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Henna Karvinen

Growth Factor Expression in Atherosclerosis and Gene Transfer for Therapeutic Angiogenesis

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UNIVERSITY OF
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HENNA KARVINEN

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ABSTRACT

Atherosclerosis and its ischemic symptoms are the leading causes of illness and deaths in Western countries. Patients with cardiovascular diseases are increasingly older and have multiple other comorbidities which limit the use of conventional treatments. Therapeutic gene transfer with proangiogenic factors may offer new treatment opportunities for these patients.

In this study, therapeutic potency of vascular endothelial growth factors (VEGFs) and closely related platelet derived growth factors (PDGFs) were studied. In the first study, we found that PDGF-C was strongly expressed in the endothelium and lesion macrophages while PDGF-D expression was more diffuse in the vessel wall in human atherosclerotic lesions. Next, therapeutic angiogenesis was studied in rabbit hindlimb ischemia model and in mouse myocardium using adenoviral (Ad) gene delivery for short-term and adeno-associated virus (AAV) for long-term gene expression, respectively. We observed that the combination gene transfer of AdVEGF-A and AdPDGF-B enhanced the proliferation of pericytes but that did not lead to the stabilization of newly formed blood vessels. However, the combination gene transfer prolonged the angiogenic effect. Thirdly, AAV-VEGF-A was found to induce a 10-fold perfusion increase compared to AAV-LacZ in rabbit hindlimb. Perfusion was followed for up to one year using a high resolution ultrasound method. In comparison, AdVEGF-A gene transfer increased muscle perfusion by 20-fold but that returned back to baseline in two weeks. As a side effect of long-term VEGF-A expression, we observed remarkable histological disorganization of AAV-VEGF-A transduced skeletal muscle. Finally, as a potential novel proangiogenic gene therapy, the effects of two hypoxia inducible transcription factors (HIFs), HIF-1α and -2α, on angiogenesis and tissue energy metabolism were studied in ischemic rabbit hindlimb. Gene transfer of AdHIF-1α and AdHIF-2α were found to increase skeletal muscle perfusion by 4-fold compared to AdLacZ control. In addition, we observed that HIF gene transfer enhanced the recovery of ischemic skeletal muscle from the energy loss induced by exercise evaluated using magnetic resonance spectroscopy (MRS).

In summary, the potential of vascular growth factors, transcription factors and different viral gene delivery vectors in proangiogenic gene therapy were studied. Enhancing angiogenesis together with improved exercise tolerance by gene therapy is a promising new treatment strategy for the patients suffering from impaired vascular function. The expression time and intensity can be controlled by choosing optimal therapeutic genes and viral vectors for a certain treatment application.

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TIIVISTELMÄ

Valtimonkovettumatauti ja sen aiheuttamat verenkierron komplikaatiot, kuten alaraajaiskemia, ovat suurin sairastavuuden ja kuolleisuuden aiheuttaja länsimaissa. Sydän- ja verisuonisairauksia sairastavat potilaat ovat yhä vanhempia ja heillä on useita liitännäissairauksia, jotka rajoittavat perinteisten hoitomuotojen käyttöä. Verisuonten uudismuodostus geeniterapian avulla voisi tarjota näille potilaille uudenlaisen hoitomahdollisuuden.

Tässä tutkimuksessa selvitettiin verisuonen endoteelikasvutekijöiden (vascular endothelial growth factor, VEGF) ja verihiutalekasvutekijöiden (platelet derived growth factor, PDGF) terapeuttisia ominaisuuksia. PDGF-kasvutekijäperheeseen kuuluvien PDGF-C:n ja PDGF-D:n osoitettiin ilmentyvän valtimonkovettumataudista kärsivissä ihmisen verisuonissa. PDGF-C ilmentyi voimakkaasti verisuonen endoteelikerroksessa ja makrofaageissa. PDGF-D:n havaittiin ilmentyvän laajemmin suonen seinämässä. Terapeuttista uudisverisuonten kasvua tutkittiin kanin alaraajamallissa sekä hiiren sydänlihaksessa. Tutkimuksessa käytettiin lyhytaikaisen geeninilmentymisen aikaansaavaa adenovirusta (Ad) ja pitkäaikaisen geenin-ilmentymisen aiheuttavaa adeno-assosioitua virusta (AAV). Havaitsimme AdVEGF-A + AdPDGF-B yhdistelmägeeninsiirron lisäävän perisyyttejä kapillaarisuonten ympärillä, mutta suotuisaa vaikutusta verisuonten pysyvyyteen verrattuna AdVEGF-A geeninsiirtoon ei saavutettu. Yhdistelmägeeninsiirto kuitenkin sai aikaan pitkäkestoisemman verisuonten muodostuksen vasteen. AAV-VEGF-A geeninsiirron osoitettiin pitävän verisuonten muodostus 10-kertaisena vuoden ajan verrattuna kontrolligeeniin. AdVEGF-A lisäsi verisuonten muodostuksen 20-kertaiseksi vaikutuksen kestäessä kaksi viikkoa. Pitkäaikaisen VEGF-A:n ilmentymisen havaittiin aiheuttavan lihaksessa sivuvaikutuksena kudosmuutoksia. Tutkimme myös kudoksen hapenpuutteessa indusoituvien transkriptiotekijöiden (hypoxia indicible factors, HIF) HIF-1α:n ja HIF-2α:n vaikutuksia uudisverisuonten muodostukseen ja kudoksen energia-aineenvaihduntaan. AdHIF-1α:n ja AdHIF-2α geeninsiirtojen havaittiin lisäävän luurankolihaksen verenkiertoa 4-kertaisesti kontrolligeeniin verrattuna. Työssä osoitettiin ensimmäistä kertaa HIF geeninsiirron parantavan verenkierron puutteessa olevan lihaksen energiavarastojen kykyä palautua rasituksesta.

Yhteenvetona voidaan todeta että kasvutekijöiden ja transkriptiotekijöiden geeninsiirrolla kahta eri viruskuljetinta käyttäen saatiin kasvatettua uudisverisuonia ja parannettua merkittävästi verenvirtauksen puutteessa olevan lihaksen rasituksen sietokykyä. Valitsemalla geeninsiirtovektori halutun ilmentymisajan ja tehokkuuden perusteella voidaan jatkossa geeniterapiaa kohdentaa lihaksen verenkierron ja hapenpuutteen hoitamiseksi.

Yleinen suomalainen asiasanasto: ateroskleroosi - - hoitomenetelmät; geeniterapia; geenitekniikka; adenovirukset; iskemia; eläinkokeet; jänikset; hiiret; kasvutekijät; verisuonet; magneettitutkimus; spektroskopia; ultraäänitutkimus

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LIST OF ORIGINAL PUBLICATIONS

I Karvinen H, Rutanen J, Leppänen O, Lach R, Levonen A-L, Eriksson U, and Ylä-Herttuala S. PDGF-C and -D and their receptors PDGFR-α and PDGFR-β in atherosclerotic human arteries. Eur J Clin Invest 2009; 39

II Korpisalo P, Karvinen H, Rissanen TT, Kilpijoki J, Marjomäki V, Baluk P, McDonald DM, Cao Y, Eriksson U, Alitalo K, Ylä-Herttuala S. Vascular endothelial growth factor-A and platelet derived growth factor-B combination gene therapy prolongs angiogenic effects via recruitment of interstitial mononuclear cells and paracrine effects rather than improved pericyte coverage of angiogenic vessels. Circ. Res. 2008;103: 1092-9.

III Karvinen H., Pasanen E., Rissanen TT., Korpisalo P., Vähäkangas E., Jazwa A., Giacca M., Ylä-Herttuala S. 2010. Long-term VEGF-A expression promotes aberrant angiogenesis and fibrosis in skeletal muscle. Gene Ther. 2011. Accepted for publication

IV Karvinen H, Honkonen K, Korpisalo P, Kansanen E, Babu M, Rissanen TT, André H, Pereira T, Poellinger L, Alitalo K, Ylä-Herttuala S. 2010. Adenovirus-mediated gene transfers of hypoxia inducible factors-1α and -2α improve ischemic skeletal muscle recovery after exercise by inducing vessel growth and blood flow. Manuscript. 2011.

Some unpublished results will be also presented.

Contents

ABREVIATIONS

AAV	Adeno-associated virus
Ad	Adenovirus
Ang	Angiopoietin
αSMA	Alpha smooth muscle actin
ATP	Adenosine triphosphate
CAD	Coronary artery disease
CAR	Coxsackie adenovirus receptor
CEU	Contrast enhanced ultrasound
EC	Endothelial cell
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
EPAS-1	Endothelial PAS domain protein-1
EPC	Endothelial progenitor cell
Flk-1	Fetal liver kinase-1 (murine VEGFR-2)
Flt-1	Fms-like tyrosine kinase-1 (murine VEGFR-1)
Flt-3	Fms-like tyrosine kinase-3 (murine VEGFR-3)
GM-CSF	Granulocyte macrophage-colony stimulating factor
GT	Gene transfer
HIF	Hypoxia inducible factor
HRE	Hypoxia responsible element
i.m.	Intramuscular
i.v.	Intravenous
KDR	Kinase domain region (human VEGFR-2)
LacZ	Betagalactosidase (marker gene)
MRS	Magnetic resonance spectroscopy
Nrp	Neuropilin
PAD	Peripheral arterial disease
PBS	Phosphate buffered saline
PCr	Phosphocreatine
Pi	Inorganic phosphate
PDGF	Platelet derived growth factor
PDGFR	Platelet derived growth factor receptor
PlGF	Placental growth factor
RT-PCR	Reverse transcriptase polymerase chain reaction
SMC	Smooth muscle cell
VEGF	Vascular endothelial growth factor
VEGFR	Vascular endothelial growth factor receptor
Vp	Viral particles

1 Introduction

Despite improved prevention and treatment of cardiovascular diseases, they are still the leading cause of morbidity and mortality in Western countries. Atherosclerosis is an arterial disease where lipid accumulation leads to inflammation and narrowing of the lumen of the blood vessel (Lusis, 2000). In peripheral arterial disease (PAD), occluded arteries cannot provide sufficient blood flow to the extremities resulting in ischemia (Kumar et al., 2004). Reduced blood flow impairs the oxygen supply and decreases aerobic energy metabolism in the ischemic tissue. At the same time, metabolites and waste products accumulate, which triggers inflammation and causes malfunction of the tissue. At present, bypass surgery and percutaneous procedures treatments are available for revascularization of the ischemic tissue. However, since many of the patients suffering PAD are old, conventional treatment strategies become more challenging due to the presence of diffuse and disseminated arterial disease, poor general health status or other medical limitations in these patients. Gene transfer of growth factors is one emerging option for the revascularization of ischemic limbs. In cardiovascular gene therapy, collateral blood vessels are opened and new vessels grown after the administration of proangiogenic growth factors (Yla-Herttuala and Alitalo, 2003). Two processes are needed to rescue ischemic tissue from irreversible tissue necrosis: angiogenesis and arteriogenesis. Angiogenesis is a process where new vessels are grown from pre-existing vessels via different mechanisms (Risau, 1997). Arteriogenesis is the remodeling of conduit vessels by making them increase their diameter, leading to increased blood flow (Schaper and Scholz, 2003). Many factors, including vascular endothelial growth factors (VEGFs), platelet derived growth factors (PDGFs) as well as hypoxia inducible transcription factors (HIFs) are involved in these processes. VEGF has been widely used to induce angiogenesis in pre-clinical and clinical cardiovascular gene therapy studies (Rissanen and Yla-Herttuala, 2007). In addition to increased angiogenesis, improvement in tissue energy metabolism is needed to support proper tissue recovery from ischemia. The aim of this thesis was to increase knowledge about the factors involved in atherosclerosis and to search for new approaches to induce blood vessel growth in ischemic limbs. In this thesis, the expression of PDGFs in human atherosclerotic lesions was characterized, and the angiogenic power of VEGF-A, PDGFs and HIFs was evaluated in animal models using short- and long-term gene expression.

2 Review of the literature

2.1 VASCULAR SYSTEM AND MECHANISMS OF VASCULAR GROWTH

2.1.1 Vascular system

The vascular system is the first organ to develop during fetal growth (Risau and Flamme, 1995). The correct structure and function of blood vessels are essential for the developing organism. Blood vessels supply oxygen and nutrients to the tissues, carry metabolites and waste products for secretion and are essential for immune defense. The cardiovascular system consists of the heart as a pumping regulator of blood flow, the arteries and veins transferring blood to the periphery and back to the heart, and the capillaries which are responsible for substance exchange in tissues (Guyton and Hall, 2006). In general terms, blood vessels are formed in the early state of ontogenesis, but also some blood vessel formation can occur in adults. Many factors are involved in the correct formation of the blood vessels and the regulation of their function. The rate of blood flow in tissues is controlled by many factors, such as the need for oxygen and nutrients (Guyton and Hall, 2006).

2.1.2 Organization of vascular system

There are three types of blood vessels, arteries, capillaries and veins and these can be differentiated by their size, structure and function. The vessel walls are composed of endothelial cells (ECs), mural cells, and connective tissue. (Figure 1) Thick walled arteries carry oxygenated blood from the heart to the periphery under high pressure, in normal conditions 80-120 mmHg (Guyton and Hall, 2006). The diameters of human arteries vary from centimeters to millimeters. The average of blood velocity in arteries is 33 cm/s. Arteries branch to smaller arteries and then to even smaller branches before becoming capillaries which are called arterioles. Arterioles control the flow of blood into capillaries (Guyton and Hall, 2006). Arterioles have a strong smooth muscle cell (SMC) layer and they are able to constrict and reduce, even close the vascular lumen or relax and dilate it, thus having a capability to respond quickly to the need of tissues (Guyton and Hall, 2006).

Capillaries, which are 4 to 9 μm in diameter, are connected to arteries and veins but their main function is to supply oxygen and nutrients into tissues and carry carbon dioxide and waste products away from the tissue. The capillary length may be only 0.3 to 1 millimeter and the velocity of blood flow is typically 0.3mm/s. Capillaries have a very thin endothelial layer enabling rapid substance exchange between the blood and the interstitial fluid (Guyton and Hall, 2006). Capillary ECs are connected to each other by intercellular junctions. Several biological factors can modulate endothelial junctions, such as inflammatory mediators and growth factors, and in that way influence permeability across the endothelium (Toborek and Kaiser, 1999).

Venules, 10 to 50 μm in diameter, collect blood from capillaries. One fourth of oxygen from red blood cells is released in capillaries to tissue and carbon dioxide as a waste product from tissue metabolism is collected for transportation. Venules become fused to form larger veins, with diameters ranging from millimeters up to centimeters in humans. Veins carry oxygen-deprived blood back to the heart at varying velocities and low pressure, in the end of vena cava the pressure is close to 0 mmHg (Guyton and Hall, 2006). Veins have the largest cross-sectional area in the cardiovascular system and they are capable of constricting and enlarging. Thus, veins provide a reservoir function for storing large quantities of extra blood, which can be taken into use whenever needed elsewhere in circulation (Guyton and Hall, 2006).

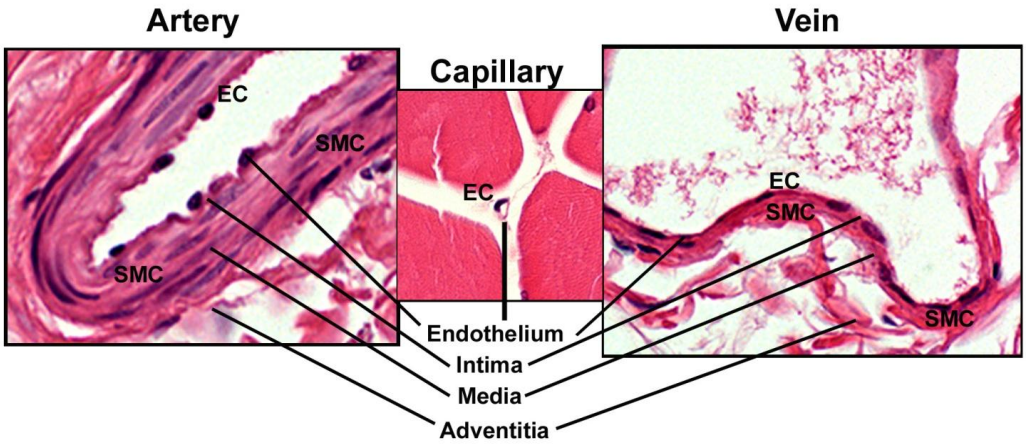

Figure 1. Histological structure of an artery wall, a capillary and a vein wall (hematoxylin-eosin staining from rabbit skeletal muscle). EC = endothelial cell, SMC = smooth muscle cell.

2.1.3 Vasculogenesis

The formation of blood vessels *de novo* during embryogenesis is called vasculogenesis (Figure 2) (Risau and Flamme, 1995). In the early state of embryogenesis, EC progenitors, mesodermal angioblasts, differentiate to vascular plexuses, which will later develop into blood vessels. Some angioblasts migrate to form vascular plexuses at distant sites. Members of fibroblast growth factor family are crucial in inducing the mesoderm to form angioblasts and hematopoietic cells (Shalaby et al., 1995a). VEGF is essential for the differentiation of ECs and the further development of the vascular system (Risau and Flamme, 1995; Carmeliet et al., 1996). Until recently, it was thought that vasculogenesis was restricted to the perinatal period. The process of differentiation of adult stem or progenitor cells and the *de novo* formation of blood vessels is called postnatal vasculogenesis (Kassmeyer et al., 2009). Endothelial progenitor cells (EPCs) have been shown to be mobilized endogenously by ischemia or exogenously by GM-CSF stimulus, and improve neovascularization of ischemic tissues (Takahashi et al., 1999). In addition, VEGF mobilizes bone marrow derived EPCs and contributes to neovascularization (Asahara et al., 1999; Kalka et al., 2000). However, the effects on vascular growth rather seem to be due to the increased levels of secreted growth factors of mobilized EPCs than to their direct incorporation into the endothelium of neovessels (Rehman et al., 2003; Purhonen et al., 2008). Additionally, hematopoietic stem cells isolated from umbilical cord vein have been shown to be able to enhance the regenerative capacity of skeletal muscle after acute ischemic injury but even though they do not directly participate in angiogenesis (Koponen et al., 2007).

2.1.4 Angiogenesis

Angiogenesis is vascular growth from pre-existing capillaries, which occurs by enlarging, sprouting, bridging and non-sprouting angiogenesis called intussusceptions (Figure 2) (Risau, 1997; Conway et al., 2001). Angiogenesis is a common mechanism in tissue regeneration, e.g. occuring in the menstrual cycle and wound healing, but it is present also in pathological conditions like diabetic retinopathy and tumor growth (Risau, 1997). Hypoxia is the principal physiological stimulator for angiogenesis, and a low oxygen level activates HIF which in turn up-regulates other pro-angiogenic factors such as VEGF (Fong, 2008). The first step in angiogenesis is the vasodilatation of capillaries followed by increases in permeability and degradation of the extracellular matrix, which leads to EC activation. Activated ECs proliferate and migrate to distant sites under the influence of many factors including FGF, VEGF and angiopoietins and their receptors (Conway et al., 2001). Migrated ECs clusters in the interstitial

space, form lumen structures and finally fuse with existing vessels to create larger vessels (Conway et al., 2001). The Branching of newly formed vessels can result from several mechanisms: new vessel branches can sprout towards ECs clusters in the surrounding mesenchyme, vessels can split to form daughter vessels by bridging of ECs, or branching can occur via intussusceptions where surrounding tissues invade the vessel lumen splitting the vessel into two new vessels (Conway et al., 2001). Newly formed vessels are often leaky because of the loose EC junctions and this leakiness can cause protein extravasation which increases the osmotic pressure in surrounding tissues leading to tissue edema (Guyton and Hall, 2006). Tissue edema cannot be avoided during efficient angiogenesis, and in fact, a high amount of extravasated protein correlates with the increase in capillary size (Rissanen et al., 2003). In addition to EC function in angiogenesis, peri-endothelial mural cells and extracellular matrix play important roles in the maturation of vessel wall (Jain, 2003). Nascent vessels recruit SMCs, which stabilize newly formed vessels by inhibiting EC proliferation and migration (Conway et al., 2001). PDGF is an important regulator of the maturation of the vessels by mediating SMC and pericyte differentiation, proliferation and migration (Lindahl et al., 1997; Hellberg et al., 2010).

2.1.5 Arteriogenesis

Arteriogenesis refers to the transformation of small arterioles into much larger conducting arteries (Figure 2) (Schaper and Scholz, 2003). Unlike angiogenesis, arteriogenesis is not induced by hypoxia (Deindl et al., 2001). Arteriogenesis is induced by physiological forces and refers to the remodeling of existing arterioles to increase their luminal diameter in response to increased blood flow (Heil et al., 2006; Conway et al., 2001). Collateral growth by arteriogenesis occurs next to an occluded artery, while angiogenesis takes place in an ischemic area distal from the occlusion (Ito et al., 1997; Schaper and Scholz, 2003). Occlusion in the artery leads to a decrease in blood pressure in the region distal from the occlusion and that in turn increases blood flow in pre-existing small collateral arteries leading to more shear stress and elevated pressure on the vessel wall. Fluid shear stress is a strong mechanical inducer of arteriogenesis (Heil et al., 2006). Shear stress activates the endothelium to express monocyte chemoattractant protein (MCP-1) which together with transforming growth factor (TGF) attracts circulating monocytes to adhere and penetrate into the intima. These adhering monocytes secrete growth factors and proteases (Scholz et al., 2000).

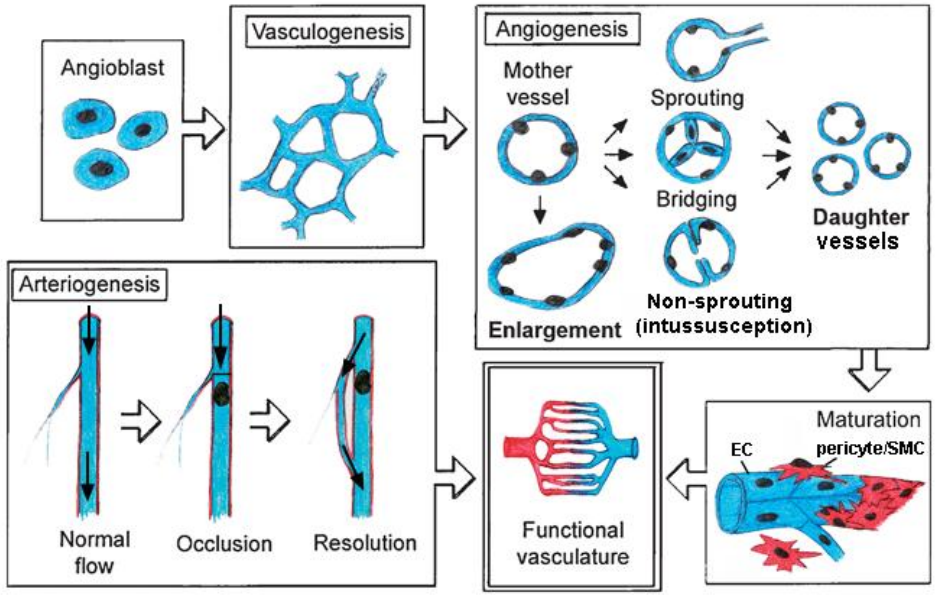

Figure 2. Mechanisms of vascular growth. Modified from Korpisalo and Ylä-Herttuala 2010

In the remodeling phase of arteriogenesis, SMCs start to proliferate, the vessel wall thickens and the intima is formed. The collaterals grow in length. Their main characteristic is their tortuous shape (Schaper and Scholz, 2003; Scholz et al., 2000). Some weeks after an occlusion only the largest collaterals remain and maintain blood flow while small collaterals have regressed ensuring the most efficient blood flow and low energy consumption (Hoefer et al., 2001).

2.2 VASCULAR GROWTH FACTORS

2.2.1 VEGFs and their receptors

Vascular endothelial growth factor A (VEGF-A or VEGF) was the first member of VEGF family cloned in 1989 (Leung et al., 1989; Ferrara and Henzel, 1989). It is a crucial growth factor for physiological and pathological angiogenesis. In adults, VEGF-A is expressed in all vascularized tissues, especially in fenestrated and sinusoidal vessels in endocrine and secretory organs as well as in blood vessels, skeletal muscle and myocardium, emphasizing its importance in

vascular homeostasis(Maharaj et al., 2006). Embryos lacking only a single allele of *vegf-a* will die *in utero* in E11 to E12 due to abnormalities in the vascular system (Carmeliet et al., 1996). VEGF-A induces vasodilatation, and vascular permeability, maintains EC survival and stimulates EC proliferation and migration (Ferrara et al., 2003). The expression of VEGF-A in the endothelium is up-regulated during tissue injury as well as by inflammation, especially under hypoxia. It is known that hypoxia can induce VEGF-A expression in tissues (Minchenko et al., 1994).

The human VEGF-A gene consists of eight exons separated by seven introns with isoforms being produced by mRNA splicing. At least seven different VEGF-A isoforms are currently known: VEGF-A_{121}, VEGF-A_{145}, VEGF-A_{162}, VEGF-A_{165}, VEGF-A_{183}, VEGF-A_{189} and VEGF-A_{206} (Tischer et al., 1991; Poltorak et al., 1997; Jingjing et al., 1999; Lange et al., 2003). The most widely studied isoform, VEGF-A_{165}, is a 45 kDa glycoprotein that binds to heparin on the cell surface and forms homodimers via cysteine bonding (Ferrara and Henzel, 1989). In addition, VEGF-A_{189} and VEGF-A_{206} bind tightly to heparin and exist mainly bound to the extracellular matrix. VEGF-A_{121} is a freely soluble isoform as it does not bind to heparin (Houck et al., 1992). VEGF-A signaling is transduced via two receptor tyrosine kinase receptors, VEGFR-1 and VEGFR-2 (de Vries et al., 1992; Terman et al., 1992). Both receptors have high affinity binding sites for VEGF-A (Waltenberger et al., 1994).

VEGF-B growth factor is particularly highly expressed in the heart and skeletal muscle, and it has about 43 % identical amino acid sequence with VEGF-A (Olofsson et al., 1996). VEGF-B has two splice variants: heparin binding VEGF-B_{167} and freely soluble VEGF-B_{186}. VEGF-B binds selectively to VEGFR-1 (Olofsson et al., 1998). The expression of VEGF-B is not regulated by hypoxia. Since it lacks the hypoxia responsive elements in the promoter area, and the mRNA levels of VEGF-B are not changed by hypoxia (Enholm et al., 1997). VEGF-B has effects on the myocardium. *Vegf-b*$^{-/-}$ mice have smaller hearts, vascular dysfunction and they recover poorly from myocardial ischemia in comparison to normal mice (Bellomo et al., 2000). Another study revealed that *vegf-b*$^{-/-}$ mice suffer an atrial conduction abnormality, but no changes in proper development of the cardiovascular system either during development or as adults (Aase et al., 2001). VEGF-B induces angiogenesis in the heart via VEGFR-1 and neuropilin-1 (Lahteenvuo et al., 2009). VEGF-B has been reported to have species specific properties to influence the growth coronary arteries (Bry et al., 2010).

VEGF-C and VEGF-D are similar in their structural and functional properties. VEGF-C was cloned in 1996 and VEGF-D in 1997 (Orlandini et al., 1996; Yamada et al., 1997; Joukov et al., 1996a). VEGF-C and –D are secreted as precursor proteins, which are proteolytically cleaved to mature forms which possess VEGF homology domains (Joukov et al., 1997; Achen et al., 1998). The mature forms of VEGF-C and VEGF-D bind predominantly to VEGFR-2 and the precursor forms to VEGFR-3 (Li and Eriksson, 2001). VEGF-C is an essential factor for normal lymphatic

development and during lymphangiogenesis in adults (Karkkainen et al., 2004). VEGF-C mRNA is mainly expressed in lymph nodes, heart, placenta, ovaries, small intestine and thyroid (Joukov et al., 1996b; Kukk et al., 1996). It has also been shown to increase vascular EC mitosis, migration and permeability and have an angiogenic effect on mouse eye (Cao et al., 1998). VEGF-D induces lymphangiogenesis via VEGFR-3 and its mRNA is mainly expressed in heart, lungs and small intestine with smaller amounts in skeletal muscle, large intestine and pancreas (Achen et al., 1998). Mature VEGF-D is also a powerfull vascular growth factor and it functions via VEGFR-2 (Rissanen et al., 2003).

VEGF-E is a VEGF-A homologue, which was found in the Orf-virus (Lyttle et al., 1994). It is similar to VEGF-A_{121} in its properties and is a non-heparin binding, freely soluble growth factor. (Ogawa et al., 1998) VEGF-E binds to VEGFR-2 and induces EC proliferation, migration and angiogenesis *in vivo* (Meyer et al., 1999). The angiogenic efficacy of VEGF-E is close to that of VEGF-A but it causes fewer side effects than VEGF-A in transgenic mice (Kiba et al., 2003).

Placental growth factor (PlGF), which belongs to the VEGF superfamily, was identified from a placental cDNA library (Maglione et al., 1991). PlGF has two protein variants: one is 149 amino acids long PlGF-1 which is cleaved to form a functional soluble protein consisting of 129 amino acids, and another the 170 amino acids containing heparin binding form PlGF-2 (Maglione et al., 1993; Hauser and Weich, 1993). PlGF is mainly expressed in the placenta, thyroid gland and lungs (Ziche et al., 1997). PlGF forms heterodimers with VEGF-A and its angiogenic properties are partly explained by this feature (DiSalvo et al., 1995). PlGF binds specifically to VEGFR-1 (Clauss et al., 1996). It has also been observed that PlGF binding to VEGFR-1 causes transphosphorylation of VEGFR-2 and enhances VEGF-A mediated angiogenesis (Autiero et al., 2003). PlGF also induces angiogenesis via upregulation of VEGF-A (Roy et al., 2005). Furthermore, it is known to evoke angiogenesis and arteriogenesis in skeletal muscle and heart almost as effectively as VEGF-A (Luttun et al., 2002). *Plgf*$^{-/-}$ mice display defects in angiogenesis and collateral vessel growth after tissue injury and this can be alleviated by a bone marrow transplantation pointing to a role of PlGF in the mobilization of bone marrow-derived cells (Carmeliet et al., 2001).

Signal transduction of the members of the VEGF family is mediated via five receptors (Figure 3). VEGFR-1 or fms-like tyrosine kinase receptor (Flt-1) was cloned in 1989 and further characterized in 1992 (de Vries et al., 1992; Shibuya et al., 1989). VEGFR-1 plays an important role in the organization of embryonic vasculature but is not necessary for the EC formation (Fong et al., 1995). It also mediates PlGF induced capillary formation (Cai et al., 2003). In addition to ECs, VEGFR-1 is expressed on monocytes where it mediates VEGF-A and PlGF induced chemotaxis and downstream signaling (Barleon et al., 1996; Clauss et al., 1996; Tchaikovski et al., 2008). VEGFR-1 is also expressed in SMCs where its phosphorylation

activates metalloproteinases and induces SMC migration (Wang and Keiser, 1998). It has also been postulated that VEGFR-1 does not directly mediate signal transduction but rather acts as a decoy receptor for VEGFR-2 ligands (Ferrara et al., 2003). It has also been claimed that VEGFR-1 can decrease VEGFR-2 expression *in vitro* and modulate VEGF-A mediated vascular growth (Rahimi et al., 2000). In addition, its soluble isoform, sVEGFR-1, inhibits VEGF-A activity (Kendall and Thomas, 1993). Recent data suggest that VEGFR-1 has an important role in EC adaptation in hypoxia. VEGFR-1:VEGFR-2 ratio is elevated under hypoxia leading to decreased VEGF-A-mediated VEGFR-2 signaling in hypoxic ECs. This mechanism may release more oxygen for viable cells which are able to renewal and repair tissue (Ulyatt et al., 2010).

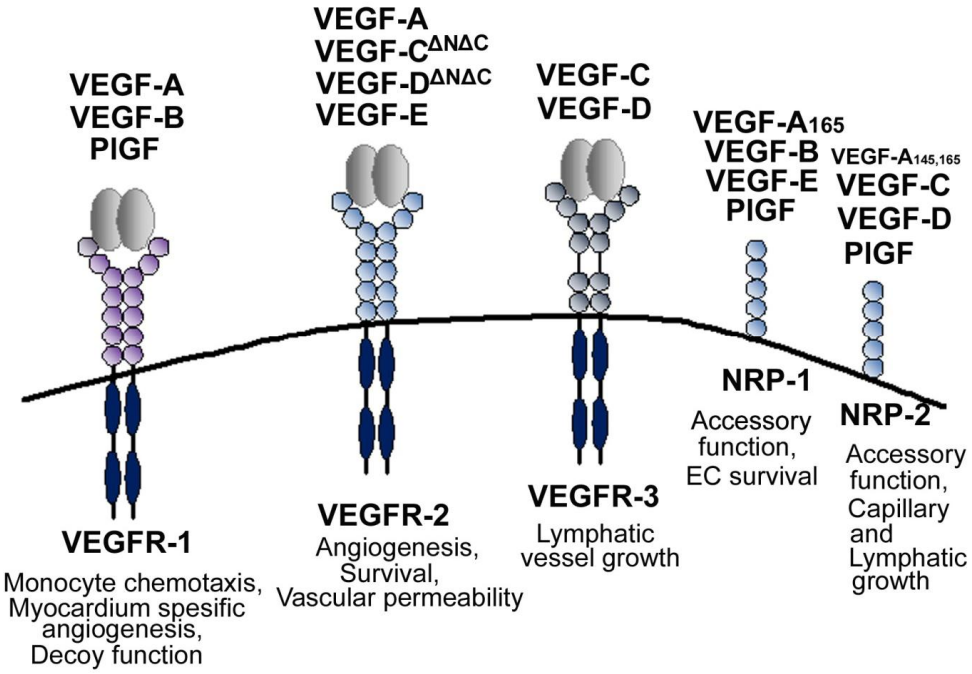

Figure 3. VEGF family members and their binding to receptors. Modified from Ylä-Herttuala et. al. 2007.

VEGFR-2 or kinase domain region (KDR or Flk1) was cloned from human EC cDNA library in 1992 (Terman et al., 1992). It is the major mediator of the mitogenic, angiogenic and permeability-enhancing effects of VEGF-A (Ferrara et al., 2003). It has a key role in developmental vascular growth and hematopoiesis and therefore *flk1*$^{-/-}$ mice die in utero

between days E8.5 and E9.5 due to the lack of vasculogenesis and the failure to develop blood islands and organized blood vessels (Shalaby et al., 1995). VEGFR-2 mediates vascular EC growth and migration *in vitro* and angiogenesis and vascular permeability *in vivo* (Waltenberger et al., 1994; Gille et al., 2001).

VEGFR-3 or fms-like tyrosine kinase receptor-4 was cloned in 1992, and its expression has been detected in many cancer cell lines, and in spleen, brain and lung in embryos but not in vascular ECs (Pajusola et al., 1992). Subsequently, the expression of VEGFR-3 was found to be restricted in the lymphatic endothelium during the development and to be increased in lymphatic sinuses, metastatic lymph nodes and lymphangiomas (Kaipainen et al., 1995). VEGFR-3 mediates signal transduction involved in growth, migration and survival of lymphatic ECs and it is critical for the lymphatic vessel growth and homeostasis. Thus, it is important in the regulation of tissue edema (Veikkola et al., 2001; Makinen et al., 2001b; Makinen et al., 2001a).

Neuropilins are closely related to VEGF signaling. Neuropilin 1 (Nrp-1) binds to VEGF-A_{165}, PlGF-2, VEGF-B and VEGF-E (Klagsbrun et al., 2002). Overexpression of Nrp-1 in transgenic mice leads to increased capillary formation and vasodilatation but can also cause cardiac malformation (Kitsukawa et al., 1995). Mice lacking *nrp-1* gene have problems in the formation of neuronal axons and blood vessels (Kawasaki et al., 1999). Nrp-1 is a co-receptor for VEGFR-2 enhancing VEGF-A binding to VEGFR-2 and this leads to increased cell chemotaxis and growth (Zachary, 2003). Nrp-1 is also an important regulator of VEGF-A induced tissue permeability (Becker et al., 2005). Neuropilin-2 (Nrp-2) is known to be involved in lymphatic vessel function (Yuan et al., 2002). For example, it has been reported that Nrp-2 binds to VEGF-C and VEGF-D and forms complex with VEGFR-3 contributing to lymphatic functions (Karkkainen et al., 2001; Karpanen et al., 2006).

2.2.2 PDGFs and their receptors

PDGFs belong to the PDGF/VEGF superfamily, of which there are four known members. PDGF-B (also known PDGF) was identified in the 1970s (Kohler and Lipton, 1974; Ross et al., 1974; Westermark and Wasteson, 1976) and PDGF-A in the mid 1980s (Antoniades et al., 1979; Betsholtz et al., 1986). They are mitogens for mesenchymal cells including SMCs, bone and cartilage cells (Heldin and Westermark, 1999). PDGF-A mRNA is highly expressed in most human tissues with the highest expression being seen in the heart, pancreas and skeletal muscle (Fredriksson et al., 2004). Deletion of the *pdgf-a* gene in mice leads to their death at three weeks of age due to defects in alveoli formation (Bostrom et al., 1996). *Pdgf-a* knock-out mice display multiple pathologies including the lack of SMCs in lungs and abnormalities in

oligodendrocytes, skin, intestine and testis (Li and Eriksson, 2003). PDGF-B has 50 % identical amino acid sequence to PDGF-A. PDGF-B mRNA is expressed in most human tissues with the highest amounts found in the heart and the placenta (Fredriksson et al., 2004). Deletion of *pdgf-b* gene causes defects in the development of kidneys and blood vessels, and bleeding at the time of birth due to the inability of the newly formed vessels to attract pericytes (Leveen et al., 1994; Lindahl et al., 1997). It has also been reported that PDGF-B affects angiogenesis mainly via SMC and pericyte recruitment (Heldin and Westermark, 1999). PDGF-B is strongly present in the atherosclerosis and has influence on inflammatory response during the atherosclerotic processes (Bohm et al., 1994; Tang et al., 2005)

PDGF-C and PDGF-D were discovered more recently and their biological functions remain somewhat unclear (LaRochelle et al., 2001; Bergsten et al., 2001; Li et al., 2000). In human tissues, high PDGF-C mRNA expression levels have been observed in the heart, liver, kidney, pancreas and ovaries, and lower levels in the placenta, skeletal muscle and prostate (Li et al., 2000). PDGF-D mRNA expression is high in several human tissues e.g. the adrenal glands, heart, pancreas and ovaries and low in some other tissues, such as adipose tissue, placenta and liver (LaRochelle et al., 2001; Bergsten et al., 2001). Both PDGF-C and PDGF-D antigens are present in renal arterial wall in SMC layer and in adventitial connective tissue (Uutela et al., 2001). Differences in the expression occur in primary vascular cells as PDGF-C was highly expressed in the coronary artery SMCs whereas PDGF-D expression was more pronounced in umbilical vein and microvascular ECs (Uutela et al., 2001). Proangiogenic effects of PDGF-C have been reported (Li et al., 2005). However, both PDGF-C and PDGF-D are more strongly linked to tissue fibrosis and SMC proliferation. PDGF-C is a strong inducer of fibroblast proliferation, and it causes cardiac hypertrophy and cardiomyopathy in transgenic mice (Ponten et al., 2003). Similar to PDGF-C, PDGF-D is a strong chemoattractant and it recruits macrophages and participates in vessel maturation during VEGFR-2 mediated angiogenesis in the mouse wound healing model (Uutela et al., 2004). In addition, PDGF-D overexpression in the heart had caused cardiac fibrosis and additionally proliferation of SMCs (Ponten et al., 2005).

PDGFs signal via two receptor tyrosine kinases PDGFR-α and PDGFR-β, and the PDGFR-$\alpha\beta$ heterodimer also exists. Ligand binding promotes receptor dimerization, which activates signal transduction. PDGFR-α signaling is involved in gastrulation, the development of neural structures, gonads, lung, intestine, skin, central nervous system (CNS) and the skeleton. PDGFR-β signaling is important in blood vessel formation and early hematopoiesis (Andrae et al., 2008). Figure 4 presents the PDGF family ligands and their binding to the receptors.

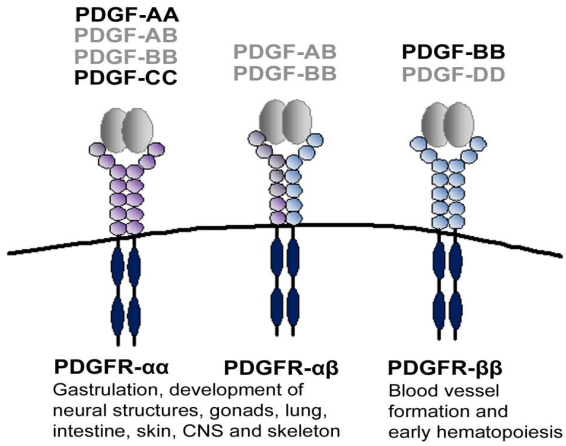

Figure 4. Platelet derived growth factors and their receptors. Ligands with proved to be important interactions in vivo are shown in black and other interactions (ligands in grey) have been demonstrated so far only in cell culture. Modified from Andrae et. al. 2008

2.2.3 HIFs

HIF-1α is a transcription factor mediating adaptive responses to low cellular oxygen levels (Wang et al., 1995). Under normal cellular oxygen conditions, HIF-1α is continuously translated but rapidly degraded in proteasomes. In the degradation process, two proline residues, P402 and 564 are hydroxylated by prolyl hydroxylases which leads to the binding of von Hippel-Lindau protein and recruitment of E3 ubiquitin-protein ligase, resulting in HIF-1α ubiquitination and proteasomal degradation (Jaakkola et al., 2001; Maxwell et al., 1999). Factor inhibiting HIF (FIH) hydroxylates the asparagine residue 803 and this prevents the binding of the transcriptional co-activator p300 (Mahon et al., 2001; Lando et al., 2002). During hypoxia, the hydroxylase activity is inhibited and HIF-1α levels rapidly increase. HIF-1α translocates to the nucleus and forms the HIF-1 heterodimer with constitutively expressed HIF-1β (Wang et al., 1995a). HIF-1 binds to hypoxia responsible elements (HREs) in a gene promoter area (Semenza et al., 1996). The Deletion of the *hif-1α* gene leads to embryonal death at day E11 due to neural tube defects, cardiovascular malformations, and marked cell death within the cephalic mesenchyme (Iyer et al., 1998). HIF-1α is the main factor governing tissue oxygen homeostasis and it regulates the expression of many genes including genes involved in angiogenesis, metabolism and erythropoiesis (Hirota and Semenza, 2006). VEGF-A is a central angiogenic gene upregulated by HIF-1α (Forsythe et al., 1996). Puryvate dehydrogenase kinase 1 (PDK1) is one of the metabolic target genes of HIF-1α. PDK1 is an important enzyme for attenuation of

mitochondrial reactive oxygen species production, maintenance of ATP levels, and adaptation to hypoxia (Kim et al., 2006; Papandreou et al., 2006).

HIF-2α or endothelial PAS Domain Protein-1 (EPAS-1) shares 48 % sequence identity with HIF-1α (Tian et al., 1997). HIF-2α mRNA is present in many tissues and strongly expressed in the heart, placenta and lung (Tian et al., 1997). The regulation of HIF-2α expression is similar to HIF-1α and it is also induced by hypoxia (Wiesener et al., 1998). In contrast to widely expressed HIF-1α, HIF-2α seems to be expressed only certain tissues (Wiesener et al., 2003). In a similar manner as HIF-1α, during hypoxia HIF-2α binds to HIF-1β and translocates to nucleus prior to DNA binding and gene activation in hypoxia (Tian et al., 1997). HIF-2α knockout mice exhibit multiple pathological and metabolic abnormalities including retinopathy, cardiac hypertrophy, skeletal myopathy and mitochondrial abnormalities (Scortegagna et al., 2003). In addition, HIF-2α is involved in regulating the expression of VEGF-A and VEGFR-1 (Takeda et al., 2004). Both HIF-1α and HIF-2α bind to HRE in DNA sequences but they differ from transactivation domain suggesting that they may have unique target genes (Loboda et al., 2010).

2.3 Cardiovascular diseases

2.3.1 Atherosclerosis

Atherosclerosis is a progressive disease characterized by the accumulation of lipids, SMCs and fibrous elements in the arteries (Lusis, 2000; Libby, 2002). There are many risk factors for this disease, e.g. elevated serum low density lipoprotein (LDL) levels, low serum high density lipoprotein (HDL) levels, elevated blood pressure, obesity, high-fat diet, smoking and lack of exercise for this common disease (Lusis, 2000). Atherosclerosis is initiated by the accumulation of LDL in the subendothelial matrix, which acts to attract monocyte influx. Monocytes differentiate into macrophages and take up lipoproteins (Lusis, 2000). The LDL is modified e.g. oxidized, before the macrophages can take it up (Steinberg et al., 1989). The lipoprotein-rich macrophages turn into foam cells and form the first lesion phase, fatty streaks, in the vessel wall (Lusis, 2000).

The accumulation of oxidized LDL stimulates ECs to produce a number of pro-inflammatory molecules and growth factors such as macrophage colony stimulating factor (M-CSF), and these factors in turn recruit monocytes and lymphocytes. Cytokines and growth factors induce SMC migration into the intima and promote their proliferation (Lusis, 2000). The intimal SMCs which have migrated from the media produce extracellular matrix and induce the formation of plaques, the second phase of the atherosclerotic lesion. With time, cells die and release their

lipid contents into the intima which gives rise to the necrotic core of the lesion (Lusis, 2000). Initially, the atherosclerotic lesion grows towards the adventitia, but after a certain point, it starts to expand inwards and obstructs the lumen. In the same lesions, the plaques become vulnerable especially if proteinases have degraded the fibrotic cap. In addition, the presence of inflammation can enhance plaque destabilization. The calcification of lesions also makes them vulnerable and in turn increases the risk for the rupture. Plaque rupture leads to thrombosis, which may occlude the artery, and cause an ischemic attack or an infarction (Lusis, 2000). The development of atherosclerotic lesion is demonstrated in figure 5.

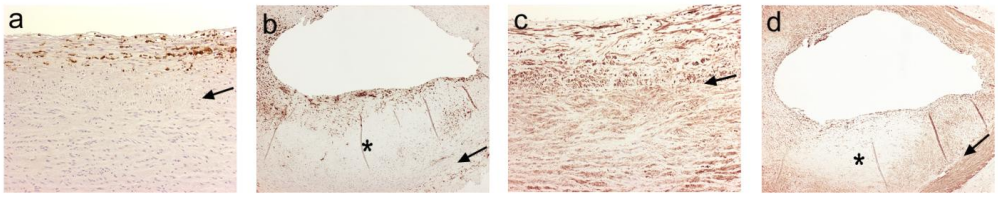

Figure 5. Development of atherosclerotic lesion in human arteries. Macrophage staining (a,b) shows infiltrated macrophages in the intima of the fatty streak lesion at the beginning of the atherosclerosis process (a) and subsequent accumulation of macrophages in the shoulder area of the advanced lesion (b). Smooth muscle actin staining (c,d) reveals the thickening of the intima in the fatty streak lesion (c) and decreased SMC number in the intima of the advanced lesion. The arrows indicate the internal elastic lamina between the intima and the media, and asterisks refer to the necrotic core of the lesion. Narrowing and the irregular structure in the lumen are seen in the advanced lesion (b,d).

2.3.2 Peripheral arterial disease

Peripheral arterial disease (PAD) is characterized by the narrowing or occlusion of lower extremity arteries, most often due to atherosclerosis. The disrupted blood flow and oxygen delivery in lower limbs leads to ischemia (Kumar et al., 2004). PAD can lead to pain during walking (claudication), ulceration, rest pain and critical limb ischemia (CLI) (Norgren et al., 2007a). Symptoms can be graded with Rutherford classification (0-6) as follows: stage 0 asymptomatic, 1 mild claudication, 2 moderate claudication, 3 severe claudication, 4 rest pain, 5 minor tissue lost, and 6 severe tissue lost or gangrene. Stages 0-3 are considered PAD and stages 4-6 the end stage of PAD, CLI (Varu et al., 2010). The prevalence of PAD ranges from 3 to 15 % in the entire population (Norgren et al., 2007; Kasapis and Gurm, 2009). Patient with CLI represents 1 % of all PAD patients (Varu et al., 2010). PAD is more common in elderly people. The known risk factors for PAD are elevated levels of serum cholesterol, smoking, high blood pressure and diabetes. The ankle-brachial index (ABI), the ratio of highest systolic ankle

pressure to the highest arm pressure (in mmHg), is the most widely used initial test to perform when suspected PAD. The ABI ratio under 0.90 is considered abnormal, 0.71-0.90 and 0.40-0.70 indicate mild and moderate obstructions, respectively. The ABI ratio under 0.40 point to severe obstructions (Kasapis and Gurm, 2009). In addition to PAD diagnosis, the ABI index has prognostic value in the progression of the disease after treatment, identifying patients who are at risk for critical limb ischemia, and cardiovascular morbidity and mortality (Hirsch et al., 2006). Exercise testing, non-invasive imaging and angiography can be used for further diagnosis of PAD (Kasapis and Gurm, 2009).

Treatment methods for PAD patients are surgical by-pass or angioplasty as well as drug therapy but many of the patients are not suitable candidates for these treatments and 10-40 % of patients may require leg amputation (Sneider et al., 2009; Ghosh et al., 2008). In addition, patients presenting PAD have often coronary artery disease (CAD), which increases the risk for cardiovascular events. Thus, new treatment strategies, such as gene therapy, are needed.

2.4 SKELETAL MUSCLE ENERGY METABOLISM

Occlusion of the main feeding artery in peripheral muscles clearly disrupts the energy metabolism within the ischemic area. Normally, skeletal muscle is able to rapidly increase its rate of energy consumption in situations where increased contraction is required. The increase in energy production can be 300-fold from resting to fully activated contraction, and this can occur within milliseconds (Sahlin et al., 1998). The fundamental energy source in muscle activation is ATP. There is very little ATP actually stored in cells (5-6mM) and this is depleted in a few seconds (Sahlin et al., 1998). Hence other metabolic pathways are needed. Energy metabolism can be divided into the anaerobic and aerobic pathways. The former occurs under low oxygen conditions and when rapid muscle activity is needed, and the latter process (aerobic metabolism) occurs when oxygen is freely available and muscle contraction is prolonged and submaximal (Guyton and Hall, 2006). The major anaerobic pathways to generate ATP are the degradation of phosphocreatine (PCr) and the breakdown of glycogen to lactate and hydrogen ions (Westerblad et al., 2010). The degradation of PCr to creatine (Cr) and ATP occurs via creatine kinase (CK). In conditions when ATP is being rapidly consumed, the PCr amount declines, and the amount of Cr and inorganic phosphate (Pi) increase. During the recovery from exercise, PCr is synthesized and the normal levels are restored (Westerblad et al., 2010c). ATP levels remain rather constant in muscle tissue, and the intracellular ATP concentration does not decrease below 60 % of the resting concentration, even during an intense exercise (Westerblad et al., 2010). Glycogen breakdown occurs by phosphorylases which release glucose which can undergo glycolysis and pyruvate conversion. Lactate dehydrogenase converts pyruvate to

lactate, and at the same time, hydrogen ions accumulate and decrease muscle pH from ~7.0 to ~6.5 (Westerblad et al., 2010). In the aerobic pathway, oxidative metabolism of carbohydrates and lipids is the main ATP-producing system (Spriet and Watt, 2003). The effects on energy metabolism can be measured with phosphorus magnetic resonance spectroscopy in both preclinical studies and in the clinic (Korpisalo et al., 2008; Greiner et al., 2006).

2.5 CARDIOVASCULAR GENE THERAPY

2.5.1 Principles of angiogenic gene therapy

Gene therapy means the delivery of genetic material into cells to achieve a therapeutic effect. In angiogenic gene therapy, the transfer of genetic material can be used to rescue tissue from hypoxia and ischemia by overexpressing factors which are intended to increase collateral vessel growth and improve perfusion (Yla-Herttuala and Alitalo, 2003). Angiogenic gene therapy is a potential treatment for patients suffering from PAD or CAD but who are not candidates for the current therapies because of diffuse CAD or poor health status. Gene therapy may also be used in conjunction with bypass surgery and angioplasty. The main advantages of gene therapy are that administration of the gene drug is local and it is endogenous proteins which are being used to treat the disease. A single administration of the gene drug can achieve a long lasting local effect without side effects which could arise from its systemic administration (Yla-Herttuala and Alitalo, 2003).

2.5.2 Gene transfer vectors and delivery routes

Gene transfer vectors and delivery routes are important factors to achieve the best gene therapy result. Different vectors have their own advantages and disadvantages. The characteristics of the most common vectors used in cardiovascular gene therapy are summarized in table 1. The first cardiovascular gene therapy studies were conducted with naked plasmid DNA. Plasmid DNA is easy to produce but unfortunately its efficacy is very low. Instead, viruses are effective tools to deliver genetic material to cells. A critical issue when using genes as therapeutic drugs is that the therapeutic gene should enter the cell nucleus and initiate transcription. It is also very important to pay attention that one has the correct dose of the gene drug. Direct extrapolation from animal experiments to man are always problematic, but they do at least suggest an effective dose for humans. However, finding the correct dose for patients still remains a key

problem (Karvinen and Yla-Herttuala, 2010). Virus production is more challenging than plasmid production. Good quality of virus preparations in preclinical studies can prevent false effects; some effects in the past may have been attributable to impurities. Extrapolation between species is another problem. Studies conducted in small rodents have often been promising, but have failed when transferred to larger animals (Yla-Herttuala and Alitalo, 2003; Markkanen et al., 2005). The target organs for proangiogenic cardiovascular gene therapy are the arteries, veins, myocardium and skeletal muscles of the lower limbs. In animal studies intravenous (i.v.) delivery of the gene drug is mostly used. This may be a convenient method, but the large blood and tissue volumes in humans reduce the usefulness of this delivery route as a clinical applications (Karvinen and Yla-Herttuala, 2010). Intra arterial gene transfer did not offered increase in transduction efficiency compared to direct intramuscular (i.m.) injections (Korpisalo et al., 2010). Gene drugs can be delivered effectively via catheters into the heart or by i.m. injection into peripheral muscles (Yla-Herttuala and Alitalo, 2003). Direct injections into myocardium and into the lower limb skeletal muscles are the most efficient delivery routes to produce high expression of transgene, but the target areas need to be carefully selected to ensure that the injections can reach the ischemic border areas where new blood vessel formation is desirable (Karvinen and Yla-Herttuala, 2010). Catheter mediated intramyocardial injections guided with an electronical 3D mapping system have recently been proven to be both safe and practical (Lahteenvuo et al., 2009; Stewart et al., 2009). Ultrasound guidance can also be used to target the injections into the lower limb skeletal muscles.

2.5.3 Viral vectors

2.5.3.1 Adenovirus vectors

Adenoviruses (Ad), 70-90 nm in diameter, are linear double stranded non-enveloped DNA-viruses which cause airway and intestine infections. They enter cells via the Coxsackie-adenovirus receptor (CAR), and integrins $\alpha v\beta_3$ and $\alpha v\beta_5$ (Bergelson et al., 1997; Wickham et al., 1993). The first generation Ad vectors have deletions in virus genome areas E1 and/or E3 which mean that they are incapable of producing new virus particles in transduced cells. The transgene capacity of the first generation adenoviral vector is about 7 kb (Russell, 2000). Ads are easy to produce in large scale cell culture systems and they have been widely used in gene therapy applications. The advantage of adenoviruses is their capacity to transduce both dividing and non-dividing cells with high efficacy. Disadvantages of adenoviruses include immunogenicity and formation of neutralizing antibodies (Russell, 2000). Modification of

immunogenicity has been achieved but the potency of modified adenoviruses in gene therapy applications in humans remains unknown (Wen et al., 2004).

2.5.3.2. Adeno-associated virus vectors

The adeno-associated virus (AAV) is a small single-stranded (25 nm) non-enveloped DNA virus which belongs to parvovirus family. The infection of wild type AAV occurs only in the presence of helper virus, adenovirus or herpesvirus. AAV can integrate site-specifically at chromosome 19 but current AAV vectors do not have this ability (Daya and Berns, 2008). Most AAV vectors are based on AAV serotype 2 which enters into a cell using heparan sulphate proteolygans as receptors (Summerford and Samulski, 1998). AAV posesses many benefits such as nonpathogenicity for humans, a wide range of infectivity depending on serotype, and a long expression time (Wu et al., 2006; Daya and Berns, 2008; Warrington, Jr. and Herzog, 2006). AAV transduction into the mouse heart can induce stable transgene expression in cardiomyocytes without evoking inflammation (Svensson et al., 1999). The gene expression after AAV injections can remain even many years (Niemeyer et al., 2009; Stieger et al., 2009). AAV serotypes have a specific biodistribution in various organs. In terms of cardiovascular gene transfer, serotypes 1, 6, 8 and 9 seem to have the best transduction efficacy for heart and skeletal muscle (Palomeque et al., 2007; Raake et al., 2008; Zincarelli et al., 2010; Xiao et al., 1999).

2.5.3.3. Lentivirus vectors

Lentivirus vectors are modified from human immunodefiency virus. Lentiviruses would be able to induce stable gene expression via integration in to the genome. This may, on the other hand, increase the risk for the activation of ongogenes. So far, the transduction efficacy of lentiviruses in myocardium and in skeletal muscle has been low. (Kang et al., 2002; O'Rourke et al., 2003) Some studies conducted with lentiviral gene transfer in cardiovascular models have revealed slightly positive effects (Niwano et al., 2008; Conklin et al., 2005). However, lenti- and retroviruses may well have use in gene therapy applications based on ex vivo gene transfer into stem cells (Karvinen and Yla-Herttuala, 2010). Additionally, lentiviruses transduce efficiently liver and neural cells, and thus may represent a useful tool for other types of gene therapy (Naldini et al., 1996; Trono, 2000).

Table 1. Characteristics of the most common vectors used in cardiovascular gene transfer. Modified from Rissanen et. al 2007

Vector	Advantages	Disadvantages
Non-viral vectors (plasmid DNA, oligonucleotides)	Easy to produce Safe	Short expression time Low efficacy
Adenoviruses	Easy to produce High transduction efficacy Relatively high transgene capacity (~7 kb) Transduces both proliferating and quiescent cells	Short expression time (~14 days) Inflammatory reactions Immunity against virus
Adeno-associated virus	Long expression time (months to years) High transduction efficacy Transduces both proliferating and quiescent cells Serotype specific tissue tropism	Limited transgene capacity (~4 kb) Laborious to produce in high quantities Safety of long-term gene expression unclear
Lentivirus	Long expression time Transduces quiescent cells Low immune response	Non-specific integration Low transduction efficiency Laborious to produce in high quantities and titers
Retroviruses	Long expression time Relatively easy to produce Low immune response	Non-specific integration Transduces only dividing cells Low transduction efficiency Laborious to produce in high quantities and titers

2.6 PRECLINICAL STUDIES

Before therapeutic gene transfer can be used to treat humans, preclinical studies are needed. Cell culture studies, where the interactions between ligands, receptors and downstream signaling can be characterized, can be used to acquire basic information. In vitro testing is also an essential tool in any investigation of the functionality of gene transfer vectors. However, in

vivo testing in animal models is crucial if one wishes to study the actual effects of gene transfer on tissues and organs in their natural environment.

There are many preclinical studies where the administration of growth factor genes, including VEGF-A, -B, C, -D, and -E, PlGF, FGF-1, -2, -4 and -5, angiopoietins (Ang)-1 and -2, hepatocyte growth factor (HGF), PDGF-A, -B, -C and –D, IGF-1 and -2, HIF-1α, and monocyte chemoattractant protein-1 (MCP-1), have been shown to promote angiogenesis (Rissanen and Yla-Herttuala, 2007). Most clinical trials for proangiogenic gene therapy have been conducted by using VEGF-A (Yla-Herttuala et al., 2007). AdVEGF-A gene transfer has been shown to form functional vessels in rabbit hindlimbs (Rissanen et al., 2005). Tissue-specific growth factors offer improved gene transfer efficacy and good safety records. For example, VEGF-B has shown cardiospecific potency in angiogenesis and also cardiospecific metabolic effects (Lahteenvuo et al., 2009). Overexpression of VEGF-A can cause significant side effects, such as tissue edema and uncontrolled vessel growth, which may limit the dose that can be administered. On the other hand, maximal VEGF doses should be used to achieve physiological benefits. In recent study, lower AdVEGF doses induced only angiogenesis while larger AdVEGF-A dose was needed to enhance tissue metabolism (Korpisalo et al., 2010). Combinations of growth factors have been studied in attempts to avoid side effects and improve physiological angiogenesis,. In particular, Ang-1 has been shown to stabilize new blood vessels (Zacharek et al., 2007; Lamont et al., 2010). Simultaneously administered AAV-VEGF-A_{165} and AAV-Ang-1 displayed an equal angiogenic effect as AAV-VEGF-A_{165} alone, but considerably reduced the leakage of the new vessels (Arsic et al., 2003). In addition to angiogenesis, VEGF gene transfer has been reported to stimulate skeletal muscle regeneration in a mouse hind limb ischemia model (Arsic et al., 2004). Recent study introduced that the drug-mediated regulation of AAV-VEGF expression improves vascularization and perfusion in ischemic hind limb and the effects were able to persist upon withdrawal of the angiogenic stimulus (Tafuro et al., 2009). Combining AAV-PDGF-B with AAV-VEGF-A achieved collateral vessel growth in rabbit hindlimb ischemia model and in pig myocardium compared to AAV-VEGF-A, which induced only angiogenesis (Kupatt et al., 2010). Even though there have been several preclinical studies, the optimal delivery dose and route of administration of VEGF-A have not yet been established. Further studies with new strategies such as screening the optimal VEGF-A dose, studying and developing the best vector for gene transfers into specific tissues and investigating long-term effects of VEGF-A gene transfer will be important steps to be taken to meet this goal.

Transcription factors, such as HIFs regulate many of the genes involved in angiogenesis (Hewitson and Schofield, 2004). Thus, virally delivered HIF could offer a more physiological approach to induce therapeutic angiogenesis in ischemic tissues. It has been shown that adenovirally delivered constitutively active HIF-1α increases muscle perfusion and collateral

blood vessel formation in rabbit hindlimb ischemic skeletal muscles (Patel et al., 2005). Additionally, in a mouse hind limb model HIF-1α delivered via AAV gene transfer has achieved angiogenesis with less tissue edema compared to the administration of VEGF-A (Pajusola et al., 2005). The potential benefits of HIF gene transfer on ischemic tissue metabolism remain unknown.

2.7 CLINICAL TRIALS

To date there are no gene drugs that have been approved for clinical use in patients with cardiovascular diseases. Most of the clinical trials have been done with VEGF-A, FGF4 and HIF-1α using adenoviruses or plasmids as vectors (Yla-Herttuala et al., 2007; Gupta et al., 2009). The first attempt to treat lower limb ischemia in the clinic by angiogenic gene therapy was performed in 1996 (Isner et al., 1996). Gene transfer was conducted via intravascular administration of the plasmid encoding human VEGF-A_{165}. Four weeks after gene transfer, improvements in collateral vessel growth and blood flow were observed. Subsequently, phase I studies done with plasmid VEGF-A_{165} showed promising results in angiography, improved ankle brachial index (ABI) and blood flow, and healing of wounds (Baumgartner et al., 1998; Isner et al., 1998; Baumgartner et al., 2000). In placebo controlled phase II trial, adenoviral VEGF-A_{165} gene therapy in PAD patients was observed to be well tolerated and to increase vascularity (Makinen et al., 2002). Dose dependent effects of adenoviral gene transfer have also been reported. The injection of a high dose of adenoviral VEGF-A_{121} increased tissue edema more than the low dose in PAD patients, i.e. evidence that virally delivered growth factor had a dose dependent biological effect (Rajagopalan et al., 2003). The results from other phase II/III trials have not been very promising. Most clinical trials using plasmid gene transfer have failed to reach their goals (Rissanen and Yla-Herttuala, 2007; Gupta et al., 2009). Only one study done with the intra-arterial administration of recombinant protein FGF-2 gene transfer (TRAFFIC) has shown a positive effect as measured by increased walking distance (Lederman et al., 2002). Clinical trials with adenoviruses have also been disappointing (Rissanen and Yla-Herttuala, 2007), with the exception of AdVEGF-A gene transfer in PAD patients (Makinen et al., 2002). Myocardial gene transfer has also been tried to treat ischemia. In the KAT trial, positive effects were obtained as adenoviral VEGF-A_{165} gene transfer increased myocardial perfusion in comparison to placebo (Hedman et al., 2003). Phase I trial (38 PAD patients) where i.m. injections of AdHIF-1α administration was used revealed good safety and evidences of of pain relief and ulcer healing (Rajagopalan et al., 2007). However, in a larger (289 patients), randomized, controlled trial in patients with severe, intermittent claudication, no significant difference in the exercise treadmill test

time was observed between AdHIF-1α treatment and control groups (not published data yet) (Gupta et al., 2009) .

2.8 SAFETY IN GENE THERAPY

Overall, angiogenic gene therapy has been shown to be well tolerated and safe in clinical trials with no link to malignancies (Yla-Herttuala and Alitalo, 2003; Gupta et al., 2009). An tragic death occurred in 1999 after adenoviral gene transfer to a patient who suffered from a genetic immune deficiency disease (Lehrman, 1999). Gene drugs, in the same way as other drugs and therapies, are associated with side effects which depend on the administered vector, dose, route of delivery, and properties of the transgene (Wirth et al., 2006). Adenoviral gene transfer has been reported to be well tolerated, causing only mild fever in PAD patients (Makinen et al., 2002). Only a few long-term follow-up studies have been conducted. After the KAT trial (Hedman et al., 2003), safety was followed for up to eight years after the primary gene delivery. There were no differences between treated groups i.e. VEGF-adenovirus vs. VEGF plasmid/liposome vs. placebo control in terms of health status or the incidence of major adverse cardiovascular events (MACEs) or cancer (Hedman et al., 2009). The clinical study where myocardial injections of adenovirus encoding VEGF-A_{121} were administered with a NOGA catheter also reveales a good safety profile (Stewart et al., 2009).

Gene therapy has mostly been used to treat elderly, no-option or end-stage cardiovascular patients (Gupta et al., 2009). Angiogenic therapy may be more effective at an early disease stage than in more severely ill patients. As reports of the safety of cardiovascular gene therapy increase, researchers may now be able to perform trials in less morbid patients (Gupta et al., 2009). Since it is a novel therapy, gene transfer is an expensive method, and only certain patients will obtain more benefit than from traditional treatments. However, gene therapy is ethically permissible for use in the treatment of complicated cardiovascular disorders. The gene transfer applications used in the proangiogenic therapy are mainly based on adenoviruses and plasmids, which induce the transient expression of transgene, and do not integrate into the genome, decreasing the risk of the vertical transmission. Traditionally, therapies for treatment of cardiovascular disorders must demonstrate improvements in morbidity or mortality; however, patients may consider diminished quality, rather than quantity, of life which is the primary detrimental factor in cardiovascular disease. Avoiding hospitalization and the other manifestations of progressive disease could well be appropriate goals for angiogenic gene therapy (Gupta et al., 2009).

3 Aims of the study

I To characterize the expression of PDGF-C and PDGF-D and their receptors PDGFR-α and PDGFR-β in human atherosclerotic lesions.

II To study the potential of AdPDGF-B gene transfer in angiogenesis and the stabilization of vessels grown by AdVEGF-A gene transfer.

III To evaluate the long-term effects of AAV-VEGF-A gene transfer in normoperfused and in ischemic hind limb skeletal muscles.

IV To identify the angiogenic potential of AdHIF-2α and to evaluate the effect of HIF-1α and HIF-2α on energy metabolism in ischemic hind limb skeletal muscles.

4 Materials and methods

4.1 HUMAN ARTERIAL SAMPLES (Study I)

Human arterial samples were collected from amputations and autopsies from 25 to 84 year old males and females. Arterial samples were immersion-fixed in 4 % paraformaldehyde and embedded in paraffin for histological analysis or snap-frozen in liquid nitrogen and stored at -70 °C for mRNA analysis. The protocol of this study was approved by the Ethical Committee of the Kuopio University and the study conforms to all principles outlined in the Declaration of Helsinki. Lesions (n=34) were classified into the following categories as: I normal, no lesion n=6, II fatty streak n=7, III plaque n= 10, and IV complicated lesion n=11. The evaluation of the sections was done in a random order independently by two experienced researchers.

4.2 REVERSE TRANSCRIPTASE-PCR (Study I)

Total RNA was isolated from snap frozen artery samples with Trizol (Invitrogen, Carlsbad, CA, USA) and RNA pellets were dissolved in sterile water containing 1 U/μl RNase Inhibitor (MBI Fermentas, Burlington, ON, Canada). cDNA synthesis and RT-PCR were conducted as previously described. Two μg of total RNA was used in cDNA synthesis. Samples were treated with DNase (Promega, Madison, WI, USA) and cDNA synthesis was undertaken by using random primers (Promega, Madison, WI, USA). Table 2 summarizes the primers used in the study. PCR reactions contained 5 μl of 10×buffer including 1.5 mM Mg (Finnzymes, Espoo, Finland), 1 μl dNTP (10 mM, Finnzymes, Espoo, Finland), 1 μl both forward and reverse primers (20 pmol), 0.5 μl Dynazyme II polymerase (Finnzymes, Espoo, Finland) added to 50 μl sterile H_2O. PCR reactions of 35 cycles were 45 s in 96 °C, 45 s in 54 °C for PDGFs or 58 °C for α-actin and 90 s in 72°C. PCR products were detected in 1.2 % agarose gel. Samples without reverse transcriptase were used as controls.

Table 2. RT-PCR primers

Gene	Primers	Sequence
PDGF-C	Forward	5'-TCACAGCCCAAGGTTTCC-3'
	Reverse	5'-GTCCAACTGCCATCTCTC-3'
PDGF-D	Forward	5'-TGACATGGCGGCTTCA-3'
	Reverse	5'-GTAACGCTTGGCATCATC-3'
α-actin	Forward	5'-ACCAACTGGGACGACATGGAAAA -3'
	Reverse	5'-GTCAGGATCTTCATGAGGTAGTC-3'

4.3 IMMUNOHISTOCHEMISTRY (Studies I-IV)

In the immunostainings, 7 μm thick paraffin sections were cut. The antibodies and specific stainings used in the study are listed in table 3. Avidin-biotin-Horse Radish Peroxidase (HRP) system with diaminobenzidine (DAB) (Vector Laboratories, Burlingame, CA, USA) was used for signal detection in single stainings and in double stainings, the second antibody was detected by alkaline-phosphatase with Vector Blue colour substrate (Vector Laboratories, Burlingame, CA, USA).

Table 3. Immunostainings

Antibody	Specificity	Clone/ code	Manufacturer	Dilution	Pretreatment	Study
CD31	Endothelium	JC70A	Dako	1:50	-	II-IV
Griffonia simplifolia lectin I	Endothelium	B-1105	Vector	1:100	-	unpublished
αSMA	Smooth muscle, pericytes	1A4	Sigma	1:500	-	II-IV
HHF-35	Smooth muscle	C34931	Enzo Life Sciences	1:50	-	I
CD68	Macrophages	KP1	Dako	1:250	-	I
RAM11	Macrophages	M0633	Dako	1:500	-	II,III
PDGF-B	PDGF-B	AF-220-NA	R&D Systems	1:500	Citrate boiling	II
PDGF-C	Human PDGF-C		U. Eriksson	0,5 μg/ml	Citrate boiling	I
PDGF-C	PDGF-C	AF1560	R&D Systems	1:100	-	I
PDGF-D	Human PDGF-D		U. Eriksson	0,5 μg/ml	Citrate boiling	I
PDGF-D	PDGF-D	AF1159	R&D Systems	1:100	-	I
PDGFR-α	PDGFR-α	Sc-338	Santa Cruz	1:250	Citrate boiling	I
PDGFR-β	PDGFR-β	Sc-6252	Santa Cruz	1:250	Citrate boiling	I,II
VEGF-A	VEGF-$A_{121-206}$	C-1	Santa Cruz	1:500	-	II-IV
VEGFR-1	VEGFR-1	sc-316	Santa Cruz	1:250	Citrate boiling	II,IV
VEGFR-2	VEGFR-2	sc-6251	Santa Cruz	1:250	Citrate boiling	II,IV
HIF-1α	HIF-1α	NB-100-123	Novus Biologicals	1:200	-	IV
HIF-2α	HIF-2α	NB100-132	Novus Biologicals	1:200	-	IV
eNOS	eNOS	610296	BD BioSciences	1:50	-	IV
Ki67	Proliferating cells	MIB-1	Dako	1:100	Citrate boiling	II-IV

Hematoxylin-eosin staining was used to characterize tissue morphology (Studies II-IV). Specific staining for glycogen Periodic acid-Schiff staining (PAS) was used to evaluate the amounts of glucose in skeletal muscle (study IV) and masson trichrome staining was used to study the formation of fibrosis (unpublished).

4.4 IN SITY HYBRIDIZATION (Study I)

The localization of PDGF-C and PDGF-D mRNA was studied by non-radioactive in situ hybridization (Roche Diagnostics Co., Mannheim, Germany) from paraffin sections (Study I). Digoxigenin- (DIG) labelled antisense and sense probes for PDGF-C and PDGF-D were synthesized according to the manufacturer's protocol. Paraffin sections were pretreated with Proteinase K (Roche Diagnostics Co., Mannheim, Germany) (10 μg/ml). Hybridization of slides was made in hybridization buffer (40 % deionized formamide, 10 % dextran sulfate, 1 × Denhardt's solution, 4 × saline sodium citrate buffer (SSC), 10 mM dithiothreitol, 1 mg/ml yeast t-RNA, 1 mg/ml denatured ssDNA) containing antisense or sense probes at a concentration of 0.33 ng/μl overnight at 58°C. After hybridization, the slides were washed in a shaking water bath at 58°C with a decreasing SCC concentration. Immunological detection of DIG-labelled probes was done according to the manufacturer's protocol (Roche Diagnostics Co., Mannheim, Germany) using anti-DIG-alkaline phosphatase Fab fragments (Roche Diagnostics Co., Mannheim, Germany). The colour reaction was detected with Nitro blue tetrazolium chloride/ 5-Bromo-4-chloro-3-indolyl phosphate, toluidine salt (NTB/BCIP) solution (Roche Diagnostics Co., Mannheim, Germany).

4.5 ISCHEMIA OPERATIONS (Studies II-IV)

Two surgically induced models of acute ischemia in rabbits were used in this thesis work (Figure 6). Ligation of arteria femoralis profunda (Rissanen et al., 2005) was used in studies II and III, and the ligation of arteria femoralis superficialis (SFA) with additional ligations to block excessive native collateral growth (Rissanen et al., 2003) in study IV. The ligation of PFA was operated according to the previous description (Rissanen et al., 2005). The procedure was done under ketamine (Ketalar 20 mg/kg, Pfizer, Finland) and medetomidine (Domitor 0.3 mg/kg, Orion, Finland) anesthesia. The rabbits were given Carprofen (Rimadyl 0.1 mg/kg, Pfizer, Finland) for post-operation pain. In this model, the thigh area becomes mildly ischemic but the calf remains normally perfused. The ligation of SFA was also carried out as in the previous study (Rissanen et al., 2003) one day before gene transfer. SFA and the re-entry branches for the collateral blood vessels growing from the lateral circumflex and deep femoral arteries were ligated. This model results in a severe ischemia in the calf region but the thigh remains normally

perfused. All animal experiments were approved by the Experimental Animal Committee at the University of Kuopio.

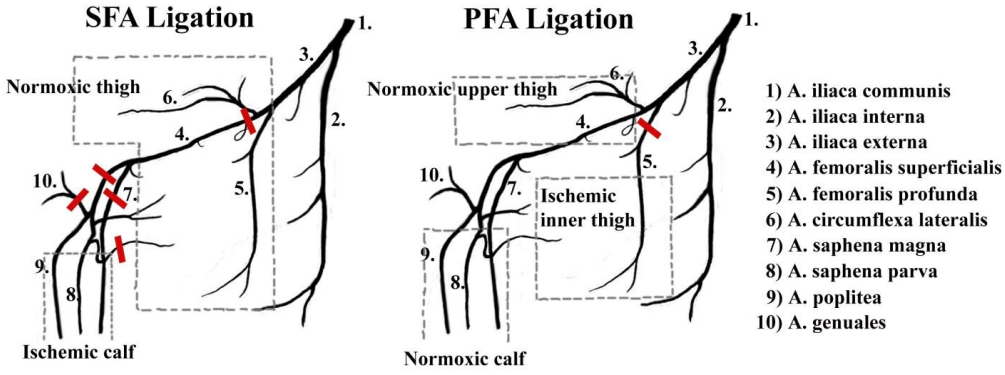

Figure 6. Ischemia operations (Korpisalo 2009)

4.6 GENE TRANSFER (Studies II-IV)

The gene transfer was performed by intramuscular (i.m.) injection into the hind limb skeletal muscles of New Zealand White rabbits (studies II and IV) under ketamine (Ketalar 20 mg/kg, Pfizer,) and medetomidine (Domitor 0.3 mg/kg, Orion, Finland) anesthesia. First generation serotype 5, adenoviral vectors, which had been tested first in vitro, were used with a viral dose 1×10^{11} vp/ml. Serotype 2 AAV viruses were used in study III with a viral dose of 1×10^{11} vg/ml. All of the viruses used in these studies have the cytomegalovirus (CMV) promoter. Intramuscular (i.m.) injections into rabbit hind limb were performed in ten 0.1 ml injections using a 1 ml syringe and a 25G needle.

The mouse myocardium model was also used in this thesis. During the myocardial injections, 57Bl6Ja mice were under the isoflurane inhalation anesthesia (induction: 4.5 % isoflurane, 450 ml air, maintenance: 2.0 % isoflurane, 200 ml air, Baxter International Inc., Deerfield, IL, USA). AdLacZ, AdHIF-1α, AdHIF-2α, AdPDGF-C and AdPDGF-D were tested in the mouse heart. Myocardial injections into the anterior wall of the left ventricle were performed under ultrasound guidance with viral dose $1x10^{10}$ to $4x10^{10}$ vp. The injections were carried out as previously described (Springer et al., 2005; Huusko et al., 2010). The gene transfer was done with a microinjector using a 50 µl Hamilton syringe and a 30G needle. Carprofen (50 mg/ml, Rimadyl, Pfizer, Finland) was given for analgesia after the procedure. The effects of the gene

transfers were studied at predetermined time points i.e. 6, 14, 28 days or 6 and 12 months after gene transfers. Table 4 summarizes the gene transfers carried out in this thesis work.

Table 4. Summary of gene transfers

Study	Animal	Ischemia operation	Gene transfer	Total dose	Transduced muscles	Time points
II	Rabbit	PFA ligation	AdLacZ AdVEGF-A AdPDGF-B AdVEGF-A + AdLacZ AdVEGF-A + AdPDGF-B	10^{11} vp 2×10^{11} vp, for combinations	semimembranosus	d6, d14, d28
III	Rabbit	PFA ligation	AAV-LacZ (control) AAV-VEGF-A	10^{11} vg	semimembranosus	1 month, 6 months 12 months
IV	Rabbit	SFA ligation	AdLacZ AdVEGF-A AdHIF-1α AdHIF-2α	3×10^{11} vp	semimembranosus cruris cranialis quadriceps femoris tibialis anterior gastrocnemius	d6, d14
Unpublished	Mouse	-	AdLacZ AdHIF-1α AdHIF-2α AdPDGF-C AdPDGF-D	10^{10} to 4×10^{10} vp	myocardium, left ventricle anterior wall	d6
	Rabbit	PFA	AdPDGF-C AdPDGF-D	10^{11} vp	semimembranosus	d6, d14

4.7 ULTRASOUND IMAGING (Studies II-IV)

Three different ultrasound approaches were used to assess skeletal muscle perfusion: Native Doppler ultrasound (studies II-IV), Contrast enhanced Doppler (CEU) (study II) and contrast pulse sequence ultrasound (studies III and IV). All ultrasound imaging was performed with the Acuson Sequoia 512 system and a linear 15L8 transducer (Siemens). In the CEU and CPS experiments, a second generation contrast agent, sulfur hexafluoride in a phospholipid shell, (diameter 2.5 μm, SonoVue, Bracco) was used. Native Doppler imaging was performed without contrast agent at the following parameters: 12.5 frames/s, power Doppler 14 MHz, dynamic range 20 dB, power -5 dB, gain 50 and depth 20 mm. For CEU transversal plane video clips of 20

s were captured using the power Doppler (mechanical index =0.6) mode at 8.5 MHz (dynamic range 10 dB, power -18 dB, mechanical index 0.60, gain 40 and depth 20 mm) with administration of 0.3 ml bolus of SonoVue via the ear vein. The CPS method uses non-linear fundamental frequencies, which result in better spatial resolution and high sensitivity to the contrast media, and this provides improved tissue subtraction than the previous ultrasound perfusion imaging techniques (Phillips and Gardner, 2004). Transversal images of semimembranosus muscles of both limbs were captured after a single bolus (0.5 ml) of SonoVue contrast microbubbles (Bracco, Milan, Italy) with the following parameters at 8.0 MHz: power -16dB, mechanical index 0.31, CPS gain -10 and depth 20 mm (Rissanen et al., 2008). Muscle perfusion was quantified with DataPro 2.13 software (Noesis, Courtaboeuf, France) and the perfusion ratio between transduced muscle and intact muscle was quantified using the maximal signal intensities as previously reported (Rissanen et al., 2008).

A high-resolution imaging system specially developed for small animal research (Vevo 770, VisualSonics Inc., Toronto, ON, Canada) was used to capture ultrasound data from the mice myocardium on the injection day (d0) as well as six days after injection (d6). A high-frequency ultrasound probe (RMV-707B) operated at 30 MHz, with a focal depth of 12.7 mm from the skin surface. Ejection fraction and fractional shortening were determined from transversal short-axis M-mode measurements and calculated by Vevo770 software (Huusko et al., 2010).

4.8 ^{31}PHOSPHORUS –MAGNETIC RESONANCE SPECTROSCOPY (Study IV)

The degree of ischemia and the level of anaerobic metabolism (anaerobic capacity) of rabbit calf muscles before the ischemia operation, and 6 days after gene transfer were evaluated using ^{31}Phosphorus –magnetic resonance spectroscopy (^{31}P-MRS) according to the previous study (Korpisalo et al., 2008a). ^{31}P-MRS was performed with a UNITYINOVA imaging console (Varian) interfaced with 4.7. T horizontal magnet (Magnex Scientific) with actively shielded gradients (Magnex Scientific). A linear surface coil consisting of two separated loops tuned to ^{1}H and ^{31}P frequencies was used for reception (^{1}H, 200 MHz and ^{31}P, 80.9 MHz, RAPID Biomedical, Rimpar, Germany). The region of interest under the surface coil was adjusted using ^{1}H signal and ^{31}P data were acquired after ~70 degree hard pulse excitation using repetition time of 1.5 s, spectral band of 10 kHz covered by 4096 data points and number of averages of 16. Small needle electrodes (27 G) were placed on both sides of the sciatic nerve on the lateral side of the rabbit thigh. During the whole duration of the experiment (20 min), ^{31}P-MRS spectra were collected, each scan taking 30 s. First, base line spectra from resting muscle were collected for 2 min. Thereafter, the sciatic nerve was electrically stimulated for 6 min at 3 Hz using a voltage of 150 V (14A11 Electromyograph, Disa) to induce maximal contraction in the calf muscles. Then the stimulation was stopped and muscle recovery was followed for 12 min. Line shape fitting analyses of spectra after pre-processing the data by discarding the two first data points, using a

DC correction of 500 and 15 line broadening were performed by using JMRui 2.1. software. The ratio between the peak areas of phosphocreatine (PCr) and the sum of PCr and inorganic phosphate (Pi) i.e. PCr/(PCr+Pi) was calculated and used as a measure of anaerobic capacity and fatigue. Results are presented as means from baseline (0-2 min), the last two minutes of the exercise (6-8 min) and the last two minutes in the end of the procedure, recovery stage, (18-20 min).

4.9 TISSUE EDEMA (Studies II-IV)

Modified Miles assay was used to evaluate the leakiness of newly formed vessels (Rissanen et al., 2005). Evans Blue dye (30 mg/kg) binding to plasma proteins was injected 30 min before euthanasia via the ear vein. After euthanasia, animals were perfusion-fixed with 1 % paraformaldehyde in 0.05 M citrate buffer, pH 3.5, via the left ventricle. Muscle samples were taken from both transduced muscles and contralateral intact muscles and weighed. Evans Blue dye was eluted in formamide and the released dye was quantified with a spectrophotometer at 610 nm. Absorbances were normalized to sample weights and the ratio between transduced and intact muscle samples was calculated reflecting the permeability of neovessels.

4.10 HISTOLOGICAL ANALYSIS (Studies II-IV)

For evaluating capillary growth, mean capillary area (μm^2) was measured. Photographs for analysis were taken with an Olympus AX70 microscope (Olympus Optical, Tokyo Japan). Ten images of 200 x magnification were taken from each CD31 stained section, and the mean capillary area (μm^2) was measured using the AnalySIS software (Soft Imaging System, Muenster, Germany). Capillary and myocyte numbers and the capillaries/myocytes ratio were counted from the same photographs. All measurements were performed in a blinded manner.

4.11 PROTEIN EXPRESSION (Studies II-IV)

Samples from transduced muscles and non-transduced safety tissues, the heart, lungs, liver, spleen, kidney, and ovaries or testis were snap frozen in liquid nitrogen and stored at -70°C. Approximately 100 mg of tissue were lysed in T-PER tissue lysis buffer (Termo Scientific) containing 1× Halt protease inhibitor cocktail (Termo Scientific) for protein expression analysis. Tissue lyser (Qiagen) was used for the homogenization of the samples. Supernatants were collected and used in ELISA assays. Anti-human VEGF-A and PDGF-B ELISA kits (R&D Systems, Minneapolis, MN, USA) were used to measure VEGF-A and PDGF-B levels, respectively, and the results were normalized to the total protein amount measured with BCA kit (Termo Scientific). The Western blot analysis for protein expression *in vitro* was done for all virus contructs used in the thesis work. In electrophoresis, 10-15µg of the total protein was

separated in SDS-PAGE gel and proteins were blotted into a nitrocellulose membrane. Primary antibodies and used dilution are the same as in the Table 3 except mab HIF-1α and -2α (1:400) and goat polyclonal PDGF-C and –D (1:500). Proteins were visualized using horseradish peroxidase-conjugated secondary antibody and ECL Plus detection system (GE Healthcare), and detected with Typhoon 9400 (GE Healthcare) scanner.

4.12 STATISTICAL ANALYSIS (I-IV)

The results are presented as mean ± standard error of mean (SEM). Kruskall-Wallis test was used to evaluate the statistical significance of the results followed with Mann Whitney-test as a post-hoc test where appropriate with non-parametric data. (I-IV). One-way Anova and t-test were used with parametric data. Significances are shown as follows *$p<0.05$, **$p<0.01$ and ***$p<0.001$. All statistical analyses were performed using GraphPad, version 4.03, software.

5 Results

5.1 GROWTH FACTOR EXPRESSION IN HUMAN ATHEROSCLEROTIC LESIONS (I)

The expressions of PDGF-C and PDGF-D and their receptors PDGFR-α and PDGFR-β in human atherosclerotic lesions were studied. Altogether 34 human arterial samples were classified into the following categories as: I normal, no lesion (n=6), II fatty streak (n=7), III plaque (n= 10), and IV complicated lesion (n=11) according to the macrophage and smooth muscle actin stainings. PDGF-C and PDGF-D immunostainings indicated that PDGF-C was expressed strongly in the endothelium and macrophages in the intima of all stages of the lesion, whereas PDGF-D was more diffusively expressed in the vessel wall (Figure 7a-d). In addition to protein immunostainings mRNA expression of PDGF-C and PDGF-D was investigated in arterial lesions since secreted growth factors could be produced elsewhere than in the arterial wall. PDGF-C and PDGF-D mRNA expressions were detected by *in situ* hybridization in plaque shoulder areas (Figure 7 inserts in b,d). RT-PCR confirmed the *in situ* hybridization results.

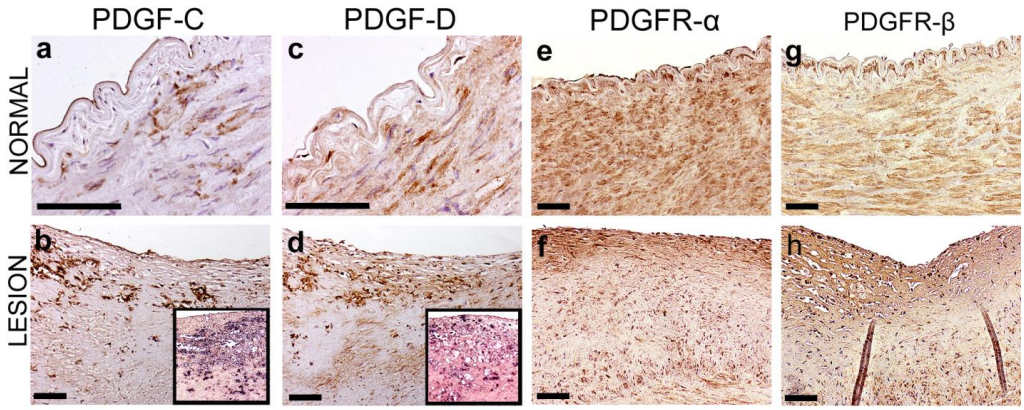

Figure 7. Expression of PDGF-C and PDGF-D and their receptors PDGFR-α and PDGFR-β in human atherosclerotic lesions. PDGF-C and –D stainings in a normal artery wall (a and c, respectively) and in a lesion (b and d respectively). The inserts in b and d reveal mRNA expression. PDGFR-α and -β stainings in a normal artery wall (e and g, respectively) and in a lesion (f and h, respectively). Scale bar 100 μm

Differences in the protein expression of PDGF-C and PDGF-D were measured by microscopical semiquantitative analysis. Table 6 summarizes data from PDGF-C and PDFG-D stainings and differences in the expression of the examined groups. PDGF-C staining was significantly increased (P= 0.041) in the intima of fatty streaks compared to the intima of normal arteries. There were no significant differences between normal and plaque groups or normal and complicated lesion groups in PDGF-C stainings. PDGF-D staining detected no significant differences between the examined groups. PDGF-C staining in normal artery ECs was significantly higher than PDGF-D staining in ECs in normal arteries (P= 0.009). The P-values for comparison between PDGF-C and PDGF-D stainings in ECs in fatty streaks was almost statistically significant (P=0.07) and between PDGF-C and PDGF-D stainings in ECs in plaques the corresponding value was 0.08.

Table 6. Mean scores of the semiquantitative evaluation of the atherosclerotic artery samples

Samples		**PDGF-C**			**PDGF-D**	
	EC	**Intima**	**Media**	**EC**	**Intima**	**Media**
Normal	2,6	1,3	2,2	1,3	1,8	2,3
Fatty streak	2,1	2,4	1,4	1,1	2,2	1,9
Plaque	1,9	2,0	1,6	0,7	2,0	2,5
Complicated lesion	2,0	1,9	1,4	1,7	2,3	2,2

Normal samples and atherosclerotic lesions were collected from aortas, coronary arteries and femoral arteries of 25-84 year-old patients (n= 34). PDGF-C and PDGF-D stainings were evaluated as percentage (%) of the microscopical section and divided into four categories; score: 0: no staining, score: 1: less than 30 % of area stained, score: 2: 30-60 % stained, and score: 3: more than 60 % stained.

PDGFR-α and PDGFR-β expressions were also investigates in normal arteries and in atherosclerotic lesions (Figure 7e-h). Positive PDGFR-α staining was seen in ECs, intima and media in normal arteries (Figure 7e). In plaques PDGFR-α expression remained strong in the EC layer and was moderate in the intima and the media (Figere 7f). Macrophages were prominently positive for PDGFR-α in plaques (Figure 7f). PDGFR-β was expressed in SMCs in normal arteries (Figure 7g). In the plaques macrophages in the shoulder area and the intima displayed intense expression of PDGFR-β but less positivity was detected in medial SMCs (Figure 7h).

5.2 GENE TRANSFER FOR THERAPEUTIC ANGIOGENESIS (II-IV and unpublished results)

In an attempt to stimulate therapeutic angiogenesis, the angiogenic effects of viral gene transfer in rabbit hindlimb model and in mouse myocardium were studied. Six different adenoviruses encoding human VEGF-A, human PDGF-B, human PDGF-C, human PDGF-D, mouse HIF-1α and mouse HIF-2α, and AAV encoding human VEGF-A were used. AdLacZ and AAV-LacZ were used as controls. The protein expressions of the viral constructs were analyzed by Western Blot. The results from gene transfer studies on angiogenesis are summarized in Table 7. Firstly, the angiogenic potency of AdPDGF-B was tested and compared to the effects of intramuscular gene transfer of AdVEGF-A or AdPDGF-B or in combination on pericyte activation and the stability of angiogenic vessels in normoperfused and ischemic rabbit hindlimbs (II). AdPDGF-B gene transfer increased perfusion significantly but remarkably less than AdVEGF-A six days after gene transfer. However, the main response to AdPDGF-B gene transfer, instead of angiogenesis, was the proliferation of cells in the muscle interstitium. AdVEGF-A was shown to induce about a twenty-fold increase in skeletal muscle perfusion in normoperfused limbs (II,IV) and an eight-fold increase in ischemic limbs compared to AdLacZ control at six days after gene transfer (II). At the six day time point, AdVEGF-A gene transfer had evoked a major increase in mean capillary size, and the most newly formed capillaries were covered with pericytes (II,IV). However, a side effect of this intense and sudden capillary growth, was the presence of a significant tissue edema (II,IV). The combination of AdVEGF-A and AdPDGF-B gene transfer increased skeletal muscle perfusion almost as much as AdVEGF-A alone at six days but this was achieved with a less extensive increase in capillary size and with a remarkabl higher degree of tissue edema compared to the AdVEGF-A administration (II). While AdVEGF-A induced perfusion returned back to the baseline, both AdPDGF-B and AdVEGF-A+AdPDGF-B induced perfusions were detectable fourteen days after gene transfer. In addition, the extend of the increase in AdPDGF-B induced perfusion remained significantly elevated 28 days after gene transfer (II). The effect of PDGF-B on the stability of blood vessels grown by VEGF-A was also examined. Surprisingly, it was noted that rather than increasing pericytes that were attached to new capillaries, administration of PDGF-B evoked the detachment of pericytes which could be seen in confocal images. In addition, scattered, interstitial located αSMA positive cells were seen in histology. For comparison, the new capillaries were properly covered with αSMA positive pericytes in muscles transduced with AdVEGF-A alone (Figure 8) (II).

Since PDGF-B showed a slight increase in angiogenesis the angiogenic potency of other PDGF ligands, PDGF-C and PDGF-D was tested but these did not induce significant angiogenesis in rabbit hind limbs, though minor increases in perfusion were observed in AdPDGF-C transduced muscles six days after gene transfer. AdPDGF-C and AdPDGF-D were also been

tested in mouse myocardium. In mouse myocardium, both AdPDGF-C and AdPDGF-D increased mean capillary size compared with AdLacZ (4.5 and 5.0 vs. 3.5 μm², $P<0.05$), but had no favorable effects on myocardial function, ejection fraction or fractional shortening (Unpublished results).

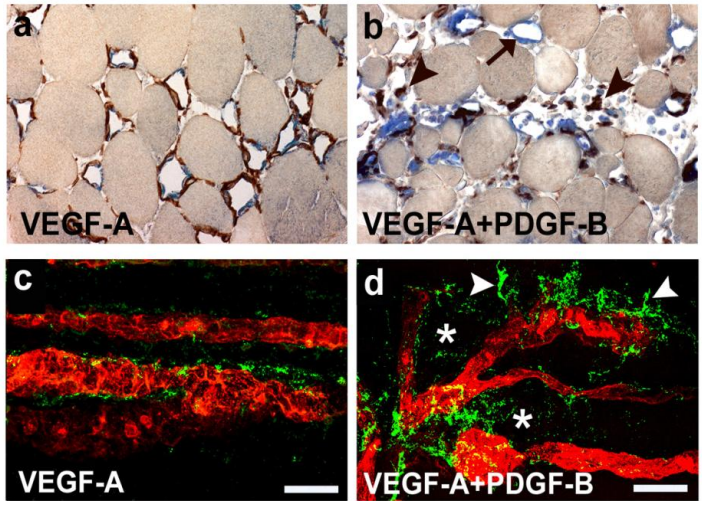

Figure 8. CD31 (blue) + αSMA (brown) double staining shows that AdVEGF-A gene transfer induced new vessels were coveraged by pericytes (a) while after AdVEGF-A+AdPDGF-B gene transfer (b) αSMA positive cells were found in interstitium (arrowheads) and some new vessels completely lack pericytes (arrow). In confocal images αSMA positive pericytes (green) are associated with vascular structures (red) of AdVEGF-A transduced muscles (c). In contrast, αSMA positive cells were often found between vascular structures (asterisks) and directing away from vessels (arrowheads) after AdVEGF-A+AdPDGF-B gene transfer.

Adenovirally delivered HIF-1α and HIF-2α caused approximately four-fold increases in perfusion and capillary size compared to AdLacZ in the rabbit hind limbs. The effects of HIFs were modest compared to VEGF-A, but newly formed capillaries were uniform in shape, and no visible tissue edema was detected in ultrasound imaging (IV). In comparison, AdVEGF-A gene transfer, HIFs did not notably recruit interstitial cells but nevertheless new capillaries were covered with αSMA positive pericytes. In mouse myocardium, adenovirally delivered HIFs significantly increased the mean capillary area (μm²) compared to AdLacZ transduced myocardium (6.0 and 6.5 vs. 3.4 μm², $P<0.01$ and $P<0.05$) but no benefits were achieved in myocardial function, ejection fraction or fractional shortening (Unpublished results). Figure 9. presents the image of the ultrasound guided injection into the mouse heart and lectin stainings from AdPDGF-C, AdPDGF-D, AdLacZ, AdHIF-1α and AdHIF-2α transduced myocardiums.

Table 7. Summary of angiogenic effects in rabbit hind limbs induced by several viral constructs

	Ad-LacZ	Ad-VEGFA	Ad-PDGF-B	AdVEGFA+ AdPDGF-B	Ad-PDGF-C	Ad-PDGF-D	Ad-HIF-1α	Ad-HIF-2α	AAV-LacZ	AAV-VEGF-A
Doppler (ratio to intact)	1.3	**34.1*** **	**4.3** **	**24.1*** **	**4.0***	**2.3**	**1.8**	**1.8**	0.1	**9.4***
CPS (ratio to intact)	1.0	**18.3***	-	-	-	-	**3.8***	**4.5***	0.3	**10.8***
Capillary area (μm^2)	6.9	**178.5** **	**13.0**	**150.7** **	**16.1**	**13.3**	**29.0***	**21.6***	13.5	**38.4***
Edema (Abs./g)	1.6	**33.8** **	**3.8***	**37.7** **	**3.3**	**2.9**	**1.9**	**2.1**	0.9	**3.3***
Recruit-ment of cells	None	**Moderate**	**High**	**High**	**High**	**High**	**Very mild**	**Very mild**	None	**Moderate**

Effects of adenoviral (Ad) constructs are at six day time point and the effects of AAV at six months after gene transfer. Recruitment of cells in the interstitial space is estimated from HE-stainings.

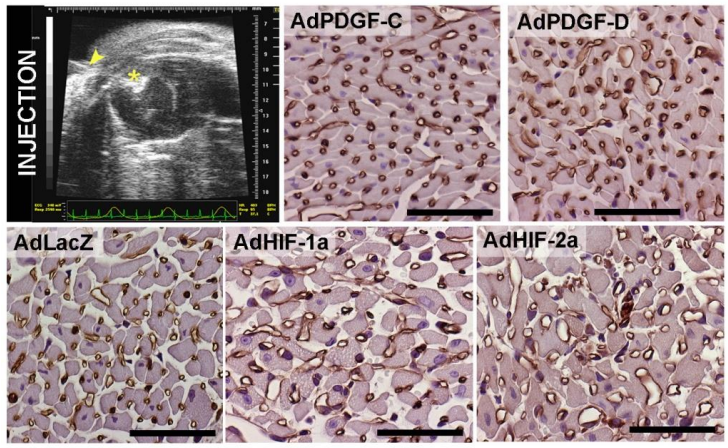

Figure 9. Gene transfers into mouse myocardium. Ultrasound guided injection into the anterior wall of the left ventricle. Arrowhead indicates the needle and asterisk injected virus solution. Lectin stainings from AdPDGF-C, AdPDGF-D, AdLacZ, AdHIF-1α and AdHIF-2α transduced myocardium at day 6. Scale bar 50 μm.

5.3 EFFECTS OF LONG-TERM VEGF-A EXPRESSION IN SKELETAL MUSCLE (III)

AAV-VEGF-A induced long-term angiogenesis in rabbit hind limbs. An approximately 10-fold increase was observed in skeletal muscle perfusion which remained elevated for up to one year (III). In addition to perfusion, other effects of the long-term AAV-VEGF-A expression were studied in skeletal muscles. Slightly increased levels of VEGF-A could be measured by ELISA from AAV-VEGF-A transduced muscles at one year, but no significant differences were found in comparison to AAV-LacZ muscles (112 ± 60 pg/mg and 85 ± 39 pg/mg, respectively). VEGF-A levels were also measured from non-transduced safety tissues: heart, lungs, liver, spleen, kidney, and ovaries or testis, but no significant changes were observed between the study groups. Immunostaining revealed VEGF-A expression in myocytes and enlarged capillaries around VEGF-A expressing myocytes one year after gene transfer (Figure 10). When quantified, increases in angiogenesis at the capillary level were detected in both the mean area of capillaries (38.4±8.8 μm^2 vs. AAV-LacZ 13.5±2.0 μm^2) and capillaries per myocyte ratio (1.6±0.05 vs. AAVLacZ 0.4±0.04) indicating the presense of both capillary enlargement and sprouting angiogenesis. Only some new vessels were covered with an αSMA layer indicating that not all formed vessels were stabilized. Additionally, long-term VEGF-A expression had recruited a number of macrophages which may have induced further endogenous VEGF-A expression.

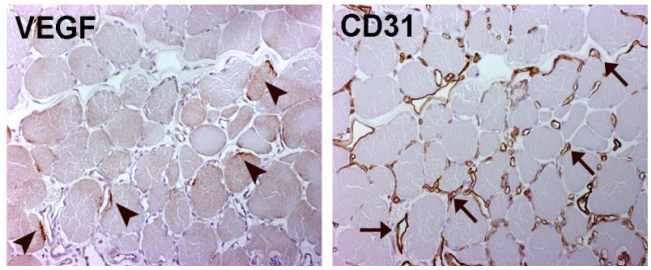

Figure 10. VEGF immunostaining showing VEGF-A expression one year after AAV-VEGF-A transduction (left) and CD31 staining showing angiogenesis at the capillary level (right). Arrowheads point to VEGF-A positive myocytes and arrows show enlarged capillaries.

Importantly, dramatic changes in skeletal muscle morphology were seen in the histological evaluation. Both atrophic and hypertrophic muscle fibers were observed in transduced areas and there were high amounts of extracellular matrix and fibrosis. Interestingly, long-term VEGF-A expression produced vascular-like structures inside the muscle fibers indicating the presence of aberrant forms of neovascularization. Vascular-like structures containing red blood cells were observed inside myocytes, but these structures did not have CD31 positive endothelium one month after AAV-VEGF-A gene transfer. On the contrary, one year after

AAV-VEGF-A gene transfer many of these vascular structures were covered by CD31-positive ECs and no red blood cells were seen, which may be due to that they were flushed away during perfusion fixation indicating connection to circulation.

5.4 STIMULATION OF PHYSIOLOGICAL ANGIOGENESIS (IV)

Due to the very intense angiogenic response of VEGF-A and side effects described above it was speculated that it may not be the most optimal therapeutic gene. Therefore the therapeutic potential of proangiogenic transcription factors HIF-1α and HIF-2α was examined. Perfusion increase after AdHIF-1α and HIF-2α gene transfer was detected by Doppler and CPS ultrasound methods, respectively. No tissue edema was observed and histology revealed enlarged capillaries after AdHIF-1α and AdHIF-2α gene transfer. Figure 11 summarize CPS perfusion and capillary enlargement after AAV-VEGF-A, AdVEGF-A, AdHIF-1α and AdHIF-2α gene transfers.

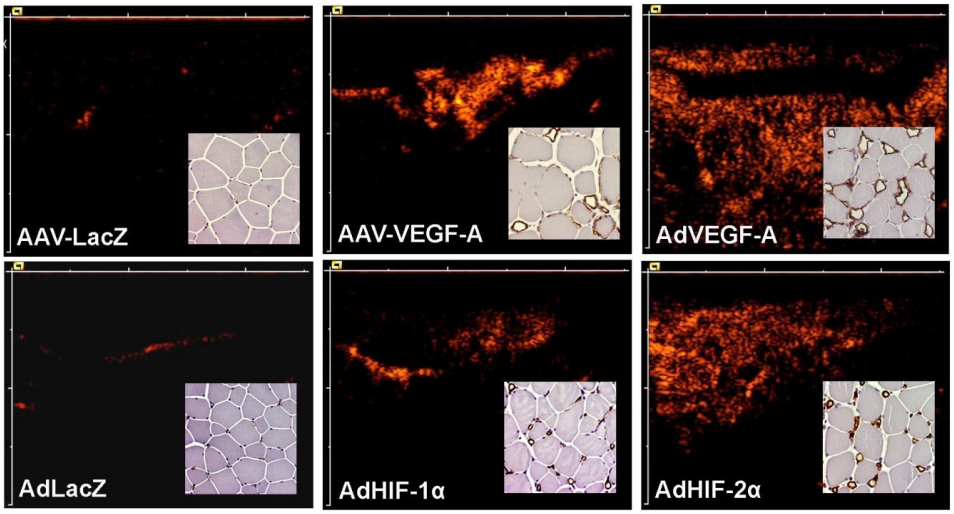

Figure 11. The CPS perfusion and the capillary enlargement after AAV-VEGF-A, AdVEGF-A, AdHIF-1α and AdHIF-2α gene transfer. AAV-LacZ and AdLacZ present the effects of the control viruses.

One aim of the AdHIF-1α and AdHIF-2α gene transfers was also to investigate if these factors are capable of enhancing skeletal muscle metabolism. Energy metabolism was measured in an ischemic rabbit hind limb skeletal muscle by ^{31}P-MRS and it was observed that AdHIF-1α gene transfer significantly enhanced the recovery of the ischemic tissue from the energy loss induced by exercise. AdHIF-2α transduced muscles recovered similarly as intact muscles. Figure 12

presents representative MRS spectra from baseline (0-2 min), exercise (2-8 min) and recovery (8-20 min). In baseline, before the electrically stimulated exercise, the high PCr peak and ATP peaks are visible (Figure 12 a). During the exercise the PCr peak declines and the PCi peak arise (Figure 12 b). In 12 minutes recovery time PCi peak declines and PCr stores are refilled increasing the PCr peak in a spectrum (Figure 12 c). In addition, quantified results from ^{31}P-MRS obtained from ischemic rabbit hind limbs after AdHIF-1α and AdHIF-2α gene transfer are shown (Figure 12 d and e). Exercise declined PCr(PCr+Pi) ratio in all the study groups compared to baseline ($P<0.001$) before and six days after gene transfer (Figure 12 d and e). At day 0 only intact muscles were able to recover while PCr(PCr+Pi) ratio in operated muscles before gene transfer remained close to the same as under exercise (operated muscles vs. intacts $P<0.01$and $P<0.001$) (Figure 12 d). Six days after gene transfer, ischemic muscles were also able to recover, but PCr(PCr+Pi) ratio in AdLacZ muscles remained still significantly under the value of intact muscles ($P<0.05$). AdHIF-1α transduced muscles recovered significantly better that AdLacZ muscles ($P<0.01$). PCr(PCr+Pi) ratio in AdHIF-2α muscles after the exercise recovered back to the baseline.

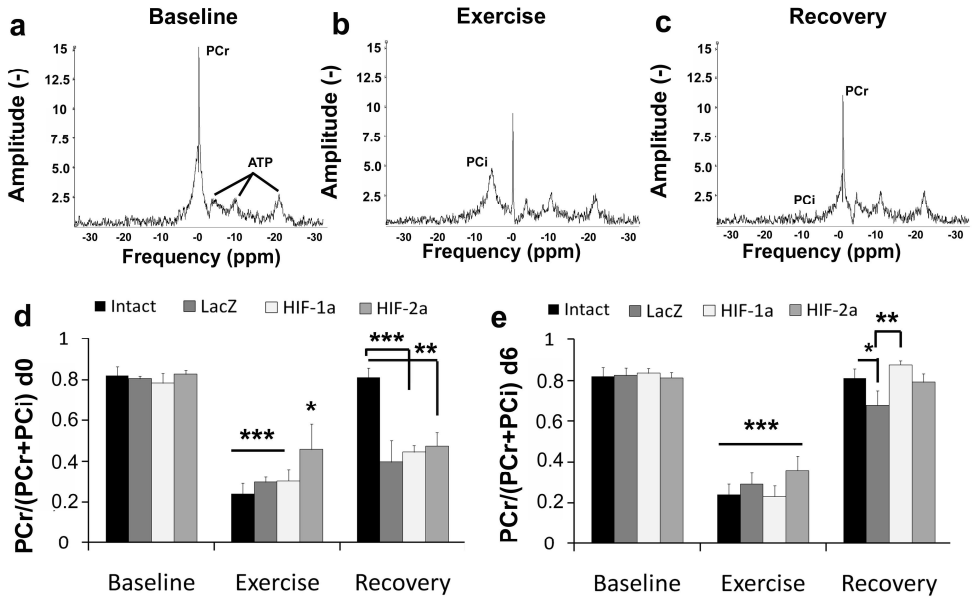

Figure 12. Representative MRS scans from rabbit hind limb skeletal muscle, Baseline (a), Exercise (b) and Recovery (c). Quantified ^{31}P-MRS data one day after ischemia operation (d0) (d) and six days after gene transfer (d6) (e).

6 Discussion

6.1 GROWTH FACTOR EXPRESSION IN ATHEROSCLEROTIC LESIONS

Atherosclerotic lesions occlude arteries in the periphery and lead to a progressive impairment of blood supply. Today, treatments for PAD patients are somewhat limited and new strategies are needed (Ghosh et al., 2008; Sneider et al., 2009). Gene therapy and the delivery of proangiogenic growth factors into ischemic tissue could be a novel treatment for PAD patients (Yla-Herttuala and Alitalo, 2003). The optimal gene to deliver should not only open the collaterals surrounding the occluded artery but it should also be able to reduce the progression of atherosclerosis in the diseased artery. Thus, studying the expression profiles of growth factors in lesions provides important information about which factors are involved in normal vascular biology and which factors are linked to diseases. This thesis has examined the expression profile of novel PDGF family members, PDGF-C and PDGF-D. It has been reported previously that PDGF-C and PDGF-D are expressed in advanced carotid atherosclerotic lesions and they have effects on monocyte migration and the expression of matrix metalloproteinase-2 and -9 (Wagsater et al., 2008). Here their expression pattern has been characterized more comprehensively and it was observed that both of these factors were intensely expressed in normal human arteries, fatty streaks, early lesions as well as in advanced lesions. The results from these studies are descriptive but important for gene therapy applications if it is considered to use PDGF-C or –D. Both of these factors seem to have a function in normal vascular wall maintenance rather than in the progression of atherosclerosis. In addition, PDGF-C may have some impact on the formation of the fatty streak because PDGF-C levels were significantly elevated in fatty streak ECs. Further studies in animal models will be needed to investigate this hypothesis in more detail. In transgenic mouse models, increased levels of PDGF-C and –D have been shown to cause pathological changes. Both PDGF-C and PDGF-D are strong stimulators of tissue fibrosis (Ponten et al., 2003; Ponten et al., 2005). Additionally, recent studies have also implicated both PDGF-C and PDGF-D in pathological angiogenesis in mice models (Hou et al., 2010; Kumar et al., 2010). The present results, as well as the results from mouse studies above, indicate that the therapeutic window of PDGF-C and -D is relatively narrow, i.e. overexpression with adenoviral gene transfer does not improve vascular function, and more efficient overexpression simply causes tissue fibrosis.

6.2 THERAPEUTIC OUTCOME OF GENE TRANSFERS

Vessel maturation is a key issue in the outcome of angiogenic gene therapy. Newly formed vessels should feed the ischemic area and the opened collateral vessels should stay patent for the rest of the patient's life. Adenovirally delivered VEGF-A expression is transient, and after VEGF-A withdrawal, angiogenesis returns back to the basal level at least in normoxic muscles (Rissanen et al., 2005; Vajanto et al., 2002). It has been reported that the stabilization of newly formed vessels occurs after a few weeks of VEGF-A expression and tissue perfusion is permanently elevated even after cessation of VEGF-A expression. (Zentilin et al., 2006b; Dor et al., 2002). Thus, growth factor expression and its effect need to be long enough to achieve a real therapeutic impact. Here the ability of combined PDGF-B and VEGF-A gene transfer to stabilize VEGF-A driven angiogenesis was studied. PDGFs recruit pericytes, and induce SMC proliferation and migration and may therefore stabilize vessels and decrease edema (Cao et al., 2003; Uutela et al., 2004). In the present study, PDGF-B prolonged angiogenesis in rabbit hind limb but this seemed to be mediated by recruitment of interstitial cells i.e. it appeared to be the paracrine effect of growth factors secreted by these recruited cells rather than direct vessel maturation. Pericyte coverage of new vessels was seen in AdVEGF-A transduced muscles and even pericyte detachment was detected in AdVEGF-A+AdPDGF-B transduced muscles. Apparently, VEGF-A and PBGF-B were not expressed in the same cells and this hampered the therapeutic effect of the combination gene transfer. Previous reports have indicated that the correct PDGF-B gradient is required for optimal pericyte recruitment and vessel maturation (Abramsson et al., 2003; Lindblom et al., 2003). Recently, the role of the two novel growth factors, PDGF-C and PDGF-D, in angiogenesis has been investigated. PDGF-C has been reported to induce angiogenesis in a mouse model (Li et al., 2005). Here capillary growth was detected in the mouse heart but in the rabbit model only very mild angiogenic effect was noted, evidence for the poor angiogenic efficacy of PDGF-C in a larger animal. There are similar observations after adenoviral PDGF-D gene transfer in the mouse heart and in rabbit hind limb. PDGF-D may have a positive effect on angiogenesis since it is known to induce vessel maturation when used in combination with VEGF-E in gene transfer (Uutela et al., 2004). Recruitment of interstitial cells, mostly fibroblasts, after AdPDGF-C and –D gene transfer was signicant in rabbit skeletal muscles, indicating a similar limitation to the therapeutic use as discussed above.

Unwanted events can occur when angiogenic growth factor is expressed constitutively in tissue. Here the effects of long-term VEGF-A expression were monitored in rabbit hind limb and it was observed that angiogenesis remained elevated for up to one year but in addition, long-term VEGF-A expression evoked severe problems in skeletal muscle morphology, formation of extracellular matrix, hypertrophy or atrophy of myocytes, infiltration of mononuclear cells and

the formation of vascular structures inside muscle fibers. It should be noted that enhanced extracellular matrix is proangiogenic, but fibrosis may also decrease the flexibility and contractility of skeletal muscle. Long-term VEGF-A expression recruited macrophages, and these cells can promote endogenous expression of VEGF-A leading to prolonged and enhanced angiogenesis. Previously, VEGF-A has been shown to be able to recruit mononuclear cells via VEGFR-1 and Nrp-1 stimulation, evidence for the positive role of the paracrine secretion of these cells (Zentilin et al., 2006; Zacchigna et al., 2008). Only a few VEGF-A positive cells were seen in the transduced muscles one year after the gene transfer but local, even low, expression of VEGF-A could be possibly sufficient to maintain active angiogenesis for long periods of time. The other option is that the vessels have been maturated as shown previously by a model of conditional switching of VEGF expression (Dor et al., 2002). The AAV-VEGF-A driven negative effects in skeletal muscle need to be taken into account. Aberrant vascular structures inside muscle fibers are not a desirable outcome of therapeutic angiogenesis. One possible explanation for these vascular lacunae could be that the AAV-VEGF-A transduced myocytes had been recognized and destroyed by the immune system and their remnants had been vascularized. Additionally, AAV has a high natural tropism towards skeletal muscle (Warrington, Jr. and Herzog, 2006b), which in this case may cause too efficient transgene expression in muscle cells leading to abnormal tissue structures. Some similar structures have been reported at shorter time points (Arsic et al., 2003; Zacchigna et al., 2007).

In comparison, adenoviral VEGF-A gene transfer increased perfusion up to 20-fold but the effect of AdVEGF-A was transient lasting about two weeks. In contrast, AAV-VEGF-A induced a 10-fold increase in perfusion, and the effect of AAV can last for years. With adenovirus, the muscle area can be efficiently transduced since it spreads evenly after an injection. The effect of AAV is very local due to AAV binding to heparan sulfate proteoglycans expressed on cell surface (Summerford and Samulski, 1998) and this may limit the efficient transduction so that it does not cover the muscle properly. Transient and a very strong AdVEGF-A expression evoked tissue edema but AAV-VEGF-A expression had side effects as well, although the edema was not significant. AAV-VEGF-A caused tissue fibrosis and muscle disorganization which are disadvantages reducing the suitability of this construct for therapeutic use. Apart from the findings at the local site of gene transfer, the general safety of the intramuscular AAV-VEGF-A gene transfer in rabbit hindlimb appeared to be acceptable. There were neither increased levels of human VEGF-A found in ectopic organs nor were any histological differences detected outside of target muscles in AAV-VEGF-A animals compared with AAV-LacZ animals. This is important for the safety of VEGF-A gene therapy because systemic long-term expression of VEGF-A has been reported to induce tumor formation and bleeding in tissues (Leppanen et al., 2006).

HIF-1α gene transfer has been shown to induce angiogenesis but have fewer side effects than VEGF-A gene transfer (Pajusola et al., 2005). The increase in permeability and tissue edema are strongly linked to capillary enlargement (Rissanen et al., 2003). In this present study, no harmful tissue edema was observed after AdHIF-1α or AdHIF-2α gene transfer. However, capillary growth and skeletal muscle perfusion were significantly improved indicating that enhanced angiogenesis can be achieved without any major tissue edema.

6.3 GENE TRANSFER FOR STIMULATING ENERGY METABOLISM

An optimal gene transfer construct for ischemic tissue would improve not only angiogenesis but also tissue metabolism, therefore the capacity of HIFs to influence ischemic skeletal muscle metabolism and recovery from exercise was evaluated. Previously, AdPlGF gene transfer has been shown to improve energy metabolism during exercise (Korpisalo et al., 2008). Both AdHIF-1α and AdHIF-2α improved the recovery from the energy loss induced by exercise in comparison to AdLacZ. The effects on skeletal muscle metabolism were evaluated by ^{31}P-MRS, and PCr and Pi levels were used to measure the skeletal muscle recovery. AdHIF-1α significantly enhanced the capacity of the muscles to refill PCr storage after exercise, whereas the recovery of AdHIF-2α transduced muscles was similar to the situation in intact muscles. PCr stores are not properly refilled in 20 minutes, which explains the smaller PCr peak in recovery stage compared to the baseline PCr peak. The positive effect on energy metabolism indicates that vessels grown under the influence of the transcription factor gene transfer function normally and the 4-fold increase in angiogenesis, as measured with ultrasound techniques and histological analysis, is believed to be sufficient to improve PCr based ATP synthesis and to restore and replenis PCr stores rapidly after exercise. The results from ^{31}PMRS indicate that HIFs have an effect on anaerobic metabolism during the recovery stage, whereas overexpression of the strong individual growth factor such as PlGF, can affect anaerobic metabolism during the exercise. These results are important, as in PAD patients have been reported to experince problems in restoring PCr levels after exercise. (Greiner et al., 2006) Since HIFs were able to accelerate the recovery of the ischemic skeletal muscle, this may represent a new therapeutic approach for improving muscle function of PAD patients. Further studies are important in determining the contribution of direct effects of HIFs on metabolism apart from those mediated by increased blood flow.

7 Conclusions

I The expression profile of PDGF-C and PDGF-D and their receptors in normal human arteries and in atherosclerotic lesions was characterized. Novel PDGF family members have different expression profiles in lesions and both of these factors are clearly present in normal arteries indicating that they are likely to be involved in vascular homeostasis.

II Adenoviral PDGF-B gene transfer induced moderate angiogenesis but the effects remained for up to 28 days. Combining PFGF-B with VEGF-A gene transfer did not improve vessel maturation. On the contrary, PDGF-B caused pericyte detachment, probably due to an improper PDGF-B gradient.

III AAV-VEGF-A is a powerful angiogenic factor and that can promote angiogene sis in the rabbit skeletal muscle. However, long-lasting expression of VEGF-A appears to evoke local unfavorable effects which will need to be considered when designing future therapeutic applications.

IV Adenoviral overexpression of HIF-1α HIF-2α induces angiogenesis in both rabbit normoperfused and ischemic skeletal muscles. Additionally, gene transfer of AdHIF-1α and -2α elevates skeletal muscle energy metabolism via improving blood flow and the oxygen supply to the ischemic area.

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Growth Factor Expression in Atherosclerosis and Gene Transfer for Therapeutic Angiogenesis

New treatment strategies for the patient suffering from cardiovascular diseases are needed. Gene therapy may offer a novel opportunity to improve vascular growth in ischemic tissue. In this thesis, growth factor expression in human atherosclerotic arteries was described. In addition, gene transfer into ischemic limb was observed to increase angiogenesis and improve the recovery of skeletal muscle from the energy deprivation induced by exercise.

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