td>
<td>66</td>
</tr>
</tbody>
</table>

* study 1: PAD stage III
  * study 2: PAD stage IV with diabetes mellitus

Abbreviations: ABI, ankle-brachial index; VAS, visual analogic scale; bl, baseline; w8, week 8; Op revasc, surgical revascularization; Bka, below knee amputation; MI, myocardial infarction; Aka, above knee amputation

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**Figure 18.** Digital Subtraction Angiography (DSA) assessing collateral count (a) and size (b) in the thigh.
Figure 19. Magnetic Resonance Imaging (MRI) assessing blood perfusion by:
(a) time to peak signal intensity after a reactive hyperemia test using a BOLD signal and
(b) time to peak signal intensity after a contrast bolus.

Figure 20. Scintigraphy was performed to evaluate blood flow during reactive hyperemia.
It is evident from clinical studies that the symptomatic treatment available for patients with PAD is not optimal and that there are room for new methods. When aiming to develop vascular growth stimulation as one such strategy it becomes obvious that our knowledge about the pathophysiological process in PAD is insufficient both in general and regarding arteriogenesis and angiogenesis per se. It is probable that the success of such new strategies will depend on the possibilities to gain information about what triggers and control vascular growth and the repair processes in ischemic tissue.

With this background the focus of this thesis, vascular growth factor expression and supplementation, could be regarded as a too small segment without proven relevance to the large pathophysiological and treatment problem in PAD. Hopefully the information gained in the studies included in this thesis can provide some valuable information.

**PATHOPHYSIOLOGICAL IMPLICATIONS FOR PAD**

**Expression of vascular growth factors**

Arteriogenesis appears to be important for a long time after onset of the PAD disease, whereas angiogenesis may be induced only in the acute ischemic situations or in border zones between ischemic and healthy tissue. This thesis, however, focus on the chronic forms of PAD, CLI and IC, where angiogenesis could be of minor importance. Another possibility is that these processes are synergistic, where arteriogenesis occurs close to the atherosclerotic lesion and angiogenesis in the hypoxic distal parts of the leg (Fig.21). According to this hypothesis, reaching the ACD might stimulate both processes in IC. In our patients we found an upregulated expression of VEGF mRNA in calf muscle which indirectly supports that exercise to ACD leads to hypoxia which stimulates angiogenesis distally (Study II).

**Figure 21.** Pathophysiological implications.
Arteriogenesis, the enlargement of small arteriole to collateral arteries bridging the stenosis has been investigated in animal models of limb ischemia where increased levels of ICAM-1 are expressed due to an activation of ECs from increased shear stress. Recruited macrophages upregulate the expression of FGF-2, TNF-α and MMPs leading to degradation of ECM and proliferation of ECs and SMCs (Arras et al 1998, Scholz et al 2000). Very little is known about this process in PAD patients.

Angiogenesis is stimulated by hypoxia and suggested to be of importance in the distal ischemic part of the leg. Increased levels of HIF-1α and VEGF have been shown in CLI patients (Rissanen et al 2002, Choksy et al 2004). We found increased levels of FGF-2 in CLI patients, probably released by inflammatory cells in the ischemic area. Increased levels of VEGF were detected after exercise. Systemic levels of inflammatory proteins and FGF-2 have been shown to be higher in PAD patients than in healthy controls (Rohovsky et al 1996, Tzoulaki et al 2005). We found elevated levels of IL-6 after exercise.
The findings are supported by other studies who observed enhanced VEGF mRNA expression in healthy subjects after exercise (Gustafsson et al., 1999; Hiscock et al., 2003). It is however unknown if this upregulation leads to actual new vessel growth in healthy or in PAD patients. The local upregulation of VEGF mRNA in our study was not reflected in plasma levels of the protein which is consistent with one earlier report (Gustafsson et al., 2005). Whether this indicates that the stimulated expression is insufficient or only a local process is also unknown.

We did not observe any indications that the arteriogenesis stimulating growth factor FGF-2 in plasma was upregulated in our IC patients. Furthermore we did not observe an upregulation of FGF-2 mRNA in the calf tissue. This is not surprising since the arteriogenic process involving activated inflammatory cells with production of FGF-2 is located to the thigh. We did find an increased expression of IL-6 after walking, which occurred both in IC patients reaching ACD and those who only exercised without reaching pain at all. This suggests that calf ischemia is not necessary to initiate an inflammatory response. Thus it is possible that exercise creates an increase in blood flow through collateral vessels bypassing the occlusion. Unfortunately we did not have any parameters reflecting collateral growth locally in the area of interest, the thigh. Percutaneous biopsies in this area would not give enough tissue and it would be difficult to know where to direct the needle. Furthermore we considered it unethical to obtain repeated samples of sufficient amount that would incorporate collateral vessels. In the literature very few studies have been able to prove the clinical importance of collateral growth in IC. While evident clinically on angiograms performed on IC patients only one study from our own group have attempted to assess collaterals in PAD patients (Fig.22) (De Vivo et al., 2005).

Whether angiogenesis and arteriogenesis are important repair processes for CLI patients is also unknown. Also for this patient group we only have indirect evidence based on growth factor expression and nothing else. VEGF and HIF-1α have been observed in distal muscle samples from amputated legs from patients with CLI (Rissanen et al., 2002; Choksy et al., 2004). In Study I we found VEGF levels higher in distal muscle in patients than in control subjects but not compared to the patients’ proximal muscle. Overall VEGF levels were not extensively upregulated locally in the ischemic leg. That the CLI patients in our study had low VEGF levels in distal tissue compared to proximal was quite surprising, especially considering our previously mentioned assumption that episodes of rest pain involve hypoxia and inflammation. The reasons why this contrasts to the previously mentioned human studies are probably differences between amputated tissue and the fact that our samples were gathered in the calf rather than in the foot (Rissanen et al., 2002; Choksy et al., 2004). Also for CLI there is a lack of studies showing actual

Figure 22. Angiography showing collateral arteries bridging a superficial femoral artery occlusion.
angiogenesis in the tissue. Rissanen et al., however, found that capillarity was increased in relation to VEGF levels indicating that angiogenesis may take place also in the chronic disease CLI (Rissanen et al., 2002). In Study I we also had the unique observation that the FGF-2 expression was increased in the calf. The source of this growth factor is probably inflammatory tissue. It is plausible that even a chronic disease such as CLI have episodes of ischemic insults accentuating inflammation, thus initiating FGF-2 expression. In our study immunohistochemistry of FGF-2 indicated enhanced staining around blood vessels, close to cells that probably were inflammatory cells. Accordingly FGF-2 has been detected in high levels in serum from patients with CAD and PAD (Rohovsky et al., 1996; Hasdai et al., 1997). The origin of this circulating FGF-2 could hypothetically be the ischemic heart and leg or from local processes of arteriogenesis but could also be generated in inflammatory cells within atherosclerotic lesions.

In summary our results and other studies suggest that angiogenesis may occur also in the chronic manifestations of PAD, IC and CLI. Data are conflicting, however, and the evidence is based on VEGF expression only. It is completely unknown if arteriogenesis occurs, but our results did provide some indirect indications that it does. Adequate methods to study this process are lacking and the tissue where arteriogenesis is likely to occur is hard to access.

Diabetes mellitus

We found evidence that patients with PAD and diabetes may have different growth factor expression patterns than patients without diabetes indicating a different pathology. Our patients had lower levels of FGF-2 in distal muscle compared to patients without diabetes. It has been suggested that the poor prognosis for patients with diabetes may be due to impaired arteriogenesis (McDaniel and Cronenwett, 1989; Schaper and Buschmann, 1999; Simons, 2005). Angiographic studies of patients with CAD as well as PAD have indicated lower number of collaterals in diabetic patients (Abaci et al., 1999; De Vivo et al., 2005). Accordingly a low growth factor production may be consistent with poor ability to form collaterals. Other mechanisms, however, may be responsible for this disturbance. In patients with diabetes monocyte migration, an important initiating event in arteriogenesis, towards growth factors such as VEGF has been shown to be impaired, possible due to a signal transduction defect downstream of the VEGFR-1 (Waltenberger et al., 2000). The mechanisms leading to impaired arteriogenesis in patients with diabetes may also be associated with hyperglycemia or metabolic consequences such as glycation of proteins or the formation of advanced glycation end products (AGE proteins) (Waltenberger, 2005).

Inflammatory markers in PAD

Inflammation is probably involved in arteriogenesis in PAD and also in angiogenesis if it occurs. In animal models, monocyte accumulation surrounds the tissue of collateral arteries after arterial occlusion (Arras et al., 1998; Hoefer et al., 2004). These cells are potent sources of inflammatory cytokines like IL-6 and TNF-α as well as vascular growth factors such as FGF-2. TNF-α has been demonstrated to act as a positive modulator of arteriogenesis which was attenuated by treatment with TNF-α inhibitors (Hoefer et al., 2002; Grundmann et al., 2005).

We found elevated levels of IL-6 in IC patients after treadmill exercise (Study II) (Fig.9). Accordingly the source of this cytokine could be a release from activated monocytes due to local increases in shear stress in collateral arteries bridging an occlusion in the superficial femoral artery. This would be in concordance with the inflammatory, monocyte-driven theory of arteriogenesis (Schaper and Scholz, 2003). Recently, IL-6 and TNF-α levels have been shown by increase in response to exercise in one study, which is in line with our findings (Signorelli et al., 2003). Furthermore, the concentration of CRP and TNF-α have previously been found not to be elevated in response to exercise (Fiotti et al., 1999). The IL-6 production could also originate from activated monocytes in atherosclerotic
lesions in general. In order to clarify where it comes from biopsies from the tissue surrounding collateral arteries needs to be analyzed for IL-6 and other inflammatory cytokines. This is planned for in future studies. Selecting and gathering muscle samples incorporating collateral arteries, however, appears to be very difficult.

As indicated, arteriogenesis and atherosclerosis share many features including the monocyte involvement with upregulation of adhesion molecules, cytokines, growth factors and matrix proteases, the stimulation of SMC migration and formation of a neointima. The main difference is that arterioles enlarge by adding SMC layers whereas larger arteries react with an intimal growth and plaque formation with subsequent narrowing of the lumen. The knowledge that atherosclerosis is an inflammatory disease has offered new opportunities for diagnosis and prediction and has indicated new anti-inflammatory treatment strategies (Libby, 2002; Ridker et al., 2005). It is possible that such treatments have negative implications for arteriogenesis.

GENE THERAPY STIMULATION OF VASCULAR GROWTH

Therapeutic arteriogenesis in animal model

Data in the literature as well as some of our results from Study I and II suggest that vascular growth factors already are upregulated in the distal leg in PAD patients (Rissanen et al., 2002; Choksy et al., 2004). Other studies have also found high levels of FGF-2 systemically in CLI (Rohovsky et al., 1996). If it originates from the leg it can be questioned why even further overexpression of this protein would be an appropriate strategy for arteriogenesis stimulation.

There is some evidence, particularly from animal models that suggest that very high concentrations of vascular growth factors are necessary for rapid and efficient vascular growth (Yang et al., 2000; Chang et al., 2003). Furthermore, focusing growth factor administration to proximal areas of collateral growth, i.e the thigh for PAD patients, where endogenous expression of growth factors not are abundant (Study I) could be more logical (Hershey et al., 2003).

Also the choice of growth factor to use for treatment is important. In preclinical studies using animal models of limb ischemia the use of VEGF was the initial strategy to try to stimulate vascular growth by stimulating ECs. The use of FGFs has been suggested as a better strategy for stimulating arteriogenesis as it stimulates proliferation of both ECs and SMCs. Several studies show beneficial effects of both recombinant protein and virus mediated gene therapy (Chleboun et al., 1992; Takeshita et al., 1994; Yang et al., 2000; Hoefer et al., 2001; Cao et al., 2003; Chang et al., 2003; Rissanen et al., 2003). In most studies, however, vascular changes are reported at relatively early time points or without evaluation of collateral growth.

The choice of FGF-4 as the primary growth factor to use for therapy is based on several facts. FGF-4 is a unique member of the FGF family because it is secreted under the control of a signalling peptide. This characteristic may improve secretion and make FGF-4 more efficient in stimulating collateral growth. This was the reason for using it in our gene therapy studies (Study III and IV). Several studies have shown that FGFs are able to promote arteriogenesis in animal models of hindlimb ischemia increasing collateral flow significantly during the first weeks after treatment (Yang et al., 1996; Stark et al., 1998; Baffour et al., 2000).

In our study (Study III) we could not detect any signs of increased arteriogenesis or angiogenesis four weeks after gene transfer with adenovirus mediated FGF-4. These results differ somewhat from a simultaneous study using the same vector and growth factor (Rissanen et al., 2003). They detected an increase in collateral diameter measured by angiography four weeks after gene therapy, but the early signs of increased capillary ratio and increased politeal blood flow did not remain at four weeks. Although angiography is often used to quantify collaterals, the lack of spatial resolution hampers its ability to accurately assess collateral count and size and should there-
fore be regarded as a complementary method only (Fuchs et al., 2001; Waters et al., 2004). While we did not have any early time points in our study the results at four weeks are similar. The lack of findings that suggest an effect of treatment could have a number of explanations (Fig.23). For example it could be associated to the gene therapy methodology; poor adenoviral transfection or low or transient expression of the gene. It could also be explained by limited arteriogenic stimulation or vessel growth of short duration. This could be a consequence of using FGF-4 and not other growth factors. The lack of signs of angiogenesis in Study III is not surprising since the primary aim of the study was to restore blood flow by stimulating arteriogenesis. As mentioned before this is probably a better strategy for treatment of limb ischemia (Herzog et al., 2002).

Overall long lasting effects of therapeutic arteriogenesis or angiogenesis has been difficult to achieve. Hoefer et al stimulated arteriogenesis by adding monocyte chemoattractant factor -1 (MCP-1) in an animal model of limb ischemia but observed no effects four weeks after treatment (Hoefer et al., 2001). Accordingly it is possible that other strategies are required to develop collaterals that are stable long term. Because monotherapy have been inadequate to induce sustained vascular growth our group have used of a combination of PDGF-BB and FGF-2 (Cao et al., 2003). Stable vessels up to one year after treatment were observed in that study. The temporal and spatial coordination of different arteriogenic stimuli may also have impact on their activity and effect.

Clinical applications

Considering the limited effect of AdFGF-4 treatment in Study III, continuing to a clinical trial using the same vector may be difficult to understand. This decision was based on the encouraging results early after treatment in the study by Rissanen et al and a positive effect of a clinical trial for CAD, AGENT, using a similar vector construct (Grines et al., 2002; Grines et al., 2003; Rissanen et al., 2003; Gao et al., 2004). The trial was anticipated to assess the optimal dose besides the usual safety aspects.

Clinical trials of therapeutic angiogenesis and arteriogenesis

There have been eight prospective, randomized, double-blind, placebo-controlled trials of stimulation of vascular growth in the myocardium (Table 4). One trial using GM-CSF (Seiler et al., 2001), four VEGF (Losordo et al., 2002; Hedman et al., 2003; Henry et al., 2003; Kastrup et al., 2005) and three FGF (Grines et al., 2002; Simons et al., 2002; Grines et al., 2003). Of these eight only two phase I trials displayed a substantial effect of treatment. In one of these two, collateral flow index determined by invasive coronary pressure measurements was augmented after treatment with GM-CSF given intracoronary once and continued subcutaneously for 2 weeks (Seiler et al., 2001). In the other trial angina class status was improved at 12 weeks after intramyocardial delivery of VEGF (Losordo et al., 2002). As mentioned before the AGENT trials administered AdFGF-4 as a single intracoronary injection and showed a strong trend towards improved exercise treadmill test time and a great reduction in size of reversible perfusion defects as assessed by SPECT (Grines et al., 2002; Grines et al., 2003). The AGENT trials were the ones that supported use of the AdFGF-4 concept also in PAD patients. The promising results from the early AGENT trials were not repeated when the strategy was tried in a large phase III study that ended in 2004. The results are to our knowledge not published yet. In the other trials that included over 500 patients all administered growth factors using gene therapy and none accomplished any proof of clinically relevant treatment effects.

Recently, some stem cell based treatments aiming to stimulate collateral growth for CAD have been reported. These are mostly small uncontrolled phase I trials (Assmus et al., 2002; Fuchs et al., 2003; Perin et al., 2003; Tse et al., 2003). Less placebo-controlled trials aiming to stimulate vascular growth have been performed for PAD. Two trials using FGF-2 (Lazarous et al., 2000; Lederman et al., 2002), one VEGF (Rajagopa-
### TABLE 4. RANDOMIZED CLINICAL TRIALS IN CAD AND PAD

<table>
<thead>
<tr>
<th>Trial</th>
<th>Therapeutic agent</th>
<th>Delivery method</th>
<th>n</th>
<th>Primary endpoint</th>
</tr>
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<tbody>
<tr>
<td><strong>CAD</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Seiler, 2001</td>
<td>rhGM-CSF</td>
<td>i.c + s.c</td>
<td>21</td>
<td>Safety (pos CFI at 14d)</td>
</tr>
<tr>
<td>Losordo, 2002</td>
<td>pVEGF</td>
<td>i.m</td>
<td>19</td>
<td>Safety (pos angina CCS at 12w)</td>
</tr>
<tr>
<td>FIRST; Simons 2002</td>
<td>rFGF</td>
<td>i.c</td>
<td>337</td>
<td>ETT time at 90d</td>
</tr>
<tr>
<td>VIVA; Henry 2003</td>
<td>rhVEGF</td>
<td>i.c + i.v</td>
<td>178</td>
<td>ETT time at 60d</td>
</tr>
<tr>
<td>AGENT; Grines 2002</td>
<td>AdFGF-4</td>
<td>i.c</td>
<td>79</td>
<td>ETT time at 4w (pos ETT in subgroup)</td>
</tr>
<tr>
<td>AGENT 2; Grines 2003</td>
<td>AdFGF-4</td>
<td>i.c</td>
<td>52</td>
<td>SPECT at 8w (pos when excluding one outlier)</td>
</tr>
<tr>
<td>KAT; Hedman 2003</td>
<td>AdVEGF</td>
<td>i.c</td>
<td>103</td>
<td>Luminal diameter at 6 m after PCI, (pos SPECT in Ad group)</td>
</tr>
<tr>
<td>Euroinject One Trial; Kastrup</td>
<td>pVEGF</td>
<td>i.m</td>
<td>80</td>
<td>RPDS at 3m (pos local wall motion)</td>
</tr>
<tr>
<td><strong>PAD</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>Lazarous, 2000</td>
<td>rFGF-2</td>
<td>i.a</td>
<td>15</td>
<td>Safety (pos calf blood flow)</td>
</tr>
<tr>
<td>TRAFFIC; Lederman 2002</td>
<td>rFGF-2</td>
<td>i.a</td>
<td>190</td>
<td>PWT at 90d</td>
</tr>
<tr>
<td>RAVE; Rajagopalan 2003</td>
<td>AdVEGF</td>
<td>i.m</td>
<td>105</td>
<td>PWT at 12 w</td>
</tr>
<tr>
<td>START; van Royen 2005</td>
<td>rhGM-CSF</td>
<td>s.c</td>
<td>40</td>
<td>PWT at 14d</td>
</tr>
<tr>
<td>Study IV, 2005</td>
<td>AdFGF-4</td>
<td>i.m</td>
<td>13</td>
<td>Safety</td>
</tr>
</tbody>
</table>

Abbreviations: i.c. - intracoronary, s.c. - subcutaneously, i.a. - intraarterially, i.m. - intramuscularly, i.v. - intravenously; CFI – collateral flow index; CCT – Canadian Cardiovascular society angina class ETT – exercise treadmill test; RPDS – reversible perfusion defect size; SPECT – single photon emission computer tomography; PWT – peak walking time

Ian et al., 2003) and one GM-CSF (van Royen et al., 2005) have been published. The first was a phase I trial evaluating intra-arterial recombinant FGF-2 protein administration into the femoral artery to patients with IC (Lazarous et al., 2000). The therapy was not related to severe side effects and patients displayed some improvement in calf blood flow. The follow-up trial, TRAFFIC, with similar study design included 190 IC patients (Lederman et al., 2002). Both active and placebo groups demonstrated an increase in ACD after 90 days, but patients receiving FGF-2 could walk longer without pain. Interestingly, the group of patients that was given a double dose of FGF-2 had similar walking distance as controls. This study is the only phase II trial of therapeutic angiogenesis/arteriogenesis in PAD to show a clear benefit of treatment in its primary efficacy measure. Statistical significance, however, was only achieved by applying a secondary intention-to-treat nonparametric analysis capturing all subjects. Also noteworthy is the fact that the placebo effect on ACD at 90 days was relatively low compared to 180 days, indicating that the entire treatment success was due to an unusually low placebo effect at 90 days.
In another trial, RAVE, a high-dose or low-dose AdVEGF, or placebo was administered i.m. to 105 patients with IC (Rajagopalan et al., 2003). All groups, including those given placebo, demonstrated an increase in ACD at 12 weeks. The final example is the START trial which applied GM-CSF or placebo subcutaneously for a period of two weeks in 40 patients with IC (van Royen et al., 2005). Also in this study no difference between the groups was observed. The only study focusing on CLI patients, besides our Study IV is a randomized, controlled trial of stemcell therapy (Tateishi-Yuyama et al., 2002). In this trial, 22 patients received i.m. injections of bone marrow mononuclear cells or placebo, which were peripheral blood mononuclear cells. The results showed a significant improvement in ABI, transcutaneous oxygen tension and rest pain symptoms at 4 weeks in the active treatment group compared to placebo. These fantastic results have to our knowledge, however, not been repeated.

In our clinical trial of AdFGF-4 administered i.m. to stimulate arteriogenesis, no clear signs of therapeutic efficacy were observed.

**Evaluation of therapeutic efficacy**

In our study, safety aspects were the primary outcome but clinical parameters such as ABI, pain, analgetic drug consumption, lesion size and number were recorded as well. A number of efficacy methods were applied for assessment of collateral growth (DSA, MRI and Scintigraphy). Unfortunately, the value of clinical efficacy assessment was limited due to the small number of patients that were included in the study and that only few underwent each investigation. Outcome data was further hampered by the progression of disease leading to amputation in one patient and revascularization in another patient before efficacy evaluation at 8 weeks. Large efforts to develop appropriate methods for collateral and distal perfusion assessment were spent on this study. DSA is the golden standard for objectively assessing collateral growth in the literature. It evaluates the number and size of the vessels but not the function and due to its poor spatial resolution it has been questioned as a tool of assessing collateral growth (Simons et al., 2000; De Vivo et al., 2005). In this study standardization of DSA was employed as far as possible. In the future CT-angiography may evolve as a better method for collateral assessment (Pearlman et al., 1997). MRI appears to be the method of the future to measure therapeutic efficacy of perfusion and perhaps also collateral size and number (Mankad et al., 2003). Incorporating BOLD and contrast techniques during and after reactive hyperemia as in our study may be a way along this line. Reactive hyperemia seems to be a promising way to gain information on flow capacity of collateral vessels. Scintigraphy was added to the methods to provide extra information on particular skin perfusion. Unfortunately there is limited experience with Scintigraphy in PAD patients in the literature and only a few patients underwent investigations in our study.

It is almost impossible to draw any conclusions regarding efficacy from our study. The group of patients was small and heterogenous. Each clinical investigation was not performed at all study centres and only a few patients were examined by MRI and Scintigraphy. One positive finding was, however, that the MRI investigation appeared to be useful and of good quality.

DSA was performed in 11 patients, of whom one or two displayed angiograms after treatment that could be interpreted as a positive effect of treatment. Similar data was available for MRI performed in 4 patients, where two patients appeared to have improved perfusion after active treatment. Scintigraphy data was very difficult to interpret because of variability. Also primary outcome parameters were difficult to evaluate (Table 3). Rest pain, evaluated by visual analogic scale (VAS), was decreased in 5 and increased in 3 patients in the active group at 8 weeks while ABI was increased in 3 and decreased in 3 patients in the active treatment group. Information on ulcers was very variable.

The primary goal of stimulating arteriogenesis in CLI patients is relief of symptoms. There is no consensus of the optimal way to evaluate therapeutic efficacy in either CAD or PAD clinical tri-
als. The lack of adequate methods is reflected in the vast number of efficacy parameters that have been used in the randomized clinical trials published (Lazarous et al., 2000; Lederman et al., 2002; Rajagopalan et al., 2003; van Royen et al., 2005). In the previously mentioned phase I study with a positive effect, calf blood flow by strain gauge plethysmography was used (Lazarous et al., 2000). In the TRAFFIC and RAVE studies, primary outcome was change in ACD and secondary outcomes were claudication onset time, ABI and quality of life measures (Lederman et al., 2002; Rajagopalan et al., 2003). No methods to assess collateral growth were employed. The START trial also used walking time as primary outcome and Laser Doppler flowmetry was added to measure local skin perfusion on the great toes before and after treatment (van Royen et al., 2005). Every one of these methods has known disadvantages and advantages.

Safety aspects
In our study (study IV) as in many other early phase trials the most important outcome to evaluate was safety. We found no changes in vital signs, ECG, physical examination, eye examinations or tumor screening at follow up. There was a tendency towards higher levels of CRP in the active treatment group compared to placebo. This could be related to an immune response to the vector or to a local inflammatory reaction at the injection site. Liver enzymes were also elevated in the active treatment group but the levels were higher already at baseline and not effected by study therapy. Three out of 14 SAEs were considered possibly related to study therapy because of a temporal relationship to treatment. One patient, a 90 year old woman died of myocardial infarction six months after treatment but this event was not considered related to treatment. AdFGF-4 was not detected in any samples (blood, throat swab, urine or faeces), nor was the FGF-4 protein detected in blood. Antibody titres appeared to increase in a dose dependent manner and were not detected in the placebo group. Overall, events were few in our patient and besides a possible relationship between AdFGF-4 treatment and aggravation of symptoms no safety concerns appeared.

Three previous studies using a similar vector and growth factor delivered intracoronary for CAD have been performed (AGENT, AGENT 2, AGENT 3) (Grines et al., 2002; Grines et al., 2003). In total, over 600 patients have been included in these trials. Including data from AGENT 3s’ 415 patients, AdFGF-4 was well tolerated, not effecting heart rate or blood pressure during intracoronary administration and no rise in cardiac enzymes, changes in ECG or signs of myocardiitis was associated to treatment. As in our study a majority of patients increased antibody titres to adenovirus but this was unrelated to any adverse events. During infusion and one hour after treatment, the vector was detected in the pulmonary artery as well as in peripheral blood in a dose dependent manner (Grines et al., 2002). No virus particles were detected in urine. FGF-4 protein was not found in plasma at any time points after treatment. In general safety assessment was similar as in our study. Examples of adverse events include 8 patients with transient fever within 24 hours after treatment and 3 patients with a transient increase in liver enzymes. Also in these studies one patient died during follow up due to myocardial infarction. More worrisome is that three patients in the initial AGENT trials who received active treatment were diagnosed with malignancy. One was a 68 year old man with heredity for colon malignancy who was diagnosed with metastatic colon cancer 9 months after treatment. A second patient had a brain tumor (glioblastoma multiforme) diagnosed about 2.5 months after treatment. The tumors were negative for AdFGF-4 by PCR and had no signs of increased vascularity. Both patients died despite surgery. A third patient was diagnosed with squamous cell skin cancer. Although an effect of the therapeutic agent can not be excluded, the malignancies were considered unlikely to have been caused by the product. The frequency of cancer among the patients in the studies was similar as in the general population for this age group. Overall AdFGF-4 gene therapy appears to be rather safe, but the associa-
tion to malignancies indicate that caution must be employed in future studies.

**Gene therapy considerations**

Experience has demonstrated that despite success with therapeutic arteriogenesis in animal models, this does not necessarily translate into positive results in humans. Our poor results in Study IV highlights the problems with efficacy, and Study III the lack of long term effect also in some animal studies. The explanations for the lack of prominent effect could arise at any of the crucial steps in the gene therapy process. Some examples are presented in Figure 23. Furthermore, a large number of questions remains to be explored both regarding the stability of the new collaterals in animal models and the conversion of the experimental findings to human trials. As previously discussed, supplementary details of the pathophysiology of PAD and collateral growth are definitely needed before new clinical trials are initiated. Regarding gene therapy per se, questions still remains: Which vector is best to use? What growth factors or cytokines should we chose? What is the optimal dose and route of administration?

**Vectors**

The most commonly used vector for gene delivery in the cardiovascular field are adenoviral vectors based on serotype 5. These viruses infects a broad range of cells but transfection of endothelial cells and smooth muscle cells is probably poor (Thirion et al., 2002; Nicklin et al., 2004). This may have reduced transfection efficacy in our Studies III and IV. Ad vectors mostly accomplish expression of proteins during the first week and peak expression occurs at 24 h after adenoviral gene transfer in muscle (Perrin et al., 2004). During the second week it declines to basal levels (Hershey et al., 2003; Gounis et al., 2005). The duration of gene expression mediated by adenoviruses may thus be insufficient to give rise to stable vessels. This, together with the fact that adenoviruses evoke an immune response which hampers repeated administration, suggests that other types of vectors or modification of existing vectors may be more efficient.

Adeno-associated viral (AAV) vectors supposedly are even more efficient for skeletal muscle transduction and thereby produce sustained transgene expression. Shimpo et al found expression of VEGF for at least 10 weeks after i.m. gene transfer in a rat hindlimb ischemia model using AAV (Shimpo et al., 2002). This virus however, has very low tropism for ECs and therefore are not ideal for cardiovascular gene therapy (Dishart et al., 2003). Several strategies have been used to modify the tropism of Ad and AAV vectors, i.e. serotype switching, genetic engineering and use of antibodies (Baker, 2004). This might improve the applicability of gene therapy as well as its safety and efficacy.

**Growth factors**

Another issue which influences the efficacy of therapeutic arteriogenesis is the choice of growth factor. Several growth factors and cytokines have been used for angiogenesis and arteriogenesis stimulation in animal models including VEGF, FGFs, but also PDGF-BB, MCP-1 and HGF (Baffour et al., 2000; Yang et al., 2000; Buschmann et al., 2001; Taniyama et al., 2001; Shimpo et al., 2002; Cao et al., 2003; Schirmer and Royen, 2004; Gounis et al., 2005). FGFs are known to stimulate EC and SMC proliferation and thus have been suggested as better candidates for therapeutic arteriogenesis than VEGF. FGF is a family of heparin-binding growth factors and signalling requires receptor tyrosine kinases that form high-affinity complexes with FGF and heparan sulphate (HS) proteoglycans at the cell surface. It has been suggested that assembly of these complexes requires simultaneous recognition of distinct sulphating patterns within the HS chain and that tissue-specific HS synthesis thereby may regulate FGF signalling (Allen et al., 2001). Allen et al showed that FGF-4 fails to activate aortic endothelial cells in culture and that FGF-2 but not FGF-4 binds HS in the heart and major blood vessels in mouse embryos at embryonic day 18, suggesting a disturbance in this regulation (Allen et al., 2001). The failure of FGF-4 to bind HS on these cells correlates with a failure of FGF-4 to stimulate these cells sug-
suggested that FGF receptors require specific HS sulphation sequences in order to recognize a specific FGF. Accordingly, our lack of effect may also have been influenced by our use of FGF-4 rather than FGF-2. The concept of HS binding impairment has, however, recently been contradicted by others (Kreuger et al., 2005). The good results of AdFGF-4 stimulating therapy in animal models of cardiac and hindlimb ischemia 2 and 4 weeks after gene transfer also questions this (Rissanen et al., 2003; Gao et al., 2004). The ideal growth factor or combination of factors remains to be identified.

**Dose**

Allocating the optimal dose of the growth factor is another important issue for efficacy and safety in gene therapy. Generally, when using recombinant protein therapy with a known quantity administered, a more precise dose-response relationship can be determined. Protein therapy, however, necessitates continued delivery to provide adequate amounts of growth factors. In gene therapy, sufficient transfection, translation and transcription are required before the protein is produced effectively. It is difficult to assess the dose eventually achieved and it is influenced by several factors. One example is that the viral vector needs to escape the immune system. Adenoviral infections are common worldwide and specific neutralizing antibodies against Ad5 are detectable in 60% of the population (Schulick et al., 1997). This immune response may reduce transfection efficacy and prevents repeated administrations. In Study IV one contributing factor to poor treatment efficacy could be that 54% of the patients had neutralizing antibodies against Ad5.

Several preclinical toxicology and dose-response studies were performed with the AdFGF-4 vector construct before the clinical trials (not published). Based on these results the dose of $10^{11}$ vp/animal was used in Study III. In the first phase I clinical trial administrating AdFGF-4 intracoronary to patients with CAD, doses from 3.3 x $10^8$ to 3.3 x $10^{10}$ vp were used without safety concerns (Grines et al., 2002). As mentioned, this study found positive effect of treatment. Based on these results the same doses were selected for our phase I/II study.

**Route and site of administration**

The agent can be administered systemically or locally. For therapeutic arteriogenesis gene therapy techniques utilized to date involve plasmid DNA or adenovirus containing the gene of interest and the route of administration is better suited for some vectors and organs than others. Adenoviral vectors, for instance, can be administered by the intravascular route while plasmid DNA rapidly would be degraded by DNases. Intramuscular delivery is probably an appropriate route of delivery for plasmids to skeletal and myocardial muscle. This requires meticulous administration so that all areas where arteriogenesis is likely to occur are targeted. Systemic administration of the therapeutic agent is attractive because of the ease of access but unsuitable because of the risk of non-specific or toxic responses. For the leg, local administration either intra-arterial or intramuscular is probably preferable.

Finding the optimal site for gene or protein delivery in PAD is complex. The significant atherosclerotic lesion is often located in the superficial femoral artery in the thigh, whereas the ischemic tissue is located in the calf muscle and foot. The arteriogenic process stimulated by increased shear stress is close to the stenosed or occluded artery and the angiogenic activity is thought to be located in the distal ischemic region or in border zones between ischemic and non-ischemic tissue. In previous clinical trials in PAD the therapeutic agent has been administered into the common femoral artery (Lazarous et al., 2000; Lederman et al., 2002), or to muscle close to the stenosed artery (Rajagopalan et al., 2002; Rajagopalan et al., 2003). The same site was chosen for our Study IV.
The E1 region of the adenoviral genome is replaced by the gene of interest (FGF-4) and integrated into a helper cell genome. Adenovirus bind via the knob domain to the CAR receptor and a second interaction between \(\alpha_v\beta_3\) or \(\alpha_v\beta_5\) integrins and the penton base of the capsid occurs that mediates endocytosis of the complex. The vector binds to nuclear pore complex and enters the nucleus where it locates episomal. Transcription and translation of the gene of interest occurs and the protein is secreted. FGF-4 binds to FGFR on ECs and SMCs leading to activation of signalling cascades with upregulation of adhesion molecules and stimulation of migration and proliferation. Cellular and humoral immunity neutralize the virus and eliminate infected cells.

**Considerations for adenoviral gene therapy**

A. Optimal dose and route of administration  
B. Transfection of the cell  
C. FGF-4 secretion and duration of gene expression  
D. Immune response to the adenovirus  
E. FGF-4 protein binding and receptor activation
FUTURE DIRECTIONS

Endogenous arteriogenesis in response to a significant stenosis or occlusion is a complex interplay of many different growth factors, cytokines and proteolytic enzymes with inflammatory cells initially playing a key role. Little information about arteriogenesis in patients and the capacity of angiographically obvious collaterals to ease symptoms is available. One example of important issues to be resolved is the effect of exercise that seems to improve walking performance in claudicants. Our results indicate that exercise in claudicants leads to a release of the pro-inflammatory cytokine IL-6 probably originating from collaterals exposed to increased shear. To verify this assumption new studies are needed where biopsies are gathered preferably from the area where this process is likely to occur, the thigh. A clinical study in this context would involve different exercising types as our results indicate that exercise to the pain limit as well as exercise without reaching pain at all is responsible for the observed inflammatory response. Interestingly, a more profound activation of arteriogenesis might be the result of low graded exercise for a long time period. Results emerging from such studies might have great implications for everyday clinical practice. Proximal inflow stenoses even in asymptomatic patients may be important to abolish as the increase in flow is essential to induce the arteriogenic process.

There is still lack of information about the state of the muscle in CLI patients. Information about muscle metabolism and necrosis should be obtained from biopsies gathered distally from specific muscle groups and locations in the calf depending on the distribution of arterial lesions. Increased knowledge about risk factors affecting collateral development is also needed. One such important risk factor is diabetes. It has been suggested that the poor prognosis for this group of patients might be connected to impairment of collateral growth. Our results indicate that patients with diabetes might have different growth factor expression patterns indicating a unique pathology in this group of patients. To find more information about the pathophysiology, biopsies and blood samples from diabetic and non-diabetic patients could be compared by exposing the patients to increased blood flow demands, i.e. by treadmill exercise or by hyperaemic tests.

Stimulating the arteriogenic process by simply adding one single growth factor is probably not a good way of establishing stable and functional collateral arteries. Taking into account the complexity of the process, it seems more realistic to add a factor that can initiate a cascade of events. This could lead to release of a number of growth factors and cytokines resembling the endogenous process of arteriogenesis. Future trials using such combinations are on its way. The optimal route of administration, however, is not known. The most attractive proposal is to administer the stimulating agent where it is thought to exert its effects, intra-arterially into small arterioles. To achieve a high local concentration it could be performed by endovascularly occluding the circulation while injecting into the deep femoral artery. It could probably also be managed by placing an occluding cuff above the knee. In future clinical trials the objective assessment of efficacy is also of great importance. It should preferably include objective methods such as MRI or CT angiography together with clinical parameters.

Another interesting issue is the relation between atherosclerosis and arteriogenesis. Both are closely connected to inflammatory events in the vascular wall. Presumably, anti-inflammatory treatment reduces the initiation and progression of the atherosclerotic disease. Such treatment might on the other hand hamper arteriogenesis. One study suggestion might be to investigate patients receiving high doses of anti-inflammatory treatment i.e. patients with rheumatic disease and compare their collateral function to other patient groups.
I. In patients with critical limb ischemia we have demonstrated an association between distal ischemia and elevated levels of FGF-2 but not VEGF in calf muscle compared to proximal muscle.

II. In patients with intermittent claudication exercise to the absolute claudication distance evokes an increase in the inflammatory cytokine IL-6 while TNF-α and CRP remained unchanged. The hypoxia in the calf caused by the treadmill test caused an upregulation of VEGF-A mRNA in calf muscle tissue two hours after exercise.

III. Intramuscular administration of adenovirus mediated FGF-4 in a rat model of chronic limb ischemia does not appear to stimulate angiogenesis or arteriogenesis detectable at 4 weeks after gene transfer assessed by capillary density, collateral count and Laser Doppler imaging. No expression of FGF-4 mRNA was detected in the treated muscle 4 weeks after gene transfer.

IV. Intramuscular administration of adenovirus mediated FGF-4 at doses up to 2.87 x 10^10 viral particles in patients with critical limb ischemia were generally well tolerated and appeared to be safe. Transfection efficacy at these concentrations may have been limited or local. The small sample size did not allow any firm conclusions regarding efficacy.
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