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MYOCARDIAL ANGIOGENESIS. ASPECTS ON ENDOGENOUS DETERMINANTS AND EFFECTS OF STIMULATION

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TO MY FAMILY

ABSTRACT

Background: Therapeutic angiogenesis using i.e. VEGF and FGF have shown beneficial effects, however, delivery routes and other modulating agents have not been thoroughly investigated. In this study the efficacy of adenovirus gene transfer was compared to plasmid gene transfer. The angiogenic effects of ephrinB2 were explored, as were the cardioprotective effects of erythropoietin and estrogen, with angiogenic effects in focus. Since hypoxia is the key regulator of angiogenesis, the studies were performed in an ischemic setting with a myocardial infarction model.

Results: Intramyocardial AdhVEGF-A_{165} transfer induced higher hVEGF-A protein expression than PhVEGF-A_{165} in rat myocardium ($p<0.001$). PhVEGF- A_{165} and AdhVEGF-A_{165} stimulated angiogenesis and improved left ventricular function to similar extent. AdhVEGF- A_{165} induced more apoptotic cells ($p<0.001$) and higher ectopic expression of VEGF-A_{165} than PhVEGF-A_{165} gene transfer.

Treatment with darbepoietin-α did not alter capillary density after myocardial infarction in a mouse myocardial infarction model. Cell proliferation was higher in the periinfarct area compared to the non infarcted area. Darbepoietin-α treatment gave a decreased cell proliferation (BrdU, $p< 0.02$) and apoptosis (TUNEL, $p<0.005$) with 30% in the periinfarct area. Darbepoietin-α and VEGF-A_{165} both induced angiogenic sprouting from cultured murine aortic rings.

The ephrin/Eph system was expressed in murine myocardium and altered as regards the ephrinB2/EphB4 expression after myocardial infarction ($p<0.005$). Modulation of ephrinB2 with fusion protein tended to increase mitosis, measured by BrdU incorporation in the periinfarct area, and also increased capillary density in the periinfarct area. EphrinB2 induced proliferation in human aortic endothelial cells ($p<0.0005$) and aortic ring sprouting ($p<0.05$) to a similar extent as VEGF-A_{165}.

In ERβKO mice the downregulation of ERα and the absence of functional ERβ and in ERαKO the absence of functional ERα and the downregulation of ERβ did not influence myocardial angiogenesis or arteriogenesis after myocardial infarction. In the periinfarct area of ERαKO mice the number of macrophages was lower compared to control ($p<0.05$).

Conclusions: AdhVEGF-A_{165} does not have any obvious superior angiogenic efficacy compared to PhVEGF-A_{165} but more side effects in a rat myocardial infarction model. Darbepoietin-α induces endothelial sprouting in a murine aortic ring culture, but in this model darbepoietin-α decreases cell proliferation and apoptosis in the periinfarct area with capillary and arteriolar densities unchanged. The ephrin/Eph system is present in the myocardium, and alters after myocardial infarction. EphrinB2 Fc tends to increase the mitotic activity and prevents a decrement in capillary density in the periinfarct area. Also ephrinB2 Fc induces endothelial cell proliferation in vitro, and stimulates angiogenic sprouting in an aortic ring model. mRNA expression of estrogen receptors are present in the myocardium. After myocardial infarction in ERβKO ERα mRNA and in ERαKO ERβ mRNA are downregulated, without any influence on angiogenesis or arteriogenesis.

Key words: Myocardial infarction, ischemia, angiogenesis, VEGF- $A_{165,}$ gene therapy, adenovirus, plasmid, ephrinB2, darbepoietin-α, apoptosis, estrogen receptors.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which will be referred to by their Roman numerals:

I. Hao X, Månsson-Broberg A, Grinnemo KH, Siddiqui AJ, Dellgren G, Brodin LÅ, Sylvén C. Myocardial angiogenesis after plasmid or adenoviral VEGF-A_{165} gene transfer in rat myocardial infarction model. Cardiovasc Res. 2007;73:481-7

II. Månsson-Broberg A, Grinnemo KH, Genead R, Danielsson C, Andersson AB, Wärdell E, Sylvén C. Erythropoietin has an antiapoptotic effect after myocardial infarction and stimulates in vitro aortic ring sprouting. Biochem Biophys Res Commun. 2008;371:75-8

III. Månsson-Broberg A, Siddiqui AJ, Genander M, Grinnemo KH, Hao X, Andersson AB, Wärdell E, Sylvén C, Corbascio M. Modulation of ephrinB2 leads to increased angiogenesis in ischemic myocardium and endothelial cell proliferation. Biochem Biophys Res Commun. 2008;373:355-9

IV. Månsson-Broberg A, Siddiqui AJ, Fischer H, Grinnemo KH, Wärdell E, Andersson AB, Inzunza J, Gustafsson JÅ, Sylvén C. Estrogen receptors do not influence angiogenesis after myocardial infarction. Manuscript

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LIST OF ABBREVIATIONS

Ad	adenovirus
Ang	angiopoetin
BrdU	bromodeoxyuridine
CMV	cytomegalovirus
DAB	diaminobenzidine
DAPI	diamidinophenylindole
EPC	endothelial progenitor cell
EPO	erythropoietin
ER	estrogen receptor
ERKO	estrogen receptor knock out
FGF	fibroblast growth factor
HAEC	human aortic endothelial cell
HIF	hypoxia inducible factor
LAD	left anterior descending coronary artery
NF-κB	nuclear factor kappa B
TRITC	tetrametyl-rhodamine-isothiocyanate
VEGF	vascular endothelial growth factor

1 INTRODUCTION

1.1 General background

1.1.1 Cardiovascular disease

In one century cardiovascular disease has evolved from causing approximately 1/10 of all deaths worldwide, to accounting for almost 1/2 of all deaths, in Sweden approximately 41% (Figure 1). In the developing countries it is an emerging problem due to changes in lifestyle, such as smoking- and dietary habits, and the incidence is increasing (World Health Statistics 2009, Lloyd-Jones 2009).

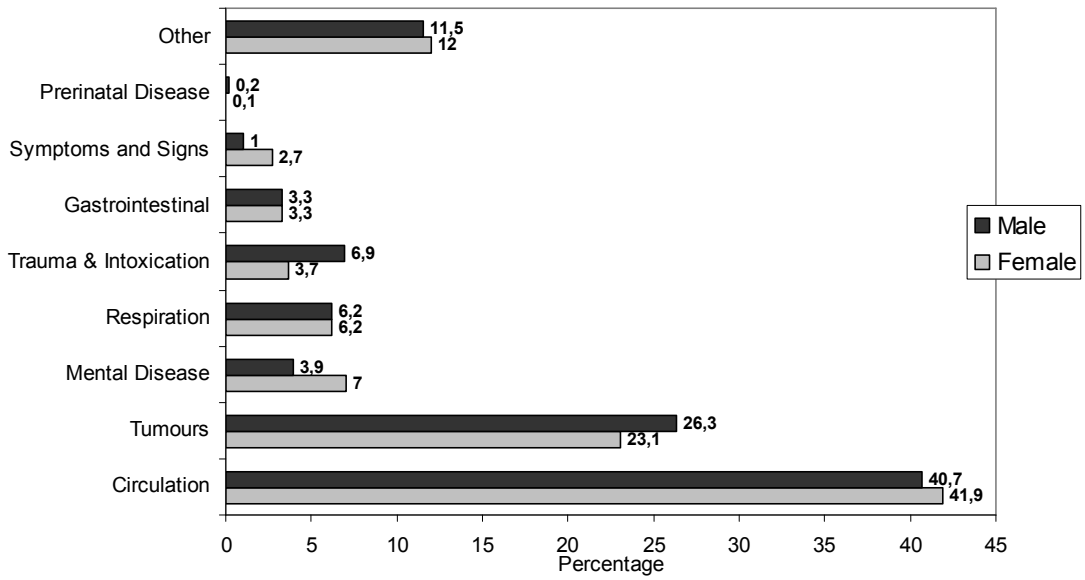

Figure 1. Causes of death in Sweden 2007 for women (47795) and men (44025), Causes of death 2007, Swedish Board of Health and Welfare (Socialstyrelsen)

The underlying atherosclerotic process has various local and systemic manifestations. Changes in the endothelial cell environment, due to hypertension, oxidative stress, inflammatory stress give rise to endothelial dysfunction that lead to changes in the arterial wall, also including the connective tissue and smooth muscle cells surrounding the vessels. (Hansson 2009, Fearon 2009).

As the arterial disease proceeds with developing atherosclerosis, the arterial function declines in that the vessel wall thickens and the vascular lumen becomes narrower and the endothelium dysfunctional, the vessels become less able to react to flow-regulating stimuli and the risk of intravascular thrombosis due to platelet aggregation increases. The arterial dysfunction gives symptoms of hypoperfusion and ischemia, either slowly such as angina pectoris upon effort or at rest, or suddenly, through acute occlusion of an artery as in acute coronary syndromes including acute myocardial infarction. Acute myocardial infarction is a life threatening cardiac condition due to a sudden occlusion of a coronary artery often because of a rupture of an atherosclerotic plaque and interrupted blood flow, and thus ischemic necrosis of myocardium. This condition has a considerable risk of sudden lethal ventricular arrhythmias or cardiac failure.
After the acute phase of the disease, if the patient survives, the morbidity is high, causing symptoms such as angina pectoris and/or congestive heart failure, both often limiting the patients' general level of function. Of all cardiovascular diseases, the leading cause of death is due to cardiac failure (Swedish Board of Health and Welfare 2007).

1.1.2 Gender perspective

The age-matched incidence of ischemic heart disease is greater in men than in premenopausal women. After menopause the incidence increases also for women, indicating that estrogen plays a protecting role in the cardiovascular system. Several clinical trials have tried this hypothesis in giving hormone replacement therapy to postmenopausal women in order to prolong the protection against ischemic heart disease, but reports on increased cancer risk and adverse cardiovascular outcomes in subgroups prompt that a better understanding of the cardiovascular effects of estrogens is needed (Grodstein 1995, Hulley 1998, Hulley 2009).

1.2 Patophysiological changes after myocardial infarction

1.2.1 Histological changes

The first hour after myocardial infarction the histological changes are few, a waviness of fibers at the border of the infarction is first observed. In the area of ischemia a coagulation necrosis develops with oedema, haemorrhage and infiltrating erythrocytes. The first 3 days the necrosis proceeds, and the myocytes begin to loose their striation and nuclei and neutrophils infiltrate the interstitium. After 5-10 days the transversal striation and nuclei of the cardiomyocytes in the necrotic area are completely lost, but the contour of the myocardial fibers is preserved. Lymphocytes and macrophages infiltrate the interstitial space. The necrotic area is surrounded and progressively invaded by granulation tissue, collagen depositions increases and cellular density is decreased. After 2 months the necrotic tissue is replaced by a fibrous scar. (Kumar V, Robbins Basic Pathology 8th edition).

1.2.2 Adaptation to hypoxia/ ischemia

After myocardial infarction there is an irreversible loss of contractile function in the necrotic area/fibrous scar. The necrotic center of a myocardial infarction is viewed to be surrounded by transition zone towards non-ischemic myocardium. In the transition zone , the periinfarct area, the myocardial contractile function is reduced in response to the reduction of myocardial blood flow, in order to maintain myocardial integrity and viability. This adaptation is referred to as hibernation, and is reversible upon reperfusion. In the hibernating myocardium morphological changes occur; the myocytes hypertrophy and disorganize, with increased myocyte width and decreased myocyte density, as well as increased interstitial fibrosis and lower vascular densities. (Rahimtoola 1999, Chen C 1996, Heusch 1996, Siddiqui 2003). Hibernating myocardium is present in clinical syndromes caused by coronary artery insufficiency

such as unstable and stable angina pectoris, acute myocardial infarction and left ventricular failure with or without congestive heart failure.

Cellular and molecular responses to hypoxia are targeted towards increased oxygen supply to the cell in order to promote tissue survival. The key regulator in inducing an adaptive response via gene expression is the transcription factor hypoxia-inducible factor-1 (HIF-1), a heterodimeric complex consisting of HIF-α and HIF-β (Semenza 1999). In normoxic conditions HIF-α is degraded, but during hypoxia it is activated (Figure 2), and regulates transcription of genes encoding proteins that will either increase oxygen delivery (VEGF, erythropoietin, endothelin-1) or achieve metabolic adaptations under conditions of reduced oxygen availability (glucose transporter-1, glycolytic enzymes) (Semenza, 2004). Sustained hypoxia leads also to the activation of other transcription factors such as nuclear factor kappa B (NF-κB) (Cummins, 2005) a key player in inflammatory and innate immune responses, closely linked to the pathogenesis of atherosclerosis (Willerson 2004).

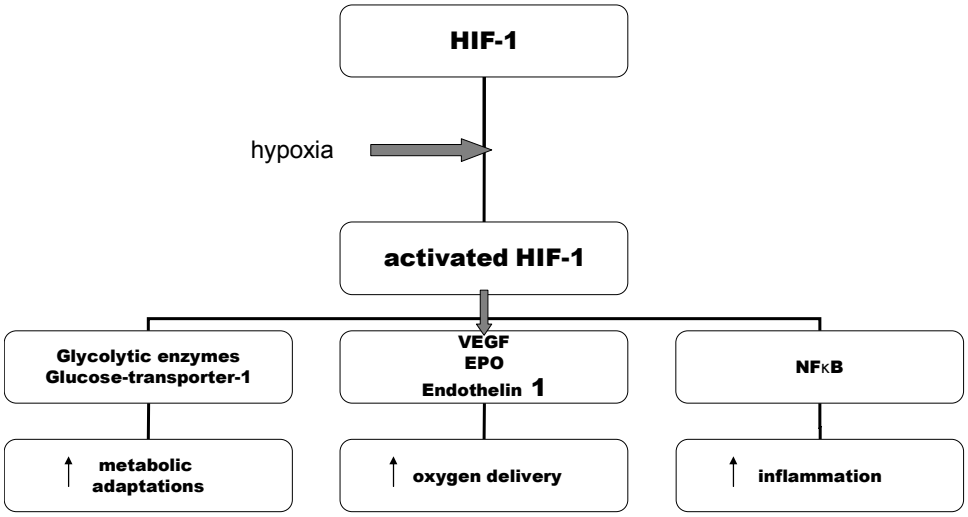

Figure 2. Actviation and actions of HIF-1.

1.3 Angiogenic processes

1.3.1 Vasculogenesis

Vasculogenesis gives rise to the first primitive vascular plexus and the heart inside the early embryo (Figure 3), where endothelial progenitor cells share a common origin with hematopoietic progenitors. These progenitors differentiate into endothelial cells and assemble into a primitive vascular plexus (vasculogenesis). In the embryo, low levels of O_2 (<25 mmHg) stimulate the differentiation of various progenitors, including that of early mesoderm into hemangioblasts (Ramirez-Bergeron 2004). Genetic studies show that HIF-1β coordinates the early steps of vessel development through paracrine release by hematopoietic cells of angiogenic cytokines, such as VEGF and angiopoietin-1 (Ramirez-Bergeron 2006).

1.3.2 Angiogenesis

Angiogenesis is growth of new capillaries from a preexisting network that develops via sprouting, branching, intussusception and remodelling into a more, mature vascular network (Figure 3). Sprouting angiogenesis, is considered to be the major type. Activation of endothelial cells and vasodilation is followed by increased vascular permeability. The basement membrane and extracellular matrix are degraded by proteases. By this degradation numerous growth factors are released, and endothelial cells can migrate to new sites where new capillaries can form (Risau 1997, Carmeliet 2003, Conway 2001). Non-sprouting angiogenesis mostly occur by intussusception. The opposite microvascular wall protrude into the capillary lumen creating a contact zone between the cells (Burri 1990, Makanya 2009). Intussusception permits rapid expansion of the capillary network and thus enlargement of the endothelial surface.

1.3.3 Arteriogenesis

Arteriogenesis is functional remodelling of a pre-existing artery or capillary (Figure 3). The process involves remodelling of endothelial cells, smooth muscle cells and

fibroblasts, (Helisch 2003, Heil 2005) and is initiated and regulated by biomechanical effects like increased blood flow, shear stress and wall tension, which induce arteriogenesis by inducing several transcription factors and augmenting NO (Resnick 1997, Nagel 1999). Also mechanical stretch affects proteases in the extracellular matrix (O'Callaghan 2000).

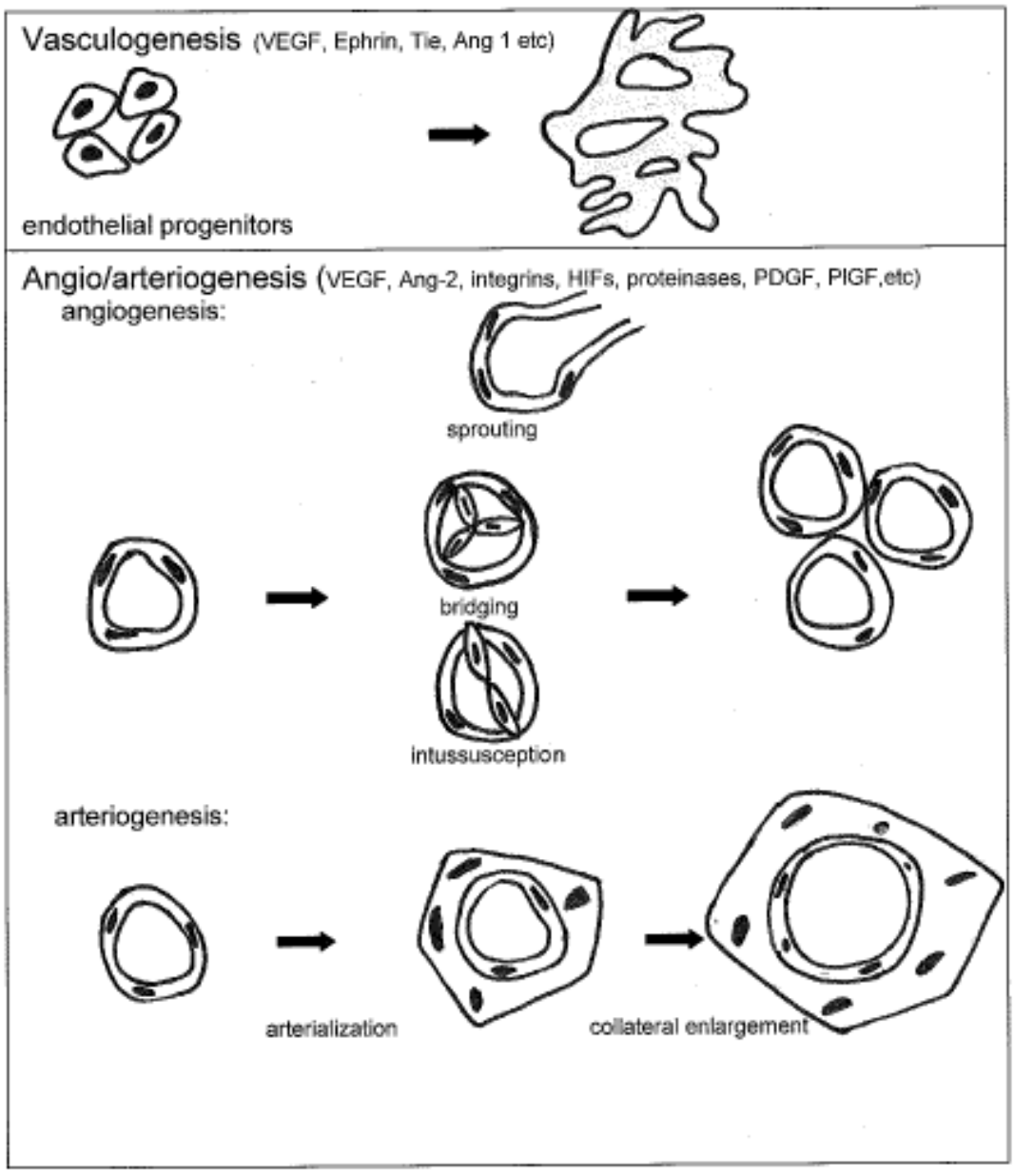

Figure 3. Vasculogenesis, angiogenesis, arteriogenesis.

1.4 Factors involved in angiogenesis

1.4.1 Angiogenic switch

The angiogenic process is regulated by intra/extracellular angiogenic factors in response to stimuli, and the balance between angiogenic stimulators and inhibitors has been referred to as the angiogenic switch (Hanahan 1996).

1.4.2 Progenitor cells

There is evidence that bone marrow derived endothelial progenitor cells (EPC) home at sites of endothelial damage to form an intact endothelium, and contribute to postnatal neovascularization (Asahara 1999, Werner 2002, Xu 2006).

1.4.3 Tyrosine kinase receptors

Both VEGF- (VEGFR 1/2) , angiopoetin- (Tie 1/2) and ephrin B2/Eph B4-receptors are tyrosine kinase receptors, which are a group of transmembrane polypetides containing a protein kinase domain in their intracellular region, a transmembrane- and an extracellular domain (Figure 4). The extracellular portion of the receptor is responsible for binding to a specific ligand, ie growth factor or signal molecule. When stimulated the tyrosine kinase receptor can initiate several cellular events such as proliferation, migration, cellular shape change, production of metalloproteinases and expression of transcription factors. The VEGF family and their VEGF receptor tyrosine kinases participate in vasculogenesis and angiogenesis and are survival factors, motility factors and potent vascular permeability agents. These VEGF functions are coordinated by angiopoietins (Ang), expressed by endothelial and perivascular cells, and by their associated endothelial tyrosine kinase receptors (Tie1 and Tie2). VEGFs affect cell-matrix interactions (through the expression of for example integrins, fibronectin and vitronectin) and affect cell-to-cell interactions (through, for example, platelet-endothelial cell adhesion molecules), mechanisms important for vasculogenesis and angiogenesis. Ephrins and Eph-receptor tyrosine kinases are part of the cell-to-cell

recognition system, differ between arteries and veins, modulate adhesion through integrins, and are also involved in remodelling of the primary vascular plexus and sprouting angiogenesis (Gale 1999).

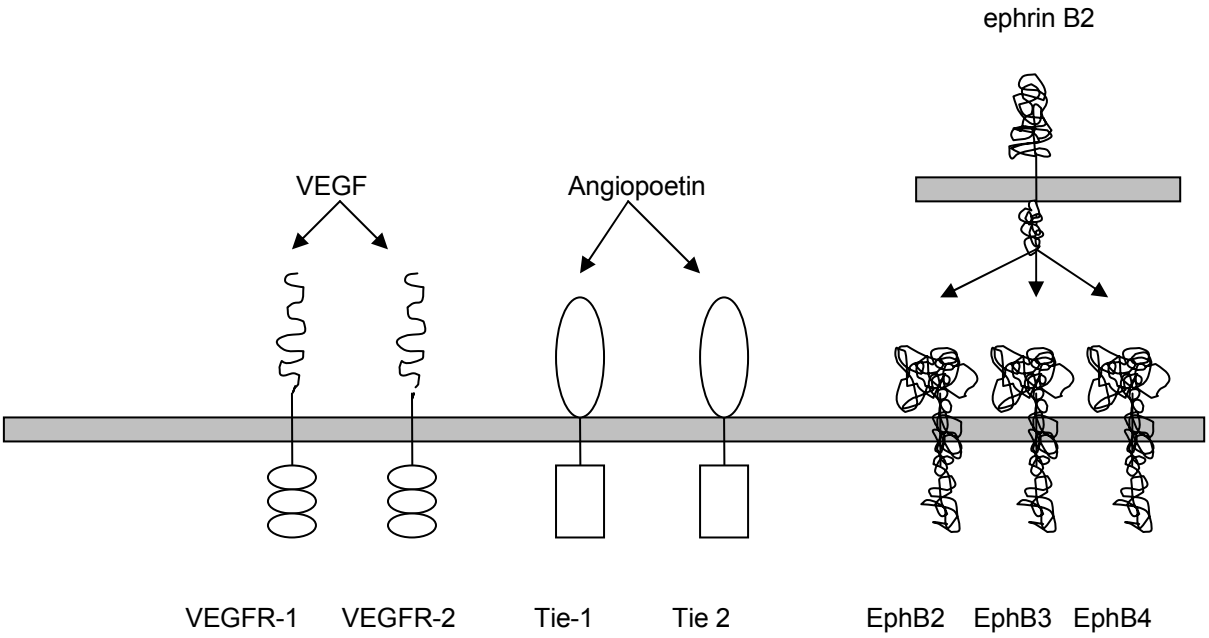

Figure 4. Endothelial tyrosine kinase receptors.

1.4.4 VEGF

The master angiogenic growth factor is vascular endothelial growth factor. It is expressed during angiogenesis, and plays an important role in wound healing, tumour angiogenesis, development of the cardiovascular system and in diseases with pathological angiogenic responses. (Folkman 1995, Risau 1997, Conway 2001). There are at least five isoforms (VEGF- $A_{-121\ -145\ -165\ -189}$ and $_{-206}$) where VEGF-A_{165} is the predominant form. The major vascular actions of VEGF are mediated by two tyrosine kinase receptors, VEGFR-1/ Flt-1 and VEGFR-2/Flk-1.

Other growth factors involved in the angiogenic process are, for example, platelet derived growth factors (PDGF), fibroblast growth factors FGF, and also involved in angiogenesis and endothelial function are selectins (E and P), integrins and adhesion molecules from the immunoglobulin superfamily (intracellular adhesion molecule (ICAM-1) and vascular cell adhesion molecule (VCAM-1). These latter two mediate rolling and adhesion of blood cells (such as leukocytes and platelets) to the endothelium. VE-cadherin (cell-to-cell interactions, sprouting angiogenesis) and Notch/Jagged family members (cell-to-cell recognition, cell fate determination and tubular morphogenesis, Jain 2000).

1.4.5 Angiopoetins

Angiopoetins (Ang 1/2) are vascular endothelial cell specific secreted proteins and share a common tyrosine kinase receptor Tie-2. Ang 1 acts locally while Ang 2 freely diffuses into the tissues. Ang 1 acts as an agonist and Ang-2 as an inhibitor on the Tie 2 receptor.

1.4.6 Eph receptors and Ephrins

Eph receptors comprise the largest subgroup of tyrosine kinase receptors including at least14 members (Vanderhaeghen 1998). The Eph receptors diverge in sequence from the other tyrosine kinase receptors and are more related to cytoplasmic tyrosine kinase receptors within their kinase domain.The ephrins also differ from typical soluble ligands in that they must normally be membrane attached to activate their receptor (Davis 1994, Gale 1997). Membrane attachment seems to promote clustering or multimerization and it seems to be important to activate receptor on adjacent cells (Figure 4). Artificial clustering can allow them to activate their receptors. (Davis 1994). The ephrins are tethered naturally to the cells in which they are expressed, and the different attachment mechanisms along with shared homology and binding

characteristics that divide them into two subgroups, the ephrin A and ephrin B subgroups (Eph Nomenclature Committee 1997).

The Eph/ephrins have pivotal effects on axon guidance, cell migration, and cell attachment during embryogenesis (Pasquale 1997, Brantley-Sieders 2004). In vitro studies reveal that EphB4 is involved in regulating embryonic stem cell responses to mesodermal induction signals (Wang 2004). They are important during neurogenesis and neural development (Flanagan JG 1998, Holmberg J 2000, Holmberg 2005, Mukouyama 2002), as well as for the development of the cardiovascular system including differentiating arteries from veins (Augustin 2003, Gerety 2002, Adams 1999, Wang 1998, Oike 2002).

EphrinB2 is expressed predominantly on arterial endothelial cells, and EphB4 on venous endothelial cells. EphrinB2 is suggested also to be of importance in postnatal and adult angiogenesis (Hayashi 2005, Zamora 2005). EphrinB2 has a stimulatory effect on the migration of microvascular endothelial cells in vitro and hypoxia is reported to upregulate the expression of ephrinB2 in mouse skin (Vihanto 2005)

1.4.7 Erythropoietin

Under hypoxic circumstances erythropoietin-production is stimulated by activated HIF complex, and the myeloproliferative effects of erythropoietin result in an elevated hematocrit and thus protection of tissues securing oxygen delivery. Erythropoietin has been reported to improve cardiac function and induces neovascularization in experimental models of chronic myocardial dysfunction with chronic heart failure (Hirata 2006, Prunier 2007, van der Meer 2005). Erythropoietin-induced neovascularization is related to increased mobilization and incorporation of bone marrow derived endothelial progenitor cells (Bahlmann 2005) and augmented expression of angiogenic factors in the myocardium (Westenbrink 2007). Some reports

show that the actions of erythropoeitin are tissue protective at doses that do not influence the red blood count (Lipsic 2008, Leist 2004).

1.4.8 Estrogen

In human adult tissue estrogen receptors (ER) ERα and ERβ have been identified in the myocardium, aorta, coronary - and carotid arteries, inferior vena cava and also in the endothelial cells of the thyroid and uterine cervix (Taylor 2000). Functional ERα and ERβ-proteins have been reported to be expressed in cardiomyocytes, cardiac fibroblasts, vascular smooth muscle cells, endothelial and epithelial cells of the kidney (Grohé 1997, Karas 1995). Cardiovascular ERs have been associated with the prevention of apoptosis (Patten 2004), with antihypertrophic effects in the heart (Babiker 2006, Pedram 2005) and with the control of cell-to-cell interactions by targeting adherence molecules both in endothelial and myocardial cells (Groten 2005, Mahmoodzadeh 2006). Furthermore estrogen has been reported to stimulate VEGF production in cholangiocarcinoma cells (Mancino 2009) and in myometrial microvascular endothelial cells (Gargett 2002).

1.5 Therapeutic angiogenesis

The human heart has restricted collateral growth of coronary arteries. By performing percutaneous coronary intervention, PCI, or on selected patients with severe ischemic heart disease such as severe symptomatic angina pectoris, 3-vessel disease or unprotected left main coronary artery, coronary artery by-pass grafting, arterial blood flow can be reestablished to the underperfused, hibernating areas of the myocardium. A large group of patients that can not be adequately revascularized remain. Therapeutic attempts to reconstitute microvasculature through stimulating vessel growth have been made with recombinant growth factors such as basic fibroblast growth factor (bFGF) and vascular endothelial growth factor (VEGF) in models of myocardial ischemia (Yanagisawa-Miwa 1992). However, maintaining adequate levels of the recombinant

protein in the target area is technically difficult and expensive. Clinical trials with gene transfer therapy with VEGF and FGF have reported beneficial effect on symptoms and perfusion, but efficiency as regards to decreased mortality and major coronary events remains to be evaluated.

2 AIMS

1. To compare the angiogenic effects of AdhVEGF-A_{165} and PhVEGF-A_{165} and adverse effects as regards apoptosis and ectopic expression of the transgene in a rat myocardial infarction model.

2. To explore if darbepoietin-α in a mouse myocardial infarction model influences periinfarct cellproliferation, apoptosis, capillary and arteriolar densities, and to explore if darbepoietin-α can induce angiogenic sprouting in a murine aortic ring culture.

3. To explore if the ephrin/ Eph system is present in the mouse myocardium and if modulation can influence cell proliferation in vivo and in vitro, and if ephrinB2 Fc can induce angiogenesis in an aortic ring culture.

4. To explore if the pattern of expression of estrogen receptors α (ERα) and β (ERβ) in myocardial tissue changes after myocardial infarction, and to explore if the presence/ absence of ERα or ERβ influences angiogenesis after myocardial infarction.

3 MATERIALS AND METHODS

3.1 Animals

Male Fisher rats weighing around 200-250 g (B&K Universal) were used in paper I. C57BL/6 mice weighing around 25 g (Taconic) were used in paper II, III and IV. ERαKO- and ERβKO-mice (Jan-Åke Gustafsson, Novum, Sweden) and C57BL/6 mice weighing around 25 g (Taconic, Denmark) were used in paper IV.

3.2 Myocardial infarction model

In paper I the animals were anaesthetized with an intraperitoneal injection of midazolam (Dormicum, 5 mg/kg, F. Hoffmann-La Roche Ltd.), and medetomidin hydrochloride (Domitor vet, 0.1mg/kg, Orion Corp.). In paper II, III and IV fentanyl (0.3mg/kg, B. Braun Medical AG) was added. After the induction of anaesthesia the animals were endotracheally intubated and ventilated using a Zoovent ventilator (Model CWC600AP, BK Universal). The heart was exposed via thoracotomy, and the left anterior descending artery (LAD) was ligated with a 7.0 polypropylene suture (Figure 5). Myocardial infarction was confirmed by pallor of the myocardium and dyskinesia of the anterior wall. The anesthesia was reversed by an intraperitoneal injection of flumazenil (Lanexat, 0.1 mg/kg, F. Hoffmann-La Roche Ltd.), atipamezol hydrochloride (Antisedan vet 5mg/kg, Orion Corp.) and buprenorphin hydrochloride (Temgesic, 0.1mg/kg, Schering-Plough Corp.).

At end of experiments animals were euthanized by cervical dislocation. All studies were approved by the local Ethics Committee.

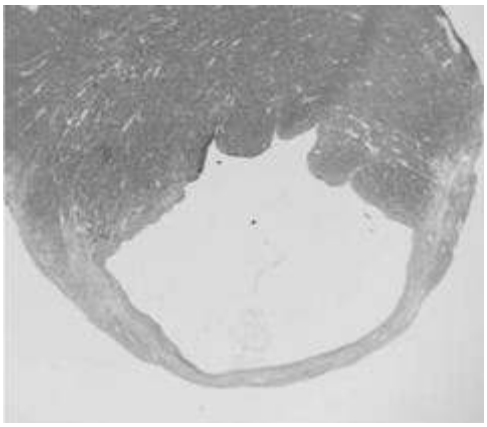

Figure 5. Transversal section of left ventricle of mouse heart 4 weeks after myocardial infarction induced by LAD-ligation.

3.3 In vivo modulation

3.3.1 AdhVEGF A_{165}, Ph-VEGF- A_{165}, AdLacZ and PLacZ

In paper I gene transfer was carried out into the heart with or without myocardial infarction and in the case of infarction one week after the infarction via e second thoracotomy. The transfer was performed intramyocardially with single or multi-spot injections. In myocardial infarction heart, periinfarct region along the border of the myocardial infarction was chosen as injection site. Human GMP-grade first-generation adenovirus (Laitinen 1998) or naked plasmid were used. Human AdhVEGF A_{165}, Ph-VEGF- A_{165}, AdLacZ and PLacZ, were transferred. Transfer volume was 100 µl. Delivery dose of adenovirus was 5 x 10^9 pfu and of plasmid 40 ug.

3.3.2 Plasmids

PhVEGF-A_{165}: PhVEGF-A_{165} is an eukaryotic expression vector encoding for the 165 aminoacid isoform of human VEGF-A, driven by a cytomegalovirus immediate early promoter/enhancer (CMV). Plasmid LacZ: the amplified LacZ gene was amplified by PCR. The amplified fragment was digested with BsiWI and BamHI and inserted into PhVEGF-A_{165} where the VEGF gene had been cut out.

3.3.3 Adenovirus

AdhVEGF-A_{165}: Human GMP-grade first-generation adenovirus encoding human VEGF-A_{165} driven by a CMV promoter.

AdLacZ: Human GMP-first-generation adenovirus encoding LacZ marker gene driven by a CMV promoter.

In order to determine the optimal doses of adenovirus or plasmid, 1×10^9, 5×10^9, 1×10^{10} pfu AdhVEGF-A_{165} or 20, 40, 60 μg PhVEGF-A165 in 100 μL saline was injected intramyocardially into the anterior wall at one spot in the normal heart. There were 5–6 animals in each group. Hearts were harvested to detect hVEGF-A expression 24 h after PhVEGF-A_{165} and 6 days after AdhVEGF-A_{165} gene transfer. These time points were previously reported to give maximum myocardial expression of the respective vector. (Sylvén 2001, Rutanen 2004).

3.3.4 Erythropoietin treatment

Paper II: Darebepoietin-α commercially available as Aranesp (AMGEN) was used. Darbepoietin-α 7.5μg/kg was in the treated groups given ip at days 0 (0-10 minutes after ligation of the LAD) and 5, or at day 5 only. This dose of darbepoietin-α has previously been reported to increase hemoglobin by 10–20 g/L/week when given once weekly (LaMontagne 2006).

3.3.5 EphrinB2-fusionprotein treatment in vivo

Paper III: Recombinant mouse EphrinB2-Fc-chimera (R&D Systems) was used. On day 2 postoperatively animals were randomized to control or to EphrinB2-Fc treatment with a single dose of 20 μg (in 100 μL PBS) recombinant mouse EphrinB2-Fc chimera (R&D systems) ip. The reason for delaying treatment for 48 h was to avoid the intense inflammation in the subacute phase following acute myocardial infarction.

3.4 Cardiac function

Paper I: Cardiac function was assessed by Tissue Doppler Echocardiography using a Vingmed Vivid 5 (Vingmed) ultrasound system equipped with a 10 MHz transducer. Echocardiography was performed 3 days after myocardial infarction, and 4 weeks after gene transfer. Color Tissue Velocity Image was performed at the apical chamber view

at frame rates close to 230 fps. The probe position was adjusted under the guidance of Pulsed Tissue Doppler to the maximum velocity of the mitral valve plane. The recording was obtained by positioning a sample volume in each basal septal left ventricular wall segment. Analysis was performed off-line from acquired cineloops with Echopac version 6.1. Peak systolic velocity was not measured due to unfavourable noise to signal ratio. Systolic velocity-time integral, a parameter for displacement of myocardium which is less noise dependent, was assessed from 2-3 beats to minimize the variability. The final value of regional systolic myocardial function was calculated as a mean value from 2-3 peak velocity-time integral at systole.

3.5 Capillary and arteriolar densities

Papers I, II, III and IV: The harvested heart was divided into two halves and the apical parts were imbedded in OCT (Histolab) and frozen in liquid nitrogen, and kept in -70 ˚C. 5 μm thick sections were prepared using a cryostat. Staining with eosin/ hematoxylin verified that the samples chosen came from the periinfarct area or from the noninfarcted area. For the analysis of capillary density the sections were incubated with Griffonia Bandeiraea Simplicifolia Isolectin B4 (GSL-I-B4, Vector Laboratories) followed by a second incubation with ABC Complex. Finally, capillaries were visualized by DAB (diaminobenzidine) with supplementation of 0.03% hydrogen peroxide (H_2O_2). Capillaries were counted at a magnification of 400x taken by an LCD camera (Olympus) connected with a microscope. Ten fields of pictures around the injection site were taken and the capillary count was analyzed blinded with an image analysis program (Micro Image, Olympus). For the analysis of arteriolar density, lectin stained sections were incubated with primary antibody against α-actin (Sigma). Then rabbit anti-mouse secondary antibody (TRITC, Dako) was used to visualize the blood vessels. Blood vessels stained around the injection site were counted under 200x

magnification in a fluorescence microscope. All analyses were performed in a blinded manner.

3.6 Human VEGF-A expression

Paper I: Human VEGF-A gene expression was determined by ELISA in hearts both with and without myocardial infarction. In addition, ectopic hVEGF-A expression was determined in kidney, lung, liver and spleen from the noninfarcted rat. Organs were minced with a homogenizor knife in homogenization buffer. Thereafter the homogenized substance was centrifuged for 10 min at 14,000 ×g at 4 °C. The supernatant was collected and frozen at -70 °C. Later all frozen samples were analyzed by ELISA for hVEGF-A by immunoassay according to the manufacturer's instructions (Quantkine, R&D system). hVEGF expression was visualized in myocardial tissue by immunohistochemistry stained with hVEGF antibody. Frozen sections were incubated with monoclonal anti-hVEGF-A (Sigma) followed by adding fluorescent-labelled (FITC) secondary antibody. Endogenous VEGF was analyzed by ELISA with detection of rat VEGF-A (Quantkine, R&D system). The heart samples were obtained as for human VEGF-A.

3.7 EphrinB2-Fc for detection in myocardial tissue

Paper III: EphrinB2-Fc (R&D Systems) or Ig-G-Fc protein was delivered intravenously to the mouse. Twentyfour hours later, after sacrifice the heart was perfused using 4% formaldehyde whereafter, the tissue was postfixed in 4% formaldehyde overnight at 4˚C, and then frozen and sectioned on a cryostat. Sections were analyzed using either Cy3 donkey anti-human antibody (1:1000), for detections of the infused chimeric protein, or GSL-1 isolectin B4 (1:200) for identification of vessels.

3.8 In vivo BrdU-labeling

Papers II and III: The mice were given 5’-bromo-2’-deoxyrubicine (BrdU) (Sigma–Aldrich), 100 mg/kg/daily, ip day 6–9 in paper II , and day 7-13 in paper III in order to

assess mitotic activity, (see below). The lectin stained sections were double stained with BrdU antibody. Unmasking of proteins was done by boiling the sections in a citrate buffer, followed by incubation with the primary mouse anti-BrdU antibody (Dako Cytomation) overnight. As secondary antibody we used polyclonal rabbit anti-mouse IgG/FITC (Dako Cytomation), dilution 1:10-1:20. The slides were mounted with mounting medium containing 4′,6-diamidino-2-phenylindole (DAPI). The amount of BrdU positive cells were counted. The counting was performed in a strictly blinded manner.

3.9 Aortic sprouting

Papers II and III: C57/BL6 mice were euthanized and the thoracic aortae were dissected and cut into 0.8 mm pieces. An average of five rings per well were cultured in Growth Factor Reduced Matrigel TM Matrix (BD Biosciences) in Human Endothelial-SMF Basal Growth Medium (GIBCO, Invitrogen Corporation, cat no 11111-044), with and without the supplement of VEGF-165 20 ng/ml and/or darbepoietin-α 0; 1; and 5 ng/mL(paper II); and/or ephrinB2-Fc 0.05; 0.5 ug/mL (paper III). Each point of determination represents the mean of five experiments. The number of endothelial sprouts were assessed by visual counting at days 5 and 12.

3.10 Cell proliferation assay

Paper III: Human aortic endothelial cells (HAEC) passage 10 to 11, (Cascade Biologics), were cultured at 5000 cells/cm^2 in EGM-2 medium (Cambrex), containing <5 ng/mL of VEGF according to the recommendation of the manufacturer. After 24 h attachment of the cells the medium was changed to the test mediums (EGM-2; or EMG-2 without VEGF). After 6 days 10μM BrdU, (Sigma-Aldrich), was added, the cells harvested after another 18 h, and the proliferation rate assessed with Cell Proliferation ELISA (Roche) according to the instructions of the manufacturer. A dose-response curve was done with 0, 0.5, 5 and 10 μg/mL of soluble ephrinB2-Fc-chimera

in the three different media. The cell cultures were kept in a CO_2-incubator at 37˚C, in room air or in a hypoxic environment with 1% oxygen.

3.11 Apoptosis assay

Papers I and III: TUNEL assay was performed on frozen sections with an ApopTag Fluorescein in Situ Apoptosis Detection Kit (CHEMICON International). Sections of heart tissue were treated with digoxigenin-dNTP and termial deoxynucleotidyl transferase followed by incubation with anti-digoxigenin conjugated with fluorescein. Counterstaining with DAPI was performed to visualize nuclei. Five fields of apoptotic positive cells were counted under 400x magnification in a blinded fashion. In paper I two areas along the border of the myocardial infarction were analyzed: the injection site and the contralateral site of the myocardial infarction where no injection had been given. In paper III the periinfarct area and the noninfarcted area were compared. After TUNEL staining, slides were incubated with anti-α-sarcomeric actin to detect if the apoptotic cells were cardiomyocytes. Then Rhodamine-labelled (TRITC, Dako) secondary antibody was added for visualization of cardiomyocytes.

3.12 mRNA extraction, cDNA preparation

Paper III and IV: Transmural ventricular tissue (cross sectional circular slice from the infarcted part of the heart) and same area of sham operated heart (baseline control) were snap frozen in liquid nitrogen and kept at -70˚C for RNA extraction and cDNA preparation. RNA was isolated with Trizol reagent (Life technologies) as described by the manufacturer. Total RNA was assayed by spectrophotometer (A260nm/A280nm) to assess purity and concentration. Integrity of total RNA was determined by 1% agarose-gel electrophoresis. RNA samples were diluted with distilled water and stored at -70˚C. RNA (2 μg) from each sample was reversely transcribed to prepare cDNA by Superscript RNase H reverse transcriptase (Superscript II, Invitrogen/GIBCO/Life Technologies) with the random hexamer

primers according to the manufacturer's specifications (Roche Diagnostics GmbH) in a total volume of 20 µl.

3.13 RT-PCR

Paper III: The cDNA was amplified by RT-PCR (Taq-polymerase, Fermenta, Sweden). EphrinB2 ligand primer sets (EphrinB2-1: ATTT GCG GCC GCC AGAT CGAT AGT TTT AGA GCC TATC. EphrinB2-2: CCGC TCG AGA CCA GCTT CTA GCT CT GG ACG TCTT) and EphB4 receptor primer sets (EphB4-1: ATTT GCG GCC GCC AGGA TGTG AT CAA TGCC ATT GAAC. EphB4-2: CCGC TCG AGTT CCTC GTAT CTT CGCAT CTT GATG) were used to amplify specific cDNAs. A primer pair for the constitutively expressed beta-actin gene was included in each assay as an internal control. The PCR products were electrophoresed in 1.5% agarose gel in TAE1-buffer containing ethidium bromide and subsequently photographed under UV light. Light intensity of RT-PCR bands (three to four samples in each group) was assessed by using image acquisition and analysis software (Photoshop, Adobe). The amplicons detected in our gels matched their predicted base pair in size.

Paper IV: Real-time PCR was used to measure mRNA (ABI-PRISM 7700 Sequence Detector, Perkin-Elmer Applied Biosystems Inc). 18s was used as an endogenous control to correct for potential variation in RNA loading or efficiency of the amplification. Primers and probes were supplied as a TaqMan® Reagents kit, 18s (4310893E) Esr1 (αER) (Mm00433149_m1) and Esr2 (ERβ) (Mm00599819_m1) from Applied Biosystems. All reactions were performed in 96-well MicroAmp Optical plates. Amplification mixes (25 µl) contained the sample 5 ul cDNA diluted 1:100 and TaqMan Universal PCR Mastermix. Thermal cycling conditions included 2 min at 50 °C and 10 min at 95 °C before the PCR cycles began. Thermal cycling consisted of 40 cycles at 95 °C for 15 s and 65 °C for 1 min. For each individual, all samples were simultaneously analyzed in one assay run. Measurements of the relative

distribution of Esr1 and Esr2 were performed for each individual, a ΔC_T value was obtained by subtracting 18S C_T values from respective target C_T values. The expression of each target was then evaluated by $2^{-\Delta\text{-}\Delta CT}$

3.14 ER immunostaining

Paper IV: The OCT embedded tissue was cut in 5 µm thick sections by cryostat. The sections were fixed and then blocked with endogenous peroxide using 0.3% hydrogen peroxide (30 min). After washing, slides were incubated during 30 min in PBS (phosphate buffered solution, 5 min each) with 0.01% Tween 20 and then, incubated (1 h) with 10% normal goat serum in PBS. After washing, sections were incubated overnight with polyclonal rabbit antibodies against mouse ERα and ERβ diluted in PBS-Tween (1:100 dilution; ERα sc-542 and ERβ sc-542, Santa Cruz Biotechnology). The specificity of these antibodies has been described previously (Cheng 2004). Samples were rinsed four times, incubated with goat anti-rabbit secondary antibody (Vector Laboratories,1:200, 1 h), washed and incubated with avidin-biotin complexed with peroxidase (Vector Laboratories, 1 h), followed by the application of 3,3-diaminobenzidine in H_2O_2 (DAB-kit, Vector Laboratories). All slides were counterstained with hematoxylin, dehydrated and mounted with Pertex (Histolab). As negative control, the primary antibody was omitted and the sections were incubated only with the solvent. Murine brain tissue served as a positive control. Analyses of ERα and ERβ expression in different tissues were performed blinded.

3.15 Cellular inflammatory response

Paper IV: The inflammatory response was evaluated by counting CD3- and CD11b-positive cells in the periinfarct area (Grinnemo 2008). After fixation with 4% formaldehyde (Histolab) the sections were blocked with 5% rabbit serum (Dako Cytomation) and 5% mouse serum (Dako Cytomation) in 1xTBS (stock solution 10x concentration: 87.66 g NaCl, 60.55 g Tris diluted to 1,000 ml in distilled water, pH:

7.4). The sections were subsequently incubated over night in a humidified chamber with the primary antibody. These included rat anti-mouse CD3 (clone KT3, Serotec) and rat anti-mouse CD11b (clone 5C6, Serotec). The slides were then incubated with the secondary antibody rabbit anti-rat-IgG (FITC, Dako Cytomation) for 2 hours in a humidified chamber and mounted with an anti-fading reagent containing 4,6-diamidino-2-phenylindole (DAPI) before visualization in the fluorescence microscope (Olympus BX60). Spleen from C57BL/6 mouse was used as positive controls.

3.16 Statistical analysis

Data are presented as mean±SEM.

Comparison between the groups was made by means of Student's unpaired or paired *t*-test, Mann–Whitney *U*-test, 1-way or 2-way Analysis of Variance (ANOVA) followed by Fishers's PLSD test, or Kruskal–Wallis test. Values were considered to be significantly different at a value of $p<0.05$.

4 RESULTS

4.1 Paper I

4.1.1 Aims

To compare the angiogenic effects of AdhVEGF-A_{165} and PhVEGF-A_{165} and adverse effects as regards apoptosis and ectopic expression of the transgene in a rat myocardial infarction model.

4.1.2 Results

AdVEGF-A_{165} induced substantially higher myocardial hVEGF-A expression than PhVEGF-A_{165} (17280±5467 versus 890±452 pg/ml in normal myocardium, ($p<0.001$); 938±290 versus 60±13 pg/ml in periinfarct myocardium, ($p<0.01$). However, AdVEGF-A_{165} and PhVEGF-A_{165} induced angiogenesis to a similar extent (Figure 6). Arteriolar density was increased 40-80% with the two vectors after 1 and 4 weeks after gene transfer (AdVEGF-A_{165} or PhVEGF-A_{165} versus PLacZ, ($p<0.05$). Capillary density was also increased 15-20% after one week ($p<0.05$, AdVEGF-A_{165} or PhVEGF-A_{165} versus PLacZ). 4 weeks after treatment, only AdhVEGF-A_{165} showed a tendency to increased capillary density. AdLacZ induced transient increment of capillary and arteriolar densities after 1 but not 4 weeks. Cardiac function deteriorated around 30 % with time with PLacZ treatment compared to baseline ($p<0.05$)(Figure 7), whereas it did not change with AdLacZ and improved with AdhVEGF-A_{165} ($p<0.05$) or PhVEGF-A_{165} ($p<0.05$) to 45-57%. After 4 weeks of treatment, ventricular velocity-time integral increased similarly both after PhVEGF-A_{165} and AdhVEGF-A_{165} delivery compared to PLacZ ($p<0.05$).

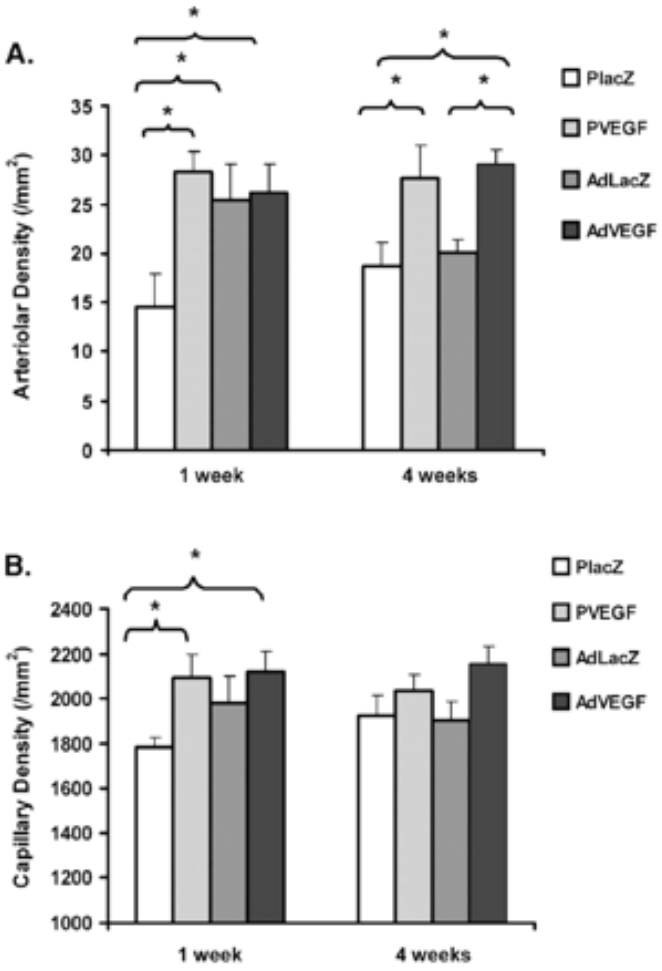

Figure 6.

*Arteriolar (A) and capillary densities (B) 1 and 4 weeks after gene transfer of PhVEGF-A_{165}, PLacZ, AdhVEGF-A_{165}, and AdLacZ. Values are mean±SEM. *p<0.05, 1 way ANOVA followed by Fisher's PLSD test.*

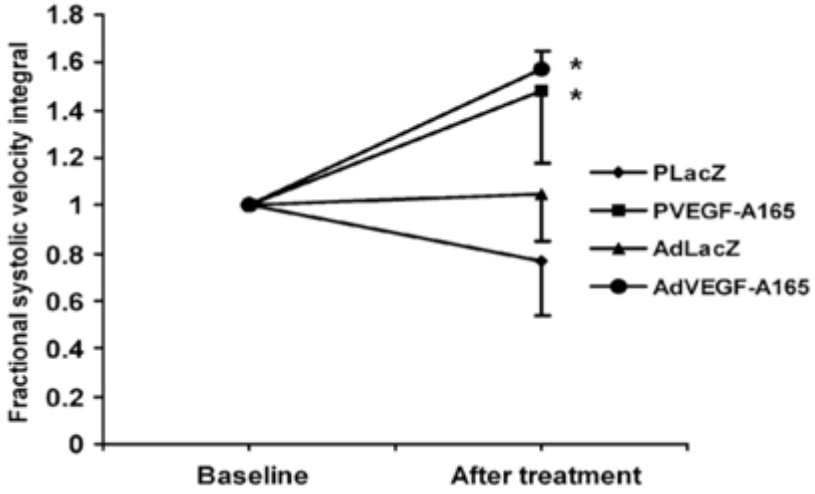

*Figure 7. Fractional change of systolic velocity–time integral 4 weeks after gene transfer of PhVEGF-A_{165}, PlacZ, AdhVEGF-A_{165}, and AdLacz. *p<0.05, versus PLacZ, ANOVA followed by Fisher's PLSD test.*

Plasmid and saline injection did not significantly increase the apoptotic cell number compared to the non-injected site, but both AdLAcZ and AdhVEGF-A_{165} induced a higher number of apoptotic cells (31±6 and 24±8 cells/field, respectively; $p<0.001$). No significant difference was observed between AdLacZ and AdhVEGF-A_{165} (Figure 8). Double staining showed that some of the cells were cardiomyocytes. Ectopic expression of VEGF was present for both AdhVEGF-A_{165} and PhVEGF-A_{165} treatment but substantially higher after AdhVEGF-A_{165} gene transfer (Figure 9).

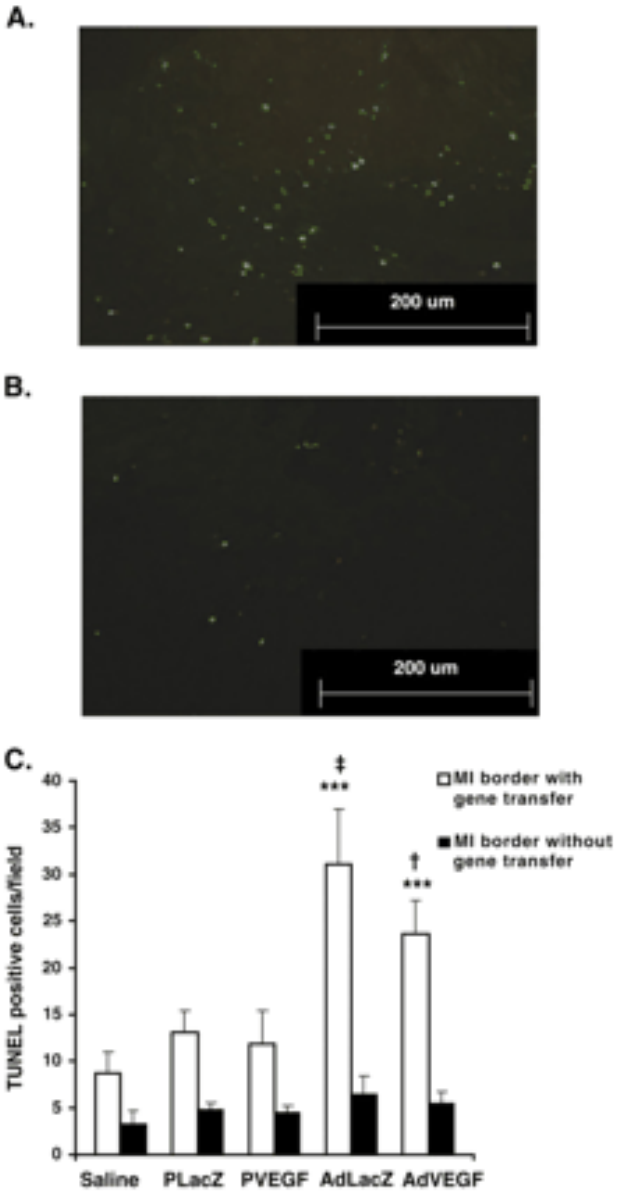

*Figure 8. Apoptotic TUNEL positive cells after treatment by AdLacZ in myocardial infarction border zones 1 week after gene transfer in injected (A)-and contralateral non-injected (B) areas 400x. (C) Counted positive cells in non-injected and injected area after treatment with saline, PLacZ, PhVEGF-A_{165} and AdLacZ and AdhVEGF-A_{165}. Values are mean±SEM. ***p<0.001. versus non-injected area, †p<0.05, p‡<0.01 versus saline.*

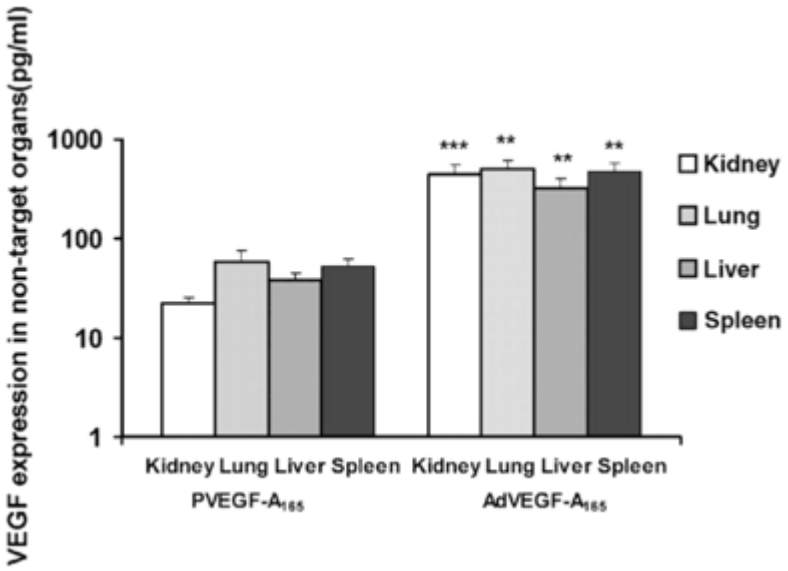

*Figure 9. Ectopic hVEGF-A-expression after intramyocardial gene transfer of PhVEGF-A_{165} and AdhVEGF-A_{165}. Values are mean±SEM. **p<0.01, ***p<0.001, AdhVEGF-A_{165} vs PhVEGF-A_{165}.*

4.1.3 Conclusions

Intramyocardial AdhVEGF-A_{165} gene transfer induced substantially higher hVEGF-A protein expression than PhVEGF-A_{165} in the myocardium. PhVEGF-A_{165} and AdhVEGF-A_{165} stimulated vessel growth and improved left ventricular function to a similar extent. AdhVEGF-A_{165} induced more apoptotic cells and also higher ectopic expression of VEGF than PhVEGF-A_{165} gene transfer. Thus at least in this myocardial infarction model AdhVEGF-A_{165} has no obvious angiogenic advantage over PhVEGF-A_{165}.

4.2 Paper II

4.2.1 Aims

To explore if darbepoietin-α in a mouse myocardial infarction model influences periinfarct cellproliferation, apoptosis, capillary and arteriolar densities, and to explore if darbepoietin-α can induce angiogenic sprouting in a murine aortic ring culture.

4.2.2 Results

Darbepoietin-α effected increased hemoglobin concentration moderately compared to control (Table 1).

	Day 0		Day 0+5		Control	
	Before	**12 d**	**Before**	**12 d**	**Before**	**12 d**
Hb g/L	154+2.2	150+2.1	148+2.5	155+4.4	154+2.5	123+2.4
Delta g/L		-19+5.6		29+2.6		-31+2.1
p vs control		<0.0001		<0.0001		

Table 1. In the three different groups darbepoietin-α (7.5 µg/kg day 5) or 2 dose darbepoietin-α (day 0 + 5) were given and the effect was compared to control mice. n=4 at each timepoint. ANOVA for the three groups $p < 0.0001$. Values are means ± SEM.

Capillary ($p < 0.01$) and arteriolar ($p < 0.03$) densities were about 80% lower in the periinfarct area (Figure 10) with no difference between the groups. Cell proliferation was about 10 times higher in the periinfarct area compared to the noninfarcted area with a darbepoietin-α dependent decrease ($p < 0.02$) of about 30% for the 2-dose regimen (Figure 11). No TUNEL staining was observed in the remote noninfarcted myocardium. TUNEL stained cells were present in the periinfarct area and the frequency of these decreased ($p < 0.005$) with darbepoietin-α (Figure 11).

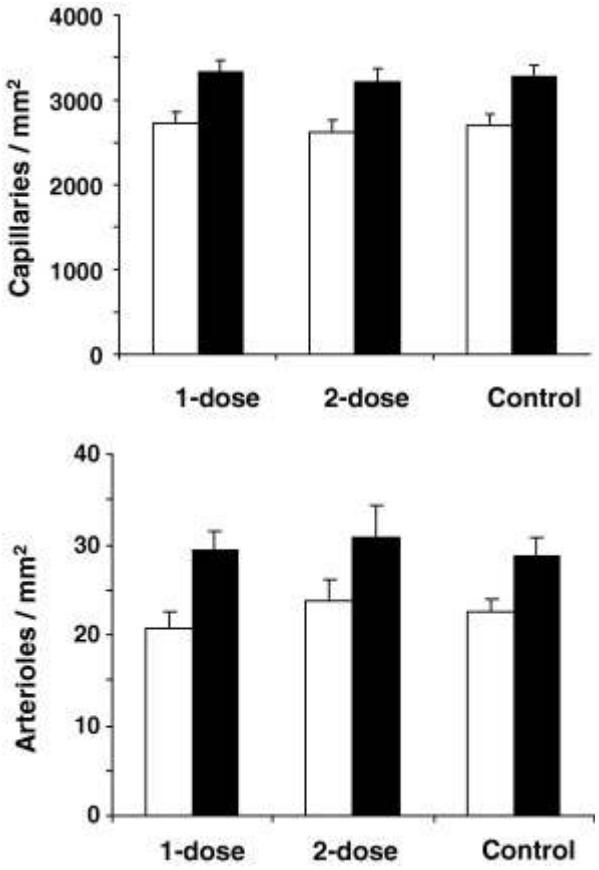

Figure 10. Capillary and arteriolar densities in periinfarct (open columns) and noninfarcted (filled columns) areas 12 days after infarction. Compared to the noninfarcted area capillary and arteriolar densities were lower in the periinfarct area ($p<0.01$ and p 0.03, respectively). Darbepoietin-α did not have any effect on the vascular densities. Values are means ± SEM.

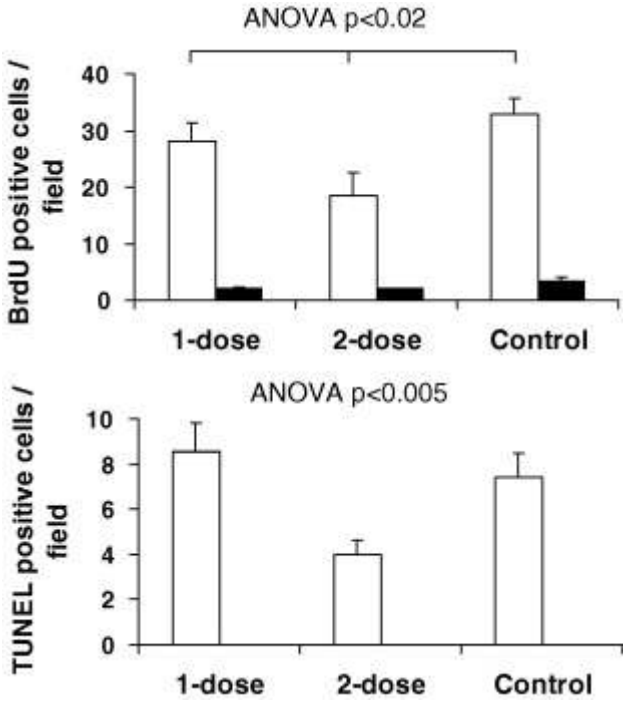

Figure 11. BrdU determined cell proliferation per field (above) in the periinfarct (open columns) and noninfarcted (filled columns) areas and frequency of TUNEL positive cells per field (below) 12 days after infarction. No TUNEL staining was present in the nonfarcted area. Values are given for the 3 groups. Values are means ± SEM.

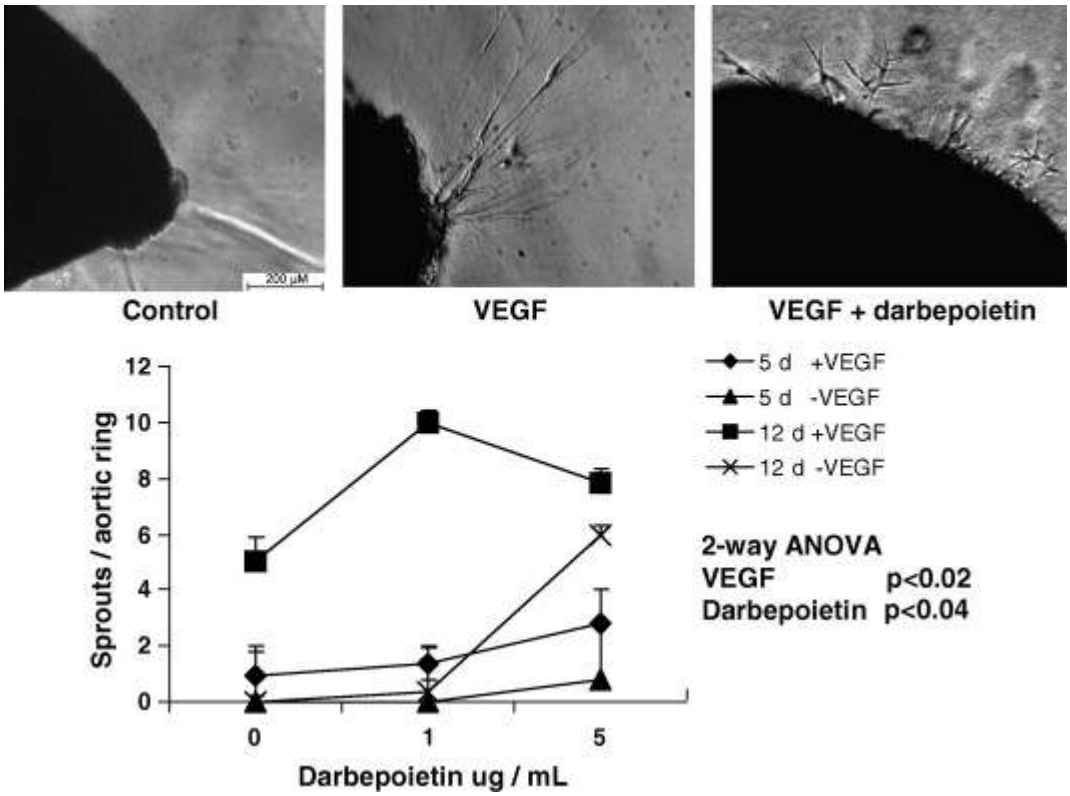

Figure 12. Sprouts from mouse aortic rings in matrigel (above) control, after addition of $VEGF_{165}$ (20 ng/mL) and $VEGF_{165}$ + darbepoietin-α after 10 days. Below quantitative results without and with $VEGF_{165}$ and darbepoietin-α 0, 1, and 5 ng/mL. N = 5 at each time point. The number of sprouts were assessed by blinded visual counting on days 5 and 12. Values are given as means ± SEM. Two-way ANOVA showed similar and independent effects of $VEGF_{165}$, $p < 0.02$ and darbepoietin-α, $p < 0.04$.

In vitro sprouting from mouse aortic rings was stimulated to a similar degree by VEGF-165 and darbepoietin-α (Figure 12).

4.2.3 Conclusions

In this study ip administration of darbepoietin-α decreases apoptosis and cell proliferation in periinfarct area, but does not affect vascular densities after LAD-ligation in mice. Darbepoietin-α has an angiogenic sprouting effect in murine aortic ring culture similar to $VEGF_{165}$.

4.3 Paper III

4.3.1 Aims

To explore if the ephrin/ Eph system is present in the mouse myocardium and if modulation can influence cell proliferation in vivo and in vitro, and if ephrin B2 Fc can induce angiogenesis in an aortic ring culture.

4.3.2 Results

EphrinB2-Fc added to human aortic endothelial cells (HAEC) culture dose-dependently increased BrdU-determined mitosis by 15%, ($p < 0.0005$). In hypoxic (1%) environment the proliferation rate was overall significantly lower than in ambient air ($p < 0.001$), and the addition of ephrinB2-Fc had only a tendency to increase the proliferation rate ($p < 0.10$). EphrinB2-Fc added to C57BL/6 aortic ring matrigel culture dose-dependently increased ($p < 0.01$) sprouting to the same level as observed after the addition of VEGF 20 ng/mL, $p < 0.05$. Counting of BrdU containing cells and of endothelial sprouting was assessed by blinded visual counting on day 7 (Figure 13).

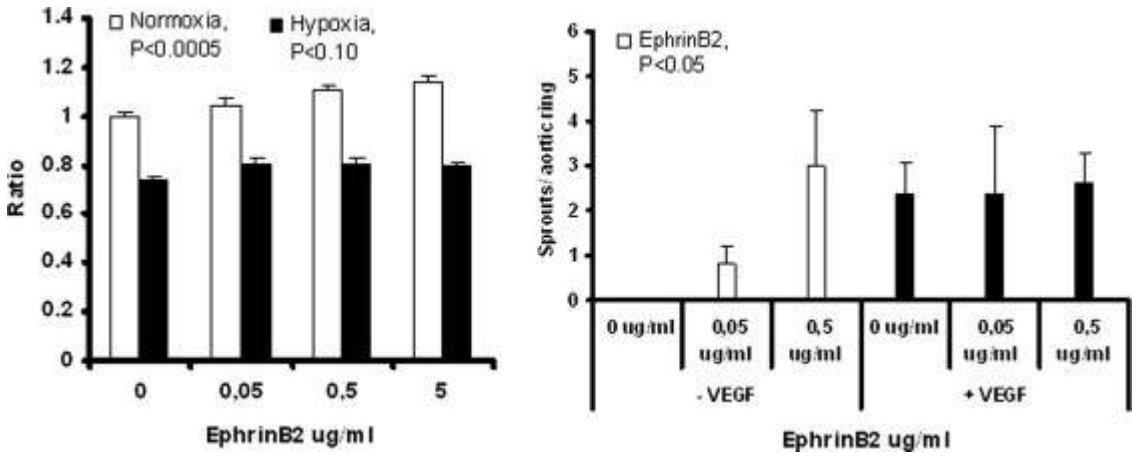

Figure 13. Left: EphrinB2-Fc chimera dose-dependently increased ($p < 0.0005$) BrdU determined proliferation of HAEC. Right: EphrinB2-Fc chimera dose-dependently increased ($p < 0.05$) aortic ring endothelial sprouting. to a level similar to that of VEGF. In the negative control, without VEGF and ephrinB2, no sprouting was seen. Values are mean ± SEM.

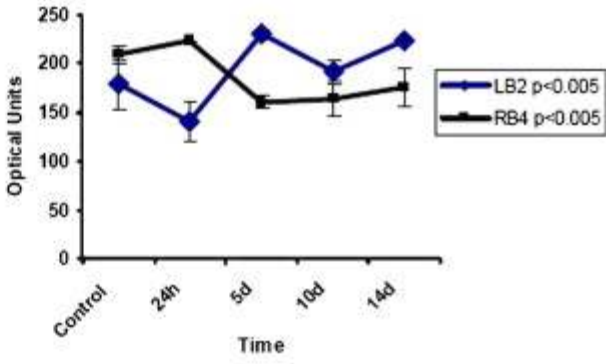

Figure 14. Myocardial mRNA of ephrinB2 (LB2), ANOVA $p < 0.005$ and EphB4 (RB4), ANOVA $p < 0.005$, analyzed by RT-PCR. Values are mean ± SEM.

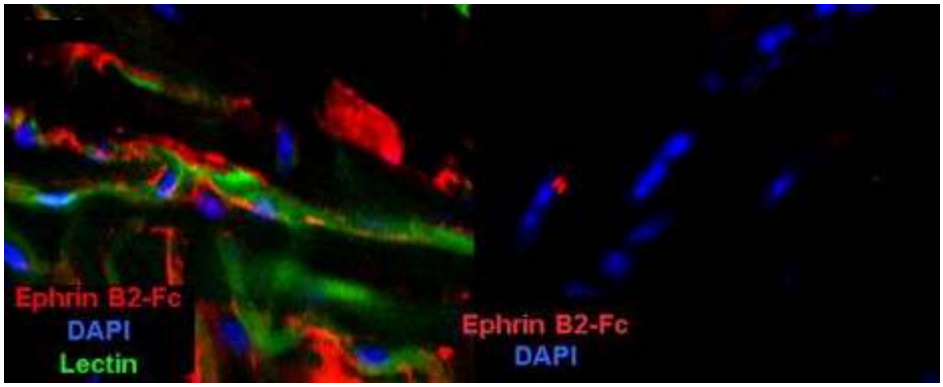

Figure 15. EphrinB2-Fc binding in myocardium. Left: Noninfarcted myocardium from mouse 24 h after i.p. injection of ephrinB2-Fc. Double staining for anti-Fc (red) and anti-GS1 isolectin B4 shows that ephrinB2-Fc binds to cells in close relation to the vessels. Right: Myocardium from control animal injected with PBS, stained with anti-Fc antibody only.

EphB4 and ephrinB2 were highly, and consistently expressed 24 h, 5, 10 and 14 days after infarction as well as in the noninfarcted control group. Samples were transmural ventricular tissue from periinfarct region in C57Bl/6 mice 0, 24 h, 5, 10 and 14 days after LAD-ligation. Each point represents n = 3–4. After an initial dip ephrinB2 was upregulated ($p < 0.005$). An opposite and dichotomous pattern of expression was seen for EphB4 ($p < 0.005$) (Figure 14). The myocardial capillary lining stained positive for ephrinB2 and lectin 24 h after i.p. injection of ephrinB2-Fc (Figure 15).

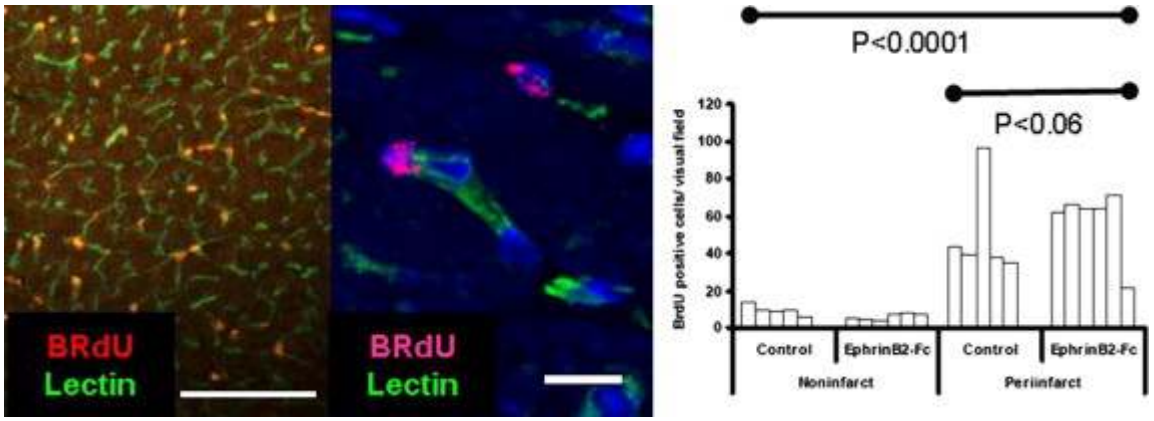

Figure 16. Increased endothelial proliferation after ephrinB2-Fc treatment post-MI.
Left: Increased proliferation adjacent to capillaries, lectin (green);and BrdU (red). Scale bar indicates 100 µm.
Middle: Increased proliferation adjacent to capillaries. Confocal microscopy of the sample described in the left figure stained for lectin (green); BrdU (red), and cell nuclei (DAPI blue). Scale bar indicates 10 µm.
Right: The number of BrdU positive cells 4 weeks after AMI in the periinfarct and non-infarcted areas. Each column represents one individual mouse. The mitotic activity in the myocardium increased ($p < 0.0001$) after myocardial infarction and with a tendency to a greater extent ($p < 0.06$) in the ephrinB2-Fc treated group. BrdU counting was done blinded.

Four weeks after myocardial infarction, the mitotic activity increased in the periinfarct region, ($p < 0.0001$), with signs of even higher mitotic activity in the ephrinB2-Fc treated group ($p < 0.06$) (Figure 16). The BrdU positive cells were located along the capillaries. EphrinB2-Fc treatment resulted in the periinfarct area in a 28% increase, ($p < 0.05$), in capillary density, not different from the capillary densities in the non-infarcted myocardium 2400 ± 253 to 3074 ± 126 capillaries/ mm^2 in the control and ephrinB2-Fc treated groups, respectively (Figure 17).

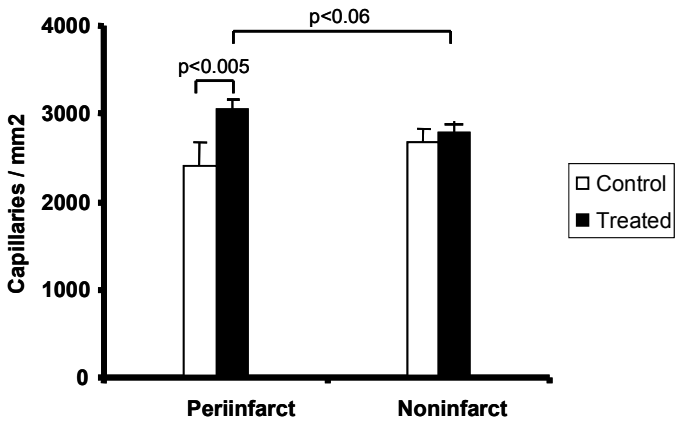

Figure 17. Myocardial capillary densities in periinfarct and noninfarcted areas 4 weeks after AMI Values are mean ± SEM. The capillary density in the peri-infarct area decreased in the control group but increased after treatment with ephrin-B2-Fc, p< 0.005. There was a tendency (p<0.06) to higher capillary density in the treated periinfarcted area compared to the noninfarcted regions in the non-treated group.

Conclusions

The ephrin/ Eph system is present in the mouse myocardium. In a mouse myocardial infarction model Ephrin-B2-Fc tends to increase the mitotic activity and prevents a decrement in capillary density in the periinfarct area. Ephrin-B2-Fc induces endothelial cell proliferation in vitro, and stimulates angiogenic sprouting in an aortic ring model.

4.4 Paper IV

4.4.1 Aims

To explore if the pattern of expression of estrogen receptors α (ERα) and β (ERβ) in myocardial tissue changes after myocardial infarction (MI), and to explore if the presence/absence of ERα or ERβ influences angiogenesis after MI.

4.4 2 Results

In ERαKO, after MI disrupted mRNA coding for ERα was higher than native ERα expression in wild type mouse ($p<0.01$)**.** Also after MI, ERβ mRNA expression was downregulated compared to wild type mouse ($p<0.01$) (Figure 18 a and b). In ERβKO-mice after MI, ERα mRNA expression was lower than in wild type mouse ($p<0.05$), while disrupted mRNA coding for ERβ in ERβKO mice was higher than native ERβ expression in wild type mouse ($p<0.01$) (Figure 19 a and b). Compared to controls (murine brain) and in ERαKO, ERβKO and wild type no expression of ERα and ERβ could be demonstrated in periinfarct myocardial tissue sections containing cardiomyocytes, capillaries and arterioles. Capillaries in the periinfarct areas were in the control-, ERαKO- and ERβKO-groups 2513±382, 2503±241, 2667±385 mm^2, and arterioles were 11±3, 11±4, 11±3 mm^2 respectively. There was no difference between the groups (Figure 20 a and b).

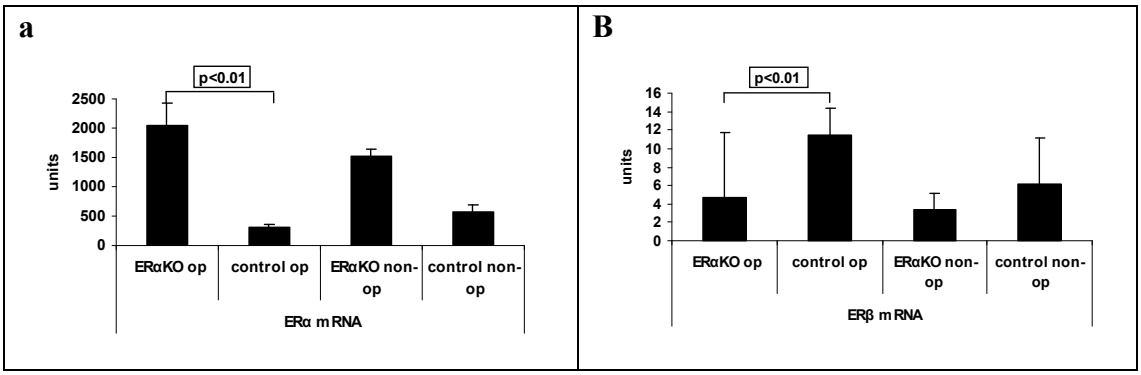

Figure 18. (a) ERα- (b) ERβ- mRNA expression in ERαKO-mice, without MI or 12 days after induction of MI, and also in littermate wildtype controls with and without MI. In the infarcted specimens periinfarct tissue was used. Animals in each group: MI operated n=5-6, non-operated n=2. Values are mean with SEM.

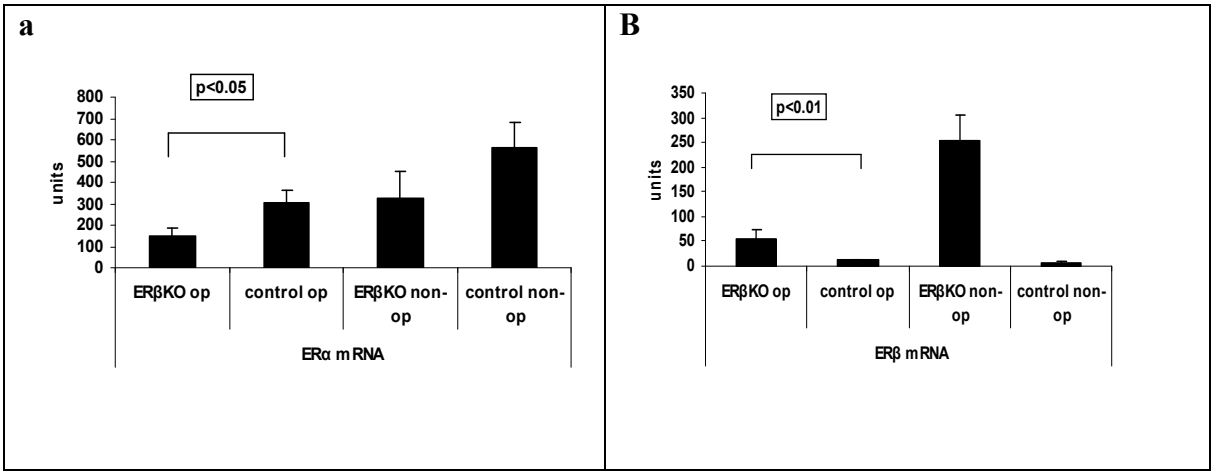

Figure 19. (a) ERα- and (b) ERβ- mRNA expression in ERβKO -mice, without MI or 12 days after induction of MI, and also in littermate controls with and without MI. In the infarcted specimens periinfarct tissue was used. Animals in each group: MI operated n=5-6, non-operated n=2.Values are mean with SEM.

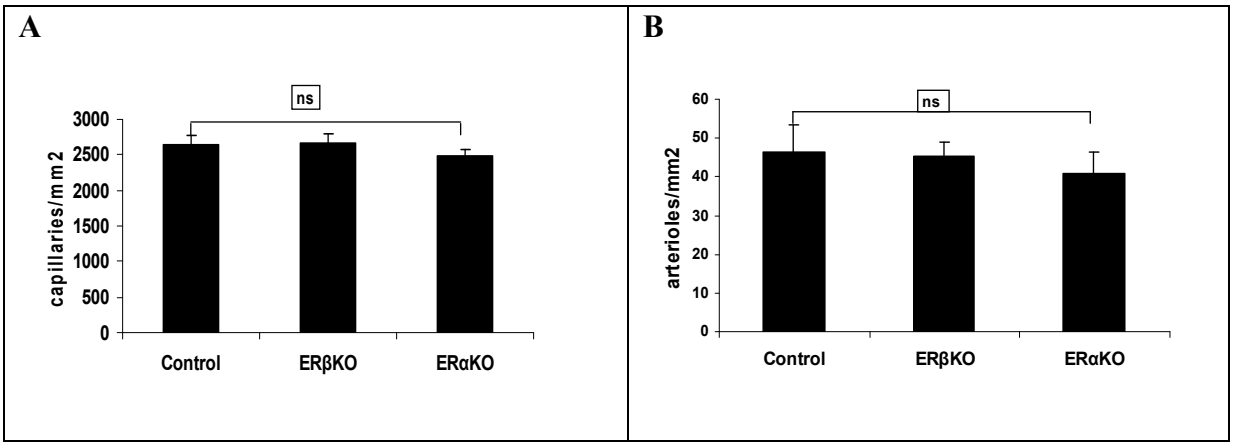

Figure 20. Periinfarct (a) capillary and (b) arteriolar densities 12 days after induction of myocardial infarction in littermate control, ERβKO and ERαKO mice. The assessment was made blinded. Values are mean with SEM.

The inflammatory response was assessed by counting CD3+ and CD 11b+ cells in the periinfarct areas. In the ERαKO group there was less macrophages (CD11b+, $p<0.05$) than in the control group (Figure 21), but there was no difference between the groups in T-cell response (CD3).

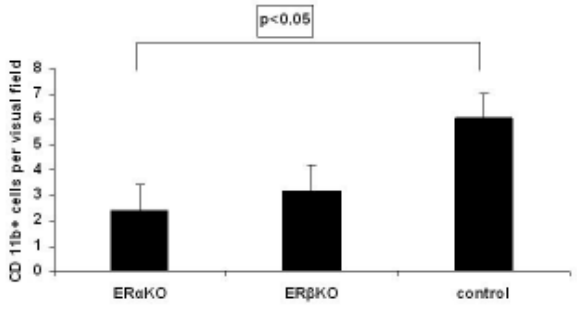

Figure 21. The number of macrophages in the periinfarct area was assessed by counting anti CD11b + cells. In the periinfarct area of ERαKO mice the number of macrophages was lower compared to control ($p<0.05$).

4.4.3 Conclusions

In ERβKO mice the downregulation of ERα and the absence of functional ERβ and in ERαKO the absence of functional ERα and the downregulation of ERβ do not influence myocardial angiogenesis or arteriogenesis after myocardial infarction.

5. General discussion

Efforts to induce an increased angiogenesis in situations where lack of vessels is the cause of serious disease has been made the past decades. Gene therapy with various angiogenic growth factors (VEGF, FGF, HIF, HGF) using different vectors and ways of delivery has been assessed in clinical studies with results showing that angiogenic therapy is feasible, at least in short term safe. The Euroinject One study (endocardial injection of naked plasmid VEGF-A_{165}) gave evidence for beneficial effects both in objective endpoints e.g. regional wall motion scores, left ventricular function and perfusion, and in subjective endpoints, eg CCS class (Kastrup 2005, Gyongyosi 2005). Post-hoc analysis of the AGENT-trials (Ad5FGF4) showed increased exercise treadmill test time for subgroups (female patient, and subjects ≥ 65 years). However, effects on mortality and major coronary events remain to be assessed with randomized controlled studies, including defining the optimal delivery modalities and vectors
In paper I hVEGF-A expression following the adhVEGF-A_{165} gene transfer was about 20 times higher than following phVEGF-A_{165} transfer in the myocardium, optimal doses chosen. This is consistent with previous studies (Laitinen 1997, Rissanen 2004). Unexpectedly, adhVEGF-A_{165} and phVEGF-A_{165} induced a similar degree of angiogenesis during the 1-month follow-up, indicating that there is not at linear relation between the level of VEGF-A and the degree of angiogenesis. Although plasmid expressed less hVEGF-A, the VEGF amount might be sufficient for the angiogenic process. AdLacZ also induced a transient increment of both capillaries and arterioles after 1 week but not after 4 weeks of treatment. This suggests that the adenoviral infection might cause an inflammatory reaction (Liu Q 2003) with secondary transient angiogenesis. We determined apoptosis 7 days after gene transfer. AdLacZ and adhVEGF-A_{165} caused a 5 to 8 fold increase in TUNEL stained cells compared with the non-injected area. It suggests that apoptosis might be induced by adenovirus in the

injection region. Adenovirus can modulate apoptosis while VEGF is known to counteract apoptosis (Thomson 2001, Meeson 1999). AdhVEGF-A_{165} showed a non-significant decrease in apoptosis compared with AdLacZ. At least some of the apoptotic cells were cardiomyocytes. Ectopic hVEGF-A gene expression in different organs was found to be around 2–5% of that in the myocardium both with adenovirus and plasmid gene transfer. This indicates a similar mechanism of systemic leakage of adenovirus and plasmid vectors from local myocardial delivery. However, AdhVEGF-A_{165} still induced much higher hVEGF-A expression than plasmid. In the myocardial infarction model used, the therapeutic window for myocardial angiogenesis appears to be saturated already with the hVEGF-A expression induced by plasmid gene transfer. This indicates that PhVEGF-A_{165} might be more applicable than AdhVEGF-A_{165} for therapeutic angiogenesis.

Erythropoietin production is triggered by hypoxia, and its main erythropoetic effect is suggested to be through decreased apoptosis of erythroid progenitor cells (Ghezzi 2004, Diwan 2007) Protection against ischemic injury, and glucose depravation in neuronal tissue are some of the cytoprotective actions described (Ghezzi 2004) also and various tissues seem to benefit from protective effects, ie, vascular smooth muscles, endothelium and cardiomyocytes (Joyeux-Faure 2007). Several animal studies show that erythropoietin has cardioprotective effects in the acutely ischemic heart, with decreased infarct size and increased neovascularisation. One possible cardioprotective mechanism is inhibited myocyte apoptosis, or modulation of the inflammatory response (Liu 2006). However, the cardioprotective effects have been demonstrated at high doses of erythropoietin, causing a substantial increment of the hematocrit. In a human context this increases the risk for serious side effects like thromboembolism, hypertension, cardiac overload and neurotoxicity, which might explain why clear benefits have not been proven in clinical trials.

In paper II therapeutically relevant doses of darbepoietin-α caused moderate increase of hemoglobin concentration and it had no effect on the periinfarct capillary and arteriolar densities in this mouse myocardial infarction model. However apoptosis was decreased in the periinfarct area. Mitosis, determined by BrdU, covaried with the degree of apoptosis (TUNEL staining) with decreased activities following darbepoietin-α administration. The cause of this is unclear. The hypothesis that this might be dependent on an antiinflammatory effect was explored with CD3 and CD11 stainings (data not shown). However, no effect of darbepoietin-α was observed on these expressions. In vitro darbepoietin-α induced sprouting from aortic rings similar to VEGF-A_{165}. As previous investigations have been made with considerably higher doses of erythropoietins (Tramontano 2003, Moon 2005) it thus appears that darbepoietin-α stimulation of angiogenesis in vivo is dose dependent. In an vitro mouse aortic ring matrigel culture with increasing doses of darbepoietin-α, did have an angiogenic sprouting effect of similar order as VEGF-A_{165}. Although any angiogenic effect could not be confirmed in the used mouse permanent occlusion myocardial infarction model, the moderate dose of darbepoietin-α used did counteract cell proliferation and apoptosis, indicating a possible mechanism for a cardioprotective effect of erythropoietin at acceptable hematocrit.

The Eph/ephrins have influence axon guidance, cell migration, and cell attachment during embryogenesis (Pasquale 1997, Brantley-Sieders 2004). Mouse embryos lacking either ephrinB2 or EphB4 die from severe malformations in the cardiovascular system with for example defects in cardiac trabeculation and poor association between the endothelium and the periendothelial cells (Wang 1998). Ephrin B2 is predominantly expressed on arterial endothelial cells and EphB4 on venous endothelial cells (Wang 1998, Adams 1999). The Eph/ephrins are expressed postnatally in neural, epidermal, and hematopoietic progenitor cells (Conover 2000). In paper III we show that EphB4

and ephrinB2 are expressed in the adult myocardium, and interestingly the level of expression changed after myocardial infarction in that EphB4-ephrinB2 showed a biphasic and opposing expression pattern following myocardial infarction. EphrinB2 was, after an initial dip, upregulated. EphrinB2-Fc increased the proliferation rate of cultured HAECs under normoxia, and a tendency to increase was also observed under hypoxia. In cultured aortic rings ephrinB2-Fc was as potent as VEGF in the induction of sprout formation. This is potentially of major significance because it reveals that ephrin signaling can be of similar importance as VEGF. This indicates a functional involvement of EphB4-ephrinB2 in the angiogenic process. In this mouse- myocardial infarction model, after treatment with ephrinB2-Fc capillary density in the periinfarct region of the myocardium was increased about 28% to the level observed in the non-infarcted myocardium. Cell division was located to the vascular and perivascular areas in the periinfarcted regions with no obvious activity in cardiomyocytes and in the normal non-infarcted myocardium. This indicates that ephrinB2-Fc specifically stimulates angiogenesis in the adult ischemic myocardium and not targets healthy muscle. The magnitude of increment of vascular densities is not different from that previously reported from our laboratory following overexpression of other tyrokine kinase receptors and angiogenic factors such as VEGF-A_{165}, PDGF-BB, and angiopoietin-1 (Siddiqui 2003, Hao 2004).

In paper IV, ER expression in ERαKO, ERβKO and wild type mice, was not detectable in the myocardium at least at the level that is detectable by immunohistochemistry. However, at the mRNA expression level non-functional disrupted both ERα and ERβ were upregulated compared to wild type control. ERβ in ERαKO and ERα in ERβKO were downregulated. These observations suggest that although no periinfarct protein receptor expression or angiogenic effects were observed adaptive effects on some undetermined ER functions may have occurred. There is ample evidence on the

vascular protective effects of estrogen in premenopausal women. Estrogen modifies for example systemic vascular tone by effects on NO mediated mechanisms (Arias-Loza 2008, Byers 2005). Such systemic effects may modify the load on the damaged heart, and consequently remodelling and prognosis. It has also been reported that estrogen can influence VEGF expression (Mancino 2009, Gargett 2002). Whether there are direct effects on the myocardium and its vascular structures have not been settled. Förster et al reported that no ER could be demonstrated in the mouse heart (Förster 2004). We therefore found it of relevance to explore if ER are expressed during stress such as acute myocardial infarction and if such expression influences angiogenesis in the ischaemic myocardium. With the mouse infarction model used we have previously reported that in the perinfarct area vascular densites are depressed, probably as an adaptive hibernating response to ischemia. Angiogenesis both at the capillary and arteriolar levels are stimulated following transient overexpression of angiogenic growth factors such as VEGF-A_{165}, PDGF-BB, FGF and angiopoietin-1 suggesting that the model is sensitive enough to detect a possible angiogenic effect of ER activation. Our results suggest that at least in this mouse infarction model ER expression is not induced. Macrophage density was depressed in the periinfarct area in ERαKO mice suggesting a decreased postinfarct inflammatory response. This effect may be viewed as an indirect ER effect on the myocardium. Although this anti-inflammatory response did not effect periinfarct angiogenesis it is in keeping with estrogen-dependent proinflammatory effects and macrophage activation (Cutolo 2004) and that this effect is related to ERα.

6. Conclusions

1. AdhVEGF-A_{165} does not have any superior obvious angiogenic efficacy to PhVEGF-A_{165} but more side effects in a rat myocardial infarction model.

2. Darbepoietin-α decreases cell proliferation and apoptosis in the periinfarct area in a mouse myocardial infarction model, but capillary and arteriolar densities are unchanged. Darbepoietin-α can induce angiogenic sprouting in a murine aortic ring culture.

3. The ephrin/ Eph system is present in the myocardium. In a mouse myocardial infarction model Ephrin B2 Fc tends to increase the mitotic activity and prevents a decrement in capillary density in the periinfarct area. Ephrin B2 Fc induces endothelial cell proliferation in vitro, and stimulates angiogenic sprouting in an aortic ring model.

4. Estrogen receptors are present in the myocardium at least at the mRNA level. After myocardial infarction in ERβKO ERα and in ERαKO ERβ were downregulated, and angiogenesis or arteriogenesis were not influenced. At least in this model ER therefore seems not to have a role in myocardial arteriogenesis and angiogenesis after myocardial infarction.

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