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TUOMAS RISSANEN

# Gene Transfer for Blood and Lymphatic Vessel Growth

Doctoral dissertation

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## ABSTRACT

Insufficient blood flow to the heart or lower limbs due to coronary artery disease or peripheral arterial disease causes severe inability and more death than any other disease in the developed countries. Conventional revascularization strategies, angioplasty and bypass surgery, are effective in improving both symptoms and prognosis of patients with ischemic disease. However, all patients, such as the elderly with comorbidity or those with extensive and severe vascular occlusions, may not be managed with these approaches. Inadequate function of lymphatics due to a genetic or acquired defect causes morbidity through swelling of extremities (lymphedema) with no proven treatment. The aim of this study was to develop novel gene therapy-based treatment strategies for ischemic disease and lymphedema via therapeutic induction of blood and lymphatic vessel growth i.e. angiogenesis and lymphangiogenesis, respectively. Firstly, we investigated gene expression in human lower limb ischemia with a DNA array to find factors involved in ischemia-induced angiogenesis. We found that vascular endothelial growth factor (VEGF), together with its major regulators, hypoxia-inducible factor-1 and -2, and the main signaling receptor VEGFR-2, were potently upregulated in skeletal muscle ischemia suggesting an important role for VEGF in revascularization of ischemic tissue. Next, a novel rabbit hindlimb ischemia model with ischemia restricted to the calf was developed in order to study the therapeutic potential of adenoviral gene transfer of VEGF family members and fibroblast growth factor-4 (FGF-4). Intramuscular injections of adenovirus were found to be superior to intra-arterial injections for gene transfer in skeletal muscle. Adenoviruses encoding the VEGFR-2 ligands, VEGF and VEGF-D<sup>ΔNΔC</sup>, as well as FGF-4, were found to be the most potent angiogenic factors and also promoted arteriogenesis i.e. growth of collateral arteries that bypass the vascular occlusion. FGF-4 upregulated endogenous VEGF expression, which may at least partly explain the angiogenic effects of FGF-4. Injections of adenoviral VEGF resulted in up to 15- and 36-fold increases in capillary vessel growth and perfusion, respectively, in rabbit hindlimb skeletal muscle six days after the gene transfer. The angiogenesis response consisted predominantly of the enlargement of preexisting capillaries via proliferation of endothelial and perivascular cells resulting in the formation of arteriole-like vessels. Vascular growth achieved with adenoviral gene transfer was transient as the majority of the effects lasted up to two weeks. No pathological blood vessel growth was observed. The NOGA catheter system-mediated intramyocardial injections of adenoviruses encoding VEGF and VEGF-D<sup>ΔNΔC</sup> stimulated efficient transmural angiogenesis and perfusion increases in the pig heart. Naked plasmid DNA was inefficient as a gene transfer vector in myocardium. As a side-effect, efficient angiogenesis increased vascular permeability, which resulted in transient edema in skeletal muscle and pericardial effusion in the heart. However, no irreversible adverse effects such as tissue damage occurred due to edema. Contrast-enhanced MRI allowed non-invasive visualization of angiogenesis-related vascular permeability. Perfusion increases in skeletal muscle and myocardium could be measured quantitatively and non-invasively with contrast-enhanced ultrasound imaging. Adenoviruses encoding the VEGFR-3 ligands, VEGF-C, VEGF-C<sup>156S</sup>, VEGF-D and VEGF-D<sup>ΔNΔC</sup>, stimulated up to 22-fold increases in lymphatic vessel growth in rabbit skeletal muscle. Nitric oxide was found to be a crucial mediator of angiogenesis but not lymphangiogenesis. The VEGFR-1 ligand VEGF-B did not promote either type of vessel growth. In conclusion, gene transfer of blood vessel growth factors may be a novel treatment for myocardial or peripheral ischemia, either alone or in combination with conventional revascularization procedures. Gene transfer of lymphangiogenic growth factors may be used to alleviate lymphedema.

National Library of Medicine Classification: QZ 50, WG 300, WG 500, WH 700

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*There are no half measures.*

*- Laphroaig, Scotland*

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Kuopio, November 2003

A handwritten signature in black ink, appearing to read 'Tuomas Rissanen', with a long horizontal stroke extending to the right.

Tuomas Rissanen

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## ABBREVIATIONS

AAV	adeno-associated virus	MAPK	mitogen-activated protein kinase
ABI	ankle brachial (blood pressure) index	mRNA	messenger ribonucleic acid
Ad	adenovirus	MCP-1	monocyte chemoattractant protein-1
Akt	serine-threonine kinase Akt (PKB)	MMP	matrix metalloproteinase
Ang	angiopoietin	MRI	magnetic resonance imaging
$\alpha$ SMA	$\alpha$ -smooth muscle actin	MRS	magnetic resonance spectroscopy
BM	basement membrane	NO	nitric oxide
CABG	coronary artery bypass grafting	NRP	neuropilin
CAD	coronary artery disease	PAD	peripheral arterial disease
CAR	coxsackie/adenovirus receptor	PAI-1	plasminogen activator inhibitor-1
cDNA	complementary deoxyribonucleic acid	PBS	phosphate buffered saline
CEU	contrast-enhanced ultrasound	PDGF	platelet-derived growth factor
CLI	critical limb ischemia	PDGFR	PDGF receptor
EC	endothelial cell	PECAM	platelet endothelial cell adhesion molecule (CD31)
ECM	extracellular matrix	pfu	plaque forming units
ELISA	enzyme-linked immunosorbent assay	PGI <sub>2</sub>	prostacyclin
eNOS	endothelial nitric oxide synthase (NOS III)	PLC $\gamma$	phospholipase C $\gamma$
EPC	endothelial progenitor cell	PIGF	placenta growth factor
FGF	fibroblast growth factor	PI3K	phosphatidylinositol-3-OH-kinase
FGFR	FGF receptor	PKC	protein kinase C
Flk-1	fetal liver kinase-1 (murine VEGFR-2)	PTA	percutaneous transluminal angioplasty
Flt-1	fms-like tyrosine kinase-1 (VEGFR-1)	PTCA	percutaneous transluminal coronary angioplasty
Flt-4	fms-like tyrosine kinase-4 (VEGFR-3)	RT-PCR	reverse-transcriptase polymerase chain reaction
GM-CSF	granulocyte macrophage-colony stimulating factor	Tie-2	tyrosine kinase with immunoglobulin and epidermal growth factor homology domains-2 (Tek)
GT	gene transfer	TNF	Tumor necrosis factor
HIF	hypoxia-inducible factor	SMC	smooth muscle cell
IGF	insulin-like growth factor	VEGF	vascular endothelial growth factor (human: VEGF <sub>165</sub> ; mouse: VEGF <sub>164</sub> )
IGFR	IGF receptor	VEGFR	VEGF receptor
iNOS	inducible nitric oxide synthase (NOS II)	vp	viral particles
i.a.	intra-arterial	VPF	vascular permeability factor (VEGF)
i.m.	intramuscular/intramyocardial		
i.v.	intravenous		
KDR	kinase domain region (human VEGFR-2)		
<i>LacZ</i>	$\beta$ -galactosidase (marker gene)		

## LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to by their Roman numerals:

- I Tiina T. Tuomisto\*, Tuomas T. Rissanen\*, Ismo Vajanto, Anna Korkeela, Juha Rutanen and Seppo Ylä-Herttua. **HIF-VEGF-VEGFR-2, TNF- $\alpha$  and IGF pathways are upregulated in critical human skeletal muscle ischemia as studied with DNA array.** *Submitted for publication.*
- II Tuomas T. Rissanen, Ismo Vajanto, Mikko O. Hiltunen, Juha Rutanen, Mikko I. Kettunen, Mari Niemi, Pia Leppänen, Mikko P. Turunen, Johanna E. Markkanen, Katja Arve, Esko Alhava, Risto A. Kauppinen and Seppo Ylä-Herttua. **Expression of vascular endothelial growth factor and vascular endothelial growth factor receptor-2 (KDR/Fik-1) in ischemic skeletal muscle and its regeneration.** *American Journal of Pathology; 160:1393-1403 (2002).*
- III Ismo Vajanto\*, Tuomas T. Rissanen\*, Juha Rutanen, Mikko O. Hiltunen, Tiina T. Tuomisto, Katja Arve, Outi Närvänen, Hannu Manninen, Heikki Räsänen, Mikko Hippeläinen, Esko Alhava, Seppo Ylä-Herttua **Evaluation of angiogenesis and side effects in ischemic rabbit hindlimbs after intramuscular injection of adenoviral vectors encoding VEGF and LacZ.** *The Journal of Gene Medicine; 4:371-380 (2002).*
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- VI Juha Rutanen\*, Tuomas T. Rissanen\*, Johanna E. Markkanen, Marcin Gruchala, Päivi Silvennoinen, Antti Kivelä, Antti Hedman, Marja Hedman, Tommi Heikura, Maija-Riitta Ordén, Steven A. Stacker, Marc G. Achen, Juha Hartikainen and Seppo Ylä-Herttua. **Adenoviral catheter-mediated intramyocardial gene transfer using the mature form of VEGF-D induces transmural angiogenesis in porcine heart.** *Circulation, in press*

\* Authors with equal contribution. Also some unpublished data are presented.



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## INTRODUCTION

A number of patients presenting with myocardial or lower limb ischemia are not suitable candidates for conventional treatments such as bypass surgery or angioplasty. Furthermore, in many cases the outcome of these therapies is not completely satisfactory even after a technically successful procedure, often due to reduced regenerative capacity of the aged patient population.

Growth of blood vessels is required for normal embryonic development, growth and tissue repair. In the recent years, regenerative medicine has emerged and introduced approaches to take advantage of nature's own tools to restore compromised blood circulation in the heart and skeletal muscle. This novel treatment to promote tissue perfusion by means of gene transfer (GT) or utilization of bone marrow-derived stem cells is called therapeutic angiogenesis. Similarly, inadequate lymphatic vasculature due to a genetic defect or surgery leads to impaired drainage of the lymph from peripheral tissues causing edema. In contrast to the beneficial role of sufficient blood and lymphatic vasculature, excessive angiogenesis and lymphangiogenesis contribute to a number of diseases, such as tumor growth and metastases, psoriasis, rheumatoid arthritis, diabetic retinopathy and atherosclerosis.

In the 1980's and 1990's the vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) families, capable of regulating blood and lymphatic vessel growth both in the good and in the bad, were discovered. At the same time, the development of recombinant DNA techniques allowed the cloning of the members of these families and other genes into naturally occurring GT vectors, viruses, which had been genetically modified to lose their replicative capability to cause disease. Viral vectors engineered to transfer the genes of potent vascular growth factors formed a tool that could be used to stimulate the growth of blood and lymphatic vessels in tissues with insufficient blood or lymph flow.

## REVIEW OF THE LITERATURE

### ISCHEMIC DISEASE AND DISEASES OF THE LYMPHATICS

#### Atherosclerosis

Atherosclerosis is the main cause of insufficient blood supply to tissues i.e. ischemia, most commonly affecting the heart, peripheral muscles and the brain (Dormandy and Rutherford, 2000; Libby, 2002; Grech, 2003). The most important risk factors for atherosclerosis are well known: genetic background, high serum LDL and low HDL cholesterol levels, high blood pressure, diabetes, smoking, low physical activity, and the male gender.

A significant stenosis of a large conducting artery leads to reduced performance and pain under exercise, and eventually also at rest (Grech, 2003). Rupture of an atherosclerotic lesion, on the other hand, may cause either a transient ischemic attack or an infarction (Libby, 2002).

#### Myocardial ischemia

Despite significant advances in its prevention, coronary artery disease (CAD) remains the leading cause of death in the Western world (Grech, 2003). When a coronary artery is narrowed by > 50% in diameter or > 75% in cross sectional area by an atherosclerotic plaque, blood flow through the vessel is reduced so much that angina may be experienced during stress depending on endogenous collateral formation (Grech, 2003; Wustmann et al., 2003).

Acute coronary events arise when endothelial injury exposes the thrombogenic core of the plaque to blood leading to initiation of the coagulation cascade and subsequent thrombus formation. A vulnerable plaque may also detach and block blood flow downstream. In acute myocardial infarction, occlusion is more complete than in unstable angina, where the occlusion is usually subtotal and may resolve.

Percutaneous transluminal coronary angioplasty (PTCA) is better than fibrinolysis for the primary revascularization of ST-segment elevation myocardial infarction in terms of death, reinfarction and stroke 30 days after the procedure (Andersen et al., 2003). Coronary artery bypass grafting (CABG) and PTCA with stenting are the primary interventional therapies of chronic stable angina (O'Toole and Grech, 2003). The long-term advantage of these treatments are limited by graft failure and post-angioplasty restenosis, although the use of internal mammary artery as a graft in CABG and the recent introduction of drug eluting stents seem to have alleviated these problems (He, 1999; Moses et al., 2003).

Revascularization of the ischemic heart has also been attempted using laser to bore holes in the myocardium, but sham-controlled randomized clinical trials have not shown any benefit of the procedure (Saririan and Eisenberg, 2003). The last treatment option of the failing heart is transplantation.

Secondary prevention of CAD includes antiplatelet drugs such as acetylsalicylic acid and clopidogrel; anti-hypertensive drugs such as  $\beta$ -blockers, diuretics and ACE inhibitors; statins and nitrates in conjunction with risk factor modification (O'Toole and Grech, 2003).

### **Lower limb ischemia**

The most common symptom of peripheral arterial disease (PAD) is pain during walking that resolves at rest (claudication). When the atherosclerotic arteries are not capable of providing sufficient blood flow to the lower limb, ischemia occurs also at rest. Chronic critical limb ischemia (CLI) is characterized by long-lasting (>14 days) rest-pain and/or non-healing ischemic ulcers and tissue loss (Dormandy and Rutherford, 2000). Acute CLI is most commonly caused by acute thrombotic occlusion of a pre-existing stenotic arterial segment or by embolus, and to lesser extent by a popliteal aneurysm or trauma. In acute ischemia characterized by complete occlusion, revascularization must be done within 6 h to save the affected parts of the limb. Acute-on-chronic CLI is

defined by deterioration of chronic limb ischemia into CLI in less than 14 days.

The age-adjusted (average age 66 years) prevalence of PAD in the US population is approximately 12% as assessed by noninvasive testing (Criqui et al., 1985). Intermittent claudication has a prevalence of 3-6% in men at the age of 60-70 years (Dormandy and Rutherford, 2000). 50-75% of these patients remain stable without any treatment, but the risk of amputation is still approximately 1% per year. The estimated incidence of chronic CLI is 500-1000 per million per year. The quality of life indices of patients with CLI are similar with those suffering from cancer at the terminal phase (Albers et al., 1992).

Patients with PAD are treated by three different approaches. The first is a conservative approach for mild or moderate claudication including the control of general risk factors, exercise therapy, drug therapy for secondary prevention of atherosclerosis, and in some cases treatment with vasodilators (Hiatt, 2001; Stewart et al., 2002). The second approach is percutaneous transluminal angioplasty (PTA) for the treatment of claudication, rest pain and non-healing ischemic ulcers. PTA is best suited for short stenoses of the iliac and superficial femoral arteries, having one year patency rate of 90% and 80%, respectively (Dormandy and Rutherford, 2000). The third option, which is reserved for patients with widespread lesions, is surgical treatment either with endovascular or open vascular surgery using vein grafts or synthetic prostheses. Aortobifemoral and femoro-popliteal bypass grafting have five year patency rates of 90% and 70%, respectively (Dormandy and Rutherford, 2000).

In spite of advances in surgical and interventional radiological techniques, about 20-30% of patients with chronic CLI cannot be treated by any conventional approach, because of the severity and extent of the disease or due to a poor general health status, and thus amputation is the only remaining option (Dormandy and Rutherford, 2000). Amputation in aged and weak patients results in a high risk of operative mortality and morbidity. Nearly 40% of patients with an amputated limb will have died within 2 years after operation (Dormandy and Rutherford, 2000).

## **Lymphedema and metastatic spread of cancer via lymphatics**

If lymphatic drainage is obstructed by infection, surgery, trauma or a genetic defect, accumulating fluid causes swelling of the affected tissues. The primary form of lymphedema, which is either hereditary or of unknown etiology, is quite rare with an estimated incidence of 1:6000 in newborns. Acquired (secondary) lymphedema as a result of tissue damage due to surgery, infection (most commonly Filariasis) or radiation therapy is relatively common. In the US alone, 3-5 million patients are estimated to suffer from secondary lymphedema (Rockson, 2001).

Normally, the remaining lymphatic vessels slowly regenerate after tissue damage (Paavonen et al., 2000). In contrast, the genetic defects causing hypoplastic cutaneous lymphatics result in persistent edema in the limbs (Rockson, 2001). In the congenital hereditary form of primary lymphedema (Milroy's disease) the superficial lymphatics are hypoplastic or aplastic while in the late onset form (Meige's disease) lymphatics are usually larger than normal (Rockson, 2001). Recently, missense mutations in the tyrosine kinase domain of VEGF receptor-3 (VEGFR-3) leading to its functional inactivity were implicated in some cases of human primary lymphedema (Karkkainen et al., 2000). Regardless of the reason, lymphedema causes tissue fibrosis, impaired wound healing and susceptibility to infections.

Although primary lymphatic neoplasms are rare, the lymphatics play a critical role mediating the metastatic spread of most cancers, which often determines their prognosis (Jeltsch et al., 2003). Experimental models suggest that the promotion of tumor lymphangiogenesis by VEGF-C or VEGF-D overexpression contributes to the dissemination of the primary tumor via the lymphatics (Karpanen et al., 2001; Skobe et al., 2001; Stacker et al., 2001). Furthermore, experimental lymphatic metastasis can be at least partly blocked by soluble VEGFR-3 (Karpanen et al., 2001). Although the clinical importance of the degree of tumor lymphangiogenesis for the metastatic spread remains to be shown, the possibility of blocking the

dissemination of cancer by inhibition of lymphatic growth factor signaling merits further research.

## **STRUCTURE AND FUNCTION OF BLOOD AND LYMPHATIC VESSELS**

Large arteries are muscular conduits with walls comprising an intimal, medial and adventitial layer divided by the internal and external elastic laminae. Arteries branch several times until reaching the size of arterioles. After further divisions, arterioles give rise to terminal arterioles (metarterioles) and finally to capillaries. From capillaries blood enters venules and then larger collecting veins until it drains into the right side of the heart.

Importantly, only small changes in vessel diameter affect blood flow rate tremendously. Poiseuille's law states that laminar blood flow is proportional to the fourth power of the vessel radius:

$$Q = \frac{\pi \Delta P r^4}{8 \eta l} \quad (1)$$

where  $Q$  is the blood flow rate,  $\Delta P$  is the pressure difference between the ends of the vessel,  $r$  is the vessel radius,  $l$  the vessel length, and  $\eta$  is blood viscosity.

### **Arterioles, capillaries and venules**

Physiological blood perfusion is tightly adapted to the metabolic needs at rest, exercise, inflammation, growth and tissue repair (Guyton and Hall, 2000a). Normally, only a portion of capillaries is perfused whilst the others stay in reserve. Contractile state of the precapillary sphincter, located at the point where the capillary originates from a terminal arteriole (metarteriole), determines whether blood cells can enter the capillary or not. Local  $O_2$  concentration is the most important factor regulating blood flow through this gate (Guyton and Hall, 2000c). Microcirculation in skeletal muscle is illustrated in Figure 1.

The regulation of tissue perfusion and the majority of peripheral resistance, and hence, blood pressure occurs at the level of

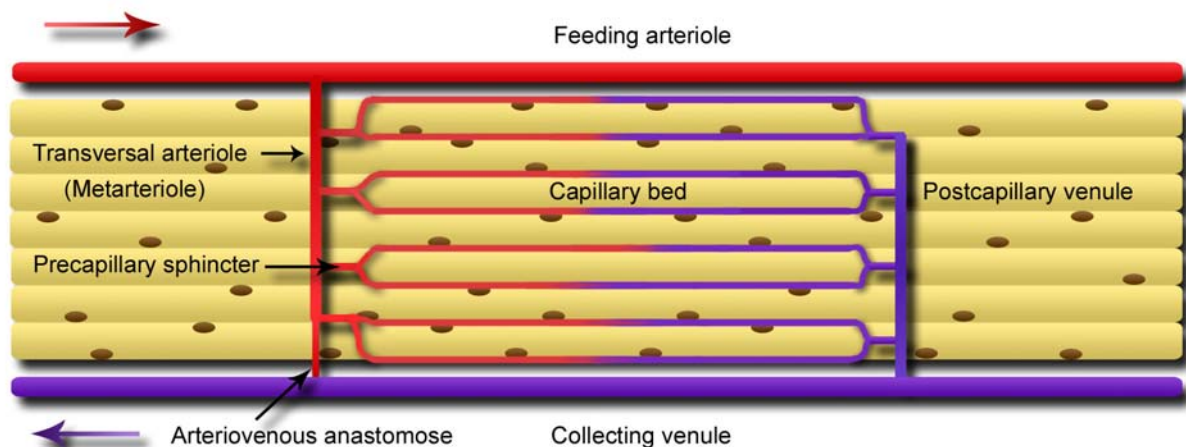
arterioles, which have a typical diameter between 10-100  $\mu\text{m}$  (Ross et al., 1995). In addition to the endothelial layer, arterioles consist of a basement membrane (BM) and one to two continuous and contractile smooth muscle cell (SMC) layers capable of changing the lumen diameter manifold (Figure 2). In the terminal arterioles the perivascular cells cover the vessel only at intermittent points.

The most important function of the circulation occurs at the level of capillaries. Capillaries are particularly well suited for the exchange of gases, nutrients, hormones, and cellular metabolites between the circulation and tissues because of the low blood velocity (approximately 0.3 cm/s), a thin wall and a close physical association with surrounding cells (Ross et al., 1995; Guyton and Hall, 2000a). Capillaries are about 0.3-1 mm in length, and the diameter is just large enough (4-9  $\mu\text{m}$ ) to allow the passage of red blood cells. Capillary endothelial cells (ECs) are attached to each other by tight junctions, surrounded by a BM and occasionally scattered perivascular smooth muscle actin-rich cells called pericytes (Figure 2). The capillary blood pressure is 30 mmHg and 10 mmHg in the arterial and venous ends, respectively, resulting in a mean pressure of about 17 mmHg (Guyton and Hall, 2000c). After passing through the capillary bed in 1-3 s,

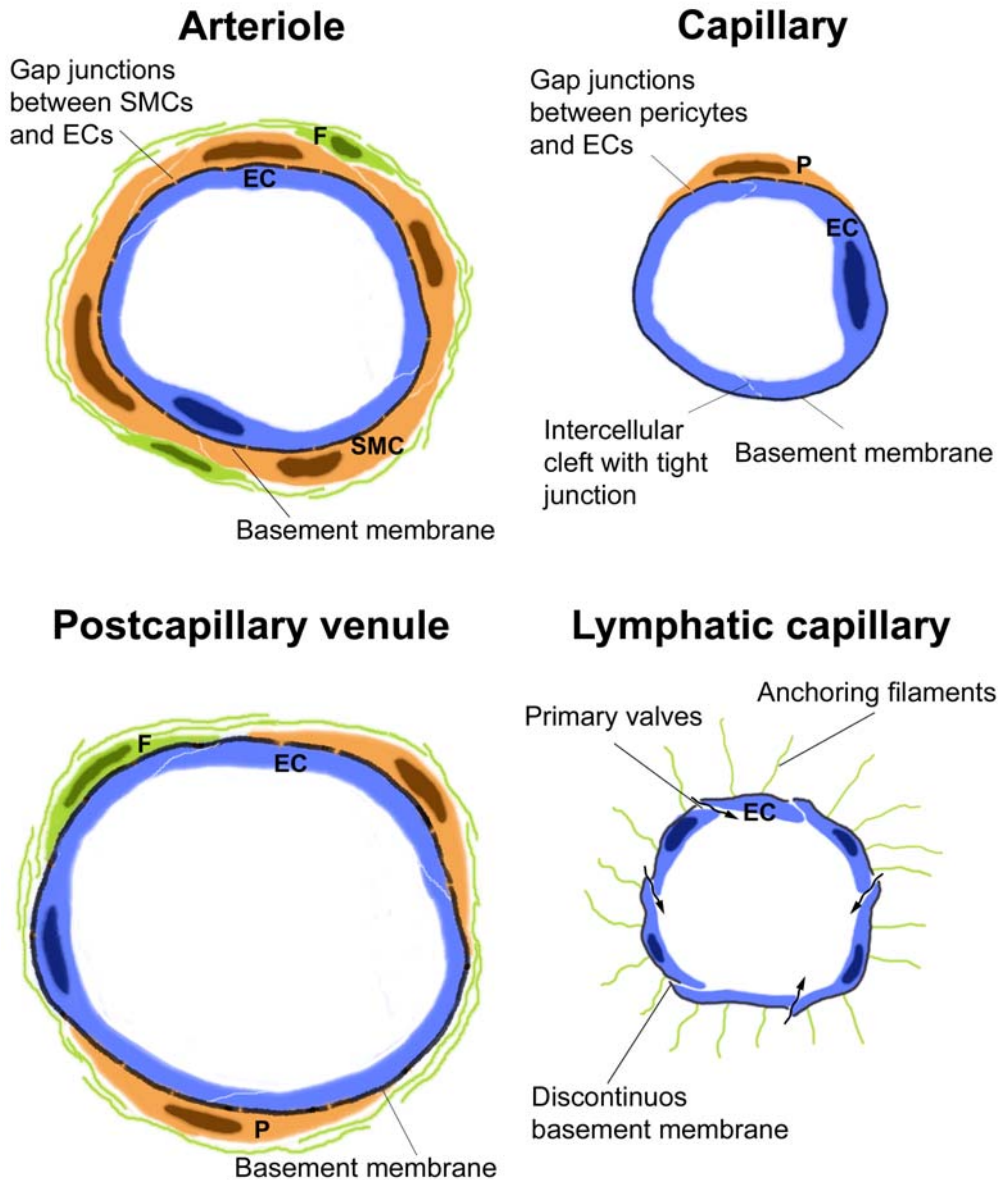
the  $\text{O}_2$ -depleted and  $\text{CO}_2$ -rich red blood cells enter post-capillary venules (Figure 2) that are 10-50  $\mu\text{m}$  in diameter and resemble capillaries in structure but have more pericytes (Ross et al., 1995). Leukocyte infiltration into tissues normally occurs mainly through the walls of post-capillary venules. In muscular venules (50-100  $\mu\text{m}$  in diameter), which drain into the smallest veins, pericytes are replaced by a continuous SMC-layer (Guyton and Hall, 2000c). Despite having a thinner layer of SMCs than arterioles, muscular venules are also capable of contracting considerably because of the low blood pressure inside them.

### Components of microvessels

Due to the functional heterogeneity, it is obvious that ECs, pericytes, SMCs and the BM, are not identical in all blood vessels but have distinct morphology and function depending on their location (Ross et al., 1995; Kalluri, 2003). It appears that both hemodynamics and specific signaling mechanisms control the differentiation of blood vessels. For example, VEGF together with the Jagged-Notch system promotes arterial differentiation at the cost of the venous phenotype even before the onset of blood flow (Lawson et al., 2002).



**Figure 1.** Schematic diagram of microcirculation in skeletal muscle. Blood flow to the capillary bed is controlled by precapillary sphincters and arteriovenous anastomoses (shunts) that guide excess blood flow directly to veins.



**Figure 2.** Schematic structure of an arteriole, capillary, postcapillary venule and lymphatic capillary (initial lymphatics). In blood vessels pericytes and SMCs regulate the tonus of the vessel and, subsequently, blood flow. Lymphatic capillaries do not have pericytes but are attached to their surroundings by anchoring filaments. In lymphatic capillaries, the primary valves formed by ECs, together with the discontinuous BM, permit high molecular weight substances to enter the lymphatics (arrow) but prevent backflow to the tissue. EC = endothelial cell, SMC = smooth muscle cell, P = pericyte and F = fibroblast. Modified from Ross et al. (1995).

The embryonic fate of vascular cells is not irreversible. Instead, they demonstrate significant phenotypic plasticity also in the adult and can transdifferentiate depending on the microenvironment. An example of this phenomenon is the reversible transformation of vein grafts towards an arterial phenotype in response to increased blood pressure and fluid shear stress (Fann et al., 1990). In most tissues, such as

skeletal muscle and heart, capillary endothelium is of the continuous type (Ross et al., 1995). Capillaries with fenestrated endothelium reside most commonly in the gastrointestinal mucosa, endocrine glands, choroid plexus and renal glomeruli. Furthermore, sinusoids within the liver, spleen and bone marrow have discontinuous endothelium that is even more permeable than the fenestrated type.

In contrast, ECs together with astrocytes constitute the tight blood-brain barrier which prevents the passage of many toxic compounds into the brain.

ECs of distinct vessel types express different enzymes and molecular markers. For example, ECs of arteries, veins and lymphatics express alkaline phosphatase, dipeptidylpeptidase and 5' nucleotidase, respectively, all of which can be histologically stained (Grim and Carlson, 1990; Matsumoto et al., 2002). Commonly used molecular markers for ECs include CD31 (PECAM), CD34, von Willebrand factor, Tie-2 and PAL-E (which is not expressed in arteries) (McDonald and Choyke, 2003). Ephrin B2 is a marker of arterial ECs and SMCs whereas Eph B4, the receptor for Ephrin B2, is expressed only in veins (Wang et al., 1998). Recently, *in vivo* phage display technology and the use of vascular "zip codes" has enabled the targeting of the endothelium of different organs or tumors with therapeutic agents (Pasqualini et al., 2002).

The BM is self-assembled by products secreted both by ECs and pericytes (Kalluri, 2003). Type IV collagen, laminin, perlecan, nidogen (entactin) and proteoglycans are the main components of the BM. ECs normally remain quiescent when they are bound to the BM, indicating that it produces signals that prevent EC proliferation (Kalluri, 2003).

Pericytes are a heterogeneous population of cells that can differentiate into other mesenchymal cell types, such as SMCs, fibroblasts and osteoblasts (Gerhardt and Betsholtz, 2003). On the other hand, fibroblasts may differentiate into myofibroblasts and subsequently into pericytes (Tomasek et al., 2002). Pericytes may have altered expression of markers in various tissues and tumors.  $\alpha$ -smooth muscle actin ( $\alpha$ SMA) is expressed both by immature and mature pericytes whereas desmin only by mature pericytes (Morikawa et al., 2002; Gerhardt and Betsholtz, 2003).

## Lymphatic vessels

The lymphatic vessels were first identified in the seventeenth century as "lactaea venae" or milky veins (Asellius, 1627). The

interstitial space of all organs except the central nervous system, cartilage, epidermis, bones and endomysium of muscles contain proper lymphatics (Guyton and Hall, 2000c).

The lymphatic system originates as lymphatic capillaries (initial lymphatics) that are EC tubes lacking SMCs and a continuous BM (Figure 2), and are thus highly permeable to proteins, triglycerides and even cells of the immune system (Casley-Smith, 1980). In skeletal muscle and intestine almost all the lymphatics are of the capillary type, while the muscular collecting lymphatics arise outside these organs (Schmid-Schonbein, 1990a). Lymphatics have two valve systems that prevent backflow of the lymph: a set of primary valves in the wall of lymphatic capillaries and a secondary valve system in the lumen of lymphatics.

The most important function of the peripheral lymphatics is to drain macromolecules and excess fluid that has escaped from the blood circulation (2-3 liters per day) and to offer a pathway to cells of the immune system back to the lymph nodes (Guyton and Hall, 2000c). Before the lymph enters larger collecting lymphatics, it passes through one or more lymph nodes where antigens are introduced to the immune system (Schmid-Schonbein, 1990b). The collecting lymphatics and large lymphatic vessels with muscular walls pump the lymph into the venous system via the thoracic duct or the right main lymphatic duct at the junctions of the left and right internal jugular and subclavian veins, respectively.

The rate of lymph flow is a result of interstitial fluid pressure, peristaltic contraction of SMCs, as well as external forces such as skeletal muscle contraction, external tissue compression and pulsation of neighboring arteries (Schmid-Schonbein, 1990b). In contrast to the effect on blood vessels, nitric oxide (NO) negatively regulates the lymph flow by relaxing the SMC layer of collecting lymphatics (Shirasawa et al., 2000).



## Vascular permeability and edema

Accumulation of water in the intracellular or extracellular space leads to formation of edema, excess fluid in tissue. Intracellular edema is usually caused by inability of cells to pump out sodium ions, leading to water retention and edema formation e.g. in infarcted tissues (Guyton and Hall, 2000b). Extracellular edema is caused either by abnormal leakage of fluid from blood vessels to the interstitial space or inability of the lymphatics to return extravasated fluid back to the circulation.

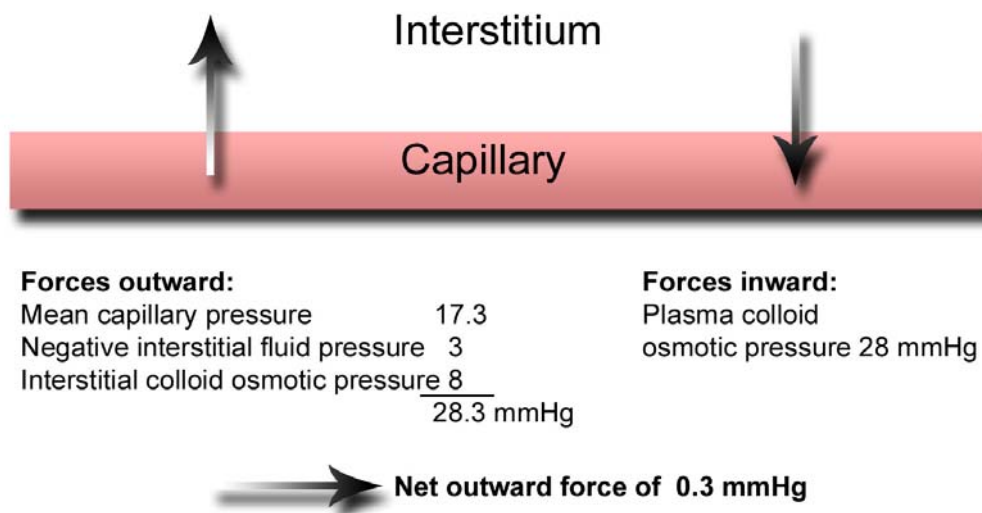
Increased plasma protein extravasation and resulting extracellular edema is firmly associated with angiogenesis (Dvorak et al., 1995). Thus, the understanding of the mechanisms controlling movement of molecules through the endothelium is important for the development of therapeutic angiogenesis. The physiological factors involved in vascular permeability induced by VEGF have previously been largely neglected (Bates et al., 1999).

The movement of substances and water between blood vessels and extravascular

space is principally controlled by two factors; the permeability properties of the endothelium and forces driving molecules (Bates et al., 1999; Guyton and Hall, 2000c). The most common cause of extracellular edema is excessive capillary fluid filtration, which can be expressed as follows (Starling equilibrium):

$$\text{Filtration} = K_f \times (P_c - P_{if} - \pi_c + \pi_{if}) \quad (2)$$

where  $K_f$  is the capillary filtration coefficient (product of permeability and surface area of the capillary),  $P_c$  is the capillary hydrostatic pressure,  $P_{if}$  is the interstitial fluid hydrostatic pressure,  $\pi_c$  is the capillary plasma colloid osmotic pressure and  $\pi_{if}$  is the interstitial fluid colloid osmotic pressure. The most important force driving fluid extravasation in the arterial end of capillaries is blood pressure (Figure 3). In the venous end, the colloid osmotic pressure of plasma, about 80% of which is caused by albumin, is of crucial importance (Guyton and Hall, 2000c).



**Figure 3.** Forces driving the movement of fluids between plasma and interstitial space in normal human capillaries according to Guyton (2000c).

Under physiological conditions in tissues containing capillaries with continuous endothelium such as skeletal muscle, about 0.5% of plasma to circulate through the extravascular space. However, the net outward filtration force of about 0.3 mmHg

causes approximately 1/10 of the extravasated fluid not to return to the circulation but to the lymphatics. Blockade of lymphatic flow causes especially severe edema because extravasated proteins have no other way to be removed.

Lipid-soluble molecules such as O<sub>2</sub>, CO<sub>2</sub> and NO can diffuse directly through cell membranes but water and water-soluble ions need to use the intercellular clefts or gaps between ECs. These clefts are 6-7 nm in width which is slightly smaller than an albumin molecule (Guyton and Hall, 2000c). However, only a minor size increase is needed to offer a route for albumin extravasation, which occurs e.g. in tumor-associated vessels (McDonald et al., 1999). Furthermore, fenestrae and vesicles such as those in the vesiculo-vacuolar organelle have been shown to contribute to the extravasation of plasma proteins, especially in tumors (Feng et al., 2000).

At least three factors in the interstitium prevent the formation of edema (Guyton and Hall, 2000b): 1) low compliance of the interstitium when the interstitial fluid pressure is in the negative range (can compensate for approx. 3 mmHg of increased capillary pressure), 2) one-way “washdown” of extravasated proteins into lymphatics (7 mmHg) and 3) the ability of lymphatics to increase their performance 10- to 50-fold (7 mmHg). Thus, capillary pressure may be theoretically increased by 17 mmHg (double the normal value) until significant edema develops. When the negative interstitial fluid pressure becomes positive as a result of excess vascular permeability, the capacity of the ECM to bind the extravasated fluid becomes exceeded and free fluid (effusion) accumulates progressively in the tissue. In the pericardial cavity the interstitial fluid pressure is normally -5 to -6 mmHg preventing the formation of effusion to some extent (Guyton and Hall, 2000b).

## **MECHANISMS OF BLOOD AND LYMPHATIC VESSEL GROWTH**

### **Vasculogenesis**

The first organ to develop in the embryo is the blood vasculature. It is formed in the beginning of the third embryonic week by the process called vasculogenesis i.e. the *de novo* formation and differentiation of blood vessels from vascular stem cells (Risau and Flamme, 1995).

In the yolk sac, the mesoderm-derived stem cells, hemangioblasts, form blood islands and give rise both to the vascular and hematopoietic cell lineages (Risau and Flamme, 1995). The cells in the interior of the primitive blood islands become hematopoietic cells whereas the outer layer of cells develop into angioblasts that further differentiate into the components of the vessel wall: ECs and mural cells. Vasculogenesis occurs similarly in the embryo.

ECs and mural cells proliferate and differentiate to assemble the early vascular plexus, which spreads by angiogenic sprouting and remodeling (Carmeliet and Collen, 1999). Extraembryonic vessels establish contacts with those inside the embryo thus connecting the embryo and placenta. Finally, the formation of a functional vascular network requires the organization of the arterial, venular and capillary circulation (Risau and Flamme, 1995).

### **Angiogenesis**

Embryonic cells initially obtain their O<sub>2</sub> by diffusion, but as the distance from the nearest capillary vessel exceeds 100 μm, new side branches are needed to supply oxygenated blood for the growing cells (Folkman, 1971; Risau and Flamme, 1995). This need is fulfilled by angiogenesis, defined as the sprouting of new capillaries from preexisting ones (Risau, 1997). Capillaries can also be split into daughter vessels by ECs (bridging) or by pericytes (intussusception) in the non-sprouting forms of angiogenesis (Risau, 1997). Wound healing, skeletal and hair growth, follicular growth and the development of the corpus luteum as well as the menstrual cycle of the endometrium are dependent on angiogenesis (Carmeliet, 2003).

Judah Folkman postulated in the early 1970's that tumors cannot grow without angiogenesis, and thus they secrete angiogenic growth factors (Folkman et al., 1971). He also proposed that tumor growth could be inhibited with anti-angiogenesis strategies (Folkman, 1971). Since then, this hypothesis has been confirmed and therapies have been designed to inhibit

tumor angiogenesis (Plate et al., 1992; Kim et al., 1993).

The adult vasculature is normally quiescent but during angiogenesis EC turnover can be rapid. Insufficient availability of O<sub>2</sub>, hypoxia, is the most important stimulus for physiological angiogenesis (Risau, 1997; Carmeliet, 2003). Metabolic stimuli such as low pH and hypoglycemia induce vessel growth, as well. Infiltrating macrophages are an important source of angiogenic growth factors in inflammation and tumors (Barbera-Guillem et al., 2002; Rehman et al., 2003).

During angiogenesis, ECs proliferate and migrate towards the stimulus, which is often VEGF expression activated by hypoxia-inducible factor-1 $\alpha$  (HIF-1 $\alpha$ ) (Semenza, 2000; Pugh and Ratcliffe, 2003). In angiogenic vessels, pericytes become loosely associated with ECs and actively participate in the formation of new capillary tubes (Morikawa et al., 2002; Gerhardt and Betsholtz, 2003). Extracellular matrix (ECM) and the BM are important for the function and survival of quiescent ECs, pericytes and SMCs providing a necessary contact surface and survival signals (Kalluri, 2003; Jain, 2003). ECM also functions as a reservoir for heparin binding growth factors which are released by proteinases (Bergers and Benjamin, 2003). In fact, the ECM binding domains of angiogenic growth factors such as VEGF<sub>165</sub> and platelet-derived growth factor-B (PDGF-B) are required for their proper action (Carmeliet et al., 1999; Lindblom et al., 2003).

ECM surrounding vessels together with the BM must be proteolytically dissolved in a balanced manner by proteinases such as MMPs (especially MMP-9) to let EC and pericytes migrate but still providing enough guidance and support (Bergers and Benjamin, 2003; Jain, 2003; Kalluri, 2003). Plasma proteins leaked from permeable, angiogenic vessels provide provisional ECM which further stimulates cell proliferation (Dvorak et al., 1995).

Factors associated with ECM turnover can be used as angiogenesis inhibitors. For example, tissue inhibitor of metalloproteinase-3 and thrombospondin-1 prevent the activation of MMPs (Rodríguez-Manzanique et al., 2001; Qi et al., 2003). Furthermore, the cleavage products of

collagen IV, VIII, XV and XVIII (endostatin) have been shown to inhibit angiogenesis and are currently being tested in clinical cancer trials (Kerbel and Folkman, 2002).

In a maturation process, ECs, pericytes, the BM as well as surrounding ECM are reorganized to form new stable capillary tubes that are less prone to regression than immature vessels (Benjamin et al., 1998). Insufficient coverage of newly formed vessels with mural cells leads to excess EC proliferation, permeability, fragility and even regression (Benjamin et al., 1998; Hellstrom et al., 2001b). Endothelium-derived PDGF-B is an important factor to control pericyte recruitment via its receptor PDGFR- $\beta$  (Lindahl et al., 1997).

In contrast to physiologically formed vessels, vasculature in tumors is often heterogeneous: disorganized, dilated, immature, leaky, prone to thrombosis and lacks pericytes and even ECs leading to excessive perfusion in some areas and insufficient in others (Bergers and Benjamin, 2003; Jain, 2003). Molecular markers that can be used for detection of angiogenic endothelium include integrins  $\alpha_v\beta_3$ ,  $\alpha_v\beta_5$  and  $\alpha_5\beta_1$  and VEGFR-2 (McDonald and Choyke, 2003).

### **Collateral artery growth (arteriogenesis)**

The presence of efficient coronary circulation confers protection from ischemia, infarction or even death after the obstruction of the main artery (Habib et al., 1991; Wustmann et al., 2003). Compromised blood flow to the ischemic region improves gradually as the preexisting arteriolar anastomoses enlarge to form collateral arteries that bypass the arterial occlusion (Schaper and Ito, 1996; Schaper and Scholz, 2003). This process is also called arteriogenesis (Schaper and Scholz, 2003).

Arteriogenesis is more important than angiogenesis in supplying blood to ischemic regions because collaterals provide bulk flow to the tissue unlike capillaries which only provide blood for the immediate cellular milieu. As discussed above, blood flow rate is proportional to the fourth power of the vessel radius according to Poiseuille's law (1). Thus, theoretically 42000 capillaries (7  $\mu$ m in diameter) provide an equivalent flow

to one collateral artery that is 100  $\mu\text{m}$  in diameter. In case of parallel collaterals with small size differences, both mathematical models and practice show that the larger ones are favored and grow whilst the smaller prune (Cornelissen et al., 2002; Schaper and Scholz, 2003).

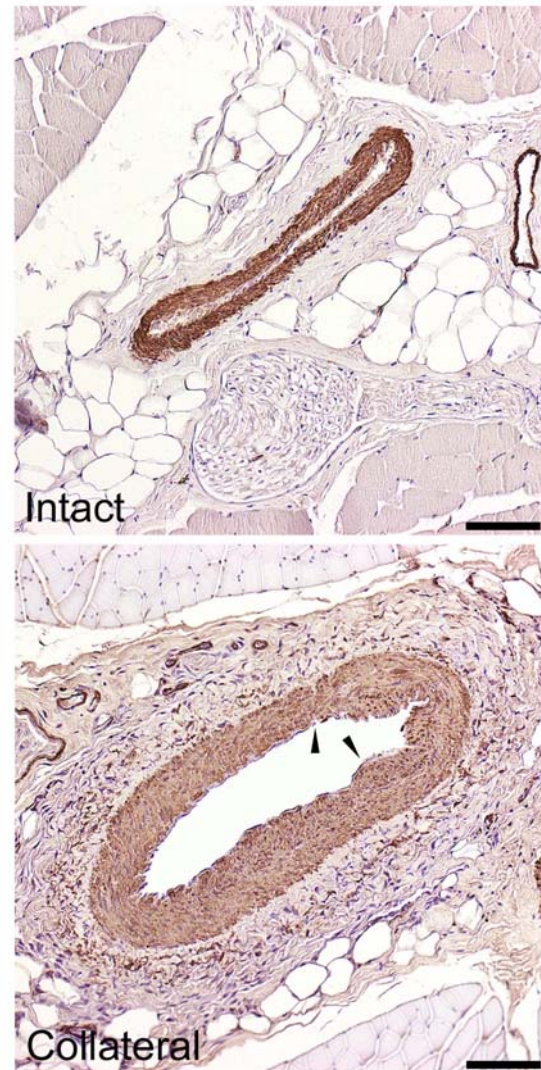
Ischemia is not a direct trigger for arteriogenesis as collaterals grow upstream to ischemic tissue (Ito et al., 1997a). Basically, two forces derived from blood flow mold the arteriolar anastomoses into bigger collaterals: circumferential wall stress against the medial layer and fluid shear stress against the endothelium (Schaper and Scholz, 2003). Circumferential wall stress is directly proportional to intravascular pressure and inversely proportional to wall thickness. On the other hand, fluid shear stress is proportional to blood flow velocity and inversely proportional to the cube of the vessel radius.

The endothelium is a crucial mediator of the vascular adaptation to blood flow (Langille and O'Donnell, 1986). However, it is not very well known how the endothelium transmits mitogenic signals to the media upon exposure to increased fluid shear stress but NO production and signaling via integrins appears to be important in this process (Nadaud et al., 1996; Muller et al., 1997; Jin et al., 2003). In fact, NO has been shown to be a crucial mediator of arteriogenesis in animal models (Matsunaga et al., 2000). Thus, an impaired endothelial function, caused e.g. by diabetes, results in decreased capacity to develop collaterals (Abaci et al., 1999).

Unfortunately, endogenous arteriogenesis stops prematurely when the conductance of <50% of normal has been reached because of the diminished fluid shear stress and circumferential wall stress due to collateral enlargement and wall thickening (Hoefer et al., 2001; Buschmann et al., 2003). Also the tortuous shape of collaterals increases resistance and is a self-limiting factor in arteriogenesis (Schaper and Scholz, 2003). As a consequence, endogenous collaterals are never as efficient as the original artery.

Cell proliferation and remodeling occur in the intima, media and adventitia in a growing collateral artery (Figure 4). Medial SMCs lose their contractile phenotype and gain synthetic activity resembling the

embryonic gene expression pattern (Schaper and Scholz, 2003). Eventually upon maturation, SMCs regain their contractile phenotype and also neointimal growth disappears. Typical collaterals are corkscrew-shaped in angiograms because they also grow lengthwise.



**Figure 4.** Collateral growth (arteriogenesis) involves remodeling of the intima, media and adventitia. Compared to an intact artery in the rabbit hindlimb, the media and adventitia of a collateral artery are clearly hypertrophic a week after excision of the femoral artery ( $\alpha\text{SMA}$  immunostaining for SMCs). The endothelium of the collateral is partially denuded, most likely because of increased shear stress, causing some neointimal growth (on the right side of arrowheads). Scale bar = 100  $\mu\text{m}$ .

Local inflammation of the vessel wall caused by the rapid increase in fluid shear stress is thought to play an important role in

arteriogenesis, especially in the initiation phase (Ito et al., 1997b; Arras et al., 1998). Cytokines such as monocyte chemoattractant protein-1 (MCP-1) and adhesion molecules have been reported to entice monocyte-macrophages to collaterals where they secrete growth factors such as FGFs (Arras et al., 1998). In support of the inflammation theory, mice with a genetic depletion of tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ) or TNF- $\alpha$  receptor p55 have impaired collateral growth after femoral artery occlusion as compared to wild-type controls (Hoefler et al., 2002). Furthermore, in diabetic patients the effect of VEGF on monocytes was shown to be attenuated leading to impaired collateral formation (Waltenberger et al., 2000).

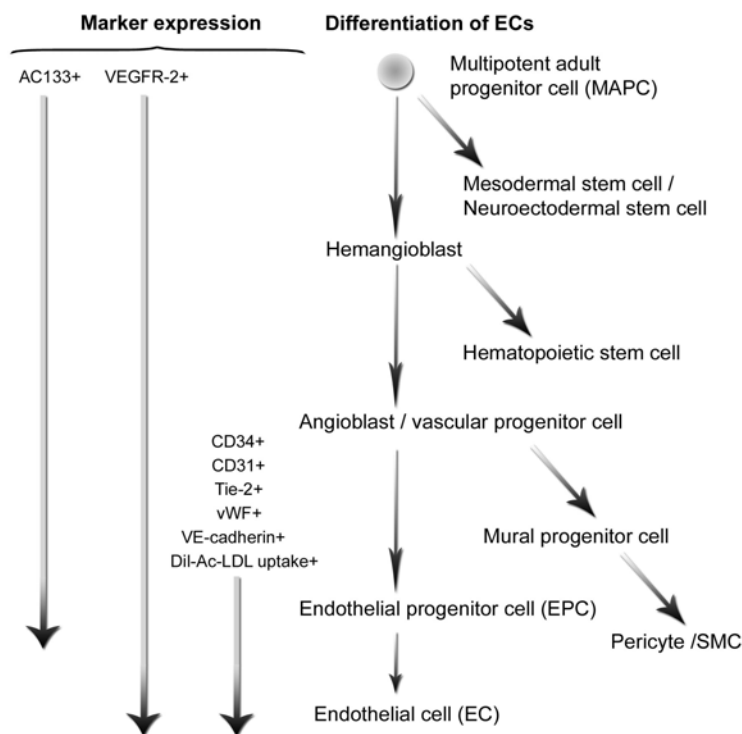
### Bone marrow-derived vascular stem cells

Blood vessel growth by stem cells was previously thought only to take place during embryonic development. However, recent evidence suggests that vascular stem cells, such as endothelial progenitor cells (EPCs), can be mobilized from the bone marrow to

circulation, home to foci of angiogenesis, differentiate to mature ECs, and thus contribute to postnatal vascular growth in wound healing, tissue ischemia and tumor growth as well as to reendothelialization of injured vessels and vascular prostheses (Asahara et al., 1997; Takahashi et al., 1999a; Lyden et al., 2001; Rafii and Lyden, 2003).

Under normal conditions, EPCs represent only 0.01% of circulating cells, whereas 24 h after burn injury or surgery, 12% of all circulating cells have been reported to be EPCs (Gill et al., 2001). It was also shown that 0.2%-1.4% of ECs in normal blood vessels are derived from vascular progenitors, but in granulation tissue up to 11% of ECs were composed of EPCs (Crosby et al., 2000).

In addition to ECs, at least embryonic VEGFR-2+ stem cells have been reported to differentiate into SMCs (Yamashita et al., 2000). Recently, EPCs were also found to be resident within skeletal muscle (Majka et al., 2003). As shown in Figure 5, progenitor cells can be differentiated and purified on the basis of expression of different molecular markers (Peichev et al., 2000).



**Figure 5.** Current view of the differentiation of ECs from progenitor cells and markers that can be used for detection and isolation of EPCs.

Cytokines and growth factors such as granulocyte macrophage-colony stimulating factor (GM-CSF), VEGF, placental growth factor (PIGF) and angiopoietin-1 (Ang-1) stimulate the proliferation, release and homing of EPCs (Takahashi et al., 1999a; Hattori et al., 2001; Hattori et al., 2002). Statins also increase the number of circulating EPCs whereas in diabetics their activity is diminished (Dimmeler et al., 2001; Tepper et al., 2002).

However, data challenging the significance of EPCs in postnatal neovascularization have been published (Springer et al., 2003). The field is further complicated by the heterogeneous definition and phenotype of the putative EPCs. Thus, it is possible that the vast majority of circulating cells thought to be EPCs are actually derived from monocyte/macrophages, do not proliferate or differentiate into ECs but secrete growth factors, which may explain their angiogenic effects (Rehman et al., 2003).

### **Development of lymphatics and lymphangiogenesis**

The most common theory to explain the origin of lymphatics was proposed a century ago and postulated that lymphatics derive from large veins (Sabin, 1902). According to the current knowledge, lymphatics may develop by multiple mechanisms (Oliver and Detmar, 2002). First, the primary lymphatic sacs bud from the endothelium of veins during early development. Prospero-related homeobox protein-1 (Prox-1) may be the master switch to cause the lymphatic phenotype in ECs and is an absolute requirement for the development of the lymphatics (Wigle and Oliver, 1999; Hong et al., 2002). Also the hematopoietic signaling protein SLP-76 is crucial for this process (Abtahian et al., 2003). After the initial budding, peripheral lymphatics spread by lymphangiogenic sprouting from preexisting ones, which seems quite analogous to angiogenesis (Jeltsch et al., 1997; Oliver and Detmar, 2002).

It has been suggested, however, that mesodermal lymphangioblasts can also participate in the development of lymphatics by differentiating into lymphatic vessels *in*

*situ* (Schneider et al., 1999). Interestingly, adult peripheral blood has been reported to contain cells that express lymphatic markers suggesting the existence of lymphatic precursors (Salven et al., 2003).

The recent identification of molecular markers specific for lymphatics has substantially contributed to the understanding of development and growth of lymphatics. In addition to Prox-1, lymphatic markers include desmoplakin, podoplanin, lymphatic endothelial hyaluronan receptor (LYVE-1) and VEGFR-3 (Karkkainen et al., 2002; Oliver and Detmar, 2002). However, at least podoplanin, LYVE-1 and VEGFR-3 may also be expressed on blood vascular ECs under some circumstances such as in tumors or even in cell types other than ECs (Valtola et al., 1999; Mouta Carreira et al., 2001; Oliver and Detmar, 2002). On the other hand, PAL-E and the transcription factor Ets-1 are expressed on blood but not lymphatic vessel endothelium (Schlingemann et al., 1985; Wernert et al., 2003).

Little was known about the molecular mechanisms regulating the growth of lymphatics until the ligand-receptor system (VEGF-C/D and VEGFR-3) was characterized, which is now thought to govern the development and growth of lymphatics (Pajusola et al., 1992; Joukov et al., 1996; Achen et al., 1998). In fact, VEGFR-3 is currently the only known growth factor receptor specific for lymphatics. Data also exist suggesting that Ang-2 is required for lymphangiogenesis since mice lacking Ang-2 have defects in the lymphatic system (Gale et al., 2002).

Analogous to the role of blood flow in the development of blood circulation, interstitial fluid flow appears important for guiding lymphangiogenesis and for lymphatic network patterning (Boardman and Swartz, 2003).

### **FACTORS INVOLVED IN VASCULAR GROWTH**

#### **Hypoxia-inducible factors (HIFs)**

HIF-1 is a mediator that couples the metabolic demand of erythrocytes and

blood flow to erythropoietin and VEGF production via O<sub>2</sub> availability (Semenza, 2002). HIF-1 is a heterodimer consisting of HIF-1 $\alpha$  and - $\beta$  subunits. The HIF-1 $\beta$  subunit is constitutively expressed while HIF-1 $\alpha$  is inducible by hypoxia (Wang et al., 1995). Both genetic inactivation of HIF-1 $\alpha$  and HIF-1 $\beta$  lead to abnormal vascular development and embryonic lethality (Kotch et al., 1999).

Among the three hypoxia-sensitive HIF- $\alpha$  isoforms of HIF-1, -2 and -3, HIF-1 $\alpha$  and -2 $\alpha$  are closely related, being capable of binding the hypoxia response elements of target genes while HIF-3 $\alpha$  appears to be a negative regulator of the hypoxic response (Pugh and Ratcliffe, 2003). In addition to erythropoietin and VEGF, the target genes for transcriptional activation by HIF-1 include VEGFR-1, insulin-like growth factor-2 (IGF-2), iNOS and plasminogen activator inhibitor-1 (PAI-1) (Forsythe et al., 1996; Gerber et al., 1997; Semenza, 2002). HIF-2 $\alpha$  regulates at least VEGF, VEGFR-2 and eNOS expression (Ema et al., 1997; Kappel et al., 1999; Coulet et al., 2003). Loss of HIF-2 $\alpha$  causes fatal respiratory distress syndrome in mice via an impaired action of VEGF on fetal lung maturation (Compernelle et al., 2002).

The most important step in the regulation of HIF-1 $\alpha$  level by cellular O<sub>2</sub> concentration is the stabilization of the protein although increased mRNA expression, nuclear localization and transactivation are also involved (Wiener et al., 1996; Sutter et al., 2000). At normal O<sub>2</sub> levels, hydroxylation of HIF-1 $\alpha$  at two prolyl and asparaginyl residues, respectively, leads to extremely rapid proteosomal destruction via interaction with the von Hippel-Lindau (VHL) E3 ubiquitin ligase (Pugh and Ratcliffe, 2003). These hydroxylases are inactive under hypoxic conditions allowing HIF-1 $\alpha$  to escape inactivation. The HIF-1 system is also induced by growth factors such as IGF-1 and oncogenic pathways such as mutant Ras and Src kinases (Pugh and Ratcliffe, 2003).

*In vivo*, HIF-1 $\alpha$  protein is usually undetectable in normoxic situations but becomes substantially upregulated in pathological conditions and may contribute to angiogenesis e.g. in perinecrotic regions of tumors, wounded skin, in the ischemic

retina and the pre-eclamptic placenta (Pugh and Ratcliffe, 2003). Tumor cells devoid of HIF-1 $\alpha$  express less VEGF and have reduced angiogenesis and growth rate (Maxwell et al., 1997). Constitutive expression of HIF-1 $\alpha$  in the skin of mice resulted in excessive angiogenesis (Elson et al., 2001). Surprisingly, the newly formed vessels were not leaky and no edema was detected. It is possible that this interesting finding can be explained by tissue adaptation to prolonged angiogenesis in response to life-long HIF-1 $\alpha$  expression.

### **Vascular endothelial growth factors (VEGFs)**

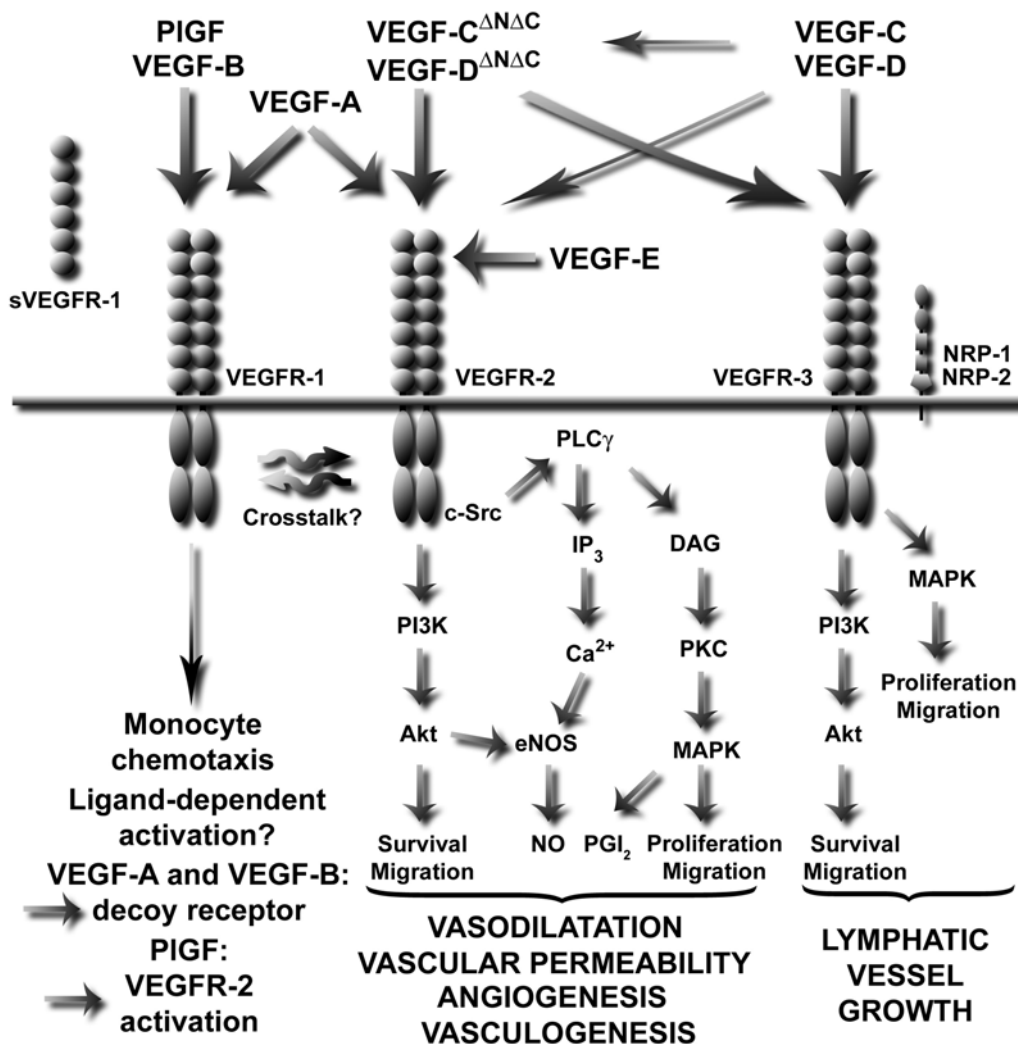
The family of VEGFs modulates a variety of EC behavior, commencing with initial embryonic vascular patterning to adult angiogenesis (Ferrara et al., 2003). Five members have been identified in the human VEGF-family: VEGF (VEGF-A), -B, -C, -D, and PlGF which differ in their ability to bind to three VEGF receptors (Senger et al., 1983; Leung et al., 1989; Maglione et al., 1991; Olofsson et al., 1996a; Joukov et al., 1996; Yamada et al., 1997; Achen et al., 1998). Also, viral VEGF homologues (collectively called VEGF-E) and snake venom VEGFs have been found (Ogawa et al., 1998; Yamazaki et al., 2003). In addition to homodimers that all VEGF family members form, generation of heterodimers such as VEGF-PlGF and VEGF-VEGF-B give even more diversity to their biological effects (DiSalvo et al., 1995; Olofsson et al., 1996a; Joukov et al., 1997; Stacker et al., 1999a). Heterodimer formation is also possible among VEGFRs, including e.g. VEGFR-1-VEGFR-2 and VEGFR-2-VEGFR-3 heterodimers (Huang et al., 2001; Dixelius et al., 2003).

### *VEGF receptors*

Three high-affinity VEGF signaling receptors (VEGFRs) have been isolated (de Vries et al., 1992; Terman et al., 1992; Millauer et al., 1993; Pajusola et al., 1992; Joukov et al., 1996). Although ECs and EPCs are the primary targets of VEGFs (Yamaguchi et al., 1993), other cell types

are also known to express VEGFRs (Ferrara et al., 2003). VEGFRs structurally belong to the PDGFR super family. They have seven extracellular immunoglobulin-like domains, a single transmembrane region and intracellular tyrosine kinase domain split by a kinase-insert domain (Pajusola et al., 1992; Petrova et al., 1999). Upon ligand binding, all VEGFRs undergo homodimerization leading to downstream signals via phosphorylation of the tyrosine kinase domain (Petrova et al., 1999).

The biological importance of VEGFRs is highlighted by the fact that knockout mice for all three VEGFRs die at an early embryonic state, presenting severe cardiovascular malformations (Fong et al., 1995; Shalaby et al., 1995; Dumont et al., 1998). In addition to signaling receptors, two co-receptors for VEGFs, neuropilin (NRP)-1 and -2 have been found (Neufeld et al., 2002). Figure 7 summarizes the interactions between VEGFs and their receptors and the resulting downstream effects.



**Figure 7.** Ligands and receptors in the VEGF family. VEGFR-2 and VEGFR-3 are the main signaling receptors on ECs of blood and lymphatic vessels, respectively. PI3K/Akt, MAPK, Ca<sup>2+</sup> and NO are key mediators of the blood vascular effects of VEGFR-2 signaling. The biological role of VEGFR-1 is currently unclear but it can act as a negative modulator of angiogenesis and exists also as a soluble form. However, VEGFR-1 activation at least by PIGF can also promote angiogenesis, perhaps through intracellular crosstalk with VEGFR-2 (Autiero et al., 2003). Also upregulation of additional growth factors in response to VEGFR-1 stimulation has been described (LeCouter et al., 2003). NRPs are coreceptors for VEGFs. Known ligands for NRP-1 and NRP-2 are VEGF<sub>165</sub>, PIGF-2, VEGF-B and VEGF-E; and VEGF<sub>145</sub>, VEGF<sub>165</sub>, PIGF-2 and VEGF-C, respectively (Neufeld et al., 2002; Karkkainen et al., 2001).



### *VEGFR-1 – the biology is unclear*

Previously, it was thought that only the extracellular domain of VEGFR-1 (Flt-1) is important because VEGFR-1 also exists as a soluble form (a decoy receptor to harvest excess VEGF) and its intracellular signaling domain was shown to be unnecessary for normal vascular development (Waltenberger et al., 1994; Hiratsuka et al., 1998; Gille et al., 2001). Knockout mice of the whole VEGFR-1 gene die *in utero* between days 8.5 and 9.5 as a result of excessive angioblast proliferation and EC organization failure, suggesting a crucial role for VEGFR-1 in the modulation of angiogenesis and vasculogenesis induced by VEGFR-2 signaling (Fong et al., 1995). VEGFR-1 activation has shown to exert even inhibitory effects on VEGFR-2-mediated proliferation (Zeng et al., 2001).

VEGFR-1 stimulation by VEGF and VEGF-B leads to weak mitogenic signals in ECs but VEGFR-1 appears to play a more potent role in monocyte chemotaxis (Waltenberger et al., 1994; Barleon et al., 1996). The affinity of VEGF to VEGFR-1 is about an order of magnitude stronger than to VEGFR-2 (Waltenberger et al., 1994). VEGFR-1 is upregulated by hypoxia in a HIF-1 $\alpha$  dependent manner (Gerber et al., 1997).

In contrast to the negative role in angiogenesis, it was recently shown that the selective VEGFR-1 ligand PIGF stimulates angiogenesis, vascular permeability as well as mobilizes EPCs and hematopoietic stem cells (Luttun et al., 2002; Gerber et al., 2002; Hattori et al., 2002). Interestingly, when the aminoacids of VEGF responsible for VEGFR-2 binding were replaced by analogous regions of PIGF, the mutant induced vascular permeability without binding to VEGFR-2 (Stacker et al., 1999b). Activation of VEGFR-1 uniquely by PIGF results in phosphorylation of specific tyrosine residues, which causes intermolecular transphosphorylation of VEGFR-2 (Autiero et al., 2003). Thus, the angiogenic and vascular permeability effects via VEGFR-1 activation by PIGF may be due to indirect VEGFR-2 stimulation.

VEGFR-1 signaling may also stimulate the release of additional growth factors. For example, VEGFR-1 activation in sinusoidal

ECs in the liver resulted in a paracrine upregulation of hepatocyte growth factor (HGF), IL-6 and other hepatotrophic molecules and subsequently enhanced liver regeneration (LeCouter et al., 2003).

These findings, that appear conflicting at the first sight, may implicate that signaling via VEGFR-1 is ligand-dependent; it is a negative modulator of VEGF-induced angiogenesis but in response to PIGF binding it is capable of promoting proangiogenic effects via indirect VEGFR-2 activation.

### *VEGFR-2 – effects on blood vessels*

VEGFR-2 (Flk-1/KDR) is considered to mediate most of the blood vessel effects by VEGFs such as mitogenesis, angiogenesis, survival, and vascular permeability as well as their effects on EPCs (Ferrara et al., 2003; Gille et al., 2001; Peichev et al., 2000). VEGFR-2 deficiency is embryonically fatal between day 8.5 and 9.5, manifested as a lack of vasculogenesis due to the differentiation failure of hemangioblasts into ECs or hematopoietic cells (Shalaby et al., 1995).

VEGFR-2 expression is regulated by HIF-2 $\alpha$  but has been reported to be both up- and downregulated by hypoxia (Waltenberger et al., 1996; Kappel et al., 1999; Gerber et al., 1997). However, hypoxia-induced VEGF expression ultimately also leads to autocrine VEGFR-2 upregulation via the phosphatidylinositol-3-OH-kinase/serine-threonine kinase Akt (PI3K/Akt) pathway (Shen et al., 1998; Jiang et al., 2000; Brogi et al., 1996). VEGFR-2 can also be triggered in a ligand-independent manner in response to fluid shear stress leading to NO production (Jin et al., 2003).  $\alpha_v\beta_3$  integrin, which is upregulated on angiogenic endothelium, enhances the growth signals via VEGFR-2 (Soldi et al., 1999).

Ligand binding to VEGFR-2 results in the activation of many intracellular mitogenic signaling cascades (Petrova et al., 1999). For example, activation of the mitogen-activated protein kinase (MAPK) pathway via protein kinase C (PKC) increases DNA synthesis, EC migration and proliferation (Kroll and Waltenberger, 1997; Takahashi et al., 1999b). The stimulation of the PI3K/Akt

pathway promotes EC migration, and cell survival together with upregulation of antiapoptotic pathways (Gerber et al., 1998b; Gerber et al., 1998a; Morales-Ruiz et al., 2000).

NO is crucial for VEGFR-2-mediated effects as NO synthase inhibition hinders angiogenesis and vascular permeability (Papapetropoulos et al., 1997; Murohara et al., 1998b). Upon VEGFR-2 activation, the increased NO production by endothelial NO synthase (eNOS) is a result of both the rise in intracellular  $Ca^{2+}$  levels, which stimulates eNOS directly, and activation of the PI3K/Akt pathway which can cause the persistent upregulation of eNOS expression in a  $Ca^{2+}$ -independent manner (Kroll and Waltenberger, 1998; He et al., 1999; Fulton et al., 1999; Dimmeler et al., 1999). Also prostacyclin is released in response to VEGFR-2 activation (Wheeler-Jones et al., 1997).

Although the downstream mediators of VEGFR-2 signaling are becoming increasingly clear, the hierarchy of the intracellular events may be more complicated than generally thought. For example, PI3K, NO and cGMP have also been suggested to act upstream to MAPK-induced EC proliferation (Parenti et al., 1998; Thakker et al., 1999).

### *VEGFR-3 – effects on lymphatic vessels*

The biological role of VEGFR-3 (Flt-4) seems clear, although the signal transduction cascade is less well characterized than that of VEGFR-2 (Petrova et al., 1999; Makinen et al., 2001b). There are two human VEGFR-3 splice variants, of which the longer isoform demonstrates more potent signaling properties than the short isoform and presumably transmits largely similar signals as VEGFR-2 (Petrova et al., 1999).

In adults, VEGFR-3 activation alone is sufficient for the growth, migration and survival of lymphatic ECs (Veikkola et al., 2001; Makinen et al., 2001b). In contrast, VEGFR-3 signaling does not stimulate blood vessel growth (Jeltsch et al., 1997; Veikkola et al., 2001; Saaristo et al., 2002). Although the MAPK, PI3K and Akt signal transduction pathways are involved, there

are also some differences between VEGFR-3 and VEGFR-2 signaling (Makinen et al., 2001b). For instance, NO is an important downstream effector of VEGFR-2 mediated signals, but it is not known whether NO is necessary for lymphangiogenesis by VEGFR-3.

Heterozygous inactivation of VEGFR-3 causes lymphedema due to a lack of cutaneous lymphatics both in man and in a mouse model (Karkkainen et al., 2000; Karkkainen et al., 2001). Moreover, expression of soluble VEGFR-3 in the skin of transgenic mice has been shown to inhibit lymphangiogenesis, promote regression of established lymphatics and cause lymphedema (Makinen et al., 2001a). In embryos, however, VEGFR-3 is also expressed on venous endothelium and is crucial for the early development of blood vessels before the emergence of lymphatics (Kaipainen et al., 1995; Dumont et al., 1998). Later, VEGFR-3 becomes restricted to lymphatic endothelium with the exception of activated blood vessels in tumors (Valtola et al., 1999).

### *Neuropilins (NRPs) – coreceptors for VEGFs*

In addition to the three tyrosine-kinase receptors, two co-receptors for VEGFs have been recently identified. NRP-1 and NRP-2, originally found to play a role in neuronal guidance, are required for normal embryonic blood and lymphatic vessel development, respectively (Neufeld et al., 1999; Kawasaki et al., 1999; Yuan et al., 2002). NRP-1 appears to be preferentially expressed in arteries and NRP-2 in veins (Herzog et al., 2001).

NRPs have not been shown to signal after binding VEGFs. However, NRP-1 amplifies the VEGFR-2 mediated signal transduction by VEGF<sub>165</sub> (Whitaker et al., 2001; Oh et al., 2002). VEGF<sub>121</sub> does not bind either to NRP-1 or NRP-2, which may at least partly explain its reduced biological potency compared to VEGF<sub>165</sub> (Whitaker et al., 2001). Like VEGFR-1, NRPs exist also as soluble (decoy) forms (Neufeld et al., 2002).

## VEGF (VEGF-A)

VEGF was the first member of the VEGF family whose cDNA sequence was published by three independent research groups in 1989 (Leung et al., 1989; Keck et al., 1989; Plouet et al., 1989). However, already in 1983 Senger, Dvorak and colleagues identified a protein, vascular permeability factor (VPF), secreted by tumors and capable of promoting accumulation of ascites fluid (Senger et al., 1983). The cDNA sequences later revealed that VPF and VEGF were the same molecule (Keck et al., 1989). Currently, VEGF/VPF is the best characterized VEGF family member with over 10000 published scientific reports by the end of year 2003 (see Figure 9 on p. 46).

The human VEGF gene consists of eight exons separated by seven introns encoding a homodimeric glycoprotein, the monomers held together with an interchain disulfide bond (Leung et al., 1989; Plouet et al., 1989). Alternative mRNA splicing was initially shown to result in four different isoforms consisting of 121, 165, 189 or 206 amino acid residues (VEGF<sub>121</sub> through VEGF<sub>206</sub>) (Tischer et al., 1991; Houck et al., 1991). The corresponding mouse and rat isoforms have one amino acid less than those of humans. VEGF<sub>165</sub> and VEGF<sub>121</sub> lack the residues encoded by the exon 6, and exons 6 and 7, respectively. Less common splice variants VEGF<sub>138</sub>, VEGF<sub>145</sub> and VEGF<sub>162</sub> have also been reported (Poltorak et al., 1997; Lange et al., 2003).

VEGF<sub>121</sub> is acidic and does not bind to heparin or heparan sulfates making it freely soluble in tissues, whereas VEGF<sub>189</sub> and VEGF<sub>206</sub> are highly basic and have a high affinity towards ECM (Houck et al., 1992). VEGF<sub>165</sub> has intermediate properties, because it is secreted but a significant fraction binds to cell surfaces and ECM. Plasmin cleaves the longer ECM-bound forms at the C-terminus generating a bioactive and soluble fragment of 110 amino acids, which however, has reduced mitogenic activity (Houck et al., 1992; Keyt et al., 1996).

Mice expressing only VEGF<sub>120</sub> but not the longer forms die within two weeks after delivery owing to the impaired distribution of ECs indicating that the ECM-bound forms

are essential for vascular branching (Carmeliet et al., 1999; Ruhrberg et al., 2002). In terms of therapeutic angiogenesis, VEGF<sub>165</sub> may have the optimal features: sufficient bioavailability with high biological potency (Ferrara et al., 2003).

VEGF is induced by hypoxia, hypoglycemia, inflammation, tissue repair and malignancy. In particular, hypoxia is a very potent inducer of VEGF expression (12- to 25-fold) by increasing its transcription and stabilizing its mRNA indicating a role for VEGF in physiological angiogenesis (Shweiki et al., 1992; Levy et al., 1995; Shih and Claffey, 1999). HIF-1 $\alpha$  is the main regulator of VEGF transcription under different O<sub>2</sub> concentrations (Forsythe et al., 1996). However, the majority of VEGF protein upregulation in hypoxia appears to be due to the increased mRNA stability (Levy et al., 1995; Levy et al., 1996). *In vivo*, hypoxia-inducible VEGF expression has been implicated in preconditioning of the heart against ischemia and in skeletal muscle in response to exercise (Kawata et al., 2001; Richardson et al., 1999). VEGF is required for "the angiogenic switch" in tumors (Bergers and Benjamin, 2003).

Actions of VEGF, including vasodilatation, vascular permeability and angiogenesis are extensively mediated by VEGFR-2 and subsequent NO production by eNOS and to a lesser extent by inducible NO synthase (iNOS) (Laitinen et al., 1997b; Murohara et al., 1998a; Matsunaga et al., 2000; Fukumura et al., 2001). VEGF promotes angiogenesis in a dose-dependent manner. For example, mouse embryos lacking even a single VEGF allele show growth retardation and die between embryonic day 11 and 12 (Carmeliet et al., 1996; Ferrara et al., 1996). Furthermore, decrease of VEGF levels by only 25% impairs perfusion in the spinal cord resulting in motor neuron degeneration, reminiscent of amyotrophic lateral sclerosis (Oosthuyse et al., 2001; Lambrechts et al., 2003). The vascular permeability actions of VEGF play a significant role in the deposition of extravascular fibrin, ascites fluid and tissue edema in tissue repair, inflammation and cancer (Dvorak et al., 1995; Detmar et al., 1998). VEGF upregulates proteases, such as plasmin and MMPs, which are important

for the degradation of ECM in the initiation of angiogenesis.

The development of tissue and organs is critically dependent on the bidirectional signals between ECs and surrounding cells (Cleaver and Melton, 2003). Consequently, disruption of VEGF, a key regulator of EC growth and differentiation, leads to fatal problems. Not only blood vessels are affected by VEGF deficiency but also several organs such as the heart, kidneys, liver and the neural system during the very early development (Carmeliet et al., 1996; Ferrara et al., 1996). After the early postnatal life the dependence of organ development on VEGF is attenuated and thereafter VEGF seems to be primarily needed in sites of active blood vessel remodeling (Gerber et al., 1999a).

Insufficient levels of VEGF contribute to pathogenesis of many common disorders. These include diabetic neuropathy, amyotrophic lateral sclerosis, retarded hair growth, preeclampsia, glomerular defects leading to nephrosis, neonatal respiratory distress syndrome, emphysema, and impaired endochondral bone formation (Gerber et al., 1999b; Kasahara et al., 2000; Oosthuysen et al., 2001; Yano et al., 2001; Compennolle et al., 2002; Maynard et al., 2003; Lambrechts et al., 2003; Eremina et al., 2003). Although the loss of VEGF-dependent EC function and impaired blood flow may be the most significant factors explaining the role of VEGF in these diseases, VEGF may also have direct effects on cells other than ECs such as neurons, osteoblasts, lung epithelial cells and HSCs, among others (Ferrara et al., 2003). As a consequence, VEGF-deficient hematopoietic stem cells and bone-marrow mononuclear cells are incapable of repopulating (Gerber et al., 2002).

Excessive VEGF levels also contribute to numerous diseases. Soon after its discovery, VEGF was connected to tumor angiogenesis, growth and ascites formation (Senger et al., 1983; Plate et al., 1992; Kim et al., 1993). Since then a cascade of other disorders have followed such as congenital heart defects, ischemia-related brain edema, polycystic ovary syndrome, endometriosis, diabetic retinopathy, collapsing glomerulopathy, age-related macular degeneration, atherosclerosis and plaque

neovascularization, as well as transplant arteriosclerosis (Aiello et al., 1994; Dor et al., 2001; Brasen et al., 2001; Celletti et al., 2001; McLaren et al., 1996; Lemstrom et al., 2002; van Bruggen et al., 1999; Krzystolik et al., 2002; Eremina et al., 2003). The contribution of VEGF to the progression of these diseases is likely attributable to its potent angiogenic, vascular permeability and proinflammatory properties. Taken together, a tight control of VEGF expression is required for normal development and homeostasis of vasculature and organ function.

Because of its major role in vascular biology, VEGF has been the favorite candidate for therapeutic angiogenesis both in preclinical and clinical studies (Rissanen et al., 2001). On the other hand, several VEGF and VEGFR inhibitors are currently being evaluated in clinical trials for the treatment of cancer (Kerbel and Folkman, 2002), likely followed by non-neoplastic diseases known to involve excessive VEGF production (Krzystolik et al., 2002). The recent studies showing VEGF deficiency in conditions such as impaired bone formation may also open new therapeutic avenues in regenerative medicine. For example, Hiltunen et al. have shown that bone formation can be stimulated by adenoviral (Ad) VEGF overexpression suggesting a novel treatment for osteoporotic fractures (Hiltunen et al., 2003).

### *VEGF-B*

VEGF-B, which has about 43% identical amino acid sequence with VEGF, is expressed from the beginning of early development to adult life particularly in the heart (Olofsson et al., 1996a; Aase et al., 1999). VEGF-B is also expressed in skeletal muscle, pancreas, adrenal gland and SMCs of large blood vessels. At least two splice variants of VEGF-B are expressed, consisting of 167 and 186 amino acid residues (Olofsson et al., 1996b; Olofsson et al., 1998). VEGF-B is a ligand for VEGFR-1 and NRP-1. VEGF-B-VEGF heterodimers can also bind to VEGFR-2 (Olofsson et al., 1996a).

In contrast to VEGF, VEGF-B is not upregulated by hypoxia or serum growth

factors (Enholm et al., 1997). VEGF-B has been reported to be an EC mitogen *in vitro* and to regulate plasminogen activator activity in ECs (Olofsson et al., 1996a). However, the significance of VEGF-B *in vivo* remains unclear as the studies of VEGF-B knockout mice have yielded controversial results. Bellomo et al. reported that VEGF-B<sup>-/-</sup> mice have smaller hearts, dysfunctional coronary arteries and impaired recovery from myocardial ischemia whereas Aase et al. showed that these mice have a subtle cardiac phenotype and that VEGF-B is not essential for development of the cardiovascular system (Bellomo et al., 2000; Aase et al., 2001). Recently, given as recombinant protein or expressed via naked plasmid DNA, VEGF-B was reported to induce angiogenesis in the mouse skin (Silvestre et al., 2003).

#### *VEGF-C and VEGF-D*

Human VEGF-C and VEGF-D were first cloned in 1996 and 1997, respectively (Joukov et al., 1996; Yamada et al., 1997). The mouse form of VEGF-D had already been sequenced in 1996 (Orlandini et al., 1996). At the amino acid level, human VEGF-C and VEGF-D are 48% identical to each other and about 30% to VEGF<sub>165</sub> (Joukov et al., 1996; Achen et al., 1998). VEGF-C and VEGF-D form a subfamily of VEGFs because of the obvious similarities to one another. Both growth factors enclose N- and C- terminal extensions that are not found in other VEGF family members. They have a similar receptor binding profile and are synthesized as large precursor forms which are proteolytically processed intra- and extracellularly into mature forms (indicated as ΔNΔC) comprising the central VEGF homology domain (Joukov et al., 1996; Achen et al., 1998; Joukov et al., 1997; Stacker et al., 1999a). Plasmin cleaves both the full length VEGF-C and VEGF-D into the short mature forms (McColl et al., 2003).

The unprocessed forms of both VEGF-C and VEGF-D preferentially signal through VEGFR-3 while only the mature forms trigger VEGFR-2 signaling efficiently (Joukov et al., 1997; Stacker et al., 1999a). In the case of VEGF-D, the proteolytically

processed mature form has approximately 290- and 40-fold greater affinity towards VEGFR-2 and VEGFR-3, respectively, compared to the full length VEGF-D (Stacker et al., 1999a). Also, the receptor binding affinity of mature VEGF-C is stronger for VEGFR-3 than for VEGFR-2 (Joukov et al., 1997). In agreement with the receptor binding profiles, the long unprocessed forms are mainly lymphangiogenic while the mature short forms are also angiogenic and promote vascular permeability (Joukov et al., 1997; Jeltsch et al., 1997; Cao et al., 1998; Marconcini et al., 1999; Veikkola et al., 2001; Byzova et al., 2002). Both VEGF-C and VEGF-D promote tumor angiogenesis, lymphangiogenesis, metastatic spread, and thus their expression in cancer may have prognostic value (Skobe et al., 2001; Stacker et al., 2001; Achen et al., 2001; Yonemura et al., 1999; White et al., 2002).

During embryonic development, the VEGF-C expression pattern is quite similar to VEGFR-3 expression, both colocalizing in regions where lymphatic vessels sprout from veins and in the lymphatic vessel-rich mesenterium (Kukk et al., 1996). In adults, VEGF-C is expressed in the lung, heart and kidney. VEGF-C<sup>+/-</sup> mice have similar defects in cutaneous lymphatic vessels as chy mice (heterozygous inactivating VEGFR-3 mutation) while VEGF-C<sup>-/-</sup> mice die because of a total lack of lymphatics (Karkkainen et al., 2001 and 2003).

The knockout studies indicate that VEGFR-3 signaling by VEGF-C is required for normal embryonic and postnatal lymphangiogenesis (Karkkainen et al., 2001 and 2003).. Because of the severe lymphatic defects in VEGF-C<sup>-/-</sup> mice, endogenous VEGF-D is not able to compensate the lack of VEGF-C. In addition to induction of lymphangiogenesis, AdVEGF-C GT has been shown to inhibit restenosis after balloon angioplasty (Hiltunen et al., 2000a). Joukov et al. have generated a mutant VEGF-C with a single aminoacid substitution (VEGF-C<sup>156S</sup>) which binds to VEGFR-3 (although with a reduced affinity than VEGF-C) but not to VEGFR-2 at all (Joukov et al., 1998; Mäkinen et al., 2001b).

Little is known about the natural biological role or regulation of VEGF-D. It is known,

however, that at least the mouse VEGF-D is induced by the proto-oncogene c-fos and cell-cell contacts (Orlandini et al., 1996; Orlandini and Oliviero, 2001). In addition to its effects on ECs, VEGF-D has been reported to have mitogenic and morphogenic activity on fibroblasts (Orlandini et al., 1996). During embryonic development, VEGF-D is expressed in several structures and organs such as limb buds, heart, kidney, lung, liver and teeth (Avantaggiato et al., 1998). In adult human tissues, VEGF-D mRNA is mostly expressed in the heart, lung, skeletal muscle, colon, and small intestine (Achen et al., 1998). Its constitutive expression in normal and atherosclerotic arteries suggests a role in vascular homeostasis (Rutanen et al., 2003). Mouse VEGF-D is different from mouse VEGF-C and human VEGF-D because it does not bind to VEGFR-2 at all (Kukk et al., 1996; Baldwin et al., 2001). It follows that, when the stimulation of lymphatic but not blood vessel growth is desired the mouse VEGF-D or the mutant VEGF-C<sup>156S</sup> could be utilized (Saaristo et al., 2002).

#### *Viral VEGFs (VEGF-E)*

The viral homologues of VEGFs, collectively called VEGF-E, are encoded by different strains of the parapoxvirus Orf (Lyttle et al., 1994; Ogawa et al., 1998; Meyer et al., 1999; Wise et al., 1999). The different forms of viral VEGF homologues have 16-27% and 41% aminoacid sequence homology with mammalian VEGFs and with each other, respectively (Lyttle et al., 1994). Viral VEGFs bind selectively to VEGFR-2 and NRP-1 but not to VEGFR-1 or VEGFR-3 (Ogawa et al., 1998; Meyer et al., 1999; Wise et al., 1999 and 2003). Consequently, angiogenesis and vascular permeability are promoted by the Orf virus skin infection and by recombinant VEGF-E (Wise et al., 2003).

Viral VEGFs resemble VEGF<sub>121</sub> as they do not bind to heparin sulfates being freely diffusible throughout the EMC (Ogawa et al., 1998). However, unlike VEGF<sub>121</sub>, viral VEGFs are almost equally potent mitogens for ECs as VEGF<sub>165</sub> both *in vitro* and *in vivo* (Ogawa et al., 1998; Meyer et al., 1999; Wise et al., 1999 and 2003). Although viral VEGFs vary from each other in their ability

to bind VEGFR-2 and NRP-1 and in the induction of vascular permeability, they are quite uniformly mitogenic for ECs (Wise et al., 2003).

Similarly with transgenic HIF-1 $\alpha$  expression, constitutive VEGF-E over-expression in the mouse skin has been reported to increase vascularization 10-fold without causing tissue edema (Kiba et al., 2003). It is possible that secondary effects to prolonged angiogenesis and vascular permeability such as increased lymph flow may explain attenuated edema in this model.

#### *Placental growth factor (PlGF)*

Maglione et al. identified a human cDNA encoding a protein related to VEGF from a term placenta cDNA library and accordingly named its product PlGF (Maglione et al., 1991). PlGF is a homodimeric glycoprotein, expressed in the placenta, thyroid, lung, and goiter, that displays a 53% homology with the PDGF-like region of VEGF (Ziche et al., 1997a). Alternative splicing of human PlGF mRNA gives rise to two isoforms, PlGF<sub>131</sub> and PlGF<sub>152</sub> (PlGF-1 and PlGF-2), which bind only to VEGFR-1 but not to VEGFR-2 (Ziche et al., 1997a; Park et al., 1994). Another splice variant, PlGF-3 (203 aminoacids), has also been reported (Cao et al., 1997). PlGF-2 is the only isoform present in mice. PlGF-1 is a diffuse, non-heparin binding protein, whereas PlGF-2 binds to ECM as well as NRP-1 and -2 (Park et al., 1994; Migdal et al., 1998).

As with the other selective VEGFR-1 ligand VEGF-B, the biology of PlGF is not yet fully understood. PlGF knockout as well as PlGF-VEGF-B double knockout mice do not display any significant vascular phenotype and are fertile (Carmeliet et al., 2001). However, Carmeliet and colleagues have suggested that impaired angiogenesis and arteriogenesis occur in PlGF<sup>-/-</sup> mice under pathological conditions such as ischemia and tumor growth (Carmeliet et al., 2001). Furthermore, PlGF has been reported to promote the recruitment of monocytes and hematopoietic stem cells from the bone marrow (Hattori et al., 2002). PlGF has little or no direct mitogenic or vascular permeability activity (Migdal et al.,

1998; Park et al., 1994; Murohara et al., 1998b), although conflicting data also exist (Landgren et al., 1998; Ziche et al., 1997a).

Chronic expression of PIGF in the skin of transgenic mice promotes vessel enlargement with an efficient SMC coverage, vascular permeability and even formation of glomeruloid bodies with upregulation of both VEGFR-1 and VEGFR-2 (Odorisio et al., 2002). Interestingly, no significant monocyte chemotaxis was observed. In the mouse skin AdPIGF-2 induced the formation of enlarged vessels (Luttun et al., 2002). In contrast to AdVEGF-induced vasculature, these vessels were reported to be very stable (>1 year) showing little or no vascular permeability.

It is now becoming evident that the VEGF-VEGFR-2 system is involved in the biology of PIGF via a number of possible mechanisms. Firstly, excess PIGF can displace endogenous VEGF from VEGFR-1, allowing VEGF binding to VEGFR-2 resulting in angiogenic signals. Secondly, activation of VEGFR-1 by PIGF may cause intermolecular transphosphorylation of VEGFR-2 (Autiero et al., 2003). Thirdly, although the underlying mechanism is still unclear, PIGF may upregulate VEGF expression (Bottomley et al., 2000). Finally, PIGF-VEGF heterodimers are able to bind also to VEGFR-2 (DiSalvo et al., 1995; Cao et al., 1996). Notwithstanding the ill-defined signaling mechanisms, PIGF appears a promising candidate growth factor for therapeutic angiogenesis.

### **Fibroblast growth factors (FGFs)**

Currently, the FGF family comprises 23 members, which share 30-70% homology with each other (Galzie et al., 1997; Klint and Claesson-Welsh, 1999). Human FGF-1 (acidic FGF, aFGF) and FGF-2 (basic FGF, bFGF), the prototype members of the family, were cloned in 1986 (Jaye et al., 1986; Abraham et al., 1986). FGFs are multifunctional proteins stimulating the proliferation of a variety of cell types of mesodermal and neuroectodermal origin, including ECs, SMCs and myoblasts (Galzie et al., 1997; Klint and Claesson-Welsh, 1999; Yablonka-Reuveni et al., 1999; Ornitz et al., 1996).

FGFs are involved in embryonic development, tissue regeneration, cell transformation, tumor growth, and angiogenesis. Theoretically, the capability of FGFs to stimulate SMCs directly could be beneficial for therapeutic arteriogenesis.

### *FGF receptors*

The cloning of FGF receptors (FGFRs) has identified four distinct genes encoding four tyrosine kinase receptors FGFR-1, -2, -3 and -4. Recently, FGFR-5 was also identified but it does not have a tyrosine kinase domain suggesting a role as a decoy receptor (Sleeman et al., 2001). Alternative mRNA splicing produces complexity to the receptor family as two isoforms (*b* and *c*) of FGFR-1, -2 and -3 exist with different affinity profiles towards FGFs, whereas FGFR-4 is not alternatively spliced (Ornitz et al., 1996; Klint and Claesson-Welsh, 1999). The receptor isoform *a* is a secreted FGF-binding protein with no signal transduction properties (Ornitz et al., 1996).

During development, FGFR-1 is expressed in the mesenchyme, FGFR-2 in several epithelial tissues, FGFR-3 predominantly in the central nervous system and FGFR-4 in several tissues of endodermal and neuroectodermal origin (Klint and Claesson-Welsh, 1999). In adult ECs, FGFR-1 and FGFR-2 appear to be the main FGFRs. It has been suggested that FGFR-1 triggers EC proliferation, migration and tube formation while FGFR-2 mediates only EC migration (Javerzat et al., 2002). However, knowledge of the exact roles and specific expression patterns of FGFRs in physiological and pathological conditions is currently sparse.

FGFRs are comprised of two to three extracellular immunoglobulin-like domains, a single transmembrane domain and two intracellular tyrosine kinase domains (Klint and Claesson-Welsh, 1999). Endogenous heparan sulfate proteoglycans such as syndecan are required for efficient receptor binding of FGFs (Klint and Claesson-Welsh, 1999; Javerzat et al., 2002). FGF-1 appears to be a universal ligand because it binds to both *b* and *c* isoforms of FGFRs (Ornitz et al., 1996). FGF-2 and FGF-4 bind essentially to isoform *c* of FGFR-1-3 and

FGFR-4. In addition, FGF-2 binds also to isoform *b* of FGFR-1 (Ornitz et al., 1996). FGF-9 binds to FGFR-2 (isoform *c*), -3 and -4.

In mice, deletion of FGFR-1 and -2 causes early embryonic lethality due to the lack of mesoderm-inducing signals (Xu et al., 1999). Blockade of FGFR-1 signaling later on in development has been shown to cause abnormal vascular network formation (Lee et al., 2000c).

The signal transduction of FGFRs is less extensively studied than that of VEGFRs. Similar to other receptor tyrosine kinases, the binding of the ligands to FGFRs leads to receptor dimerization and tyrosine kinase autophosphorylation (Klint and Claesson-Welsh, 1999). However, signaling thereafter appears somewhat different than that of VEGFRs. For example, FGF-2 promotes cell growth via MAPK involving Raf but independently of phospholipase C $\gamma$  (PLC $\gamma$ ) and Ca<sup>2+</sup> (McLaughlin and De Vries, 2001; Klint and Claesson-Welsh, 1999). It is unclear whether the PI3K pathway is efficiently activated by FGFR-signaling (Klint and Claesson-Welsh, 1999). The requirement of NO for downstream vascular effects promoted by FGFs is also controversial (Wu et al., 1996; Ziche et al., 1997b).

## FGFs

*In vitro*, FGF-1, -2, -4, and -9 are the most potent mitogens (Ornitz et al., 1996). Among these FGFs, the overexpression of FGF-1, FGF-2 and FGF-5 has been shown to induce angiogenesis *in vivo* (Muhlhauser et al., 1995; Ueno et al., 1997; Giordano et al., 1996). Upon administration to quail embryonic chorionallantoic membrane, FGF-2 stimulated the growth of small but not large arteries (Parsons-Wingter et al., 2000).

FGF-1, -2 and -9 lack a signal peptide, but can still be released from cells, to some extent, by an alternative secretion pathway (Javerzat et al., 2002). These FGFs bind to the BM and ECM and are liberated upon tissue injury. Most of the other FGFs are secreted via a conventional signal sequence. The lack of efficient secretion of FGF-1 and -2 may imply that, in addition to

paracrine actions, their angiogenic effects are partly mediated by intracellular signaling (Javerzat et al., 2002). Interestingly, FGF-2 signaling upregulates endogenous VEGF expression, and accordingly VEGFR-2 antagonists inhibit FGF-2-induced angiogenesis both *in vitro* and *in vivo* (Stavri et al., 1995b; Tille et al., 2001). Furthermore, FGF-2 induces lymphangiogenesis via VEGF-C induction (Kubo et al., 2002).

Members of the FGF family have crucial roles in embryonic development such as mesoderm induction, organogenesis, bone growth as well as limb outgrowth and patterning. Thus, the deletion of FGF-3, -4 and -8 is embryonic lethal (Xu et al., 1999). Although FGF-1 and -2 are angiogenic *in vivo*, mice lacking these growth factors, either alone or both in combination, survive, are fertile and have no gross vascular phenotype suggesting functional redundancy for these prototype FGFs in developmental vascular growth (Miller et al., 2000). Only subtle impairments in wound healing and hematopoiesis were reported but ischemia-induced angiogenic and arteriogenic responses were not affected in FGF-2<sup>-/-</sup> mice (Sullivan et al., 2002). Furthermore, transgenic overexpression of FGF-1 in the mouse heart caused no obvious vascular phenotype although a moderate increase in the number and branching of small arterioles, but not capillaries, was reported (Fernandez et al., 2000). Similarly, transgenic overexpression of FGF-2 in the retina did not promote angiogenesis, at least without a concomitant tissue injury (Yamada et al., 2000). Thus, FGF-1 and -2 are not essential for normal development of the circulation at least in mice.

## FGF-4

FGF-4 is encoded by the *hst-1* oncogene and shows ~40% aminoacid sequence homology to FGF-1 and -2 (Yoshida et al., 1987). FGF-4 is a transforming growth factor that induces proliferation, migration, and survival of several cell types, including ECs and fibroblasts (Yoshida et al., 1987; Delli-Bovi et al., 1988). FGF-4 is crucial for development because embryos lacking FGF-4 die on embryonic day 5 due to



severely impaired proliferation of the inner cell mass (Feldman et al., 1995). In adults, FGF-4 is expressed primarily during tumorigenesis but also to some extent in the nervous system, intestines, and testes (Yamamoto et al., 2000). FGF-4 is efficiently bound by ECM-associated heparan sulfates (Aviezer et al., 1999).

Although the mitogenic effects of FGF-4 on ECs are not as potent *in vitro* compared to FGF-2, they are strongly enhanced by heparin via an increase in the affinity of FGF-4 toward FGFRs (Dell'Era et al., 2001; Aviezer et al., 1999). Under restricted heparan sulfate conditions FGF-4 preferentially binds to FGFR-2, whereas at high concentrations of heparan sulfates, FGF-4 can also activate FGFR-1 (Aviezer et al., 1999). However, it is currently unknown which of the FGFRs are the most important for the angiogenic activity of FGF-4.

FGF-4 is efficiently secreted (Delli-Bovi et al., 1988), which may confer an advantage over other FGFs for therapeutic neovascularization. AdFGF-4 GT has been shown to increase peripheral platelet counts and thus could also be used for prevention of thrombocytopenia caused by irradiation or chemotherapy (Konishi et al., 1995). Similarly with FGF-2, angiogenesis induced by FGF-4 may be mediated by VEGF upregulation (Dell'Era et al., 2001; Deroanne et al., 1997). In summary, FGF-4 has clear therapeutic potential for the treatment of ischemic disease.

### **Insulin-like growth factors (IGFs)**

Two known members, IGF-1 and -2, constitute the IGF-family. IGF-1 is a polypeptide of 70 amino acids and homologous to proinsulin (Rinderknecht and Humbel, 1978). IGF receptor (IGFR) is a cell membrane tyrosine kinase that is related to the insulin receptor and mediates similar downstream cascades. In fact, at high concentrations IGFs can bind to the insulin receptor and *vice versa* (Siddle et al., 2001). Furthermore, IGFR can form heterodimers with the insulin receptor in addition to IGFR homodimers. IGF-1 has been shown to induce hypertrophic and antiapoptotic signals in cardiomyocytes as well as SMC proliferation and migration via

activation of the PI3K/Akt and MAPK pathways (Matsui et al., 1999; Duan et al., 2000; Mehrhof et al., 2001). Both IGF-1 and IGF-2 are expressed in regenerating skeletal myocytes (Levinovitz et al., 1992).

The genetic deletion of IGFs or IGFR leads to severe consequences (Liu et al., 1993). The majority of IGF-1<sup>-/-</sup> mice and all IGFR<sup>-/-</sup> mice die perinatally because of respiratory failure caused by impaired development of the diaphragm and intercostal muscles. Those with a deletion of either IGF-1 or IGF-2, or both in combination, suffer from progressive growth retardation and organ hypoplasia affecting the central nervous system, bones and skeletal muscle (Liu et al., 1993). The greatest growth deficiency occurs with the combined IGF-1 and IGF-2 knockout (30% of normal size). IGF-1 deletion in humans causes similar severe growth and mental retardation as well as insensitivity to exogenous growth hormone suggesting that the majority of the peripheral actions of growth hormone are mediated by IGF-1 production from the liver (Woods et al., 1996; Laron, 2001).

Consistent with an anabolic role, IGF-1 overexpression via adeno-associated virus (AAV) has been shown to increase muscle mass by 15% and 27% in young and old mice, respectively, and enhance muscle regeneration via the activation of satellite cells (Barton-Davis et al., 1998). IGF-1 has been demonstrated to upregulate VEGF expression involving the PI3K, MAPK and possibly HIF-1 $\alpha$  pathways (Fukuda et al., 2002). Consequently, IGF-1 acts in concert with VEGF in diabetes-induced retinal neovascularization and in retinopathy of prematurity (Smith et al., 1999; Hellstrom et al., 2001a). Adenoviral GT of both IGF-1 and IGF-2 promoted neovascularization *in vivo* in a Matrigel model (Su et al., 2003).

### **Angiopietins (Angs)**

Ang-1 has been implicated in the remodeling, maturation, stabilization and survival of blood vessels via triggering Tie-2 receptor on ECs (Davis et al., 1996; Thurston et al., 2000; Hawighorst et al., 2002; Papapetropoulos et al., 2000). Ang-1 does not directly promote EC proliferation *in*

*vitro* but has been reported to increase EC migration, mobilize EPCs as well as attenuate inflammation (Davis et al., 1996; Chae et al., 2000; Gamble et al., 2000; Hattori et al., 2001). Vascular defects lead to embryonic lethality both in Ang-1 and Tie-2 knockout mice (Sato et al., 1995; Suri et al., 1996). Ang-2 is a natural antagonist of Ang-1 by binding to but not activating Tie-2, which results in apoptosis of ECs and disruption of angiogenesis (Maisonpierre et al., 1997; Lobov et al., 2002). However, in the presence of other growth factors such as VEGF, vessel destabilization by Ang-2 has been proposed to be involved in the initiation of vascular sprouting (Lobov et al., 2002). Data also exist indicating that sometimes Ang-2 may act as a Tie-2 agonist (Gale et al., 2002).

Ang-1 has been shown to stabilize vessels and counteract vascular permeability without increasing EC proliferation (Thurston et al., 1999). These actions can be considered rather anti-angiogenic than angiogenic. Thus, it is somewhat surprising that naked plasmid encoding Ang-1 has been reported to stimulate angiogenesis and collateral growth *in vivo* (Shyu et al., 1998; Chae et al., 2000). Also other studies on Ang-1 are controversial or at least show that the effects of Ang-1 and Ang-2 are strongly context-dependent. For example, despite sharing the same signaling pathways such as PI3K/Akt and NO (Babaei et al., 2003), the actions of VEGF and Ang-1 appear to be either synergistic or antagonist *in vivo* depending on the model.

Overexpression of Ang-1 has been reported to enhance and suppress angiogenesis in the skin and in the heart, respectively (Suri et al., 1998; Visconti et al., 2002). Similarly, Ang-1 has been shown both to promote and inhibit tumor growth (Hawighorst et al., 2002; Shim et al., 2002). Extraordinarily, although blood vessels in the skin enlarged in response to Ang-1 overexpression, they were not leaky (Suri et al., 1998). In cardiac allografts, Ang-1 inhibited transplant arteriosclerosis (Nykanen et al., 2003). Different from the proposed anti-inflammatory role, constitutive overexpression of Ang-1 in the lung caused pulmonary hypertension by medial SMC hypertrophy via the induction of serotonin (Sullivan et al., 2003). In combination with

VEGF overexpression, Ang-1 has been suggested to create non-leaky blood vessels (Thurston et al., 1999 and 2000; Arsic et al., 2003).

The interesting finding that multimerization of Ang-1 at least into tetramers is required for the activation of Tie-2 may account for these different findings achieved with Ang-1 (Davis et al., 2003). Nevertheless, the true potential of this system for therapeutic angiogenesis is currently unclear due to these conflicting results.

### **Hepatocyte growth factor (HGF)**

HGF (also called scatter factor) is distantly related to plasminogen and was originally characterized as a highly potent mitogen for parenchymal hepatocytes (Nakamura et al., 1989). Today, it is recognized as a pleiotropic growth factor involved in embryonic development, skeletal muscle regeneration, angiogenesis and tumor growth (Jennische et al., 1993; Matsumoto and Nakamura, 1997).

Targeted disruption of HGF is embryonic lethal due to impaired organogenesis affecting the liver, placenta and skeletal muscle (Matsumoto and Nakamura, 1997). HGF has been shown to promote cell survival, proliferation, migration and induce transformation and protease production via its cell membrane receptor which is encoded by c-met proto-oncogene (Bottaro et al., 1991; Matsumoto and Nakamura, 1997; Xiao et al., 2001). In addition to ECs, SMC and pericytes express c-met. Similar to other receptor tyrosine kinases, triggering of c-met results in activation of PI3K/Akt and MAPK pathways (Xiao et al., 2001).

HGF is secreted from mesenchymal cells such as fibroblasts and SMCs as an inactive precursor protein which is cleaved extracellularly to a biologically active form in a similar way with the conversion of plasminogen to plasmin, (Matsumoto and Nakamura, 1997). Transgenic overexpression of HGF in the skin increased granulation tissue formation, angiogenesis and VEGF levels (Toyoda et al., 2001). Both PI3K/Akt and MAPK pathways have been shown to contribute to VEGF upregulation by HGF (Dong et al., 2001). Thus, in addition to direct effects, HGF may have

VEGF-dependent action on angiogenesis. However, data also exist that question the importance of VEGF in HGF-induced angiogenesis (Sengupta et al., 2003). HGF delivered with plasmid HVJ-liposome complexes has been reported to augment vascular growth both in non-ischemic and ischemic rat heart (Aoki et al., 2000).

### **Platelet-derived growth factors (PDGFs)**

The family of PDGFs currently comprise four members, PDGF-A, -B, -C and -D, that bind to PDGF receptors PDGFR- $\alpha$  and - $\beta$  (Collins et al., 1985; Bonthron et al., 1988; Li et al., 2000b; Bergsten et al., 2001). Both the ligands and receptors form heterodimers that modify the biology of ligand-receptor interaction. PDGFs, especially PDGF-B, are crucial for pericyte and SMC recruitment in capillaries and bigger vessels via PDGFR- $\beta$  activation as evidenced by PDGF-B-deficient mice that die of microaneurysm formation due to a lack of pericytes (Leveen et al., 1994; Lindahl et al., 1997).

Targeting both PDGFRs and VEGFRs with tyrosine kinase inhibitors has been shown to result in a more efficient inhibition of angiogenesis in a tumor model than either inhibitor alone (Bergers et al., 2003). PDGFs are also involved in the pathogenesis of atherosclerosis and restenosis after blood vessel injury via their SMC-stimulating properties. Thus, PDGFR-inhibition reduces the restenosis rate at least in animal experiments (Leppanen et al., 2003). PDGF-C overexpression in the mouse heart causes a phenotype of hypertrophic and dilated cardiomyopathy in male and female mice, respectively, as well as dilation of microvessels and vascular leakage possibly via VEGF upregulation (Ponten et al., 2003).

Recombinant PDGF-B protein given in combination with VEGF has been reported to result in more mature and stable blood vessels than monotherapy with either factor alone (Richardson et al., 2001). Administration of PDGF-B protein together with FGF-2 was also shown to produce vessel networks that remain stable in the rat cornea for more than a year after the withdrawal of the growth factors (Cao et al., 2003). Also recombinant PDGF-C protein

delivery has been shown to stimulate angiogenesis (Cao et al., 2002). However, given the controversy over the efficacy of recombinant protein administration for vascular growth, the potential of PDGFs for therapeutic vascular growth remains enigmatic.

### **Other vascular growth factors**

Recently, an endocrine-gland-derived vascular endothelial growth factor (EG-VEGF) was identified that promoted angiogenesis only in endocrine glands but not in skeletal muscle (LeCouter et al., 2001). Despite the name, EG-VEGF is not a member of the VEGF family. The discovery of its receptors as well as knock-out and knock-in experiments will hopefully shed light on the very interesting specificity of EG-VEGF.

Adenoviral GT of tissue kallikrein was shown to induce angiogenesis in mouse skeletal muscle through NO and prostacyclin production (Emanuelli et al., 2000). AdeNOS was reported to increase perfusion in a rat model of hindlimb ischemia two to four (but not one) week after gene delivery (Chao et al., 2002). These results are somewhat unexpected knowing that adenoviral gene expression peaks approximately at one week.

Therapeutic use of transcription factors might activate a number of angiogenic growth factors simultaneously. In addition to HIF-1 $\alpha$ , such potential transcription factors include early growth response factor-1 (EGR-1) and Ets-1 (Bryant et al., 2000; Aoki et al., 2000). PR39 is a small molecule secreted by macrophages and is capable of stabilizing HIF-1 $\alpha$ , which results in an angiogenic response presumably by VEGF and other HIF-1 $\alpha$ -dependent factors (Li et al., 2000a).

## **ANIMAL MODELS**

### **Animal models of myocardial ischemia**

The pig (domestic or minipig) model of myocardial ischemia has recently become more popular than the dog model because

of the financial and social pressures (Verdouw et al., 1998). In young pigs, the size of the heart and the anatomy and physiology of the coronary circulation is quite similar to man, particularly the minimal pre-existence of coronary collateral circulation. In dogs and baboons coronary collaterals are innately better developed (Schaper and Ito, 1996; Verdouw et al., 1998). In small animal models, the induction of a consistent degree of ischemia and reliable measurement of endpoints such as collateral formation or myocardial function are technically much more difficult to obtain (Verdouw et al., 1998).

One approach to induce myocardial ischemia is achieved by surgical ligation of the left anterior descending or circumflex branch of the left coronary artery. A more sophisticated induction is performed by a catheter-mediated embolization coil to block the coronary flow, avoiding the need for thoracotomy (Watanabe et al., 1998). Usually, only animals with an established coronary circulation such as dogs tolerate proximal ligations whereas in pigs even a distal occlusion may require precondition (Kivelä A et al., unpublished). A clear advantage of an acute occlusion model is that the exact time of induction of ischemia is known. However, an acute occlusion leads to acute myocardial infarction in the territory of the occluded coronary but the region of the myocardium at risk, and thus the target of therapeutic interventions, will likely be small.

Progressive coronary occlusion with an ameroid constrictor is a better model of an atherosclerotic process and has been the most popular model of chronic myocardial ischemia (Banai et al., 1994; Villanueva et al., 2002). An ameroid constrictor, composed of hygroscopic material such as casein encased within a steel sleeve, is most often implanted around the origin of the left circumflex branch. A complete occlusion is reached usually over a very variable period of 2 to 8 weeks (Hughes et al., 2003). However, some animals do not develop a complete occlusion at all. The mortality rate may be up to 30% because of a defibrillation resistant ventricular fibrillation (typical for pigs) perioperatively or due to later ischemia (Hughes et al., 2003). The large variation in both the time frame for

occlusion to occur and the size of ischemic region hamper the usefulness of the ameroid model. A better control on the timing of ischemia could be achieved by preconditioning with an ameroid implantation followed by a later coronary ligation or embolization.

In addition to the progressive occlusion model, a fixed stenosis can be generated with a hydraulic occluder with the possibility to adjust coronary blood flow by an external inflator (Hughes et al., 2003). However, the major disadvantage of this model is the technical complexity and difficulty with long-term animal maintenance.

Regardless of the model used, myocardial perfusion and function should also be measured during exercise because the perfusion deficit may be small at rest. Moreover, both pre- and post-treatment assessment should be done to minimize the effect of interindividual differences in the degree of ischemia.

### **Animal models of lower limb ischemia**

The most frequently used animal model of lower limb ischemia is the rabbit hindlimb model. In this model, the femoral artery is totally excised from its proximal origin to the site where it bifurcates into saphenous and popliteal arteries, combined with the proximal ligations of lateral femoral circumflex and deep femoral arteries (Pu et al., 1994; Tsurumi et al., 1996). Subsequent ischemia is acute and severe, as superficial necrosis of the foot was reported to occur in 32% of cases (Pu et al., 1994). A decrease in peripheral blood pressure persisted at least until postoperative day 90.

There are some limitations in the usefulness of the original rabbit hindlimb model. Firstly, animals often develop extensive muscle necrosis because of the acute ischemia. Later, there is a tendency toward spontaneous improvement in collateral growth and tissue perfusion. Regional blood flow in the ischemic legs has been reported to be similar to the unoperated legs three weeks after surgery, although the distal blood pressure is only 20% of the normal value four weeks after the operation (Gowdak et al., 2000a). The discrepancy between blood flow and

pressure is likely due to collaterals that are much smaller than the excised femoral artery, thus attenuating systolic blood pressure. On the other hand, if only the superficial part of the femoral artery is ligated, the numerous small collaterals ensure that the leg does not develop ischemia at all (Ito et al., 1997a). In a variation of the rabbit model, an ameroid constrictor is implanted around the femoral artery producing a progressive occlusion (Baffour et al., 2000).

Other animals that have been used as models for peripheral ischemia are the mouse and rat (Couffignal et al., 1998; Mack et al., 1998a). However, these models suffer from smaller peripheral vessels impeding the reliable assessment of efficacy of therapeutic interventions. A pig model of collateral growth induced by ligation of the superficial femoral artery has also been introduced (Buschmann et al., 2003). However, induction of true hindlimb ischemia in pigs requiring additive resection of the femoral artery branches would likely lead to animal welfare issues.

## **Lymphedema**

Heterozygous inactivation of VEGFR-3 or VEGF-C causes lymphedema in mice due to a lack of cutaneous lymphatics and can be used as a model of hereditary lymphedema (Karkkainen et al., 2001 and 2003). Surgical lymphedema lasting at least for 14 weeks can be created by ligation of the tail lymphatics in mice and rats (Slavin et al., 1999; Yoon et al., 2003). Similarly to the tail model, destruction of lymphatics in the rabbit ear mimics post surgical lymphatic insufficiency (Szuba et al., 2002; Yoon et al., 2003).

## **GENE TRANSFER IN SKELETAL MUSCLE AND MYOCARDIUM**

### **Principles of gene transfer**

Gene therapy is a rapidly growing field with at least 636 clinical trials completed or ongoing with 3496 patients by the year 2003 (<http://www.wiley.co.uk/wileychi/genmed/clin>

ical). Therapeutic GT can be defined as the transfer of nucleic acids to the somatic cells of an individual with a resulting therapeutic effect (Yla-Herttuala and Alitalo, 2003). The total effectiveness of GT is determined by the efficacy of gene delivery to the target tissue, the entry of genetic material into cells and the expression level of the transduced gene in the target cells (Yla-Herttuala and Alitalo, 2003). The first GT in man was performed in lymphocytes using a retroviral approach in 1989 (Rosenberg et al., 1990). In addition to therapy, GT of growth factors and cytokines provides mechanistic insight into their biology *in vivo*.

In comparison with administration of recombinant growth factors, GT offers many advantages. Most importantly, the therapeutic levels of growth factors in tissue are higher and last longer. Secondly, a GT vector can be modified genetically to generate tissue- or cell-specific effects (Wickham, 2000). The emerging regulated gene expression systems such as the tetracycline-regulated lentiviral GT may overcome the difficulties in the dosing of protein levels after *in vivo* GT (Koponen et al., 2003).

### **Gene transfer vectors**

The ideal GT vector would be one that combines efficient transduction with long-term stable and regulated gene expression only in the target tissue without side effects such as inflammation. Unfortunately, such a vector does not exist yet. In vascular gene therapy, the most widely used vectors have been plasmid DNA despite its very low efficacy and adenoviruses (Rissanen et al., 2001; Yla-Herttuala and Alitalo, 2003). Both of them can be easily produced in large quantities, are capable of transfecting non-dividing cells, and generate transient expression.

However, it is becoming evident that plasmid DNA may not be efficacious for clinical gene therapy. Thus, viruses have been extensively utilized as GT tools (Table 1). In viral vectors, sequences essential for replication are replaced by treatment and regulatory sequences rendering the virus replication deficient.

**Table 1.** Characteristics of different gene transfer vectors.

Vector	Advantages	Disadvantages
Naked plasmid DNA and complexes	Easy to produce Safe	Extremely low efficacy Transient expression
Adenovirus	Very high efficacy Relatively high capacity Easy to produce in high titers Transduces proliferative and quiescent cells Tropism for multiple cell types	Immune and inflammatory response Transient expression (<2 weeks) Repeated administration impossible Limited tropism for skeletal myocytes
Adeno-associated virus	Long expression (months) Transduces proliferative and quiescent cells Tropism for skeletal muscle and myocardium Low inflammatory responses	Limited DNA capacity (4-5kb) Difficult production
Baculovirus	High capacity Easy to produce in high titers Rapid construction of recombinant viruses Does not cause disease in mammals	Transient expression <i>In vivo</i> toxicity Moderate efficacy Limited tropism
Herpes simplex-virus	High efficacy High DNA capacity Easy to produce	Unable to transduce non-dividing cells Cytotoxicity
Epstein-Barr-virus	High capacity Stable gene expression Extrachromosomal replication	Non-specific integration in the genome Low efficacy except in hematopoietic cells Difficult production Low titers
Lentivirus	High DNA-capacity Transduces proliferative and quiescent cells Stable gene expression Specific integration in the genome Low inflammatory responses	Low efficacy except in hematopoietic cells Difficult production Low titers
Retrovirus	Stable gene expression Relatively easy to produce	Non-specific integration in the genome Unable to transduce non-dividing cells Low efficacy Limited capacity Low titers

Adenovirus and AAV are the most efficient vectors in skeletal muscle and myocardium and are currently the most promising candidates for therapeutic purposes in these tissues. Modified from (Rutanen et al., 2002).

### *Naked plasmid DNA and complexes*

Experiments performed in the early 1990's gave promise to simple injections of naked plasmid DNA as an efficient GT tool because gene expression was reported to last for months in mouse skeletal muscle (Wolff et al., 1990; Wolff et al., 1992). It was even claimed that naked plasmid DNA was better than adenoviral GT in skeletal muscle (Davis et al., 1993). However, it is now known that only very low GT efficiency is achieved with naked plasmid DNA due to the poor uptake of plasmid DNA into cells. Furthermore, only a small fraction of the DNA taken up by cells enters the nucleus, where it remains extrachromosomal and directs a transient expression lasting a couple of weeks in most tissues (Tripathy et al., 1996).

Despite the low efficacy, naked plasmid DNA has previously been the favorite vector in proangiogenic gene therapy experiments (Tsurumi et al., 1996; Baumgartner et al., 1998; Vale et al., 2001). Carrier molecules such as fractured polyamidoamine dendrimers, polyethylenimines or liposome complexes can be used to moderately increase the transfection efficiency of plasmid DNA (Turunen et al., 1999; Laitinen et al., 1997a).

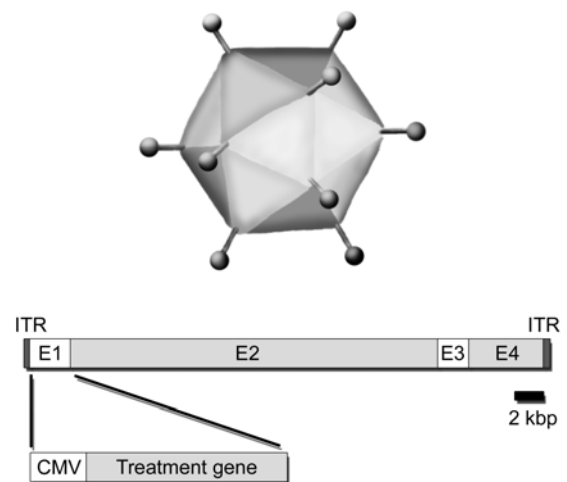
Although plasmid DNA has not traditionally been linked to major safety concerns, it may cause significant inflammation in skeletal muscle (McMahon et al., 1998). In the rabbit heart intracoronary injection of plasmid-liposome complexes was ineffective but caused false positive  $\beta$ -galactosidase (*LacZ*) signal via microinfarctions (Wright et al., 1998). Furthermore, in humans transient fever was observed in 39% of patients receiving intracoronary plasmid/liposome complexes (Hedman et al., 2003). However, severe adverse effects were not observed.

Other means to improve the efficacy of plasmids than carrier molecules have been introduced, as well. Tissue damage and regeneration caused by toxic agents or ischemia enhances transfection, presumably via permeabilization of cell and nuclear membranes (Vitadello et al., 1994; Takeshita et al., 1996). Intravascular injection of naked plasmid using high pressure and volume has been shown to

result in 10% GT efficiency in rat skeletal muscle (Budker et al., 1998). More elegantly, electric currents and ultrasound, either alone or in combination, have been used to aid the entry of plasmids to cells and reported to increase transfection efficacy substantially (Mathiesen, 1999; Yamashita et al., 2002). However, clinical use of these modalities requires the uncoupling of significant tissue damage from efficient transfection.

### *Adenoviral vectors*

Adenoviruses constitute linear double-stranded DNA of approx. 36 kbp surrounded by a capsid (Figure 8) (Kovesdi et al., 1997; St George, 2003). Wild type human adenoviruses exist in at least 47 different serotypes divided in six subgroups and are a general cause of respiratory and other infections.



**Figure 8.** Upper: adenoviruses are non-enveloped icosahedral particles approximately 70-100 nm in diameter. Penton fibers that project from each apex mediate the attachment to the cell surface. Modified from <http://web.uct.ac.za/depts/mmi/stannard/adeno.html> by Linda Stannard. Lower: in the first generation adenoviral vectors, the treatment (or marker) gene is usually inserted in the deleted E1 region of the adenovirus genome. Also the E3 region is partially or totally deleted. The cytomegalovirus (CMV) promoter is most often used to drive the expression of the foreign gene. In the first generation vector the capacity for the expressed gene is < 7 kbp depending on the extent of E3 deletion. ITR = inverted terminal repeat.

Adenovirus has several advantages as a GT vector which makes it currently the most used vector in clinical cardiovascular trials (<http://www.wiley.co.uk/wileychi/genmed/clinical>). Most importantly, it is a very efficient GT vehicle *in vivo* capable of transducing non-proliferating cells (Kovesdi et al., 1997; St George, 2003). Furthermore, adenovectors can be produced in high titers. In humans, adenovirus has not been associated with oncogenic transformation. The first generation adenoviral vector (serotypes 2 or 5 from group C) with the deletion of regions E1, or E1 and E3 of the viral genome (Figure 8) drives a transient gene expression from an extrachromosomal position usually lasting 1-2 weeks in immunocompetent animals (Hiltunen et al., 2000b). This property can be considered either as an advantage or disadvantage depending on the application.

Most adenoviral serotypes enter cells via coxsackie-adenovirus receptor (CAR) and integrins such as  $\alpha_v\beta_3$  and  $\alpha_v\beta_5$ , which mediate the primary attachment and internalization, respectively (Bergelson, 1999). After internalization, the viral DNA is released from the disassembled capsid in endosomes and it reaches the nucleus within 45 min after the infection. CAR, which is a component of the tight junction (Cohen et al., 2001), is abundantly present in myocardium, hepatocytes, and epithelial cells but is downregulated in skeletal muscle during maturation together with integrins, which hampers transduction of mature skeletal myocytes (Nalbantoglu et al., 1999). Satellite cells/myoblasts are more susceptible to adenoviral infection due to increased CAR expression (Nalbantoglu et al., 1999). Thus, muscle regeneration induced by myonecrotic agents or ischemia may improve transduction efficiency.

Modification of capsid proteins to eliminate CAR binding and redirecting the attachment to tissue-specific receptors may lead to improved targeting and a reduced immune response (Wickham, 2000). Transcriptional targeting e.g. with hypoxia-inducible or cardiac-specific promoters (Griscelli et al., 1998; Binley et al., 1999) may further decrease the risk for transduction of unwanted sites such as non-target organs or even oocytes (Hiltunen et al., 2000b; Laurema et al., 2003).

The problems associated with the use of first generation adenoviral vector are related to immunological and inflammatory reactions (St George, 2003). For example, in skeletal muscle excessive amounts of adenovirus may result in toxicity and loss of contractile force (Yang et al., 1998). In comparison with the first generation adenoviral vector, the high capacity "gutless" adenoviruses with less viral sequences (E1, E3, and E2 or E4 deleted) have a foreign gene incorporation capacity of up to 30 kbp and may provide a longer gene expression along with reduced inflammation (Chen et al., 1999). However, these improvements have also been questioned (Wen et al., 2000).

The decline in adenoviral gene expression a week after GT is due to inactivation or loss of vector DNA in transduced cells. Also mechanisms other than cellular and humoral attack on transduced cells have been proposed to contribute to the diminished gene expression (Chen et al., 1999; Wen et al., 2000). The repeated administration of adenovirus in skeletal muscle is effective in mice but not in larger animals because of the formation of neutralizing antibodies. This, however, can be circumvented by using a different adenoviral serotype (Chen et al., 2000; Zoltick et al., 2001).

Although the only death directly associated with human gene therapy was caused by an enormous dose of adenovirus given to a immunocompromised patient (Lehrman, 1999), clinical phase I/II trials suggest that adenovirus with a relevant dose is safe and well tolerated despite an occasional transient fever and hepatopathy (Makinen et al., 2002; Grines et al., 2002; Hedman et al., 2003; Rajagopalan et al., 2003).

#### *Other viral vectors*

Adeno-associated virus (AAV) has many appealing properties for therapeutic targeting of skeletal muscle and myocardium. AAV is a small (diameter 20-25 nm) single-stranded DNA parvovirus that exists at least in seven different serotypes. It displays a natural tropism for muscle cells, transduces quiescent cells, drives long-



term expression lasting several months, is little immunogenic and the wild-type virus is not known to cause disease in humans (Kessler et al., 1996; Svensson et al., 1999; Pajusola et al., 2002). Progressive gene expression pattern after AAV transduction reaching the maximum 3-6 weeks with lower peak levels is clearly different from the expression pattern of the adenoviral vector (Kessler et al., 1996; Poliakova et al., 1999). The wild type virus incorporates into chromosome 19 in man, but AAV vectors do not appear to integrate significantly despite quite stable gene expression (Schnepp et al., 2003). Problems with AAV are related to the difficult production of purified lots and a small capacity (< 5 kbp). AAV appears highly promising for induction of sustained therapeutic vascular growth in skeletal muscle, myocardium and perhaps in other tissues (Bohl et al., 1998; Arsic et al., 2003).

Retroviruses (usually murine leukemia virus, MuLV) have been used both in pre-clinical and clinical studies of cardiovascular diseases (Laitinen et al., 1997a; Pakkanen et al., 1999; Mann et al., 1999). After entering the cell, the genomic RNA is reverse-transcribed to DNA that integrates into the host genome of proliferating cells leading to permanent gene expression. The demand for cell proliferation has made retroviral GT feasible mainly in *ex vivo* applications, especially in hematopoietic cells (Mann et al., 1999). Random integration of the transgene causes the risk of oncogene activation. Indeed, a major setback affecting the whole gene therapy field was recently faced in a clinical trial of severe combined immunodeficiency (SCID), as 2/11 of the children developed leukemia after otherwise successful treatment of CD34+ bone marrow cells (Marshall, 2003).

Thus, lentiviruses (human, simian and feline immunodeficiency viruses) will likely replace MuLV-type retroviruses in gene therapy for genetic disorders because they seem to be safer and also transduce non-dividing cells (Buchsacher and Wong-Staal, 2000). Regulated gene expression will likely be a prerequisite for growth factor therapy with lentiviruses (Koponen et al., 2003). Unfortunately, transduction efficiency of skeletal muscle and myocardium with lentiviruses seems to be low (Kang et al., 2002; O'Rourke et al., 2003).

Other viruses that have been tested for GT in skeletal muscle or myocardium include Herpes simplex viruses and sendaiviruses but their feasibility for therapeutic angiogenesis needs further research (Akkaraju et al., 1999; Shiotani et al., 2001). Baculoviruses have been shown to successfully transduce blood vessels and choroid plexus in the brain but their usefulness in skeletal muscle or myocardium is currently unknown (Airenne et al., 2000; Lehtolainen et al., 2002). Other GT vehicles developed so far are vectors derived from Sindbis virus, Semliki Forest virus as well as a combination of adeno- and retroviruses (Wahlfors et al., 2000; Zheng et al., 2000).

### **Gene transfer routes**

The most commonly used route for gene delivery for therapeutic vascular growth is achieved by direct intramuscular (i.m.) injection (Baumgartner et al., 1998; Rosengart et al., 1999; Symes et al., 1999). In the heart direct i.m. injections are more difficult to perform than in skeletal muscle because thoracotomy is usually required, but may be practical during by-pass surgery. Ultrasound may help guide injections via minimal thoracotomy (Esakof et al., 1999). Feasibility of the i.m. injection strategy in the heart has been substantially improved by the introduction of percutaneous catheter-mediated injection systems such as NOGA (Gepstein et al., 1997; Kornowski et al., 1999; Vale et al., 1999; Vale et al., 2001). Myocardial GT guided by the NOGA system has been reported to be as efficient as direct injections at thoracotomy (Kornowski et al., 2000). Pericardial delivery is also a possibility, but will not result in effective transduction of the myocardium without concomitant treatment with proteases (Lazarous et al., 1999; Fromes et al., 1999).

The intra-arterial (i.a.) route has also been used for therapeutic angiogenesis in humans (Makinen et al., 2002; Grines et al., 2002; Hedman et al., 2003), but it is limited by very low GT efficacy in tissue surrounding the blood vessel (Magovern et al., 1996; Wright et al., 1998; Wright et al., 2001; Lee et al., 2000a). Furthermore, i.a. administered vector leads to more extensive

biodistribution than direct injection into tissues (Hiltunen et al., 2000b). Nevertheless, the i.a. route is still appealing because it may provide more widespread transduction of the target tissue than i.m. injections. However, interruption of blood flow, long incubation time, permeabilization of endothelium, or modulation of hydrostatic and osmotic pressures are required for successful GT in tissue via the i.a. route (Cho et al., 2000; Logeart et al., 2000; Davidson et al., 2001; Wright et al., 2001).

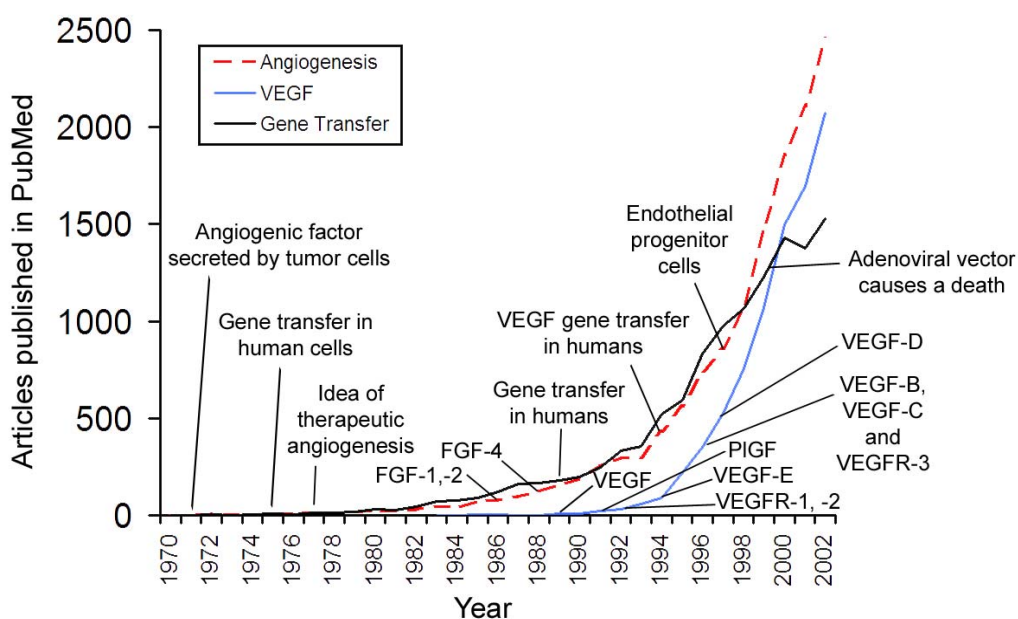
In the *ex vivo* strategy, cells, blood vessels or organs are isolated and transduced outside the body, and then returned back to the same subject resulting in high transduction efficacy and a reduced risk of vector leakage into the circulation (Mann et al., 1999; Lemstrom et al., 2002). Since myoblasts are relatively easy to culture and transduce *in vitro* coupled with their ability to fuse with myofibers *in vivo*, these precursor cells have been used for successful gene delivery into skeletal muscle and myocardium (Springer et al., 1998; Lee et al., 2000b). However, i.m. injection of transplanted cells may lead to sub-optimal spreading and, together with unregulated gene expression, to excessive growth factor concentrations and even fatal side-effects in the myocardium (Springer et

al., 1998; Lee et al., 2000b). Thus, the i.a. route may be preferable for implantation of transplanted cells throughout the myocardium (Suzuki et al., 2000).

## THERAPEUTIC VASCULAR GROWTH

Natural endogenous angiogenic and arteriogenic responses are usually inadequate to fully compensate for the consequences of occlusions within large conducting arteries. For example, the major stimulus for endogenous VEGF expression is hypoxia (Forsythe et al., 1996). However, collaterals grow typically in the non-ischemic regions resulting in a discrepancy between the need and supply of this potent vascular growth factor.

The concept of therapeutic blood vessel growth to augment tissue ischemia originated from studies investigating tumor angiogenesis factors in the 1970's (Folkman et al., 1971; Svet-Moldavsky and Chimishkyan, 1977). However, only after sequencing the cDNA of FGF-1 and -2 and VEGF in 1986-89, it was possible to produce purified growth factors and proceed to animal experiments. Thereafter, research into therapeutic angiogenesis has proceeded with an accelerated pace (Figure 9).



**Figure 9.** Historical perspective and the landmarks of therapeutic vascular growth. Curves represent scientific articles published during 1970-2002 according to the PubMed database including the word "angiogenesis", "VEGF/VPF" or "gene transfer" in the title or abstract. The indicated points refer to the cloning of the growth factor or a major event in the field.

The era of therapeutic angiogenesis can be divided into the periods of recombinant protein therapy, GT with naked plasmid DNA, and the currently ongoing chapter of viral-mediated GT and transplantation of vascular stem cells. Although several clinical trials for therapeutic angiogenesis have been judged positive, hard clinical endpoints such as mortality, myocardial infarction, need for revascularization or amputation is currently lacking (Yla-Herttuala and Alitalo, 2003). Analogous to therapeutic angiogenesis, GT of VEGFR-3 ligands could be used to promote lymphangiogenesis in patients suffering from lymphedema. A completely different approach to "therapeutic vascular growth" may be achieved by growth of arteries *in vitro* for bypass grafts (Niklason et al., 1999).

### Recombinant growth factor therapy

Before the development of GT vectors for sustained production of angiogenic growth factors, i.a. or i.m. administration of recombinant proteins was a popular mode of delivery in animal models and until recently also in clinical trials. Promising results were reported both for the treatment of myocardial and peripheral ischemia with FGF-1, FGF-2 and VEGF given once or repeatedly via i.a., i.m., s.c. or i.v. routes (Baffour et al., 1992; Yanagisawa-Miwa et al., 1992; Pu et al., 1993; Takeshita et al., 1994a and 1994b; Banai et al., 1994; Bauters et al., 1995; Villanueva et al., 2002).

The results of larger placebo controlled double-blinded randomized clinical trials were disappointing because recombinant VEGF or FGF-2 administration in patients with CAD did not improve the primary endpoints, exercise tolerance or myocardial perfusion at 60 days (VIVA trial n=178) or 90 days (FIRST trial, n=337) (Henry et al., 2003; Simons et al., 2002). In the TRAFFIC trial (n=190) a single i.a. administration of recombinant FGF-2 improved peak walking time by 1.77 min in claudicants 90 days after the treatment but the group given the second FGF-2 dose at 30 days did not differ from the placebo group (0.60 min), which

made the interpretation on the true efficacy difficult (Lederman et al., 2002).

Recombinant protein therapy for angiogenesis is fundamentally limited by the short half-life of most growth factors *in vivo* (e.g. serum half life of FGF-2 is approx. 50 min) (Lazarous et al., 1997). After a single intradermal injection, the biological activity of VEGF protein was significantly attenuated after 60 min (Dafni et al., 2002). In fact, a single injection of recombinant growth factor protein could be even compared to the strategy to treat diabetes with a single shot of insulin because both insulin and vascular growth factors are peptides that bind to tyrosine kinase receptors and launch similar cell signaling cascades such as PI3K/Akt (Jiang et al., 2000).

The theoretical advantage of recombinant protein therapy is further impaired by the low uptake of growth factors in myocardium (3-5%) even after a direct intracoronary injection (Lazarous et al., 1997). Furthermore, high doses of VEGF and FGF-2 induce hypotension via NO production and FGF-2 may cause severe proteinuria (>1g/day in 8-31% of patients treated with FGF-2) (Hariawala et al., 1996; Henry et al., 2003; Cooper, Jr. et al., 2001; Simons et al., 2002; Lederman et al., 2002). Thus, apart from nephrotoxicity the beneficial effects of a single administration of VEGF or FGF-2 seem quite similar to the effects obtained by nitrate treatment for an ischemic attack; the vasodilating effects are short-term and high doses cause hypotension.

A continuous i.a. infusion of cytokines such as MCP-1, GM-CSF and PIGF has been shown to promote collateral artery growth in animal experiments (Ito et al., 1997b; Buschmann et al., 2001; Arras et al., 1998; Pipp et al., 2003). Although the continuous infusion is a clear improvement over a single injection strategy, also this approach is hampered by the low uptake of cytokines into the growing collaterals. In a small randomized, double-blind, placebo-controlled pilot study, a single intracoronary injection of GM-CSF combined with a two week s.c. GM-CSF treatment improved a collateral flow index (Seiler et al., 2001). The effects of GM-CSF were thought to be attributable to the recruitment of bone marrow-derived monocyte-macrophages or

EPCs. However, it is possible that leukocytosis and a rise in CRP levels caused by GM-CSF (Seiler et al., 2001) or by other similar cytokines may be detrimental by evoking further ischemic events and by contributing to progression of atherosclerosis.

### **Gene transfer with naked plasmid DNA**

Because of the disadvantages of recombinant protein therapy, the next step in therapeutic angiogenesis was to utilize the simplest GT vector, naked plasmid DNA. In animal models, naked plasmid DNA encoding various factors including VEGF, FGFs, Ang-1, HIF-1 $\alpha$  and HGF has been reported to promote therapeutic vascular growth (Tsurumi et al., 1996; Shyu et al., 1998; Vincent et al., 2000). Although these studies reported increases in capillary density, collateral growth, blood pressure and perfusion in the treated muscles, they did not significantly contribute to our understanding of angiogenesis because the mechanisms of vascular growth were not addressed. Furthermore, vascular permeability and edema, which both indicate efficient VEGF production in tissue (Poliakova et al., 1999; Pettersson et al., 2000), have not been reported to occur after naked plasmid mediated VEGF GT in animals (Tsurumi et al., 1996).

The phase I clinical studies also suggested that naked plasmid DNA-mediated GT of VEGF and VEGF-C is effective for therapeutic vascular growth as these studies reported increases in myocardial perfusion in CAD patients and improvements in ankle brachial blood pressure indices (ABI) in PAD patients as well as alleviation of symptoms (Baumgartner et al., 1998; Symes et al., 1999; Vale et al., 2001; Shyu et al., 2003). However, a recent phase I open-label study using naked plasmid encoding VEGF-C in CAD patients with no other treatment option did not show angiographic evidence of collateral growth (Fortuin et al., 2003).

Limb edema was observed in 34% of patients injected i.m. with naked plasmid VEGF (Baumgartner et al., 2000). However, the lack of a control arm made it impossible to conclude if edema was due to successful

delivery of VEGF, because of the i.m. injections themselves, or inflammation caused by plasmid DNA (McMahon et al., 1998). Very modest increases in serum VEGF<sub>165</sub> levels have been used as to demonstrate the efficacy of naked plasmid-mediated VEGF production, but they are most likely due to variable baseline serum VEGF concentrations in patients with CAD and PAD (Baumgartner et al., 1998; Symes et al., 1999). Such increases were not observed after the i.a. or i.m. delivery of AdVEGF<sub>165</sub> despite a 15-18-fold increase in tissue VEGF<sub>165</sub> concentration with the i.m. route (Magovern et al., 1996; Hedman et al., 2003).

Because of the substantial placebo effects in angiogenesis trials, the data obtained thus far in phase I open-label studies can only be used to demonstrate the safety and feasibility of the treatment. Currently, there are no published randomized, double-blind, placebo-controlled trials using i.m. injections of naked plasmid DNA encoding VEGFs or FGFs but preliminary data of a such study using the NOGA system to deliver naked plasmid VEGF into ischemic myocardium appear negative (Kastrup et al., 2003).

### **Viral gene transfer**

Because of the theoretical and practical limitations of both recombinant protein and naked plasmid DNA, viral vectors are replacing these approaches in efforts to stimulate blood vessel growth. It is now clear that growth factor production lasting at least for a few days is required for efficient induction of blood vessel growth. Preclinical experiments using viral vectors have provided evidence for the usefulness of VEGF and FGF-1 and -2 towards vascular growth *in vivo* (Pettersson et al., 2000; Mack et al., 1998b; Gowdak et al., 2000b; Muhlhauser et al., 1995; Ueno et al., 1997). In contrast to naked plasmid VEGF, adenoviral VEGF GT induces vascular permeability and edema approximately a week after GT both in rabbits and mice (Poliakova et al., 1999; Pettersson et al., 2000).

The simplest, although not the most efficient, approach to deliver the vector into the myocardium or lower limbs is achieved

by catheter-mediated i.a. injection. In a randomized double-blinded phase II study (n=54) using injections of AdVEGF<sub>165</sub> or plasmid-liposome VEGF<sub>165</sub> into infrainguinal arteries, evidence for increased angiographically detectable arteries was obtained in comparison to saline-treated controls after three months (Makinen et al., 2002). In patients with CAD (n=103), hints of improved myocardial perfusion were observed six months after AdVEGF<sub>165</sub> given into coronaries i.a. immediately following PTCA (Hedman et al., 2003). Intracoronary injection of AdFGF-4 showed efficacy only in a subgroup of patients in terms of improved exercise tolerance time at four weeks in a multicenter, randomized, double-blinded study of 79 patients with chronic and stable angina (Grines et al., 2002).

According to knowledge gained from animal studies, currently the most efficient way to induce angiogenesis is obtained by i.m. administration of adenovirus or AAV encoding VEGF (Magovern et al., 1996; Poliakova et al., 1999; Pettersson et al., 2000; Arsic et al., 2003). In agreement with experimental data, AdVEGF<sub>121</sub> i.m. ( $4 \times 10^8$  or  $4 \times 10^{10}$  particle units) induced dose-dependent lower limb edema in patients with intermittent claudication. The incidence of macroscopic edema was 19% and 28% with the two AdVEGF doses, respectively, and 9% in the placebo group in the phase II, randomized, double-blind, placebo-controlled RAVE trial (n=105) (Rajagopalan et al., 2003). Despite edema, i.m. administration of AdVEGF<sub>121</sub> into ischemic muscles was well tolerated. However, 12 weeks after GT the peak walking time was not improved over placebo treatment (Rajagopalan et al., 2003).

In CAD patients with no other treatment option, time to 1 mm ST-depression during exercise test was significantly improved at 26 weeks but not at 12 weeks in patients that received AdVEGF<sub>121</sub> ( $4 \times 10^{10}$  particle units) into the myocardium at thoracotomy compared to those on maximal standard drug therapy (Stewart, 2003). Other endpoints, such as onset of angina and total exercise tolerance, were improved also at 12 weeks. A contribution of a placebo effect in these results cannot be ruled out due to thoracotomy.

## Transplantation of vascular stem cells

In animal models of peripheral and myocardial ischemia injections of bone marrow-derived cells into the ischemic tissue have augmented neovascularization (Takahashi et al., 1999a; Kocher et al., 2001; Rafii and Lyden, 2003). In open-label clinical pilot studies, administration of autologous circulating or bone marrow-derived mononuclear or progenitor cells into the infarcted myocardium has been reported to be safe and improve left ventricular function (Assmus et al., 2002; Strauer et al., 2002). Similar preliminary efficacy was also shown in a randomized double-blind pilot study (n=22) in which the transplantation of bone marrow-derived mononuclear cells into ischemic limb muscles of CLI patients improved ABI by 0.1 in comparison to patients receiving peripheral blood-derived mononuclear cells (Tateishi-Yuyama et al., 2002). The contribution of true stem cells in these findings is unknown because of the lack of appropriate controls i.e. purified monocytes/ macrophages.

Recently, purified bone marrow-derived CD133+ stem cells were injected into infarcted myocardium of six patients, resulting in an improvement of cardiac function in four patients (Stamm et al., 2003). However, keeping in mind the significant placebo effects in published angiogenesis trials (Simons et al., 2002; Henry et al., 2003; Rajagopalan et al., 2003), it is worth awaiting placebo-controlled double-blinded randomized trials before conclusions can be drawn about the clinical usefulness of bone marrow-derived cells for the treatment of peripheral or myocardial ischemia.

There are significant problems and unanswered questions regarding vascular stem cells. For example, it is currently unclear what proportion of the isolated bone marrow-derived and circulating cells thought to be EPCs and other stem cells are actually monocyte-macrophages (Rehman et al., 2003). Bone-marrow cell populations may also contain cells that promote fibrosis or arrhythmias or other harmful effects when injected into myocardium (Rafii and Lyden, 2003). Little is known about the optimal dose and route of administration of these cells. Also, it is not known why the strong

endogenous mobilization response of EPCs observed after tissue injury is insufficient but additional EPC administration is needed (Gill et al., 2001; Rafii and Lyden, 2003).

No evidence of EPC incorporation into angiogenic blood vessels was found in skeletal muscle engineered to constitutively express VEGF (Springer et al., 2003). EPCs neither significantly contributed to the blood vessel growth and remodeling in regenerating lung vasculature (Voswinckel et al., 2003). Recently, it was shown that transplantation of unfractionated bone marrow into postinfarction scars failed to induce any differentiation of grafted cells into cardiomyocytes or ECs, which correlated with the lack of a functional benefit (Bel et al., 2003). As with almost all new and exciting approaches, the true biology of EPCs seems to be hidden by the huge hype aroused by them.

### **Gene transfer for lymphedema**

Lymphedema is an attractive target for therapeutic lymphangiogenesis (Yla-Herttuala and Alitalo, 2003). In contrast to therapeutic blood vessel growth, only a few preclinical studies with a disease model and no clinical studies exist. In the chy mouse model of lymphedema, adenoviral and AAV-mediated GT of VEGF-C promoted growth of functional lymphatics in the transduced ears (Karkkainen et al., 2001). It was noteworthy that AAV-mediated lymphatics were stable at least seven weeks after GT thus facilitating a sustained therapeutic effect. Also naked plasmid VEGF-C was suggested to restore lymphatic function and reduce lymphedema both in a rabbit ear model and mouse tail model (Yoon et al., 2003). In a rabbit ear model of chronic post-surgical lymphatic insufficiency, a single s.c. dose of recombinant VEGF-C protein was reported to promote lymphangiogenesis and ameliorate lymphatic function 7-8 days after the treatment (Szuba et al., 2002). Specific VEGFR-3 ligands such as VEGF-C<sup>156S</sup> or murine VEGF-D may be preferable for the treatment of lymphedema because of the lack of VEGFR-2-mediated blood vessel effects such as vascular permeability and edema (Saaristo et al., 2002).

## **AIMS OF THE STUDY**

The aim of this thesis was to study the potential of gene transfer of vascular growth factors for the treatment of peripheral and myocardial ischemia and lymphedema. This goal was divided into specific aims as follows:

1. Profile gene expression patterns in ischemic human skeletal muscle in order to obtain information on the molecular mechanisms of tissue responses to ischemic stress and factors inducing angiogenesis (I and II).
2. Analyze the degree of ischemia and pathological changes, VEGF and VEGFR-2 expression in the previously established rabbit hindlimb ischemia model and develop a new model with restricted limb ischemia and necrosis (II, III and IV).
3. Compare naked plasmid DNA and adenovirus as well as routes of administration for gene transfer into skeletal muscle and myocardium (III, IV and VI).
4. Determine the angiogenic, lymphangiogenic and vascular permeability properties of different VEGF family members and FGF-4 in rabbit hindlimbs (III-V).
5. Study the duration and side-effects of adenoviral gene transfer of VEGFs and FGF-4 (III-VI).
6. Test the feasibility of magnetic resonance imaging (MRI) and power Doppler ultrasound imaging in the assessment of increased vascular permeability and tissue perfusion, respectively (IV-VI).
7. Determine whether NO production is crucial for angiogenesis and lymphangiogenesis (V).
8. Test the feasibility and efficacy of catheter-mediated intramyocardial adenoviral VEGF gene transfer for vascular growth in the pig heart with the NOGA electromechanical mapping system (VI).

## MATERIALS AND METHODS

### Ischemic skeletal muscle

#### *Human skeletal muscle samples (I and II)*

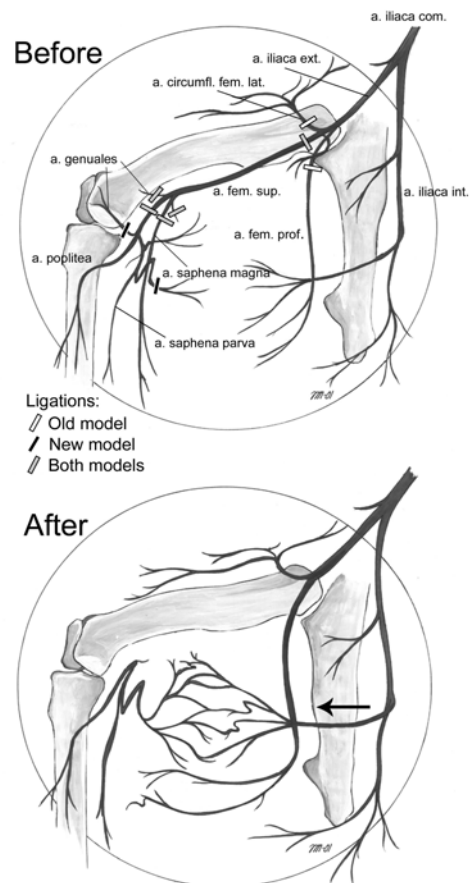
Ischemic human skeletal muscle samples were collected from lower limb amputation operations. The donors (n=8) suffered either from chronic critical limb ischemia (CLI) caused by PAD with rest-pain and/or tissue loss, or acute-on-chronic CLI of 24-48h in duration secondary to an embolism or a major thrombus affecting the native artery or prosthesis. Upon amputation, all patients had ABI of 0-0.3 indicating critical ischemia. In the acute-on-chronic CLI cases, the underlying ischemia was milder than in the chronic CLI cases on the basis of walking distance and ABI measurements 4 weeks before amputation. Samples were collected from two regions of each amputee. One set of samples was collected from the amputation stump and served as controls. The other set of samples was harvested from a region that was ischemic, but still viable, on the basis of macroscopic and microscopic investigation. Samples were not collected near to infected parts of chronically ischemic limbs. Studies of human tissues were approved by the Ethical Committee of Kuopio University Hospital.

#### *Rabbit models of hindlimb ischemia (II-V)*

Before surgery, New Zealand white rabbits (2.0-2.5kg) were anesthetized with fentanyl-fluanisone (Hypnorm, Janssen, 0.2 ml/kg) and midazolame (Dormicum, Roche, 1.5 mg/kg). All animals received anticholinergic glycopyrronium (Gastrodyn, Leiras, 0.05 mg/kg) and cefuroxim (Zinacef, Glaxo-Wellcome, 125 mg) before surgery. Carprofen (Rimadyl, Pfizer, 0.1 mg/kg) or buprenorphine (Temgesic, 0.03 mg/kg) was used for post-operative analgesia. For MRI and contrast-enhanced ultrasound (CEU) imaging, rabbits were anesthetized with s.c. injections of ketamine (10 mg/kg, Ketalar, Pfizer) and haloperidol (1 mg/kg, Serenase, Orion) and inhalation of N<sub>2</sub>O and O<sub>2</sub> (2:1).

Two different operations were performed to induce ischemia in the rabbit hindlimb. In

the total excision model (II-IV), the right femoral artery was completely excised combined with ligations of the lateral circumflex and deep femoral arteries as previously described (Takeshita et al., 1994a; Tsurumi et al., 1996). In the novel modified model developed in this study (IV and V), the superficial part of the femoral artery was excised, and in order to induce ischemia in the calf the re-entry branches for the collaterals growing from the lateral circumflex and deep femoral arteries were ligated. The ligations in both models are shown in Figure 10. All rabbit experiments were approved by the Experimental Animal Committee, University of Kuopio.



**Figure 10.** Rabbit hindlimb ischemia models. Before: bars indicate ligations in the two models. After: post-operative collateral growth pattern in the new modified rabbit hindlimb ischemia model. Collateral arteries mainly originate from the internal iliac artery (arrow) because collateral growth from a. circumflexa femoris lateralis and a. femoralis profunda is prevented by ligations of the re-entry branches (a. genuales and the sidebranch of a. saphena parva). The thigh is normoperfused in this model. Modified from study IV with permission.

## mRNA expression

### DNA array (I)

LifeGrid 1.1 filters (Incyte Genomics) containing 8400 double-spotted human cDNA clones from the Incyte Genomics' library were used to profile gene expression changes in acute-on-chronic (n=2) and chronic (n=2) CLI. mRNA was isolated using the Micro-FastTrack 2.0 kit (Invitrogen) from snap-frozen tissue samples. After prehybridization, the filters were hybridized with  $^{33}\text{P}$ -dCTP-labeled cDNA probes at +42 °C for 16 h and thereafter washed according to the manufacturer's instructions (Incyte Genomics). Phosphorimager (Storm 860, Molecular Dynamics) was used for signal detection.

Quantification of signals was performed with the ArrayVision software (Imaging Research). Signals were normalized using the GeneSpring software (Silicon Genetics). Gene expression was considered to be up- or downregulated when the intensity ratio ischemia/normal was  $\geq 2.0$  or  $\leq 0.5$ , respectively. To reach the 0.05 significance level  $|\text{Int}_{\text{ischemia}} - \text{Int}_{\text{normal}}|$  must be  $\geq 4.3\sigma_{\text{normal}}$ , where  $\text{Int}_{\text{ischemia}}$  and  $\text{Int}_{\text{normal}}$  are the averages of normalized signal intensities in ischemic and normal muscles in repeated experiments and  $\sigma$  is the distribution of signal intensities in normal muscle (Claverie, 1999). Only those genes that had signal intensity of  $\geq 1\,000 \times$  background were included for further analyses.

### RT-PCR (I and III)

Total RNA was extracted from tissue samples using Trizol® reagent (Gibco-BRL). After DNase I treatment cDNA synthesis was performed using 3 µg of total RNA, random hexamers (Promega) and M-MuLV reverse transcriptase (MBI Fermentas). RT-PCRs for  $\beta$ -galactosidase (*LacZ*) marker gene, endogenous and transduced VEGF, IGF-1, IGF-2 and GAPDH (control) were then performed as previously described (Hiltunen et al., 2000b) using specific primers (I and III). For *LacZ* nested PCR was also performed.

### In situ hybridization (II)

The spatial expression of VEGF and VEGFR-2 mRNA was studied in human and rabbit ischemic muscles by *in situ* hybridization using either radioactive [ $^{33}\text{P}$ ]UTP-labeled or non-radioactive DIG-labeled riboprobes. A riboprobe covering the whole coding region of human VEGF<sub>165</sub> cDNA and a 377 bp fragment of human VEGFR-2 cDNA were used with corresponding sense probes as controls as previously described (Ylä-Herttuala et al., 1990). For non-radioactive *in situ* hybridization cDNAs were transcribed using DIG RNA Labeling Kit (Boehringer Mannheim GmbH, Germany) in order to get DIG-labeled run-off transcripts. Signal was then detected on paraffin embedded sections using NBT/BCIP as the color substrate (Breitschopf and Suchanek, 1996).

## Protein expression

### Immunohistochemistry and other histological stainings (I-VI)

For immunohistochemistry, skeletal and myocardial samples were immersion-fixed in 4% PFA/15% sucrose (pH 7.4) for 4 h, rinsed in 15% sucrose (pH 7.4) overnight and embedded in paraffin. Other set of samples were frozen in isopentane cooled down with liquid nitrogen. Immunohistochemistry was performed with the avidin-biotin-horseradish peroxidase system (Vector Laboratories) using the 3'-5'-diaminobenzidine (DAB, Zymed) color substrate on 6 µm thick sections. For double immunostainings, the alkaline-phosphatase system with Vector Blue color substrate (Vector) was also used. The primary antibodies used for immunohistochemistry are listed in Table 2. The proliferation marker BrdU (20mg/kg, Sigma) was given i.v. 3h before sacrifice. In the study V, the tyramide signal amplification system was used for VEGFR-2 and  $\alpha_v\beta_3$  immunostainings on frozen sections (TSA-kit, NEN Lifescience). Controls for immunostainings included incubations with irrelevant class- and species-matched immunoglobulins and incubations in which the primary antibody was omitted.



**Table 2.** Antibodies used in immunohistochemistry.

Antibody	Specificity	Code/clone	Species	Ig isotype	Protocol	Company
CD31/ PECAM1	Endothelium	JC/70A	mAb mouse anti-human	IgG <sub>1,κ</sub>	1:100, P	DAKO
RAM11	Macrophages	RAM11	mAb mouse anti-rabbit	IgG <sub>1,κ</sub>	1:100, P	DAKO
CD68	Macrophages	KP1	mAb mouse anti-human	IgG <sub>1,κ</sub>	1:200, P	DAKO
HIF-1α	HIF-1α	Ab-4, H1alpha67	mAb mouse anti-human	IgG <sub>2b,κ</sub>	1:100, P+C	Neomarkers
VEGF	VEGF <sub>121-206</sub>	sc-7269	mAb mouse anti-human	IgG <sub>2a</sub>	1:500/ 1:200, P	Santa Cruz
VEGFR-1	VEGFR-1	sc-316	pAb rabbit anti-human	IgG	1:500, P/F	Santa Cruz
VEGFR-2	VEGFR-2	sc-6251/ RDI-MFLK1 Cabm	mAb mouse anti-human	IgG <sub>1</sub>	1:500, P/ F+TSA	Santa Cruz/ Research Diagnostics
VEGF-D	VEGF-D	78923.11	mAb mouse anti-human	IgG <sub>1</sub>	1:200, P	R&D
IGF-1	IGF-1	18773	pAb goat anti-human	IgG	1:50, P+C	Sigma
FGF-4	FGF-4	26019.11	mAb mouse anti-human	IgG <sub>1</sub>	1:200, P	R&D
HHF35	Skeletal, cardiac and smooth muscle α/γ- actin	HHF-35	mAb mouse anti-human	IgG <sub>1</sub>	1:50, P	DAKO
αSMA	α-smooth muscle actin	clone 1A4	mAb mouse anti-human	IgG <sub>2a</sub>	1:250, P	Dr. Giuliano Gabbiani/ Sigma
α <sub>v</sub> β <sub>3</sub>	α <sub>v</sub> β <sub>3</sub> integrin	LM609	mAb mouse anti-human	IgG <sub>1</sub>	1:200, F+TSA	Biogenesis
BrdU	Proliferating cells	Clone Bu20a	mAb mouse anti-human	IgG <sub>1,κ</sub>	1:100, P+HCl	DAKO
PCNA	Proliferating cells	PC10	mAb mouse anti-human	IgG <sub>2a,κ</sub>	1:500, P	Neomarkers

mAb=monoclonal antibody, pAb=polyclonal antibody, P=paraffin embedded sections, F=frozen sections, C=citrate buffer boiling, TSA=tyramide signal amplification and HCl=HCl pretreatment.

Specimens for X-Gal staining to visualize *LacZ* marker gene activity were immersion-fixed in 4% paraformaldehyde (PFA)/phosphate-buffered saline (PBS), pH 7.4, for 30 min, rinsed in PBS, embedded in OCT compound (Miles Scientific) and stored

at -70 °C (III and VI). X-Gal staining was then performed on 10-15 μm thick sections as previously described (Hiltunen et al., 2000b). Sections for histological detection of extravasated plasma proteins stained with Evans blue dye were deparaffinized in

xylene, mounted with Dapi (Vector) for nuclear counter staining, and viewed in fluorescence microscopy using a 610 nm excitation filter. 5'-nucleosidase staining for lymphatic vessels was performed as previously described (Kato et al., 1993). Photographs of histological sections were taken using Olympus AX70 microscope (Olympus Optical) with analySIS imaging software (Soft Imaging System) and were further processed for publication with Adobe Photoshop 5.0-7.0 (Adobe). In study II, immunostainings were graded as follows: absent "-" (0% of section area showed staining), weak "+" (0-10%), moderate "++" (10-50%) or strong immunostaining "+++" (50-100%) (Hakkinen et al., 2000).

#### *ELISA (III, IV and VI)*

Specific ELISA assays were performed for quantitative measurement of the transduced human FGF-4 and mouse VEGF<sub>164</sub> in rabbit skeletal muscle and plasma (III, IV), and human VEGF-D<sup>ΔNΔC</sup> and VEGF<sub>165</sub> in pig myocardium and plasma (VI) (Quantikine, R&D Systems). Snap-frozen skeletal and myocardial muscle samples were homogenized in the following buffer: 2.5 M NaCl in 15 mM phosphate buffer, pH 7.2, 0.2% NP-40, 10% glycerol, and protease inhibitors (Complete Mini Protease Inhibitor, Roche). After centrifugation twice at 20000 rpm/min for 5 min, the supernatant was collected and diluted 1/10 in the homogenization buffer. Thereafter, the total protein concentration was measured and ELISA analyses were performed. The results are expressed as pg of growth factor/mg of total muscle protein. According to the manufacturer, the sensitivities of mouse VEGF<sub>164</sub>, human VEGF<sub>165</sub>, human VEGF-D and human FGF-4 ELISAs are 3, 9, 11 and 30 pg/ml, respectively. Furthermore, the mouse VEGF<sub>164</sub> ELISA does not cross-react with human VEGF<sub>165</sub> or rabbit endogenous VEGF.

## **Gene transfer**

### *Gene transfer in rabbit skeletal muscle (III-V)*

Human clinical grade first generation serotype 5 replication deficient (E1, partially E3 deleted) adenoviruses produced under GMP conditions in 293 cells and analyzed to be free from endotoxin and microbiological contaminants were used throughout the studies (Laitinen et al., 1997a). All growth factors were encoded by the cytomegalovirus (CMV) promoter. Altogether 207 rabbits were used in the studies. All operations and constructs have been approved by the Finnish Board of Gene Technology.

Seven days after the modified hindlimb ischemia operation, animals received i.m. injections of adenovirus (Ad) encoding murine VEGF<sub>164</sub> (Laitinen et al., 1997b) (III and IV) or human FGF-4 (Yoshida et al., 1987), supplied by Berlex Biosciences, Richmond, CA (IV). In study V, human VEGF<sub>165</sub> (Leung et al., 1989), human VEGF-B<sub>167</sub> (Olofsson et al., 1996a), full-length human VEGF-C (Joukov et al., 1996), full-length or mature human VEGF-D (Achen et al., 1998), or the VEGFR-3 specific mutant VEGF-C, VEGF-C<sup>156S</sup> (Joukov et al., 1998) were used. Also adenovirus encoding eNOS was used (Lamas et al., 1992). Adenovirus expressing the full-length VEGF-D contains the sequence of the unprocessed prepropeptide of VEGF-D including both N- and C-terminal propeptides whereas the adenovirus encoding the fully processed mature form (VEGF-D<sup>ΔNΔC</sup>) lacks these domains and consists only of the central VEGF homology domain (Achen et al., 1998; Stacker et al., 1999a). Injections of adenovirus encoding a marker gene (β-galactosidase, AdLacZ, III-V) or PBS (III and IV) were used as controls.

GT with i.m. injections was performed into the semimembranosus and quadriceps femoris muscles (III); in the adductor, semimembranosus, quadriceps femoris and abductor cruris cranialis muscles (IV); or into the semimembranosus and abductor cruris cranialis muscles of the thigh (V). In study III, the soleus and tibialis anterior muscle of the calf were also injected. The

adenoviral concentration was  $10^{10}$  (IV) or  $10^{11}$  (IV and V) or  $5 \times 10^{11}$  (III) viral particles/ml (vp/ml). The injection volume was either 4 ml divided in 4 longitudinal i.m. injections using a 22-gauge needle (III) or 1 ml divided in 10 vertical i.m. injections using a 25-gauge needle (IV and V). I.a. route for GT in the hindlimb was studied by injecting AdVEGF<sub>164</sub> in 2 ml of saline selectively into the external iliac artery via a 4F right coronary catheter (Cordis, Johnson & Johnson) over 2 min (IV).

Two different timepoints were chosen to elucidate the effects of the growth factors. The maximal effects of the transduced growth factors on capillary and lymphatic vessel growth, regional perfusion and vascular permeability were studied six days after the injections (IV and V) because macroscopic edema after AdVEGF GT has been shown to peak at six days (Poliakova et al., 1999). On the other hand, collateral artery growth, which is a longer process (Schaper and Ito, 1996), was assessed four weeks after GT (III and IV).

#### *Catheter-mediated gene transfer in pig myocardium with the NOGA system (VI)*

Electromechanical mapping of the left ventricle was performed with the NOGA system (Biosense-Webster, Johnson & Johnson) (Gepstein et al., 1997; Kornowski et al., 2000; Vale et al., 2001) to guide the injections to the anterolateral wall of the left ventricle in domestic pigs weighing 25-30 kg (n=45). Pigs were sedated with azaperone (4 mg/kg, Stresnil, Janssen-Cilag Pharma) and anesthetized with a continuous infusion of propofol (15 mg/kg/h, Propofol, Alparma) and inhalation of N<sub>2</sub>O and O<sub>2</sub> (1:1). For inhibition of ventricular arrhythmias, animals were given fentanyl (10 µg/kg/h, Fentanyl, Orion), metoprolol (5 mg, Spesicor, Leiras), MgSO<sub>4</sub> (600 mg, Fresenius Kabi) and lidocain (100 mg, Lidocain). The animals also received atropin (0.05 mg/kg, Atropin, Leiras), heparin (5000 ie, Leo Pharma) and cefuroxim (500 mg, Zinacef, GlaxoWellcome). Mapping was carried out with an 8F NOGA injection catheter (Johnson & Johnson) filled with heparin to avoid clotting of the needle during the mapping procedure.

After mapping at least 70 points, ten intramyocardial injections (5-6 mm in depth, 0.2 ml each, total volume 2.0 ml, at least 5 mm apart from each other, during 30 s) were performed to the anterolateral wall. Human GMP-grade first generation adenoviruses with two concentrations ( $10^{11}$  or  $10^{12}$  vp/ml) or naked plasmids (0.5 mg/ml) encoding *LacZ* marker gene, human VEGF<sub>165</sub> (Leung et al., 1989) or the mature form of human VEGF-D, VEGF-D<sup>ΔNΔC</sup> were injected (Achen et al., 1998).

Six days or three weeks after GT, perfusion was studied with myocardial contrast echocardiography and microspheres immediately prior to sacrifice. Vascular permeability was assessed with the modified Miles assay. After sacrifice, myocardial samples were collected for histological analyses. All pig experiments were approved by the Experimental Animal Committee, University of Kuopio.

#### **Assessment of blood and lymphatic vessel growth**

##### *Peripheral angiography for collateral artery growth (III and IV)*

Selective angiography of ischemic hindlimbs was performed immediately before GT and four weeks later just before sacrifice with a 4F right coronary artery catheter (Cordis) introduced into the common carotid artery. Two methods were used to assess collateral growth quantitatively. In study III, an overlay composed of two straight lines 5 mm apart was placed over the angiogram at the level of mid-thigh and upper calf regions. All arterial vessels crossing over this 5 mm wide area were counted in a blinded manner and the results were averaged. However, since collateral blood flow is critically dependent on the size rather than the number of the vessels, the increase (mm) in the combined diameter of all collateral arteries in the mid thigh during the four weeks follow-up period was calculated in a blinded manner using analySIS software in study IV (Soft Imaging System).

### *Histological blood and lymphatic vessel measurements (III-VI)*

In studies IV-VI, the effects of VEGFs and FGF-4 on microvessel growth and morphology were evaluated outside the needle track in non-ischemic regions in order to exclude the confounding effects of endogenous growth factors induced by injection trauma or ischemia. In study III, measurements were done within the ischemic regions. In rabbits (III-V), capillary density (capillaries/mm<sup>2</sup> or capillaries/myocytes), mean capillary area ( $\mu\text{m}^2$ ), and the total capillary area of muscle area (%) were measured from 10 different fields of CD31 immunostained sections of semimembranosus muscles at 200X magnification in a blinded manner. Capillaries were not counted from the interstitial tissue between muscle bundles. In pigs (VI), the respective calculations were done from  $\alpha$ SMA immunostained sections from five different fields of each section of the myocardium. Total capillary area (%) denotes the percentage of muscle area occupied by capillary lumens and demonstrates the total angiogenic activity of the growth factor.

The total lymphatic vessel area of muscle area (%) was calculated to compare the lymphangiogenic effects by VEGFs (V). Except for large collecting trunks, lymphatic vessels can be distinguished from blood vessels by the lack of pericytes (Casley-Smith, 1980). Thus, completely  $\alpha$ SMA-negative but CD31-positive vessels in the connective tissue between muscle bundles were counted as lymphatics from  $\alpha$ SMA-CD31 double immunostainings at 100X magnification.

### **Perfusion measurements**

#### *Microspheres (IV-VI)*

In rabbits (IV and V), regional perfusion in transduced semimembranosus muscles was measured at rest with red fluorescent microspheres ( $2 \times 10^6$ , 15  $\mu\text{m}$  in diameter, FluoSpheres, Molecular Probes) injected into the left ventricle six days or four weeks after GT. After sacrifice, microspheres were extracted from muscle samples with the

sedimentation method according to the manufacturer's instructions. The perfusion ratio between the transduced and contralateral intact muscle was then calculated on the basis of the amount of fluorescence in each sample and using yellow-green microspheres as an internal control for pipeting errors. In pigs (VI), the perfusion ratio between the transduced anterolateral and intact apical control regions was calculated at rest and during dobutamine stress (10-80  $\mu\text{g}/\text{kg}/\text{min}$  until a heart rate  $\times 2$  of rest was achieved; Dobutrex, Lilly) using red and yellow-green microspheres, respectively, injected into the left ventricle near the mitral valve via a 5F left coronary catheter (Cordis, Johnson & Johnson).

#### *Popliteal artery blood flow by ultrasound (IV)*

Collateral vessel dependent blood flow (ml/min) in the popliteal artery of ischemic and normal legs was measured with an Acuson Aspen ultrasound device (Siemens). The measurements were performed at rest using a L10 transducer before GT and seven, 14 and 21 days after GT. Three separate measurements were done for each rabbit at every timepoint and the results were averaged.

#### *Skeletal muscle perfusion by ultrasound*

Perfusion in transduced and contralateral intact rabbit semimembranosus muscles was imaged with Acuson Sequoia 512 and 15L8 transducer (Siemens) at 14 MHz six days after AdLacZ or AdVEGF<sub>165</sub> GT (both  $10^{11}$  vp) using the power Doppler mode with or without administration of contrast agent. Transversal plane video clip (2 s, 12.5 frames/s) without contrast agent was first obtained (power Doppler at 14 MHz, dynamic range 10 dB, power 0 dB, mechanical index 0.63, gain 50 and depth 20 mm). Then, two consecutive longitudinal plane video clips of 10 s (power Doppler at 8.5 MHz, dynamic range 10 dB, power -18 dB, mechanical index 0.6, gain 40 and depth 20 mm) were captured starting at the time of 0.3 ml bolus injection of the second generation contrast agent (sulphur

hexafluoride in a phospholipid shell, approx.  $2 \times 10^8$  bubbles/ml, mean diameter 2.5  $\mu\text{m}$ , Sonovue, Bracco) via the ear vein.

It has been previously shown that power Doppler signal enhancement with microbubbles correlates linearly with blood flow both *in vitro* and *in vivo* (Heidenreich et al., 1993; Porter et al., 1995; Correias et al., 2000). The power Doppler signal intensity (dB) on the video clips was quantified with Datapro 2.13 program (Noesis) and was expressed as a function of time (Orden et al., 2003). The perfusion ratio between the transduced and contralateral intact muscles was calculated from the native power Doppler clips by comparison of the average signal intensities during the 2 s video clip. From the CEU data, the peak intensity or the area under the signal intensity curve during the 20 s imaging period in the transduced muscle were compared to the values of the contralateral intact muscle. Furthermore, the time from i.v. injection to the arrival of contrast agent in the transduced muscle and to the peak signal intensity was derived from the data.

#### *Echocardiography (VI)*

Modified long-axis images were acquired with Acuson Sequoia 512 and 3V2c transducer (Siemens) to detect pericardial effusion six days or three weeks after GT. Myocardial contrast echocardiography for the assessment of perfusion in the injected region of the heart was performed at 3.5 MHz and receiving the second harmonic echo. Real-time reperfusion images (22 Hz, power -19 dB, gain -3, MI 0.16) were obtained at the short-axis mid-papillary level after destruction of the i.v. administered contrast agent (1.0 ml, Sonovue, Bracco) with a high-energy Doppler wave. The end-systolic images which represented maximal reperfusion of the treatment area, compared to untreated segments of the left ventricle, were chosen for analysis.

## **Vascular permeability and edema**

### *Magnetic resonance imaging (MRI, II, IV and V)*

MRI was performed using a Varian UNITY INOVA (Varian) imaging console interfaced with a 4.7T horizontal magnet (Magnex Scientific) and actively shielded gradients. Contrast-enhanced MRI was used to demonstrate vascular permeability effects and subsequent edema caused by adenoviral GT of VEGFs and FGF-4 five days after GT (IV and V). Furthermore, ischemic changes induced by the total excision hindlimb ischemia model were imaged one and six weeks after the operation (II). MRI data were acquired 3 min after i.v. injection of gadodiamide-contrast medium (GdDTPA-BMA, Omniscan, Nycomed, molecular weight 574 Da, 0.25 mmol/kg). A custom built surface coil (50mm in diameter) was placed between the thighs of the animal and a 3D flow-compensated  $T_2^*$ -weighted gradient-echo sequence (FOV 6x8x6  $\text{cm}^3$ , matrix 256x128x64,  $\text{tr}=25\text{ms}$ ,  $\text{te}=80\text{ms}$ ) was acquired.

### *Modified Miles assay (IV-VI)*

The standard Miles assay was used to assess the acute vascular permeability effects of recombinant FGF-4 and mouse VEGF<sub>164</sub> protein in the rabbit skin (IV). Escalating doses of recombinant human FGF-4 (50pg, 500pg, 50ng or 500ng in 0.1ml of PBS, R&D Systems) or mouse VEGF<sub>164</sub> (10pg, 100pg, 10ng or 100ng in 0.1ml of PBS, R&D Systems), or PBS (0.1 ml) were injected into the rabbit dorsal skin using a 25-gauge needle. Immediately after the protein injections, Evans Blue dye (30 mg/kg, Sigma) was injected i.v. Rabbits were then sacrificed 30 min after the injection of Evans blue and were perfusion-fixed with 1.5 L of 1% paraformaldehyde in 0.05M citrate buffer (pH 3.5) administered via the left ventricle.

For quantitative determination of plasma protein extravasation in transduced skeletal and myocardial muscles (Modified Miles assay, IV-VI), Evans Blue (30 mg/kg) was

injected i.v. 30 min prior to sacrifice either six days or three weeks after GT. After sacrifice the rabbits or pig hearts were perfusion-fixed as in the standard Miles assay. Extravasated Evans Blue dye bound to plasma proteins was extracted from transduced (semimembranosus muscle in rabbits and anterolateral wall of the left ventricle in pigs) and intact control (contralateral semimembranosus muscle and apex of the heart, respectively) samples by incubation of the tissue in formamide at 60°C for 24-48h. The amount of Evans Blue was determined by the absorbance at 610 nm, and the ratio between the transduced and control samples was calculated.

### Statistical analyses

Results are expressed as means  $\pm$  SEM. Statistical significance was evaluated using one-way ANOVA followed by independent samples *t*-test or Kruskal-Wallis test followed by Mann-Whitney U-test where appropriate. Correlation analyses were performed by the Pearson test.  $P < 0.05$  was considered statistically significant. Microsoft Excel and SPSS 10.0 were used to perform the statistical analyses.

### Other methods

#### *In vitro* angiogenesis assays (IV)

Rabbit aortic SMCs (RaaSMCs) were transduced with AdFGF-4, AdVEGF or AdLacZ (MOI=500). The supernatants were collected 36 h after transduction and were added to ECs (EAhy926) growing in Matrigel. For negative controls the supernatant from non-transduced RaaSMCs and DMEM (no virus supernatant) were used. Tube formation was quantified as previously described (Hiltunen et al., 2000a). Proliferation assays were performed on HUVECs (Clonetics) with AdFGF-4-derived supernatant, recombinant human FGF-4 (R&D Systems) and VEGF (Sigma). Cell proliferation was determined using 4% Alamar Blue™ (BioSource International) according to the manufacturer's instructions.

#### *Inhibition of NO synthases in rabbits (V)*

N $\omega$ -Nitro-L-arginine methyl ester (L-NAME, an NO synthase inhibitor) (Laitinen et al., 1997b) was used to study whether the angiogenic and lymphangiogenic signaling pathways of the full-length and mature forms of VEGF-D were dependent on NO. L-NAME (Sigma, 100mg/kg/day) was administered twice daily *per os*.

#### *Magnetic resonance spectroscopy (MRS, IV)*

The bioenergetic condition of ischemic gastrocnemius muscles was evaluated at rest using non-localized  $^{31}\text{P}$ -MRS (np=4096, sw=10kHz, tr=1.5s, nt=64) at 4.7T and the Perch spectral analysis program (University of Kuopio) seven days after surgery (IV). The phosphocreatine/ATP ratio was used to compare the degree of ischemia between the two rabbit hindlimb ischemia models.

#### *Clinical chemistry (III and V)*

Plasma and serum creatinine, C-reactive protein, alanine aminotransferase, lactate dehydrogenase, alkaline phosphatase, Troponine T, creatinine kinase, creatinine kinase-MBm were measured at the Department of Clinical Chemistry, Kuopio University Hospital. Protein concentration measurements and electrophoresis analyses were performed on both the pericardial effusate and plasma samples (VI).

## RESULTS

### Gene expression in skeletal muscle ischemia

#### *Activation of HIF-1 $\alpha$ -VEGF-VEGFR-2 and TNF- $\alpha$ pathways in acute-on-chronic ischemia*

In human acute-on-chronic ischemia 291 genes were significantly upregulated and 174 downregulated when compared to the control sample of the same limb, representing a change in 5.5% of all genes (I). The most prominently upregulated angiogenic growth factor was VEGF (7.2-fold) with induction of its regulator under hypoxia, HIF-1 $\alpha$  (3.1-fold), and its main angiogenic receptor VEGFR-2 (2.2-fold). Also HIF-2 $\alpha$  was upregulated (3.5-fold) as well as other factors involved in angiogenesis such as Ephrin-A1, B3 and B6 (2.7-, 2.2-, and 3.2-fold, respectively), Ang-1 receptor Tie-2 (4.2-fold), PAI-1 (2.0-fold), hepatocyte growth factor receptor (c-met, 2.0-fold) and matrix metalloproteinase-23 (2.7-fold). A few molecules reported to be angiogenesis inhibitors, such as interferon proteins and tissue inhibitor of metalloproteinase-3 were also upregulated in acute ischemia.

VEGF upregulation was further confirmed at the mRNA level by RT-PCR (I). The cell-specific expression patterns of HIF-1 $\alpha$ , VEGF and VEGFR-2 were studied by immunohistochemistry. In patients suffering from acute-on-chronic CLI, VEGF and VEGFR-2 were diffusely expressed in ischemic muscle with HIF-1 $\alpha$  colocalizing in the nuclei of the same cells (I and II). VEGF, VEGFR-2 and HIF-1 $\alpha$  immunostaining was not detectable in the myocytes of control samples (I and II). Consistent with known histopathology of acute-on-chronic CLI, the ischemic muscles contained thrombotic blood vessels laden with macrophages and prominent interstitial edema.

Another striking pathway to be upregulated in acute-on-chronic CLI was TNF- $\alpha$  with its downstream signaling machinery. The members of the pathway that were found to be induced were TNF- $\alpha$  (3.7-fold), TNF- $\alpha$  convertase (15.3-fold) and TNF- $\alpha$

receptor-1 (2.9-fold) as well as the intracellular signaling components FADD (6.8-fold) and TRAF2 (1.6-fold).

#### *Upregulation of IGF-1 and -2 in chronic ischemia*

In human chronic CLI changes in gene expression patterns were much less striking than in acute-on-chronic CLI as 74 genes were significantly upregulated and 34 downregulated when compared to the control samples representing a change in 1.3% of all genes (I). In chronic ischemia the potent survival and anabolic factors IGF-1 (9.8-fold) and IGF-2 (2.4-fold) were induced (I). These findings were confirmed by RT-PCR and immunohistochemistry (IGF-1).

In chronic ischemia IGF-1 expression was found in atrophic and regenerating myocytes whereas, in agreement with the DNA array data, IGF-1 immunostaining was not detected in acute-on-chronic ischemia or in controls (I). The same atrophic and regenerating myocytes also expressed VEGF and VEGFR-2 mRNA and protein as determined by *in situ* hybridization and immunohistochemistry, respectively (I and II). HIF-1 $\alpha$  was also present in the atrophic cells although at a lower level than in acute-on-chronic ischemia. Interestingly, areas positive for VEGF and IGF-1 had increased blood vasculature (I and II). Typical of chronically ischemic muscle, adipose cell and macrophage infiltration was associated with atrophic regions. In addition to the currently known genes, many unknown expressed sequence tags (ESTs) were highly upregulated under ischemia (I).

#### *VEGF and VEGFR-2 are induced in skeletal muscle regeneration*

VEGF was expressed in regenerating skeletal myocytes in patients with chronic CLI, especially in fibers where regeneration had recently begun (II). Similarly with humans, the strong, diffuse and widespread VEGF expression at three days was followed by VEGF and VEGFR-2 expression restricted to regenerating muscle one week after the total excision

operation in rabbit hindlimbs (II). Enlarged capillaries and macrophage infiltration were associated with regenerating VEGF-positive regions (II). VEGF expression was diminished with only occasional myocytes with central nuclei remaining positive for VEGF immunostaining six weeks after induction of hindlimb ischemia (II).

#### *The rabbit hindlimb ischemia models induce different levels of ischemia*

Extensive ischemic damage was present in rabbit hindlimb muscles a week after the total excision ischemia operation (II and III). Only the abductor muscle compartment on the lateral thigh, where the majority of collaterals grow, was not severely affected. Although the ischemic muscles had partially regenerated, they were still clearly atrophic and showed abnormal vascular permeability by contrast-enhanced MRI six weeks after the operation (II and III).

The consequences of widespread and severe ischemia in this previously established model led to the development of a modified rabbit hindlimb ischemia model with ischemia restricted to the calf (IV). Similar with the total excision model, in the modified model collateral arteries mainly originate from the internal iliac artery, grow inside the abductor cruris cranialis muscle on the lateral side of the thigh and are of similar size. Five weeks after surgery, pathological changes (necrosis, atrophy, or fibrosis) were found in the calf muscles in 48% of animals (n=60) but never in the thigh (IV). Perfusion was also not impaired in the thigh muscles after the modified operation (IV and V).

Bioenergetic condition (phosphocreatine/ATP ratio) at rest was significantly worse in the gastrocnemius muscle of the calf after the modified operation than in intact limbs but similar to that in the total excision model as measured with  $^{31}\text{P}$ -MRS one week after surgery (IV). Together, these findings demonstrate that in the modified rabbit hindlimb ischemia model the calf but not the thigh is ischemic at rest.

## **Gene transfer for therapeutic angiogenesis**

### *Angiogenic activity of VEGF and FGF-4 in vitro*

*In vitro*, supernatant from RaaSMCs transduced with AdVEGF<sub>164</sub> but not AdFGF-4 promoted EC tube formation efficiently in matrigel. However, both recombinant FGF-4 and AdFGF-4 supernatant stimulated EC proliferation in a similar dose-dependent manner.

### *Dose-dependent protein production in vivo after i.m. adenoviral gene transfer*

Before the *in vivo* experiments, Western blotting (Hiltunen et al., 2000a) was used to determine that rabbit aortic SMCs transduced with the different adenoviruses secreted proteins with the correct molecular weights (data not shown). RT-PCR, immunohistochemistry and ELISA demonstrated efficient *in vivo* expression of the transduced VEGF<sub>164/165</sub>, VEGF-D<sup>ΔNΔC</sup>, and FGF-4 (III, IV and VI). The protein levels achieved with i.m. adenoviral GT in rabbit skeletal muscle were high, for example 1029±335 pg FGF-4/mg total muscle protein six days after AdFGF-4 GT as measured by ELISA. FGF-4 was not detectable in controls by ELISA. Transduced human VEGF<sub>165</sub> could not be detected by ELISA from homogenized skeletal muscle or myocardial samples, apparently due to strong ECM-binding properties (Ferrara, 1999). Instead, transduced VEGF was readily detected with immunohistochemistry (VI).

No deaths occurred during the NOGA catheter-mediated i.m. injections into the pig myocardium or during the follow-up (VI). Injections of AdVEGF-D<sup>ΔNΔC</sup> with the NOGA system resulted in dose-dependent myocardial VEGF-D<sup>ΔNΔC</sup> production ( $10^{11}$  vp/ml and  $10^{12}$  vp/ml: 1929±579 and 4397±633 pg/mg total protein, not detectable in controls). After naked plasmid DNA-mediated GT, VEGF-D<sup>ΔNΔC</sup> was not detected at all in the pig myocardium by ELISA. Also i.a. injection of adenovirus proved inefficient for transduction of rabbit hindlimb skeletal muscle (IV).



Despite the high protein levels in muscles, ECM-bound VEGF<sub>164/165</sub> and FGF-4 did not significantly leak into the circulation after adenoviral GT into skeletal muscle or myocardium (IV and VI). In contrast, the soluble VEGF-D<sup>ΔNΔC</sup> was also detected in the plasma after intramyocardial adenoviral GT (VI).

Only little or no inflammation was observed in skeletal and myocardial muscles transduced with the GMP-grade adenoviral vector at the titer of 10<sup>11</sup> vp/ml or less (IV-VI). At higher titers, some infiltration of mononuclear cells and lymphocytes was detected (III and VI). No significant changes were observed in the clinical chemistry parameters after adenoviral GT into rabbit skeletal muscle or pig myocardium (III and VI). In addition to the target muscles, some ectopic gene expression after adenoviral i.m. GT was found in the spleen, liver, lung and extra-tubular tissue in testis (III).

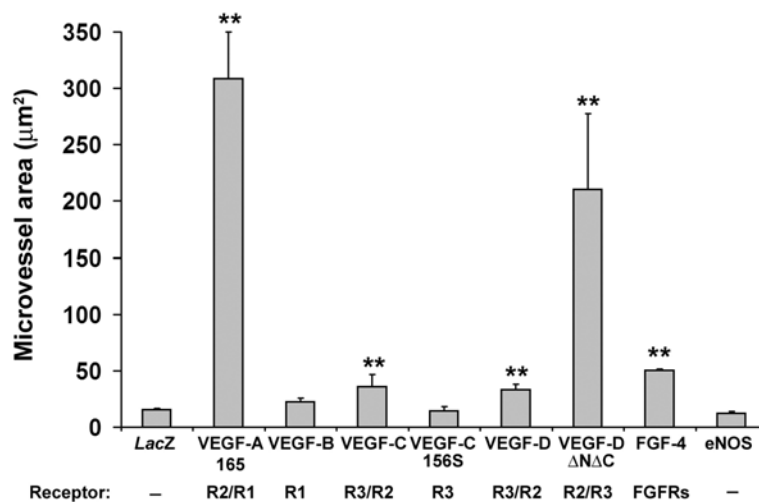
*Microvessel enlargement is the predominant feature of angiogenesis in skeletal muscle and myocardium*

The vascular response to adenovirally delivered VEGFR-2 ligands and FGF-4 both in skeletal muscle and in the myocardium

was characterized by a remarkable enlargement of pre-existing microvessels. In non-ischemic rabbit skeletal muscle, adenoviral GT (10<sup>11</sup> vp/ml) of VEGF<sub>164/165</sub>, VEGF-D<sup>ΔNΔC</sup> as well as FGF-4 enlarged the capillary mean area 3.9- to 14-fold, in comparison to AdLacZ control six days after GT (Figure 11, IV and V).

The size of the enlarged microvessels sometimes exceeded that of surrounding skeletal myocytes (diameter > 50 μm). VEGFR-2 and α<sub>v</sub>β<sub>3</sub> integrin were upregulated on the endothelium of the expanded vessels (V). Although intense, these effects on capillaries were restricted to the area surrounding the i.m. injection sites.

Also adenoviruses encoding weaker VEGFR-2 ligands promoted some enlargement: full length VEGF-C (2.5-fold) and VEGF-D (2.2-fold), while the selective VEGFR-1 and VEGFR-3 ligands AdVEGF-B and AdVEGF-C<sup>156S</sup>, respectively, had no effect on blood vessel morphology (V). Although L-NAME (NO synthase inhibitor) significantly blocked capillary enlargement induced by AdVEGF-D<sup>ΔNΔC</sup> (V), AdeNOS GT alone did not promote angiogenesis (unpublished data).



**Figure 11.** Mean area of CD31-positive microvessels in rabbit semimembranosus muscle six days after GT with adenoviruses encoding human VEGF-A<sub>165</sub>, VEGF-B, VEGF-C, VEGF-C<sup>156S</sup>, VEGF-D, VEGF-D<sup>ΔNΔC</sup>, FGF-4 or eNOS using 10<sup>11</sup> vp. “Receptor” indicates the VEGFR-specificity of the growth factors except for FGF-4 which binds to FGFRs. The data are from study V except for the VEGF-A<sub>165</sub> group which consists of all AdVEGF<sub>165</sub> animals done throughout this thesis, FGF-4 group which is from study IV, and eNOS group which is unpublished data. In statistical analysis, the groups were considered to be independent of each other. \*\* P<0.01 vs. AdLacZ.

As part of the strong capillary enlargement response, about 6% and 8% of the AdVEGF<sub>165</sub> and AdVEGF-D<sup>ΔNAC</sup>-transduced skeletal muscles, respectively, were covered by microvessel lumens in comparison to 0.7% of AdLacZ treated muscle (V).

In the pig myocardium, AdVEGF<sub>165</sub> and AdVEGF-D<sup>ΔNAC</sup> at 10<sup>11</sup> vp or 10<sup>12</sup> vp increased the mean capillary area 1.7- to 2.3-fold, respectively, six days after GT (VI). The capillary enlargement, at its best, led to a 13.5% total capillary area of the myocardium transduced with AdVEGF 10<sup>12</sup> vp group (6.7% in AdLacZ group). Naked plasmid DNA encoding VEGF<sub>165</sub> or VEGF-D<sup>ΔNAC</sup> did not induce any changes in capillary morphology or size in the heart. The control AdLacZ virus also did not induce any enlargement of the microvessels in either skeletal muscle or myocardium (IV, V and VI).

The microvessels enlarged via active EC and pericyte proliferation as shown by BrdU labeling and PCNA immunostaining (V and VI). While only a small proportion of the normal capillaries [normally about 20% of capillaries stain with pericyte markers in skeletal muscle (Morikawa et al., 2002)] had αSMA positive pericytes, nearly all enlarged capillaries had a complete or almost complete αSMA-positive pericyte coverage in AdVEGF<sub>165</sub> and AdVEGF-D<sup>ΔNAC</sup> transduced skeletal muscle and myocardium (V and VI).

Sprouting angiogenesis and formation of new capillary branches were rarely observed six days after adenoviral GT of VEGFs or FGF-4 into non-ischemic muscle. Consequently, capillary density in the transduced muscles was only slightly elevated. ECM-bound VEGF<sub>164/165</sub> and FGF-4 increased capillary density moderately while the soluble VEGF-D<sup>ΔNAC</sup> did not change the capillary number (III-V). In non-ischemic pig myocardium, even AdVEGF<sub>165</sub> did not induce any increase in the capillary number (VI). However, in the total excision hindlimb ischemia model, AdVEGF<sub>164</sub>, and to a lesser extent also AdLacZ at a very high titer (5x10<sup>11</sup>vp/ml), appeared to increase capillary density over PBS

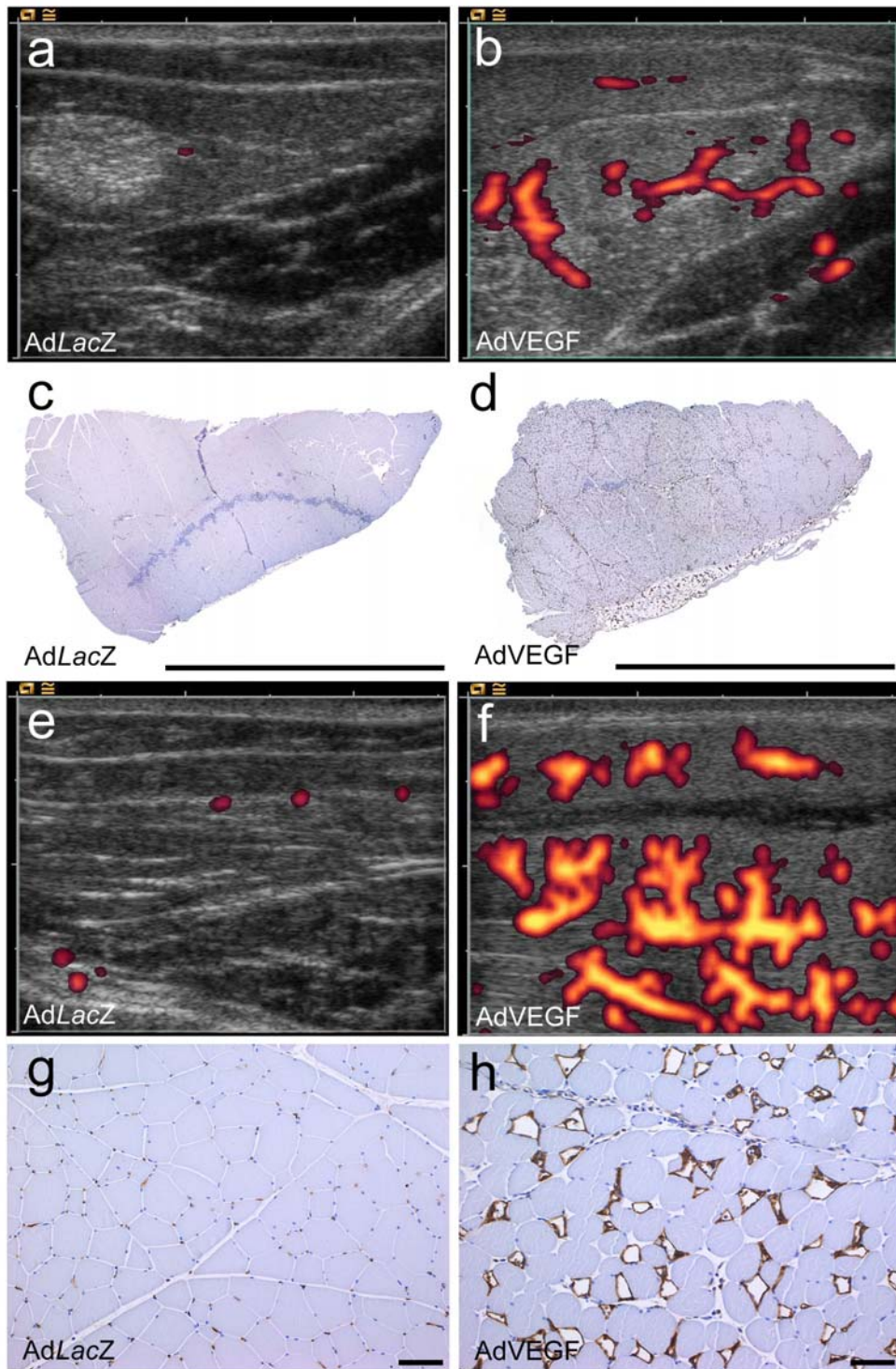
injections as studied four weeks after GT (III).

There were obvious differences in the angiogenesis patterns promoted by the ECM-bound VEGF<sub>164/165</sub> in comparison to soluble VEGF-D<sup>ΔNAC</sup>. Both in skeletal muscle and myocardium, the strongest effects of AdVEGF<sub>164/165</sub> treatment often occurred in the connective tissue in between muscle bundles, whereas angiogenesis stimulated by AdVEGF-D<sup>ΔNAC</sup> occurred diffusely throughout the transduced tissue (IV-VI). Furthermore, AdVEGF<sub>164/165</sub> generated more glomeruloid bodies, clusters of tortuous vessels (Sundberg et al., 2001), than AdVEGF-D<sup>ΔNAC</sup> (IV-VI). AdFGF-4 did not cause this kind of extreme capillary growth, although endogenous rabbit VEGF was upregulated in AdFGF-4-transduced muscles (IV).

### **Perfusion increases in skeletal muscle and myocardium**

#### *Perfusion measurement with microspheres*

Abundant capillary enlargement resulted in a marked increase in perfusion within non-ischemic skeletal muscle and myocardium six days after GT. The microsphere method showed 3.2- to 4.0-fold perfusion enhancement in rabbit skeletal muscle transduced with AdVEGF<sub>164/165</sub>, AdVEGF-D<sup>ΔNAC</sup> and AdFGF-4 when compared to AdLacZ (IV and V). In myocardium, AdVEGF<sub>165</sub> and AdVEGF-D<sup>ΔNAC</sup> resulted in 1.6- to 2.0-fold perfusion increases, respectively, in the transduced region (VI). During dobutamine stress the perfusion ratios between the transduced and apical control regions were at the same level in all groups. Although a positive correlation was found between the total capillary area and skeletal muscle perfusion (r=0.61, V), the microsphere method may underestimate real perfusion in transduced muscles, particularly for potent angiogenic growth factors because microspheres (diameter 15 μm) may not lodge in greatly enlarged capillaries (diameter >15 μm).



**Figure 12.** Ultrasound imaging of perfusion in rabbit hindlimbs six days after AdVEGF GT. **a, b)** Transversal plane power Doppler ultrasound images of non-ischemic semimembranosus muscles (triangle-shaped muscle) without contrast-enhancement. Gracilis muscle lies on top. **c, d)** CD31 immunostaining of the transduced semimembranosus muscles at the same orientation shows increased vasculature in AdVEGF-transduced muscle. In AdLacZ muscle, only the needle track but not normal-sized capillaries can be observed. Scale bar = 1 cm. **e, f)** Longitudinal plane images of the same muscles at the time of maximal enhancement after i.v. administered contrast agent demonstrate dramatically increased perfusion in AdVEGF-treated muscle. Note also enhanced gray-scale signal intensity in AdVEGF-transduced muscle due to abundant interstitial edema. **g, h)** Higher magnification images of CD31 immunostainings demonstrate strongly enlarged capillaries in AdVEGF-treated muscle. Scale bar = 50  $\mu\text{m}$ .

### Ultrasound imaging of perfusion

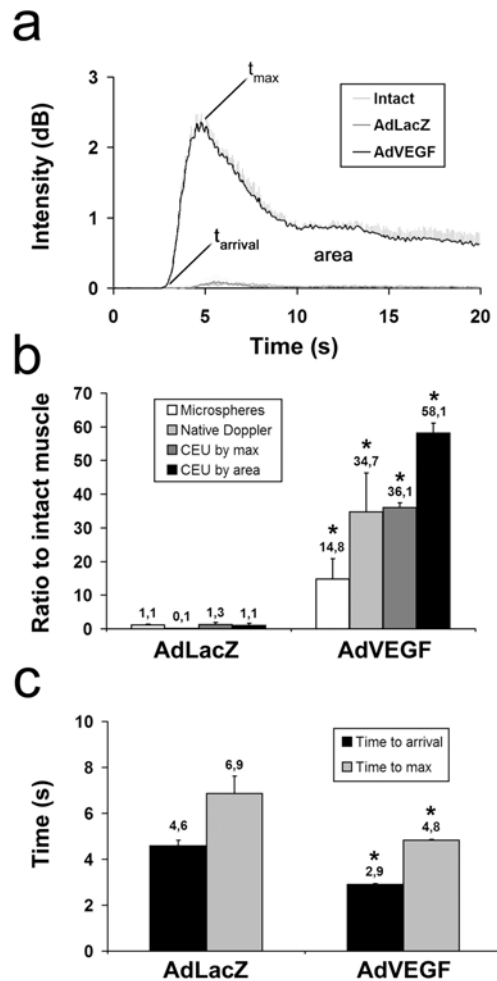
The angiogenic effects were even stronger in the rabbit experiments done for ultrasound imaging (unpublished data) than in the previous published experiments presumably because of an improved i.m. injection technique: AdVEGF<sub>165</sub> (10<sup>11</sup> vp) increased capillary mean size 20.1-fold compared to AdLacZ resulting in 10% microvessel coverage (0.7% in AdLacZ) of the treated muscle. Power Doppler ultrasound imaging both without (Figure 12a and b) and with contrast agent (Figure 12e and f) demonstrated strongly enhanced perfusion in AdVEGF<sub>165</sub>-transduced rabbit muscles. Histology of the same muscles showed abundant capillary enlargement and also remodeling of bigger blood vessels (Figure 12c, d, g and h).

Signal intensity curves after i.v. administration of the contrast agent illustrate the magnitude of difference in perfusion between AdVEGF-treated and control muscles (Figure 13a). Quantitative analysis of native power Doppler ultrasound without contrast agent showed a 34.7-fold perfusion increase in AdVEGF<sub>165</sub>-transduced muscles compared to the contralateral muscle of the same animals (Figure 13b).

Using contrast agent (CEU imaging), comparison of the peak signal intensity and area under the curve between AdVEGF<sub>165</sub>-transduced and the contralateral muscle demonstrated 36.1 and 58.1-fold perfusion increases, respectively (Figure 13b). In the same animals, the microsphere assessment also showed higher perfusion values than in the earlier experiments (14.8-fold increase). The histological total microvessel area of the same muscle correlated best with the perfusion ratio calculated using the peak signal intensity of CEU ( $r=0.77$ ) rather than with the area under the CEU signal intensity curve ( $r=0.69$ ), native power Doppler ( $r=0.61$ ), or microspheres ( $r=0.68$ ).

In addition to perfusion measurement, CEU can be used to study the kinetics of blood flow. This analysis showed that in AdVEGF-transduced muscles the average time from i.v. injection to the arrival of the contrast agent and to the maximal contrast-enhancement was significantly shorter than in AdLacZ muscles: 2.9 s and 4.8 s vs. 4.6 s and 6.9 s, respectively (Figure 13c).

Myocardial contrast echocardiography showed perfusion increases in the regions injected with AdVEGF<sub>165</sub> and AdVEGF-D<sup>ΔNΔC</sup> (10<sup>12</sup> vp/ml) in the pig heart (V).



**Figure 13.** Quantitative analysis of perfusion with power Doppler ultrasound imaging after AdVEGF treatment of the non-ischemic rabbit semimembranosus muscle. **a)** Signal intensity (+ SD) from the transduced and intact muscle as a function of time after administration of the contrast agent via ear vein.  $t_{arrival}$  = time to the arrival of contrast agent,  $t_{max}$  = time to the peak (maximal) signal intensity and area = area under the intensity curve. **b)** Perfusion ratios between the transduced and contralateral intact muscle as measured with microspheres; without contrast agent (native power Doppler); at the peak signal intensity after contrast agent administration (CEU by max); and comparing the areas under the signal intensity curve (reflects the total cumulative amount of contrast agent, and thus, blood flow in the muscle during 20 s imaging period). **c)** Time after i.v. administration of the contrast agent to the arrival and to the peak (maximal) signal intensity. \* $P < 0.05$  vs. AdLacZ.

## Vascular permeability, tissue edema and pericardial effusion

### *Modified Miles assay as a tool demonstrate vascular permeability*

Macroscopic hindlimb edema after i.m. injections of AdVEGF<sub>164/165</sub>, AdVEGF-D<sup>ΔNAC</sup> and AdFGF-4 was dose-dependent and reached its maximum 5–6 days after GT and diminished thereafter in 3-4 days (III, IV and V). The modified Miles assay showed that AdVEGF<sub>164/165</sub>, AdVEGF-D<sup>ΔNAC</sup>, and AdFGF-4 given i.m. caused 10- to 24-fold increases in the extravasation of plasma proteins six days after GT (IV and V). The modified Miles assay turned out to be a crucial tool for the macroscopic demonstration of muscle regions with maximal angiogenesis effects. NO synthase inhibition potently blocked increases in vascular permeability after AdVEGF-D<sup>ΔNAC</sup> GT (V).

Importantly, the vascular leakage did not involve extravasation of erythrocytes. Edemic muscles contained increased numbers of macrophages but no differences in the degree of inflammation after adenoviral GT of VEGFR-1 (VEGF<sub>164/165</sub>) and VEGFR-2 (VEGF-D<sup>ΔNAC</sup>) ligands was found (data not shown). Hindlimbs injected with PBS, AdLacZ, AdVEGF-B, AdVEGF-C, AdVEGF-C<sup>156S</sup> or AdVEGF-D i.m., or with AdVEGF<sub>164</sub> i.a. did not show significantly elevated vascular permeability (IV and V). Unlike VEGF<sub>164</sub>, recombinant FGF-4 protein did not induce acute vascular permeability in the rabbit skin although AdFGF-4-transduced muscles showed strongly increased vascular permeability (IV).

The modified Miles assay was also essential for the macroscopic demonstration of the biological activity of transduced VEGFs in myocardium, (VI). For example, it showed that the angiogenic effects with the NOGA system mediated adenoviral GT were transmural with the strongest response located in the epicardium despite the intraventricular route of injections.

There was a dose-dependent vascular permeability effect with AdVEGF<sub>165</sub> (4.8-fold increase with 10<sup>12</sup> vp/ml) whereas the both doses of AdVEGF-D<sup>ΔNAC</sup> caused similar levels of plasma protein

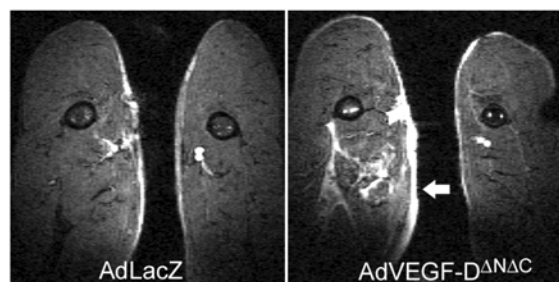
extravasation (3.8- to 4.3-fold) in pig myocardium.

Microvessel hyperpermeability with the higher dose (10<sup>12</sup> vp/ml) but not with the lower dose (10<sup>11</sup> vp/ml) of both AdVEGF-D<sup>ΔNAC</sup> and AdVEGF<sub>165</sub> resulted in substantial pericardial effusion and in some cases in the tamponade of the right atrium (VI). No effusion was observed after AdLacZ control virus (10<sup>12</sup> vp/ml). However, in spite of marked myocardial edema, no increases in Troponin T, creatinine kinase or creatinine kinase-MBm were detected (VI). The total protein and fibrinogen concentration of the effusate was smaller than in plasma of the same animals (total protein 38±3 vs. 49±4 g/l). Using specific ELISAs, high amounts of VEGF-D<sup>ΔNAC</sup> (6320±687 pg/ml) and VEGF<sub>165</sub> (796±108 pg/ml) were found in the effusate.

Interestingly, plasma protein extravasation correlated with mean capillary size in both skeletal muscle (r=0.81) and myocardium (r=0.69) (V and VI).

### *Contrast-enhanced MRI visualizes vascular leakage after angiogenic gene transfer*

Increased vascular permeability in transduced muscles was detected as extravasation of GdDTPA-BMA contrast agent 3 min after i.v. injection in T<sub>2</sub>\*-weighted MRI five days after AdVEGF, AdVEGF-D<sup>ΔNAC</sup>, AdFGF-4 GT (Figure 14, IV and V).



**Figure 14.** Contrast-enhanced MRI of angiogenesis-related vascular permeability. Transversal GdDTPA-BMA enhanced T<sub>2</sub>\* weighted MRI of rabbit mid-thighs 5 days after AdLacZ and AdVEGF-D<sup>ΔNAC</sup> GT. Transduced limb on the left. Arrow denotes the vascular leakage in the AdVEGF-D<sup>ΔNAC</sup>-transduced semi-membranosus muscle.

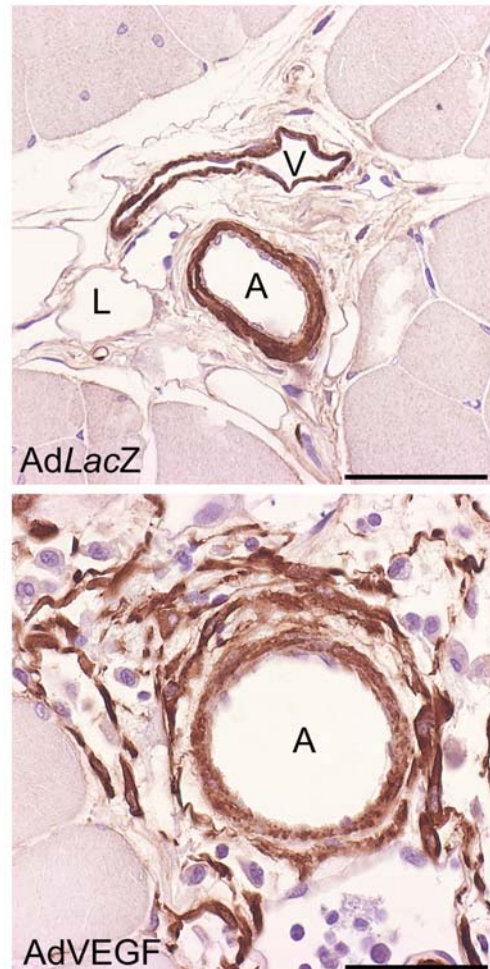
Extravasated contrast agent was observed under the skin, in the semimembranosus muscle and in its fascia, and also in the fat tissue between the medial and lateral muscle compartments. The same regions contained high amounts of transduced growth factors and enlarged capillaries in histology (IV and V). AdLacZ or other AdVEGFs did not promote significant GdDTPA-BMA extravasation as visualized by MRI.

### Arteriogenesis

BrdU labeling showed more proliferating ECs, SMCs and adventitial cells in the collaterals of muscles transduced with adenoviruses encoding the VEGFR-2 ligands VEGF<sub>164/165</sub> and VEGF-D<sup>ANΔC</sup> as well as FGF-4 (IV and V). Increased cell proliferation occurred especially in regions where expression of transduced VEGFs and FGF-4 was high on the basis of immunohistochemistry and histological detection of plasma protein extravasation (IV and V). Figure 15 illustrates the strong remodeling response of a small collateral artery six days after AdVEGF<sub>165</sub> GT in rabbit skeletal muscle.

Collateral growth was quantified from hindlimb angiograms taken just before GT and four weeks later. During the follow-up period, AdVEGF<sub>164</sub> at the doses of 10<sup>10</sup> and 10<sup>11</sup> vp/ml given i.m., but not i.a., promoted 2.1- and 2.4-fold increases in the combined diameter of all angiographically visible collateral arteries in the mid thigh in comparison to controls (IV). With AdFGF-4, the respective increases were 1.8- and 1.7-fold.

Consistent with the angiography data, Doppler ultrasound measurements showed a significant increase in collateral-dependent popliteal blood flow after AdVEGF i.m. and AdFGF-4 i.m. GT (IV). In study III, the number of collaterals instead of the diameter was calculated. A trend towards more numerous collaterals in the thigh of AdVEGF<sub>164</sub>-treated animals than in control animals was found ( $P=0.06$ ). Furthermore, a positive correlation between capillary density and the number of angiographically visible collaterals in the thigh region was observed ( $r=0.52$ , III).



**Figure 15.** Strong arterial remodeling in response to AdVEGF<sub>165</sub> treatment.  $\alpha$ SMA-immunostaining six days after AdLacZ or AdVEGF<sub>165</sub> i.m. GT in rabbit semimembranosus muscle. A = artery, V = vein and L = lymphatic vessel. Scale bar = 50  $\mu$ m.

### Lymphangiogenesis

Histochemical staining of lymphatic ECs for 5' nucleotidase activity (Kato et al., 1993) and an i.a. injection of Ricinus Communis lectin revealed that the CD31-positive but  $\alpha$ SMA-negative vessels in the interstitial connective tissue between the skeletal muscle bundles were lymphatics, not blood vessels (V, data not shown).

Adenovirus encoding the full-length VEGF-D promoted the strongest lymphatic vessel growth as almost all the interstitial connective tissue in the transduced muscle was filled with lymphatic vasculature six days after GT (total lymphatic vessel area 2.6%, V). In AdLacZ control muscles the

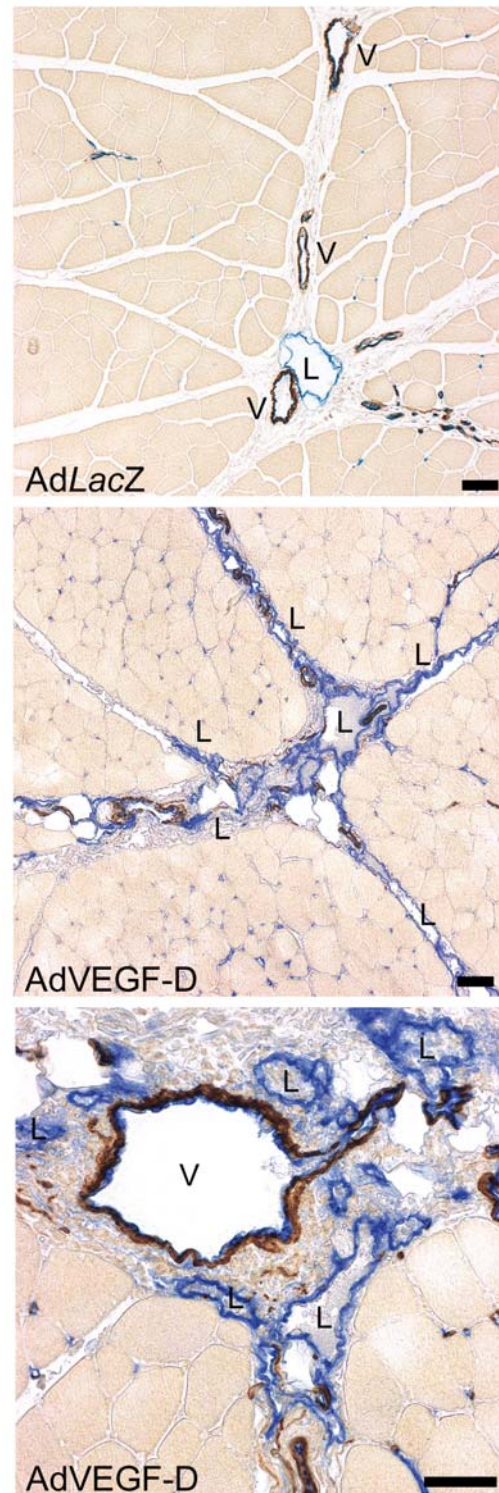
total lymphatic area was 0.12% of muscle area. Lymphatic vessels sprouted efficiently in the muscles treated with VEGFR-3 ligands (Figure 16), which was a clearly different vascular growth mechanism than that observed with blood capillaries. Furthermore, unlike angiogenesis, NO synthase inhibition did not affect lymphangiogenesis induced by full length VEGF-D.

AdVEGF-D<sup>ΔNAC</sup> (1.4%), AdVEGF-C (1.5%), and AdVEGF-C<sup>156S</sup> (1.3%) were also potent inducers of lymphangiogenesis. Remarkably, the adenovirus encoding the VEGFR-3 selective mutant VEGF-C<sup>156S</sup> stimulated exclusively lymphangiogenesis whereas AdVEGF<sub>165</sub> and AdVEGF-B had no effects on lymphatic vessels (V).

#### Duration of vascular growth with adenoviral gene transfer

Although intense, the angiogenic effects of adenoviral GT of growth factors in skeletal muscle and myocardium were mainly transient lasting up to two weeks. After AdLacZ marker GT in rabbit skeletal muscle, gene expression had completely diminished by four weeks as determined by X-Gal staining and RT-PCR (III). Furthermore, three weeks after adenoviral GT in the pig heart (VI), myocardial VEGF-D<sup>ΔNAC</sup> production was no longer detectable with ELISA (data not shown).

In agreement with transient gene expression, increases in mean capillary area, vascular permeability, and perfusion promoted by AdVEGFs or AdFGF-4 had returned to baseline by three and four weeks after GT in non-ischemic skeletal muscle and myocardium (IV and VI). In fact, muscle perfusion returns to baseline by 14 days after adenoviral GT according to our unpublished data, in parallel with the resolution of macroscopic edema. In contrast to the regression of excess capillary enlargement and perfusion in non-ischemic muscles, collateral artery growth was significantly increased four weeks after adenoviral GT of AdVEGF<sub>164</sub> and AdFGF-4 in comparison to AdLacZ (IV). Moreover, in the ischemic rabbit muscle, the increase in capillary density appeared to persist four weeks after AdVEGF<sub>164</sub> GT (III).



**Figure 16.** Lymphangiogenesis in skeletal muscle is stimulated by AdVEGF-D.  $\alpha$ SMA (brown)+CD31 (blue) double immunostaining of rabbit semimembranosus muscle six days after AdLacZ or AdVEGF-D GT. Lymphatic vessels (L) are devoid of SMCs and stain blue while blood vessels (V=vein) have SMCs and stain brown. Note abundant sprouting of lymphatics in between skeletal muscle bundles in AdVEGF-D treated muscle. Scale bar = 50 $\mu$ m.

## DISCUSSION

### Gene expression in ischemic skeletal muscle and its regeneration

In recent years, DNA array technology has offered a new powerful tool for research into molecular pathophysiology of diseases such as cancer and atherosclerosis (Hiltunen et al., 2002; Stears et al., 2003). We utilized DNA array to gain more information on ischemia-induced angiogenesis in human skeletal muscle, which is an approach that has not been published before. Although DNA array has potential especially as a screening method, there are numerous caveats, especially in its application for human tissue.

Pathological human tissues are usually characterized by interindividual differences in the etiology and progression of the disease. In order to avoid at least some of these confounding factors, the case and control samples should be collected from the same individuals and preferably from similar muscle groups. However, it is difficult and unethical to obtain non-ischemic muscle samples in critically ill CLI patients for ideal control material. Also, acutely ischemic human skeletal muscle that is devoid of underlying chronic atherosclerotic disease is rare.

Despite the difficulties in obtaining the ideal ischemic and control samples from humans and the low number of comparisons, our DNA array results are in agreement with previously published experimental data as they show the upregulation of the HIF-1 $\alpha$ -VEGF-VEGFR-2 and TNF- $\alpha$  pathways in acute-on-chronic CLI and IGF-1 and IGF-2 in chronic CLI (Paoni et al., 2002; Raghavendra Rao et al., 2002; Li et al., 1996). The most important DNA array results were confirmed with immunohistochemistry and RT-PCR. Furthermore, the overall percentage of genes differentially expressed under ischemia, 5.5% and 1.3% in acute and chronic CLI, respectively, is similar obtained in animal models (1.0-9.4%) (Kim et al., 2002; Onody et al., 2003; Stanton et al., 2000). The spatial localization of gene expression with immunohistochemistry is important because the differences in global

gene expression may originate from different cell populations in the biopsy samples (e.g. increased adipose tissue and inflammatory cell infiltration in ischemic samples compared to controls) and not from changes in gene expression levels in individual cells.

The most important finding from the DNA array screen covering 8400 human genes was that VEGF was the most prominently upregulated growth factor in acute ischemia, together with its major regulators under hypoxia, HIF-1 $\alpha$  and HIF-2 $\alpha$ , and the most important angiogenic signaling receptor VEGFR-2 (Pugh and Ratcliffe, 2003; Ferrara et al., 2003). The therapeutic potential of other angiogenesis-related molecules found with the DNA array is currently unclear. These molecules include for example Tie-2 (receptor for Ang-1 and Ang-2), Ephrin-B6, Ephrin-B3, Ephrin-A1, PAI-1 and c-met (receptor for HGF) (Suri et al., 1996; Adams et al., 1999; Bajou et al., 1998; Bussolino et al., 1992). Interestingly, many growth factors, which were represented on the array and that have previously been connected to angiogenesis, did not significantly change their expression patterns either in acute or chronic ischemia. These factors include FGF-1, FGF-2, their receptors FGFR-2 and -3; PIGF-1, and -2; VEGF-B, -C, and -D and their receptors VEGFR-1 and VEGFR-3; and HGF.

### Therapeutic perspectives of DNA array findings

The DNA array findings together with the predominant role played by VEGF in the development of blood vasculature (Ferrara et al., 1996), and strong vascular growth achieved with adenoviral VEGF in skeletal muscle and myocardium suggest that VEGF is the main natural regulator of the "angiogenic switch". HIF-1 $\alpha$ -VEGF-VEGFR-2 expression was diffuse throughout the affected muscle in acute-on-chronic CLI while in chronic CLI it was restricted to atrophic and regenerating myocytes indicating that in chronic CLI some regions of muscles were either not ischemic or had an attenuated response to ischemia. When interpreting these findings for therapeutic angiogenesis, the most significant need for



exogenous VEGF might be in chronically ischemic but not yet necrotic regions of the limb and in the proximal muscles containing potential arterial anastomoses for developing collaterals.

The activation of IGF and/or HGF in regenerating myocytes may upregulate VEGF and VEGFR-2 expression in an autocrine manner via the PI3K/Akt pathway (Levinovitz et al., 1992; Jennische et al., 1993; Shen et al., 1998; Jiang et al., 2000; Fukuda et al., 2002). Interestingly, activation of PI3K/Akt has been previously shown to induce myogenic differentiation (Shen et al., 1998; Jiang et al., 1999; Jiang et al., 2000). Furthermore, the PI3K/Akt pathway has been shown to protect cardiomyocytes against hypoxia-induced apoptosis (Matsui et al., 1999). In regenerating regions, VEGF is likely expressed to increase blood flow to the muscle being repaired but it is also possible that the autocrine VEGF expression may directly contribute to the survival and regeneration of myocytes. In support of the latter, VEGF was recently found to promote myoblast survival and enhance their migration five-fold *in vitro* (Germani et al., 2003).

The anabolic survival factors IGF-1 and IGF-2 expressed in atrophic and regenerating myocytes are also likely crucial for survival and regeneration of skeletal muscle and perhaps also for angiogenesis in synergism with VEGF (Barton-Davis et al., 1998; Levinovitz et al., 1992; Smith et al., 1999). Thus, combined therapeutic overexpression of VEGF and IGFs might yield favorable effects on angiogenesis and regeneration of ischemic muscles. In contrast to beneficial effects, the potent vascular permeability-inducing properties of VEGF may contribute to prominent, possibly harmful, interstitial edema found in acute-on-chronic CLI.

Another therapeutic intervention could be the suppression of the TNF- $\alpha$  cascade in acute ischemia because TNF- $\alpha$  is a potent promoter of inflammation, cell-death and implicated in ischemia-reperfusion injury (Baker and Reddy, 1998).

### **The novel rabbit hindlimb ischemia model**

Total removal of the superficial femoral artery and ligation of the deep and circumflex femoral arteries leads to ischemia and myonecrosis both in the rabbit thigh and calf. Widespread muscle necrosis, inflammation and VEGF expression three days after the operation are replaced by myocyte regeneration with strong VEGF expression in myotubes one week after surgery, and finally at six weeks histology is reveals infiltration by adipose and fibrotic tissue. Thus, this model initially resembles acute CLI, but at later timepoints similarities with chronic CLI develop.

The modified hindlimb ischemia model developed in this study produces ischemia only in the calf but not in the thigh with a similar collateral growth pattern as observed in the total excision model. Thus, in this new model the thigh can be utilized to study the influence of transduced growth factors both on angiogenesis and arteriogenesis in non-ischemic conditions and the calf can be used to assess the effects of these factors under ischemia. However, also this model is limited by a considerable interindividual variation in the degree of calf ischemia.

According to the experience obtained throughout these studies, healthy tissue should firstly be used to study the biology of novel growth factors *in vivo* to avoid misinterpretations about their (angiogenic) potency. Intact rabbit skeletal muscle is suitable for these kind of studies because of the ease of i.m. injections, simple structure of blood and lymphatic vasculature and the practical size for many diagnostic procedures and imaging modalities. Of course, the use of ischemia models is then important in order to address the functional benefit of the therapy. It is also possible that the vascular response to growth factors is different in ischemic tissue because of hypoxia, upregulation of cytokines, different blood flow kinetics and the real need for blood vessel growth.

## Gene transfer in skeletal muscle and myocardium

RT-PCR, immunohistochemistry and X-Gal staining are frequently used methods to measure GT efficacy (Hiltunen et al., 2000b; Turunen et al., 2002). Although these methods are useful for offering qualitative information, quantitative data of the transduced proteins in tissues and circulation is crucial when gene-based clinical therapies are designed, especially in the case of potent growth factors that are capable of causing side effects. Thus, ELISA appears to be the preferred method for demonstrating therapeutic growth factor levels in both the target tissues and serum.

Injection of AdVEGF i.a. did not lead to detectable biological effects such as increased plasma protein extravasation or vascular growth in rabbit hindlimbs. These findings together with published data indicate that this route with the adenoviral vector is inefficient for transduction of skeletal muscle and myocardium without manipulation of EC permeability (Magovern et al., 1996; Wright et al., 2001; Lee et al., 2000a).

I.m. injections of adenoviruses produced growth factor levels of 1000-5000 pg/mg of muscle protein suggesting that currently this should be the preferred GT route for therapeutic angiogenesis. In agreement with the tropism of adenovirus (Nalbantoglu et al., 1999), the transduced cells were mostly confined to the connective tissue and muscle fascia. In myocardium, however, where CAR expression is preserved (Nalbantoglu et al., 1999), GT efficacy was also high in cardiac myocytes. The effects of vascular growth factors seem to be local; as a rule of thumb only the area covered by the injectate will be treated although secondary blood vessel remodeling may also occur up- and downstream in response to increased blood flow. This paradigm seems to apply particularly for ECM-bound growth factors such as VEGF<sub>164/165</sub> and FGF-4 while soluble growth factors such as VEGF-D<sup>ANAC</sup> may diffuse further and also be secreted into the blood stream.

Consistent with previous findings with different GT routes (Hiltunen et al., 2000b), the unmodified adenovirus transduces non-target organs such as liver, spleen, lung and

testes also after i.m. injections. Although potent growth factors such as VEGF may cause severe side-effects if produced locally in excessive amounts as demonstrated in this study and by others (Lee et al., 2000b; Thurston et al., 2000), the levels of transduced proteins have been shown to be very low in non-target organs after intramyocardial adenoviral GT (0.1% of myocardial levels) (Magovern et al., 1996). Furthermore, most of the angiogenic effects appear to resolve after VEGF expression has returned to baseline, which should alleviate the safety concerns related to the promotion of pathological angiogenesis.

Although adenoviral VEGF GT is unlikely to promote pathological angiogenesis in dormant tumors, this issue should be carefully addressed in experimental tumor models and in clinical trials. This is especially important for the safe usage of long-term gene expression vectors such as AAV engineered to produce soluble growth factors that leak into the circulation from the target tissue. Furthermore, female patients of reproductive age should be excluded from clinical trials using adenoviruses, because adenoviral vector given i.a. may cause unwanted transduction of oocytes (Laurema et al., 2003). Eventually, tissue targeted vectors and promoters should reduce the potential risk of unwanted production of angiogenic growth factors in non-target organs.

Naked plasmid DNA injections i.m. neither produced detectable protein levels nor any vascular growth in non-ischemic pig myocardium. Gene expression levels after both naked plasmid DNA and viral GT seem to be much higher, and the duration longer in mouse skeletal muscle than in larger animals (Wolff et al., 1990; Chen et al., 1999). Although tissue damage caused artificially or by ischemia may apparently improve naked plasmid DNA GT also in larger animals (Vitadello et al., 1994; Takeshita et al., 1996), the use of naked plasmid DNA for therapeutic angiogenesis in humans should be reconsidered. Also, it is becoming evident that single dose recombinant growth factor injections are not an efficient way to induce vascular growth (Simons et al., 2002; Henry et al., 2003).

## Blood and lymphatic vessel growth profiles of VEGFs

Adenoviruses encoding VEGFR-2 ligands VEGF<sub>164/165</sub> and VEGF-D<sup>ΔNΔC</sup> promoted angiogenesis whereas the VEGFR-3 ligands the full-length VEGF-C and VEGF-D, VEGF-D<sup>ΔNΔC</sup> and VEGF-C<sup>156S</sup> stimulated lymphangiogenesis in rabbit skeletal muscle. These findings agree with *in vitro* and recent *in vivo* work about the proposed roles of VEGFR-2 and VEGFR-3 in vascular biology (Achen et al., 1998; Gille et al., 2001; Saaristo et al., 2002; Makinen et al., 2001b). Our results show that regardless of the context (ischemia/normoperfused, rabbit/pig, skeletal muscle/myocardium) VEGFR-2 ligands always promote blood vessel growth and vascular permeability. These findings also indicate that ischemia is not a prerequisite for efficient blood vessel growth as suggested earlier (Isner et al., 1996). This is important because in order to augment blood flow in patients with intermittent claudication or stable angina pectoris, which comprise the majority of patients with PAD and CAD, vascular growth is also needed in regions where ischemia develops only during exercise.

The special requirement of NO for blood vessel growth and vessel permeability but not for lymphangiogenesis reveals one of the first major differences between VEGFR-2 and VEGFR-3 mediated signaling. The effects of eNOS-derived NO on SMCs are known to be important for vascular homeostasis and growth (Matsunaga et al., 2000; Fukumura et al., 2001). However, overexpression of eNOS alone did not promote angiogenesis suggesting that the mitogenic pathways such as MAPK activated by VEGFR-2 signaling are also required for vascular growth. The special requirement of NO in angiogenesis but not lymphangiogenesis can be likely explained by the fact that NO production is crucial for the relaxation of perivascular cells during capillary enlargement leading to increased blood flow. In contrast, lymphatic capillaries are devoid of pericytes and SMCs, and thus NO production is not required for lymphangiogenesis.

Important knowledge is still lacking regarding the vascular role and signaling mechanisms of VEGFR-1. VEGFR-1 has

previously been associated with monocyte and macrophage chemotaxis and progression of atherogenesis (Clauss et al., 1996; Celletti et al., 2001). However, no differences in the degree of macrophage infiltration after adenoviral GT of VEGFR-1 and VEGFR-2 ligands or evidence of progression of atherosclerosis was found in the present study. The VEGFR-1 ligand VEGF-B had no effect on blood or lymphatic vessel growth in rabbit skeletal muscle and did not promote inflammation.

Recombinant VEGF-B injected in Matrigel into mouse skin and naked VEGF-B plasmid DNA injected into ischemic mouse skeletal muscle followed by electroporation produced an angiogenic response 14 and 28 days after the treatment, respectively (Silvestre et al., 2003). Inflammation and tissue damage after Matrigel implantation and electroporation may affect these results. Some of these conflicting results may also be explained by the formation of VEGF-VEGF-B heterodimers upon inflammation, tissue injury or ischemia which all upregulate endogenous VEGF expression (Olofsson et al., 1996a; Ferrara et al., 2003). In any case, the exact biological role of VEGFR-1 and its ligands still needs dissecting.

## Treatment of lymphedema

Adenovirally delivered human VEGFR-3 ligands promoted lymphangiogenesis efficiently in rabbit skeletal muscle. Thus, GT of these VEGFs could be used to treat genetic or acquired lymphedema (Karkkainen et al., 2001). This study and previously published data suggest that especially the selective VEGFR-3 ligand VEGF-C<sup>156S</sup> has a favorable lymphangiogenic profile with no angiogenic properties (Saaristo et al., 2002). Also the full length VEGF-C and VEGF-D showed much more activity on lymphatic vessels than on blood vessels. As expected, lymphatic vessels did not respond to VEGF-A<sub>165</sub>, a ligand for VEGFR-1 and -2. In only one previous study AdVEGF<sub>164</sub> was found to stimulate lymphangiogenesis, but this effect was most likely secondary to prolonged tissue edema caused by long-term

adenoviral VEGF expression in immunodeficient mice (Nagy et al., 2002).

The inherited form of lymphedema usually affects the cutaneous lymphatics which do not have a muscular layer (Casley-Smith, 1980; Rockson, 2001), and thus the stimulation of collecting lymphatics may not be crucial for the treatment of this disorder. Nevertheless, an interesting biological question is whether VEGFR-3 stimulation is able to promote the growth of large collecting SMC-rich lymphatic trunks besides lymphatic capillaries or whether other growth factors are required. The SMC-positive collecting lymphatics have been shown to express VEGFR-2 rather than VEGFR-3 (Veikkola et al., 2003), and thus they may be more efficiently stimulated by ligands that also have affinity towards VEGFR-2 than those binding only to VEGFR-3 alone. It is also possible that increased peripheral lymph flow stimulated by VEGFR-3 ligands may indirectly promote enlargement of collecting lymphatics, analogous to the secondary effects of blood flow on blood vessel remodeling.

Perhaps the most difficult practical problem regarding gene therapy for lymphedema is how to achieve the necessary widespread transduction of large tissue areas, which are devoid of functional lymphatics, in patients with primary lymphedema.

### **FGF-4 is a blood vessel growth factor**

FGF-4 increased the number and size of capillaries and enhanced vascular permeability in the rabbit hindlimb ischemia model. AdFGF-4 also promoted arteriogenesis and collateral-dependent blood flow with an efficacy comparable to AdVEGF<sub>164</sub>. However, in contrast to VEGF<sub>164</sub>, recombinant FGF-4 protein neither induced vascular permeability in rabbit skin nor did it stimulate EC tube formation *in vitro*. Because of these biological differences we sought to examine whether endogenous rabbit VEGF was upregulated in AdFGF-4-transduced muscles, which turned out to be true.

Thus, VEGF upregulation may at least partly explain the late vascular permeability effects and perhaps also vascular growth

observed after AdFGF-4 GT. This concept is supported by earlier *in vitro* studies where FGF-4 upregulated VEGF and FGF-4-induced angiogenesis could be blocked by VEGF antibodies (Dell'Era et al., 2001; Deroanne et al., 1997).

Interestingly, VEGF-inducing properties are not limited to FGFs or PIGF (Bottomley et al., 2000). In fact, there is growing evidence that also other growth factors such as HGF, IGF and PDGF upregulate VEGF, possibly via the PI3K-Akt and MAPK pathways (Stavri et al., 1995a; Fukuda et al., 2002; Gille et al., 1998; Toyoda et al., 2001; Jiang et al., 2000). These findings support the concept of VEGF as the master regulator of physiological angiogenesis.

### **Nature of vascular growth induced by AdVEGFs**

Although the efficacy of AdVEGF and VEGF-D<sup>ΔNΔC</sup>-stimulated angiogenesis in skeletal muscle and myocardium exceeded all expectations, perhaps the most impressive feature of this biological response was the rapid and remarkable microvessel enlargement involving abundant EC and pericyte proliferation. For example, AdVEGF<sub>165</sub> enlarged the preexisting capillaries up to 20-fold in rabbit skeletal muscle compared to AdLacZ, six days after GT. This enlargement led to total microvessel coverage of 10% in the transduced muscle whereas in AdLacZ controls total microvessel coverage was 0.7%.

Similar enlargement effects in response to AdVEGF<sub>164</sub> have been previously described in mouse skeletal muscle, where the formed vessels were called "mother vessels" (Pettersson et al., 2000), but this study is the first to show microvessel enlargement by VEGFs in larger animals. With naked plasmid DNA encoding VEGF, microvessel enlargement has not been reported to occur, likely reflecting the very low GT efficacy (Tsurumi et al., 1996; Shyu et al., 1998; Vincent et al., 2000).

The common paradigm in the field has been that capillary density is significantly elevated after angiogenic gene therapy. In this study sprouting angiogenesis leading to formation of new daughter vessels was

rarely observed after overexpression of angiogenic growth factors. Only modest increases in capillary density were noted in rabbit skeletal muscle after AdVEGF and AdFGF-4 GT, but not with AdVEGF-D<sup>ΔNAC</sup>. In the pig myocardium increases in capillary density were not observed at all.

It is possible that only the first step in angiogenesis, vessel enlargement and plasma protein extravasation, is achieved with adenoviral VEGF expression (Dvorak et al., 1995; Pettersson et al., 2000), and longer VEGF expression is needed for daughter vessel formation and maturation (Dor et al., 2002; Arsic et al., 2003). It was very interesting that lymphatic capillaries sprouted efficiently after therapy with VEGFR-3 ligands. It may be that pericytes restrict sprouting in blood capillaries but not in lymphatic capillaries because the latter are devoid of pericytes.

### **Angiogenesis in the myocardium**

To our surprise, catheter-mediated intramyocardial injections with the NOGA system stimulated transmural effects in the pig heart. The unexpected pattern of angiogenic effects is probably due to an intramyocardial pressure gradient; the systolic subendocardial pressure is greater than the subepicardial pressure. Thus, the GT solution and secreted growth factors move towards the epicardium. These results suggest that the epicardium can be achieved for therapeutic angiogenesis using a catheter-mediated approach without performing a thoracotomy. NOGA-mediated injections themselves have not been associated with adverse effects (Kornowski et al., 1999; Vale et al., 2001). Thus, with the correct adenoviral dose angiogenic therapy with the NOGA mapping system appears feasible, safe and efficient.

Although having some similarities, there were also differences in the angiogenic response in the pig myocardium compared with rabbit skeletal muscle. For efficient angiogenesis, a higher dose of adenovirus was generally needed in the pig myocardium. Moreover, the increases in microvessel mean size (up to 2.3-fold with AdVEGF) and total coverage (13.5% with AdVEGF vs. 6.7% in controls) as well as

perfusion (2.0-fold) were not as striking as the corresponding responses in skeletal muscle.

The reason why angiogenesis was stronger in skeletal muscle than in myocardium may be due to the fact that myocardium is innately better vascularized than skeletal muscle: total baseline microvessel coverage was 0.7% vs. 6.7% in rabbit skeletal muscle and pig myocardium, respectively. Furthermore, although CAR is more abundantly expressed in myocardium than in skeletal muscle (Nalbantoglu et al., 1999), there is also a possibility that pig tissues are more resistant to adenovirus than those of rabbit's or human's. The increases in plasma protein extravasation were also much less prominent in myocardium than in skeletal muscle which may partly be due to better endogenous mechanisms against formation of edema in the heart such as the negative pericardial pressure.

### **Matrix-binding properties of growth factors modulate angiogenesis**

Despite all being extremely potent proangiogenic factors, histological examination revealed differences between ECM-bound growth factors VEGF<sub>164/165</sub> and FGF-4 in comparison to soluble VEGF-D<sup>ΔNAC</sup>. Angiogenesis stimulated by AdVEGF<sub>164/165</sub> and AdFGF-4 was most pronounced in the interstitial ECM between myocytes and in the connective tissue both in skeletal muscle and myocardium. Furthermore, in skeletal muscle AdVEGF<sub>164/165</sub> and AdFGF-4 moderately increased the capillary number and AdVEGF<sub>164/165</sub> also generated glomeruloid bodies, clusters of tortuous vessels (Sundberg et al., 2001). In contrast, vascular growth in response to AdVEGF-D<sup>ΔNAC</sup> was more diffuse and uniform within the transduced tissues.

After i.m. injections of adenovirus in the heart, VEGF-D<sup>ΔNAC</sup> but not VEGF-A<sub>165</sub> or FGF-4 was found in blood. Consistent with our results, serum VEGF<sub>164/165</sub> levels have not been reported to be increased after pericardial, i.m. or i.a. adenoviral GT (Magovern et al., 1996; Lazarous et al., 1999; Makinen et al., 2002; Hedman et al.,

2003). Lack of systemic leakage with an ECM-bound growth factor may be an advantage over soluble factors such as VEGF-D<sup>ΔNΔC</sup>, which, on the other hand, may promote more diffuse angiogenesis throughout the tissue.

### **Increases in muscle perfusion**

From the therapeutic point of view, the improvement in tissue perfusion is more important than the demonstration of histological vascular growth. The microsphere technique first showed that AdVEGF<sub>164/165</sub>, AdVEGF-D<sup>ΔNΔC</sup> and AdFGF-4 can increase perfusion in non-ischemic skeletal muscle 3- to 4-fold compared to the contralateral intact limb. Even though a notable improvement, it was not completely consistent with the histological measurements performed on the transduced muscle (Pearson correlation was 0.61 between microsphere perfusion and total microvessel coverage).

The microsphere method is impaired by a fundamental technical limitation because microspheres (15 μm in diameter) may not lodge in enlarged microvessels that can be up to 50 μm in diameter. Thus, power Doppler ultrasound imaging with (CEU) or without contrast enhancement was utilized to evaluate muscle perfusion. CEU is not dependent on the microvessel size, is non-invasive, can be used in humans and provides quantitative data on perfusion and blood flow kinetics. CEU showed remarkable perfusion increases in non-ischemic rabbit skeletal muscle six days after AdVEGF<sub>165</sub> GT. The perfusion ratio calculated from the peak CEU signal intensity (36-fold increase) showed the best correlation with the histological total microvessel coverage suggesting that this may be the most accurate parameter to assess perfusion with Doppler ultrasound imaging.

To the best of my knowledge, perfusion increases of this magnitude have not been described with any treatment in skeletal muscle. Even stronger perfusion increases may have been anticipated because of the strong increase in microvessel diameter in accordance with Poiseuille's law (1). However, the biomechanics of blood flow are far more complex in the microcirculation

than in simple cylindrical vessels and thus blood flow is not increased to the fourth power of vessel radius (Schmid-Schonbein, 1999).

Interestingly, in the pig myocardium the perfusion difference disappeared between the transduced anterolateral and apical control regions during pharmacological stress. This may be due to the larger capacity of normal microvessels to increase their size in response to stress than those already maximally enlarged by VEGFs.

In addition to dramatic perfusion increases, CEU showed that the arrival time of the contrast medium to the AdVEGF-transduced muscle was significantly shorter than in controls indicating decreased peripheral resistance. These findings are similar to adnexal tumors that have high blood velocity, possibly because of the presence of arteriovenous shunts (Orden et al., 2003). From the therapeutic point of view, AdVEGF treatment combined with CAGB or peripheral bypass surgery could be useful to avoid cases where high peripheral resistance may lead to "poor run-off" and compromised patency of the grafts (Louagie et al., 1998). Furthermore, reduced peripheral resistance may lead to increased blood flow and subsequent enlargement of upstream collaterals.

### **Capillary arterialization**

The phenotypical changes in the enlarged vessels are so drastic that it may not be appropriate to call these vessels capillaries. The increase in diameter (up to 50 μm) and enhanced coverage with pericytes suggested that vessels resembling arterioles, venules or arteriovenous shunts develop in both skeletal muscle and myocardium. At the time of these studies, similar, but perhaps more complete, blood vessel transformation was observed with long-term VEGF expression systems (Arsic et al., 2003; Springer et al., 2003).

The vessel wall structure in the enlarged channels resembled that described in tumor vasculature, where vessels are permeable and most cannot be classified into the conventional hierarchy of arterioles, venules and capillaries. In human tumors the diameter of blood vessels with capillary-like

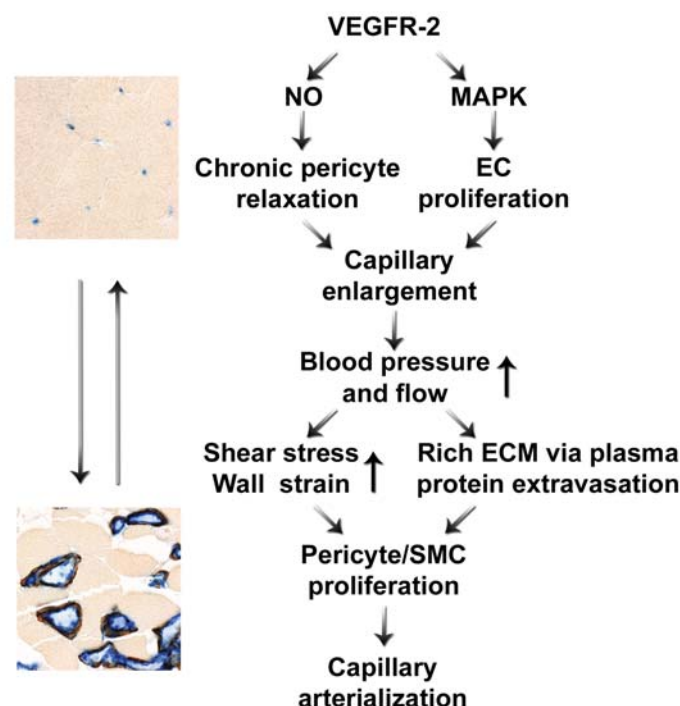
wall structure has been reported to range from 6 to 50  $\mu\text{m}$  (Konerding et al., 1995). Furthermore, in tumors pericytes may exist in multiple layers, be loosely associated with ECs, and grow cytoplasmic processes away from the vessel (Morikawa et al., 2002; Abramsson et al., 2002).

However, the most important difference between tumor vasculature and that stimulated by vascular growth factor overexpression is that blood flow in tumors is often slow and turbulent, which may result in regions of ischemia within the tumor despite ongoing angiogenesis (Bergers and Benjamin, 2003), whereas in AdVEGF-treated muscles blood flow is faster than in intact non-ischemic muscles as shown by CEU.

Although VEGFR-2 also appears to be expressed in cell types other than ECs, pericyte and SMC mitogenesis has not been demonstrated to occur directly upon VEGF stimulation *in vitro*. In fact, Plouet and colleagues noted already in 1989 that VEGF selectively induced proliferation of ECs but not SMCs (Plouet et al., 1989). SMC

migration, however, has been reported in response to VEGF (Grosskreutz et al., 1999). This leads to the question that how is VEGF able to promote the proliferation of pericytes and SMCs, and how does long term VEGF exposure lead to formation of stable vessels (Pettersson et al., 2000; Arsic et al., 2003; Springer et al., 2003)?

Blood flow is a critical determinant of vessel maintenance and durability, possibly via EC-derived signals involving VEGFR-2,  $\alpha_v\beta_3$  integrin and NO (Langille and O'Donnell, 1986; Muller et al., 1997; Sho et al., 2001; Jin et al., 2003), which were all also closely associated with the capillary enlargement in the present study. It is likely that both mechanical factors such as increased fluid shear stress and circumferential wall stress, and biochemical stimuli such as VEGFR-2 signaling are needed for capillary transformation (Figure 17). In contrast, VEGF and other growth factors may induce formation of non-functional SMC-poor vessels in tumors with sluggish blood flow (Jain, 2003).



**Figure 17.** Proposed summary of factors involved in the transformation of capillaries towards vessels resembling arterioles in response to VEGF overexpression. VEGFR-2 signaling on ECs lead to direct EC proliferation via the MAPK pathway and pericyte/SMC proliferation likely through increased blood flow. Enhanced plasma protein extravasation probably also contributes to this process via the establishment of stimulating ECM in the transduced muscles. The enlarged vessels regress to baseline if VEGF expression is transient.

In agreement with our results, mice deficient of VEGF<sub>120</sub> and VEGF<sub>164</sub> show impaired development of retinal arteries but not veins, and transgenic cardiac over-expression of VEGF<sub>164</sub> leads to increased arteriolar and decreased venular capillary formation (Stalmans et al., 2002; Visconti et al., 2002). Although VEGF may also have a direct role in arterialization of blood vessels since VEGF has been shown to promote the arterial fate even before the onset of circulation (Lawson et al., 2002), the expression of VEGFRs on both venular and arterial ECs tend to favor blood flow as the major determinant of postnatal capillary vessel remodeling and transformation towards the arterial phenotype.

Elevated circumferential wall stress may force vessels exposed to increased blood flow to strengthen their wall by hypertrophy of their pericyte and SMC coverage. It has been recently shown that increased hemodynamic stresses increase the number of mature SMCs in arterioles and venules (Van Gieson et al., 2003). Furthermore, extravasated plasma proteins create stimulating ECM that may promote pericyte and SMC proliferation.

### **Vascular permeability is an integral part of angiogenesis**

The strong angiogenic effects induced by AdVEGF or AdFGF-4 in both skeletal muscle and myocardium was always associated with interstitial edema 4-7 days after GT and resolved almost completely by day nine. Interestingly, a positive correlation was found between the microvessel mean size and plasma protein extravasation.

The development of edema after angiogenic GT is likely to result from a contribution of all major factors affecting plasma protein extravasation (2), including increased capillary pressure, colloid osmotic pressure of the interstitium as well as permeability properties of the capillary wall (Bates et al., 1999; Guyton and Hall, 2000c). Furthermore, VEGFR-2 ligands and FGF-4 induce proliferation of capillary ECs and pericytes creating intercellular clefts that leak proteins. The surface area of the endothelium is also increased manifold. It is noteworthy that the vascular leakage was

specific for plasma proteins and did not cause hemorrhage.

If the blood pressure of arterioles (approximately 35 mmHg) extends to the venous ends of capillaries (10 mmHg) due to capillary enlargement, estimated fluid extravasation rate will be about 60 times greater than normal according to the Starling equilibrium (2) (Guyton and Hall, 2000c). VEGF has also been shown to have direct effects on EC ultrastructure via formation of fenestrae and pinocytotic vesicles (Feng et al., 2000) which may be important especially during the acute phase vessel leakiness occurring in minutes after exposure to VEGF.

While diminished adenoviral expression is presumably the most important reason for the resolution of edema, also the increased interstitial fluid pressure and enhanced lymph flow in edemic muscles are also likely to oppose additional accumulation of edema (Guyton and Hall, 2000c). However, it is also possible that lymphatics are compressed resulting in their impaired function during the periods of maximal edema.

Edema, although abundant, was irreversible and did not cause tissue damage in the transduced rabbit thighs or pig myocardium. However, it is conceivable that strong edema could result in the muscle compartment syndrome and even rhabdomyolysis in the calf muscles surrounded by tight fascias. Although healthy pigs tolerated the pericardial effusion, which sometimes manifested as cardiac tamponade, gross edema and pericardial fluid may prove hazardous in the ischemic human heart. In other reported animal experiments, excessive VEGF production in the myocardium has been fatal for mice and pericardial fluid accumulation was associated with one death in a canine model after AdVEGF<sub>165</sub> GT ( $6 \times 10^9$  pfu) given into the pericardial space (Lee et al., 2000b; Lazarous et al., 1999). After intramyocardial AdVEGF<sub>121</sub> GT at high titers ( $10^9$  or  $10^{10}$  pfu) effusion was not detected (Patel et al., 1999), presumably because of pericardial fibrosis caused by thoracotomy or due to the reduced potency of VEGF<sub>121</sub> in comparison with VEGF<sub>165</sub> (Whitaker et al., 2001). The potential for severe adverse effects



emphasizes the need for preclinical dose-escalation studies for the selection of a safe but effective adenoviral dose before entering human trials.

Because lymphatics remove extravasated plasma proteins, stimulation of lymph-angiogenesis with VEGFR-3 ligands could be a potential approach to reduce edema after angiogenic GT. Compression of the edemic limb or exercise could also be used to increase lymph flow (Guyton and Hall, 2000c). Furthermore, corticosteroids such as dexamethasone could be administered in case of threatening edema as they are in clinical use e.g. for the treatment of brain edema. Although previously proposed (Baumgartner et al., 2000), use of diuretics may not be the optimal treatment of angiogenesis-related edema because the removal of fluid-attracting extravasated plasma proteins is not accelerated from the edemic tissue.

### **Imaging of angiogenesis and vascular permeability**

Imaging of blood vessel growth is important for successful clinical development of therapeutic angiogenesis. Current clinical vascular imaging modalities such as X-ray, CT and MRI angiography, however, are usually capable of detecting vessels that are larger than 100  $\mu\text{m}$  (McDonald and Choyke, 2003). PET or SPECT used for imaging of perfusion, blood volume or tissue metabolism are limited by poor spatial resolution. Microscopic imaging technologies used in animals are invasive and too small to be practical in humans. Thus, new modalities are needed for monitoring the angiogenesis effects quantitatively and non-invasively (McDonald and Choyke, 2003).

As shown in this study, CEU perfusion imaging appears a very promising tool to assess muscle perfusion quickly, minimally invasively, quantitatively and without ionizing radiation or nephrotoxic contrast agents. Furthermore, CEU perfusion measurements correlated better with anatomical blood vessel growth than the microsphere method, which is currently viewed as the gold standard for perfusion measurements in experimental models.

Gadolinium-based contrast agents are widely used to increase the MRI signal from various pathological conditions such as tumors and ischemic tissue (Padhani, 2002; Saeed et al., 2001). As shown here, gadolinium-enhanced MRI appears a feasible method to detect changes in vascular permeability after angiogenic GT. Thus, contrast-enhanced MRI could be used to monitor VEGF activity and ongoing angiogenesis after clinical gene therapy. In addition to detecting changes in vascular permeability, MRI techniques capable of functional imaging of newly formed blood vessels are under intensive research (Neeman, 2002).

### **Adenoviral gene transfer promotes transient angiogenesis**

In non-ischemic skeletal muscle and myocardium the effects on microvessel enlargement, perfusion and vascular permeability of AdVEGFs and AdFGF-4 were transient lasting no longer than two weeks. However, collaterals appeared to resist regression possibly because they provide crucial blood flow to the distal parts of the leg and they are formed from structurally mature preexisting arteriolar anastomoses.

It is evident that blood perfusion usually matches the metabolic need in healthy tissues. Thus, at least in normal skeletal muscle and myocardium, increased blood flow alone is insufficient to protect the enlarged microvessels from regression without the presence of growth factors. Mathematical models also suggest that fluid shear stress alone does not lead to stabilization of vessels but additional factor(s) must be present (Hacking et al., 1996). Despite pericyte recruitment, which has been shown to promote vessel maintenance (Benjamin et al., 1998; Benjamin et al., 1999), the enlarged vessels shrunk rapidly, probably by apoptosis, to their original size within two weeks after adenoviral GT. This may indicate that these vessels still are immature despite of pericyte proliferation. The marker used for pericyte and SMC detection,  $\alpha\text{SMA}$ , is expressed both in immature and mature pericytes (Van Gieson et al., 2003), and thus no

conclusions on the maturity of pericytes can be made on the basis of this staining.

The rapid growth and regression of microvessels by exogenous VEGFs and FGF-4 can be considered to be both an advantage and disadvantage. In case of regulated vectors, this feature may provide a tight control of angiogenesis but, on the other hand, a longer gene expression than what adenovirus can offer seems to be required for persistent perfusion increases (Dor et al., 2002; Arsic et al., 2003). This view is supported by the lack of efficacy on walking time at 12 weeks in the first randomized double-blind placebo-controlled trial using adenoviral VEGF in patients with PAD (Rajagopalan et al., 2003). These results may also imply that the adenoviral dose has to be higher than what was used in that study (highest dose  $4 \times 10^{10}$  pu).

### **Towards better therapeutic angiogenesis**

It is difficult to imagine a ligand-receptor system that could significantly add to the angiogenesis effects induced by VEGFR-2 signaling. Furthermore, long-term (>30 days) VEGF overexpression alone has been shown to orchestrate the development of persistent vessels and permanently increase tissue perfusion (Dor et al., 2002; Arsic et al., 2003). However, modulation of the angiogenic response achieved in this study would be highly desirable.

At present, the gene expression kinetics from the adenoviral vector do not appear optimal as the rapid increases in vascular growth result in gross tissue edema 4 to 7 days after GT and only a few days later almost all effects have disappeared. Vectors such as AAV with a progressive gene expression pattern and lower peak levels appear more ideal for human therapy. Indeed, three months after i.m. GT with AAV-VEGF<sub>165</sub>, muscle perfusion measured with microspheres was increased 2.5-fold and vascular permeability was increased only moderately (by 6-fold) (Arsic et al., 2003). Importantly, no hemangiomas were observed in that study and the new vessels had a SMC coverage suggesting the formation of arterioles.

In addition to vector development, approaches using cocktails of different

growth factors have been suggested to generate better blood vessels than what is achieved by VEGF alone. For example, strategies have been designed to stimulate growth of more functional vessels with VEGF combined with PDGF to induce pericytes or Ang-1 to reduce vascular leakage (Richardson et al., 2001; Thurston et al., 2000).

However, the true potential of these combinations for therapeutic angiogenesis therapy is currently unclear. Also the mechanism by which Ang-1 decreases plasma protein extravasation is poorly understood (Thurston et al., 1999). In fact, in the case of increased capillary pressure in response to vessel enlargement, Ang-1 should not only normalize the vascular permeability properties of the endothelium but actually make it hyperresistant to plasma protein extravasation to overcome the effect of increased blood pressure (2). Inhibition of vascular permeability may also be difficult during active angiogenesis because intercellular clefts between proliferating ECs leak proteins. In light of current knowledge, the best way to avoid edema would be achieved by gene expression systems that produce lower peak but longer VEGF expression such as AAV.

## **CONCLUSIONS AND FUTURE DIRECTIONS**

It has been shown here that adenoviral GT of VEGF<sub>164/165</sub>, VEGF-C, VEGF-C<sup>156S</sup>, VEGF-D, VEGF-D<sup>ANAC</sup> and FGF-4 have clear potential as vascular therapeutics. The most suitable growth factor can be chosen depending on the need for blood or lymphatic vessel growth, or both, on the basis of the receptor-binding specificity.

The pioneers in therapeutic angiogenesis, recombinant protein therapy and naked plasmid DNA seem to be inefficient for vascular growth in humans. Adenoviral GT of angiogenic growth factors in animal models has established the proof of principle that sustained production of vascular growth factors can improve blood and lymph flow. The first clinical trials using adenoviral GT for therapeutic angiogenesis

have predominantly shown the safety and feasibility of the therapy. For efficient production of therapeutic growth factors, i.m. injections of the GT vector should be used instead of i.a. injections. Currently, there is no experience on clinical gene therapy for lymphedema. A major difficulty in such trials could be the achievement of widespread gene transfer in large tissue areas lacking functional lymphatics.

The next and much bigger step in the development of clinical angiogenic gene therapy is to demonstrate improved tissue perfusion and clinical condition. To divide this goal into smaller steps, evidence of increased tissue perfusion in man with the adenoviral vector should be first confirmed at an early timepoint (5-7 days) because of the transient adenoviral expression kinetics.

In addition to perfusion, the effect of angiogenic GT on tissue metabolism should be addressed because angiogenesis in skeletal muscle and myocardium involves formation of quite extraordinary type of vessels whose benefit to the transfer of nutrients and oxygen is unclear. Furthermore, the role of blood flow in the subsequent remodeling of blood vessels stimulated by angiogenic growth factors should be addressed.

Due to the profound placebo effects observed in clinical angiogenesis trials, only placebo-controlled, randomized double-blind trials will determine if patients with ischemic disease or lymphedema will benefit from these growth factors. The major side effect, edema, may hamper the introduction of therapeutic angiogenesis into clinical practice. Furthermore, aged patients with endothelial dysfunction due to diabetes and/or hyperlipidemia may not respond as efficiently to angiogenic growth factors as healthy animals. For safe and efficient human therapy, future experimental research should especially focus on the development of an efficient, long term, regulated and tissue-targeted GT vector.

In conclusion, gene transfer for therapeutic angiogenesis and lymphangiogenesis may provide a novel treatment for patients that do not benefit from traditional therapies. Genes encoding vascular growth factors could also be tested in combination with conventional revascularization strategies.

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