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Myocardial Angiogenesis Induced by Plasmid VEGF-A$_{165}$ Gene Transfer

Experimental and Clinical Studies

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Always look on the bright side of life

Monthy Python´s Life of Brian

ABSTRACT

New therapeutic options have emerged for patients with medically intractable angina who are not candidates for conventional revascularisation techniques, from increasing understanding of the biology of vessel formation and how different angiogenic growth factors participate in the process. Administration of vascular endothelial growth factor-A_{165} (VEGF-A_{165}), a key regulator of vessel growth, has been shown to augment myocardial collateral growth. The research programme behind this thesis focuses on angiogenic gene therapy using plasmid encoding VEGF-A_{165} as a vector.

In study I the bioactivity of phVEGF-A_{165} after intramyocardial injection in normoxic and hypoxic rats was examined morphologically. A dose-response correlation between the number of VEGF-A expressing cells as well as the angiogenic effect in terms of microvessel density was observed. Hypoxic conditions had a tendency to enhance both plasmid expression and angiogenesis in rat myocardium.

In study II the morphological localization of gene expression following catheter-based transendocardial injection of plasmid encoding VEGF-A_{165} or a reporter protein in pig hearts was examined. After three days plasmid transfection and protein expression was macroscopically localised only in the target areas and microscopically localised at all levels of the left ventricular wall. VEGF-A_{165} and reporter protein were expressed to the same degree.

In study III the behaviour of plasmids encoding VEGF-A_{165} or a reporter gene injected directly into rat hearts was explored. Transfection was localized only at the injection site, with minimally detected systemic spread. The time kinetics of different plasmids was similar, with a rapid though short gene expression independent of which promotor was used. Dose-response expression of different genes was likewise similar, with an increase in protein levels detected only up to a certain dose injected plasmid after which protein expression plateaued.

In study IV plasmid uptake and protein expression was compared after injection of the same dose of the same reporter plasmid into skeletal and cardiac muscle in rats. Plasmid DNA levels one day after gene transfer was approximately 3-fold higher in skeletal than in cardiac muscle, but had by day three abruptly decreased to levels significantly lower than in cardiac muscle. Luciferase activity in cardiac muscle was 40-fold higher than in skeletal muscle one day and 10-fold higher three days post-transfection. The efficacy of plasmid gene transfer hence differs between tissues.

In study V seven patients with end-stage angina pectoris were included in an open phase I trial testing the safety and efficacy of intramyocardial injection of phVEGF-A_{165} via a mini-thoracotomy as sole therapy. A transient increase in plasma VEGF-A levels was observed. Two months after gene transfer improved perfusion in the target region was detected in four patients and improved function in all patients. Furthermore, a significant symptomatic relief that was retained under the twelve-month follow-up period was documented.

Conclusions: These results suggest that positive therapeutic effects can be achieved by angiogenic myocardial gene therapy using plasmid vectors. Catheter-based intramyocardial gene transfer is feasible to accomplish with high anatomical precision, making it possible to design randomized, blinded clinical trials. The results also emphasize the importance of continued research on the pharmacokinetics and pharmacodynamics of plasmid vectors as well as improvement and refinement of plasmid constructs in order to amplify their clinical utility.

Keywords: coronary artery disease, vascular endothelial growth factor, therapeutic angiogenesis, gene transfer, plasmid DNA, gene expression.

Contents

PAPERS I-V

List of Original Papers

This thesis is based on the following papers, referred to in the text by their Roman numerals:

I. Sylvén C, Sarkar N, Wärdell E, Jamsa A, Drvota V, Blomberg P, Bin Islam K. Protein and angiogenic dose-response expression of phVEGF-A(165) gene in rat myocardium. J Thromb Thrombolysis. 2001 Oct;12(2):151-6.

II. Sylvén C, Sarkar N, Insulander P, Kennebäck G, Blomberg P, Islam K, Drvota V. Catheter-based transendocardial myocardial gene transfer. J Interv Cardiol. 2002 Feb; 15(1): 7-13.

III. Sarkar N, Blomberg P, Wärdell E, Eskandarpour M, Sylvén C, Drvota V, Islam KB. Nonsurgical direct delivery of plasmid DNA into rat heart: time course, dose response, and the influence of different promoters on gene expression. J Cardiovasc Pharmacol. 2002 Feb; 39(2): 215-24.

IV. Sarkar N, Gustavsson T, Norman B, Wiik A, Drvota V, Blomberg P, Sylvén C. Transgene expression efficiency does not directly reflect plasmid DNA uptake efficiency in skeletal and cardiac muscle in rats. Submitted.

V. Sarkar N, Rück A, Källner G, Y-Hassan S, Blomberg P, Islam KB, van der Linden J, Lindblom D, Nygren AT, Lind B, Brodin L-Å, Drvota V, Sylvén C. Effects of intramyocardial injection of phVEGF-A165 as sole therapy in patients with refractory coronary artery disease -12-month follow-up: angiogenic gene therapy. J Intern Med. 2001 Nov;250(5):373-81.

List of Abbreviations

Ad	adenovirus
CABG	coronary artery by-pass grafting
CAD	coronary artery disease
CCS	Canadian Cardiovascular Society
CEA	chorioembryonic antigen
CM	cardiac muscle
CMV	cytomegalovirus
cDNA	recombinant DNA
pDNA	plasmid DNA
ECM	extracellular matrix
EGFP	enhanced green fluorescence protein
EPC	endothelial progenitor cells
ETT	exercise treadmill test
aFGF	acidic fibroblast growth factor
bFGF	basic fibroblast growth factor
HIF-1	hypoxia inducible factor-1
ic	intracoronary
im	intramyocardial
PCI	percutaneous coronary intervention
PSA	prostate specific antigen
RT-PCR	real time-polymerase chain reaction
SM	skeletal muscle
SPECT	single photon emission computerized tomography
TVI	tissue velocity imaging
VEGF-A_{165}	vascular endothelial growth factor isoform A of 165 amino acids
phVEGF-A_{165}	plasmid encoding human VEGF-A_{165}
VEGFR-1	vascular endothelial growth factor recptor-1
VEGFR-2	vascular endothelial growth factor recptor-2
VPF	vascular permeability factor

INTRODUCTION

Background

Coronary artery disease (CAD) is the leading cause of morbidity and mortality worldwide [1]. Despite continued advances in the prevention and treatment of CAD there is a growing group of patients with substantial myocardial ischemia, who remain inadequately treated by anti-angina medication and are not amenable to further revascularisation by percutanueous coronary intervention (PCI) or coronary artery by-pass grafting (CABG) [2].

It has long been known that patients with CAD tend to develop natural by-pass vessels, i.e. collaterals. The importance of well developed coronary collateral circulation has been documented in several clinical studies [3-5]. Presence of collaterals modifies the detrimental effects of coronary artery occlusion, lowering the incidence of myocardial infarction, improving the clinical outcome and long-time survival [6]. Neovascularization is the natural response in organs subjected to ischemia, in an attempt to minimize tissue damage and maintain organ function. The interindividual variation in this adaptive response to myocardial ischemia is however large, and up to two-thirds of CAD patients do not have sufficient collateralization to prevent myocardial ischemia [7].

Increasing knowledge during the last two decades of the biological process of vessel growth, together with the uncovering of the ability of vascular growth factors to alter vessel formation as well as the isolation and cloning of these factors, has given rise to a new potential treatment strategy for CAD patients. Based on initial data, Hockel et al 1993 proposed the term "therapeutic angiogenesis" to describe the induction or stimulation of new vessel growth for the treatment or prevention of clinical disorders characterized by local hypovascularity [8]. Delivery of vascular growth factors to ischeamic tissue has subsequently in several experimental studies and a few clinical trials been documented to enhance collateral vessel formation [9, 10]. Vascular endothelial growth factor-A_{165} (VEGF-A_{165}) is one of many angiogenesis stimulating factors so far isolated. VEGF-A_{165} appears to be a key regulator of vessel growth with its mitogenic activity primarily directed towards vascular endothelial cells, making it an appealing candidate to use for therapeutic angiogenesis [11].

The research programme behind this thesis focuses on the use of plasmid DNA as a vector for myocardial VEGF-A_{165} gene therapy.

History of angiogenesis factors

In the late 1960s two reports hypothesized that the angiogenesis necessary for tumour growth was mediated by diffusible angiogenic factors derived from tumour cells [12, 13]. Judah Folkman in 1971 first launched the innovative idea of developing angiogenesis inhibitors to treat tumours [14], and the same year in a second pivotal paper reported the finding of a soluble factor that stimulated blood vessel growth, tumour angiogenesis factor (TAF) [15]. Numerous reports on the identification of various multifunctional growth factors and their angiogenic effect appeared during the following years [16]. Two drew much attention: basic fibroblast growth factor (bFGF) identified 1974 by Gospodarowicz and acidic FGF (aFGF) in 1979 by Maciag [17, 18]. The FGFs (today a large family) were shown to have pleiotropic effects, with mitogenic activity directed to a broad spectrum of cells [19, 20]. The definitive sequencing of aFGF and bFGF was reported in 1985 [21, 22], and cDNA cloning of both the following year [23, 24]. Unexpectedly, it became clear they lacked secretory signal

sequences and could not be efficiently secreted [19]. So the search for diffusible angiogenesis factors continued.

In 1983 Dvorak´s group studying the physiological properties of tumour blood vessels, identified a protein that induced vascular leakage, vascular permeability factor (VPF) [25]. The authors had no reason to suspect it had mitogenic activity. In 1989 Ferrara´s group presented a protein showing growth-promoting activity only towards endothelial cells, isolated and sequenced to be distinct from FGF [26]. They hence named it vascular endothelial growth factor (VEGF) [26]. Meanwhile, Connolly et al in 1989 had independently isolated and sequenced human VPF [27]. By the end of 1989 both groups simultaneously reported cDNA cloning of VPF and VEGF [28] [29]. These papers revealed that VPF and VEGF were one and the same and importantly, contained a secretory signal sequence. The VEGF family now, apart from the native VEGF (VEGF-A), includes placental growth factor [30], VEGF-B [31], VEGF-C [32, 33], VEGF-D [34] and VEGF-E [35].

Therapeutic angiogenesis

Once the ability of VEGF to induce growth of new blood vessels *in vivo* was reported [36], investigation of the potential use of angiogenesis factors for the treatment of ischemic organs became the obvious next step. Taking the lead Banai et al 1991 demonstrated that administration of aFGF induced angiogenesis in a dog ischemic heart model [37]. Next, intra-coronary delivery of bFGF after myocardial infarction 1992 [38], and VEGF-A$_{165}$ in ischemic myocardium 1994 [39] were reported to augment neo-vascularization and preserve left ventricular function in canine models, while intraarterial delivery and intramuscular injection of VEGF-A$_{165}$ increased collateralization in a rabbit hind-limb ischemia model [40] [41]. The hypothesis that angiogenesis stimulation had therapeutic potential was thus confirmed. The gene therapy approach was introduced in 1996 when intramuscular injection of plasmid DNA encoding for VEGF-A$_{165}$ (phVEGF-A$_{165}$) was reported to improve tissue perfusion in the same ischemia model [42]. Proof of principle in humans was established in 1998 when intramuscular injection of phVEGF-A$_{165}$ in patients with critical limb ischaemia was shown to enhance collateral growth [43].

Many more factors involved in vessel growth have been identified [44], but the FGFs and VEGF-A are so far the most extensively investigated in both preclinical and human trials [9, 45]. The use of VEGF-A for therapeutic angiogenesis is appealing, as endothelial cells, in contrast to the FGFs, are the primary targets for its activity.

Vessel growth

Postnatal vessel growth is, except in the female reproductive system and in wound healing, normally a rare event. It is, however, a defining feature of several pathological conditions [46]. Adult neo-vascularization predominantly occurs through angiogenesis and arteriogenesis [47]. It has recently been discovered that adult neovascularization also involves vasculogenesis [48]. An array of angio-genic and anti-angiogenic factors and their receptors have been identified to interact with each other and with multiple cells in the process of vessel growth (Figure 1) [47].

Angiogenesis

Angiogenesis is a complex multistep process by which new capillaries sprout and differentiate from pre-existing post-capillary networks [47]. The first step is thought to be activation of endothelial cells and vasodilatation of the parent vessel, followed by increased vascular permea-bility, largely mediated by nitric oxide and VEGF-A [49, 50]. Plasma protein leakage degrades the basement membrane and extracellular matrix (ECM), which liberates sequestered growth factors and provides a temporary support for migrating

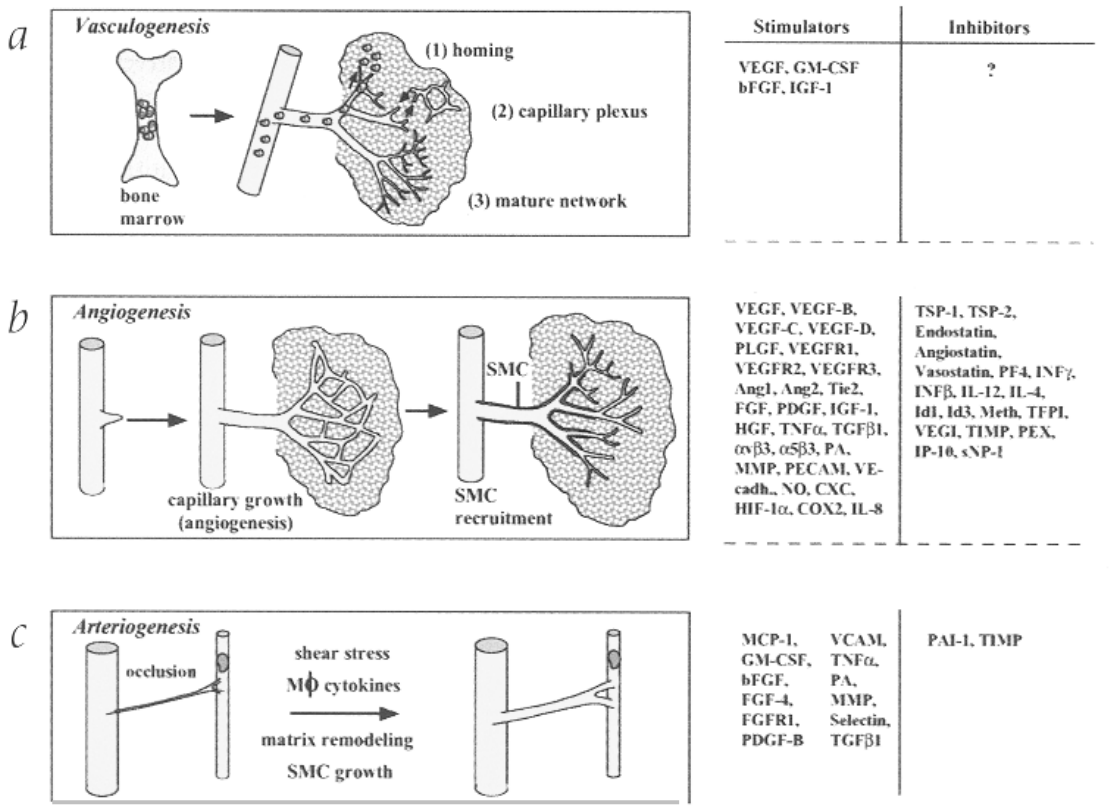


Figure 1. *Adult vessel growth may occur via: a) vasculogenesis, i.e. mobilisation and in situ differentiation of endothelial precursor cells, b) angiogenesis, i.e. capillary sprouting, c) arteriogenesis, i.e. remodelling of pre-existing vessels. SMC=smooth muscle cells. (Adapted from ref 46.)*

endothelial cells [50]. Activated endothelial cells then migrate to the site where angiogenesis is needed, proliferate, assemble into solid cords forming anastomotic connections with each other and acquire a lumen [51]. Next, pericytes are recruited to subsequently surround the new vessels in variable layers, and a new basement membrane is formed [51]. Finally a re-modeling of the vascular network occurs, with maturation and stabilization of the vessels and re-differentiation of endothelial cells into a quiescent phenotype [52]. Once the final step is completed, new vessels can survive for several years [52].

Hypoxia or ischemia after arterial occlusion is the natural stimulus for angiogenesis in adults. The angiogenic response to acute hypoxia is at least in part mediated by enhancement of VEGF-A expression by the binding of the transcription factor hypoxia inducible factor-1 (HIF-1) to the hypoxia response element in the promotor region of the VEGF-A gene [53]. Hypoxia also stabilises VEGF-A mRNA [54].

Arteriogenesis

Arteriogenesis is a further functional modification of already formed conduit arterial vessels, in order to adapt to the demand of different tissues. Post-natal arteriogenesis, in contrast to angiogenesis, seems to be independent of hypoxia and instead related to shear-stress induced up-regulation of angiogenic and inflammatory factors [55]. After occlusion of a supply artery in the myocardium, pre-existing conduit arterioles can increase 20-fold in size and calibre. In response to the increase in blood flow and consequent increase in shear stress in the lumen, activated endothelial cells by expressing chemokines recruit monocytes that infiltrate and proteolytically remodel the vessel wall

[55]. Smooth muscle cells, recruited by PDGF-BB and VEGF-A, migrate along and cover the pre-existing vessels with a thick muscular layer, subsequently providing them visco-elastic and vasomotor properties [56]. This process finally results in the formation of functional arteries [55, 56].

Vasculogenesis

Vasculogenesis was previously thought to be restricted only to embryonic vessel development. Recent studies have however identified endothelial precursor cells (EPC) in bone marrow and circulating in peripheral blood in adults [57, 58], and demonstrated that they are recruited for *in situ* vessel growth [58-60]. VEGF-A and granulocyte-monocyte colony-stimulating factor (GM-CSF) have been identified to stimulate EPC differentiation and mobilisation to sites of neovascularization [59, 61].

VEGF-A

Structural and biochemical features

The VEGF-A gene is organized as eight exons separated by seven introns [62, 63]. Originally four VEGF-A isoforms of 121, 165, 189 and 206 amino acids (VEGF-A_{121}, VEGF-A_{165}, VEGF-A_{189}, VEGF-A_{206}) were identified to be derived from the single gene by alternative splicing [62, 63] (Figure 2). Later the less frequent VEGF-A_{145} and VEGF-A_{183} isoforms were found [64]. VEGF-A_{165} is the predominant isoform produced by a variety of cells, VEGF-A_{121} and VEGF-A_{189} are detected in most cells expressing the VEGF gene, while VEGF-A_{206} is extremely rare [11, 64].

All VEGF-A isoforms have a secretory signal sequence permitting active secretion from intact cells. Differing binding ability to heparin and heparan-sulfate on the cell surface and in the extracellular matrix distinguishes the different VEGF-A isoforms [65]. VEGF-A_{121} does not bind to heparin and is freely diffusible, VEGF-A_{165} is diffusible though a significant fraction remains bound to the cell surface and the ECM, while VEGF-A_{189} is almost completely sequestered in the ECM [65]. The ECM-bound isoforms can be released by proteolytic cleavage into a soluble active fragment of 110 amino acids, though with a substantial loss of mitogenic activity [66].

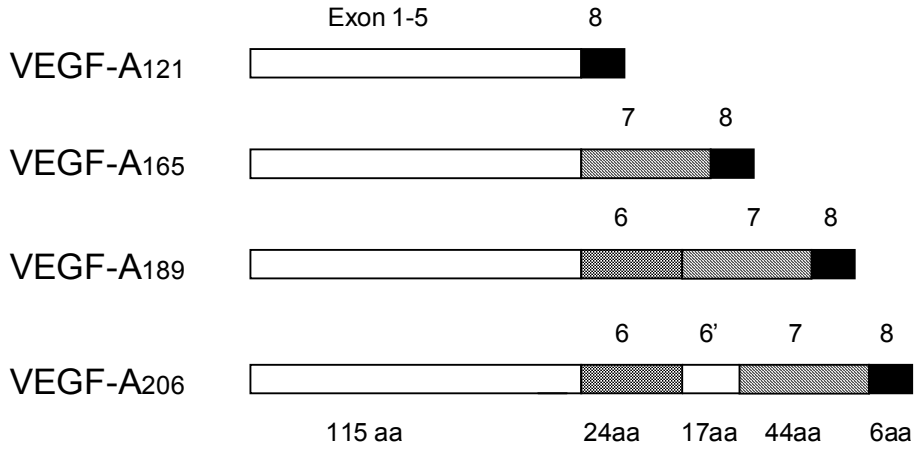


Figure 2. *Comparison of the structures of the VEGF-A isoforms*

VEGF-A receptors

There are two known related receptor tyrosine kinases with high binding affinity to VEGF-A, VEGFR-1 (flt-1) and VEGFR-2 (KDR/Flk-1) [11]. Both VEGFR-1 and VEGFR-2 have seven immuno- globulin-like loops in the extracellular domain, a single transmembrane region and intracellular ligand-stimulatable tyrosine kinase domains [11] (Figure 3). The VEGFR-1 and VEGFR-2 receptors are expressed almost exclusively on vascular endothelium cell, but also occur on bone marrow-derived cells [11]. An alternatively spliced, soluble form of VEGFR-1 is an inhibitor of VEGF-A activity [67].

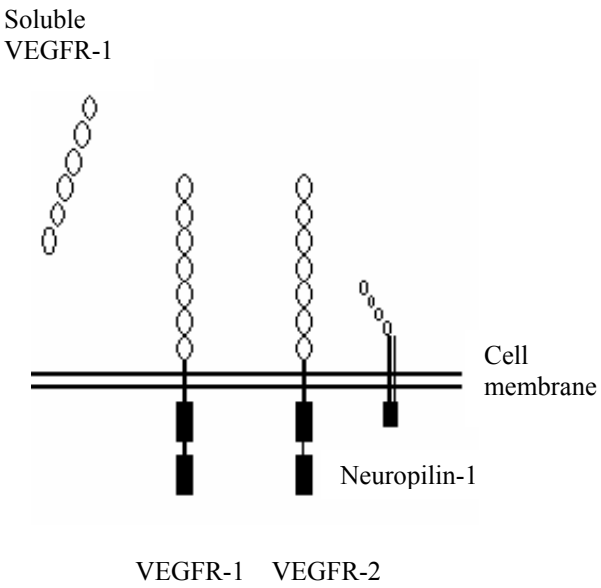


Figure 3. *VEGF-A receptors*

VEGFR-1 but not VEGFR-2 expression is upregulated by hypoxia [67]. The role of VEGFR-1 is disputed, but its main function appears to be induction of monocyte migration and maybe recruitment of EPC [67]. VEGFR-2 is the major mediator of the mitogenic, angiogenic and permeability enhancing effects of VEGF-A [67].

Neuropilin-1 (NP-1) is a VEGF-A_{165} specific endothelial cell receptor [68]. NP-1 functions as a co-receptor to VEGFR-2, enhancing VEGF-A_{165} binding to VEGFR-2 and VEGF-A_{165} mediated chemotaxis [68].

Biological properties of VEGF-A and its receptors

VEGF-A, unlike other known angiogenic growth factors, has a unique combination of qualities. Its secreted isoforms exert a highly specific effect on endothelial cells via the receptors VEGFR-1 and VEGFR-2 [11]. VEGF-A_{165} expression is upregulated by hypoxia, with promotion of angiogenesis in ischaemic tissue [53, 54, 69]. VEGF-A induces a pleiotropic response, increasing vascular permeability, stimulating endothelial cells to proliferate, migrate and assemble into tubes [11, 50]. It has a paracrine effect [11]. It is a survival factor for endothelial cells [70-73]. It promotes monocyte chemotaxis [74] and induces colony formation by granulocyte-macrophage progenitor cells [75].

VEGF-A is a key factor in the development and differentiation of the vascular system. Mice lacking only one of the VEGF-A alleles died before birth due to defects in the development of the cardiovascular system [76, 77]. Inactivation of the VEGFR-2 gene in mice led to deficient development of hematopoietic and endothelial cell lineages, while inactivation of the VEGFR-1 gene resulted in severe abnormalities in blood vessel formation, both resulting in embryonic death [78, 79].

Therapeutic myocardial angiogenesis

Rationale for therapeutic angiogenesis in CAD patients

The natural response to acute ischaemia caused by arterial occlusion includes upregulation of angiogenic growth factors, such as VEGF-A. This response is sometimes not robust enough to mediate sufficient new vessel growth. Angiogenesis in response to ischemia has been shown to be impaired by old age [80],

diabetes [81, 82] and hypercholesterolemia [83, 84]. All these factors are associated with endothelial dysfunction that at least in part is due to reduced VEGF-A expression. Interindividual differences in response to ischemia may constitute another reason for impaired neovascularisation. Schultz et al for example reported that patients with angiographically visible collaterals had greater monocyte VEGF production in response to hypoxia compared to those with reduced collaterals [85].

Inadequate production of angiogenic growth factors or an attenuated response to them might explain why some CAD patients have insufficient collateralization. They often are older, have diabetes and hypercholesterolemia. Treatment with VEGF-A gene transfer partially corrected the angiogenic response to ischemia in animal models of diabetes and hypercholesterolemia [81, 83, 86]. CAD patients may similarly benefit from administration of exogenous angiogenic growth factors, as even marginally increased levels might be sufficient to generate a biological response, alleviating ischemic symptoms and improving myocardial function.

Protein therapy versus gene therapy

Two strategies can be used to achieve an over-expression of angiogenic growth factors to induce angiogenesis: delivery of recombinant proteins or delivery of genes encoding the angiogenic protein, systemically or locally to the targeted tissue. The advantage with protein therapy is that it probably obtains a more precise dose-effect relationship than gene therapy. The disadvantage is that the short half-life of angiogenic proteins makes it unlikely that their delivery in doses avoiding toxic side effects will result in sufficient myocardial uptake or residence time to achieve biological effects [87, 88].

The theoretical advantage of gene therapy over protein therapy is that delivery of vectors encoding the angiogenic protein results in sustained expression of the therapeutic agent in the target tissue after a single administration. Thus, gene therapy potentially achieves a greater local therapeutic effect while minimizing adverse systemic by-effects.

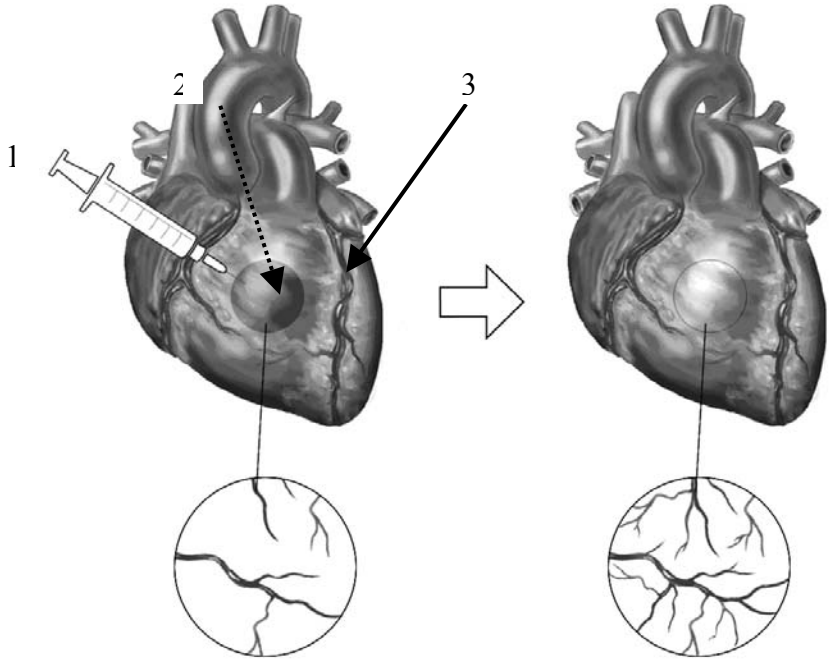


Figure 4*. With the purpose to enhance new vessel growth angiogenic growth factors can be delivered to the heart by a 1) transepicardial, 2) transendocardial or 3) intracoronary approach.*

Delivery routes to the heart

Various routes, such as intravenous, intracoronary, intramyocardial and intrapericardial administration, can deliver angiogenic growth factors to the myocardium [89] (Figure 4). Administration routes that localise the effect of angiogenic agents to the myocardium, as well as deliver a sufficient quantity of a therapeutic agent locally for a sufficient duration, are ideal. Intravenous delivery of recombinant proteins is associated with several limitations, including low bioavailability, *in vivo* instability, high hepatic and renal clearance rates and systemic toxicity [87, 90-92]. Intracoronary delivery is an alternative. 3-5% of the injected protein dose was recovered from the myocardium after intracoronary compared to 0.5% after intravenous delivery [88] while much higher first-pass myocardial up-take was reported with an Ad vector [93]. Intrapericardial delivery resulted in a substantially higher uptake of delivered protein than intravenous or intracoronary injection [94], it is however doubtful if such a delivery route will be applicable in clinical practice. There is a paucity of reports on plasmid up-take after endo-vascular delivery. Recently retrograde coronary venous delivery of plasmid DNA reportedly achieved efficient myocardial transfection [95].

The low protein uptake in the myocardium after endovascular delivery as well as the fact that a significant amount of the gene delivered intracoronarally will not be taken up during the first pass, entails the risk of stimulating promiscuous angiogenesis in remote sites. Injection of angiogenic growth factors directly into the target tissue could thus be preferable. Successful intramyocardial delivery of both protein and genes by transepicardial injection via thoracotomy and by catheter-based transendocardial injection has been reported [96-100]. In this case different techniques, such as sustained-release polymers, are needed to increase the localized efficacy of protein therapy in the target tissue [96, 97]. Gene vectors, on the other hand in themselves provide sustained protein expression.

Gene therapy

Gene therapy can simply be defined as a method for providing somatic cells with the genetic material required for over-expression of specific proteins, in order to correct or modulate a disease. Recombinant genes, cDNA, contain only the protein coding sequences, exons, lacking self-expressing ability and capacity to invade cells. cDNA is therefore linked to transport vehicles, viral or non-viral vectors, with the ability to transfect and express cDNA in host cells [101]. The vector transfers the cDNA into targeted cells, which then produce the corresponding gene product. Currently, only plasmid DNA and adenoviral vectors have been used in clinical trials of myocardial gene therapy [45].

Plasmid DNA

Plasmids occur naturally in bacteria and yeast as small, extrachromosomal circular molecules of double-stranded DNA, replicating independently as the host cells proliferate. Non-viral gene therapy is based on DNA plasmids propagated in *Escherichia coli* [102]. They are easily separated from the host cell, purified, cut with a restriction enzyme, and annealed to cohesive human DNA fragments, resulting in recombinant DNA vectors (Figure 5). In addition to the therapeutic gene, DNA transfer plasmids may contain introns, polyadenylation sequences and transcript stabilisers to control protein stability and secretion from the host cell [103]. Strong promoters are necessary to drive cDNA expression efficiently. The human cytomegalovirus (CMV) early promotor is most commonly used [103]. To ensure replication and maintenance of the plasmid during growth and division of the bacterial host cell a replication origin and a antibiotic-resistance gene is needed [102]. By reinserting the plasmids and culturing the host cells in media containing antibiotic, only cells carrying plasmids will

survive and large cell populations, clones, with concomitant plasmid replication producing several cDNA copies are obtained [102]. As some plasmids anneal without the cDNA insert (Figure 4), radioactive nucleic acid probes with a sequence complementary to the desired DNA sequence and gel electrophoresis is used to identify clones containing the recombinant DNA [102]. Collection of plasmids from host cells involves cell lysis and purification from macromolecular contaminants (i.e. genomic DNA, RNA, protein and endotoxin) [102].

The limitation with plasmid gene transfer is low transfection efficiency. After delivery, much is rapidly degraded in the ECM and only a small amount by yet unknown mechanisms passes into the cell [104, 105]. A substantial amount of pDNA is sequestered in the cytoplasm, also there prone to significant degradation, and only a fraction of the plasmid vectors finally enter the nucleus. There they remain extra-chromosomal and direct a transient transgene expression (Figure 6) [105, 106].

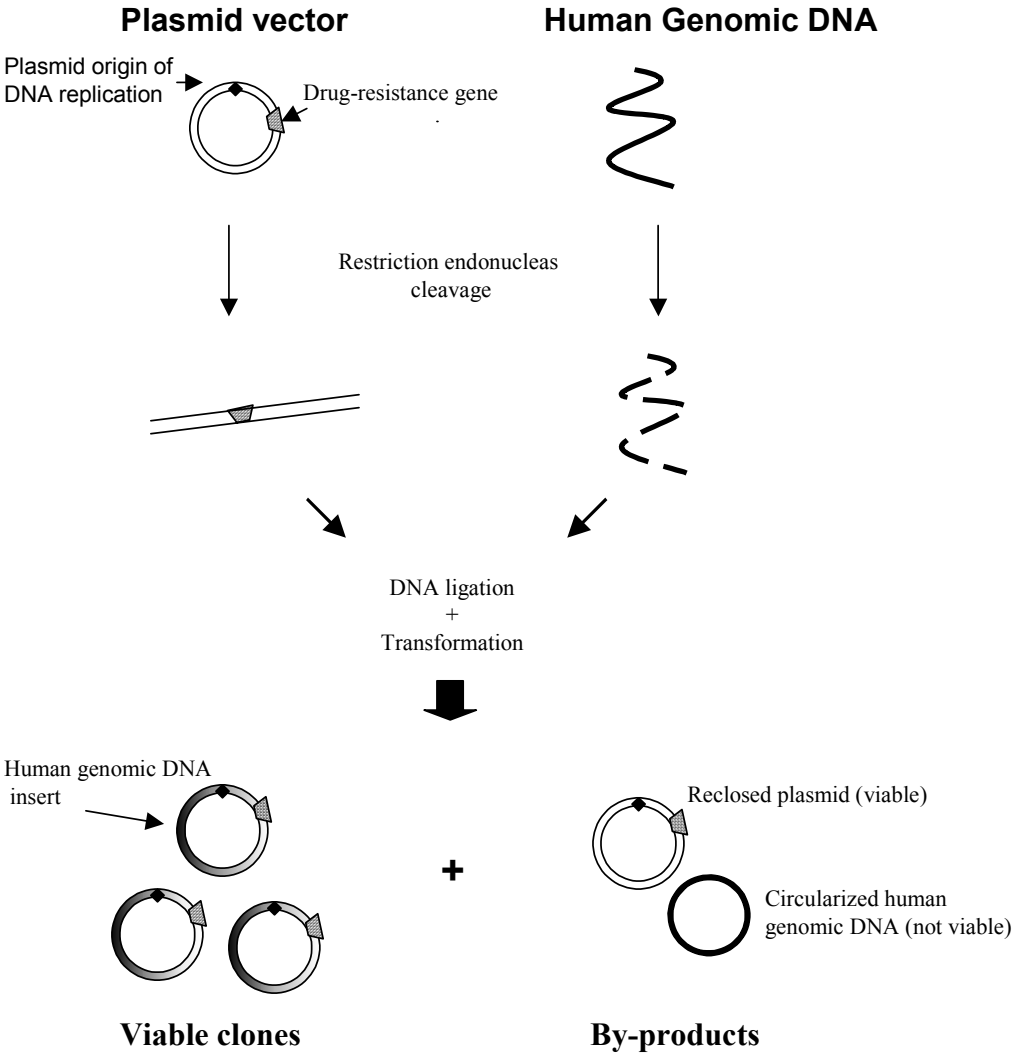


Figure 5. *Construction of hybrid plasmid DNA vectors*

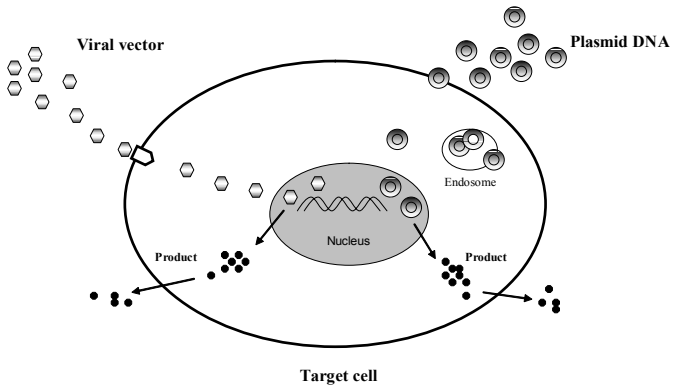


Figure 6. *Plasmid and viral vector gene transfer*

Viral vectors

Viral vectors enter target cells via specific receptors, escape endosomal degradation by a viral mechanism, and generally via nuclear targeting peptide signals migrate into the nuclei (Figure 5).

In Table 1 the differing properties of some viral vectors are briefly summarized. Ad vectors are by far the most often used in myocardial gene therapy. First generation Ad vectors, created by deleting E1 and E3 genes that rendered the virus replication-incompetent and provided room for the cDNA insert, evoked strong host immune response [101]. Newer second- and third-generation Ad vectors have demonstrated significant improvements [101]

Vectors	Advantages	Disadvantages	Duration of expression
Retrovirus	Long-term expression.	Transduces only replicating cells. Integrates into host genome. Risk of insertional mutagenesis.	Months
Lentivirus	Long-term expression. Transduces dividing and non-dividing cells.	Integrates into host genome. Inefficient large-scale production. Human immunodeficiency virus origin.	Months
Adenovirus	Transduces dividing and non-dividing cells. Relatively high transfection efficiency. Remains extrachromosomal. Easy to generate high-titre virus stocks.	Strong immune response. Widespread tropism. High pre-existing immunity.	Few days-2 weeks
Adeno-associated virus	Transduces dividing and non-dividing cells. Relatively high transfection efficiency. Long-term expression.	Inefficient large-scale production. Low packaging capacity. Integrates into host genome. Risk of insertional mutagenesis.	Months

Table 1. *A schematic comparison of viral vectors frequently used in gene therapy.*

Clinical trials on therapeutic myocardial angiogenesis

Phase I/II clinical trials

As a local administration of angiogenic proteins to the target region probably is ideal, clinical trials on myocardial angiogenic gene therapy have focused on intracoronary or intra-myocardial delivery routes. The initial trials reported in the late 1990's were all small, open studies primarily designed to provide information regarding the safety and feasibility of using gene transfer for treatment of CAD.

Dose-escalating intramyocardial injection of plasmid DNA encoding the VEGF-A_{165} gene via a mini-thoracotomy in 20 patients with refractory angina pectoris unsuitable for conventional revascularization, resulted in marked symptomatic improvement and improved perfusion detected with SPECT and coronary angiography [98]. Intramyocardial injection of adenoviral vector carrying the VEGF-A_{121} gene in 15 patients undergoing CABG and as sole therapy in 6 patients via a mini-thoracotomy resulted in symptomatic improvement in both groups [99]. Perfusion images, however, remained unchanged in the group receiving only AdVEGF-A_{121}. Intramyocardial implantation of two doses polymer-released bFGF or placebo in double-blind randomized trial on 24 patients undergoing CABG, resulted in significantly improved perfusion and symptoms in the high-dose bFGF group [96]. Dose-escalating catheter-mediated intramyocardial injection of plasmid DNA encoding the VEGF-C gene in a double-blind randomized trial in 19 patients with inoperable coronary artery disease, resulted in improved CCS class in the VEGF-C group [107]. Increase in exercise duration versus placebo was not significant but a trend favouring the VEGF-C group has resulted in an on-going larger randomized trial.

Phase II/III clinical trials

During the last years, five relatively large, double-blind randomized placebo-controlled trials with predefined end-points have been reported (Table 2). In the FIRST trial 337 patients with chronic angina were randomized to receive three doses of intracoronary bFGF protein or placebo [108]. There was no difference in exercise time, nuclear perfusion or quality of life between the groups after 90 days. The VIVA trial randomized 178 patients with reversible nuclear perfusion defects to receive a single dose intracoronary followed by three doses intravenous hVEGF-A_{165} protein or placebo [109]. There was no difference in exercise tolerance or angina class between the groups, interestingly both groups instead improved equally. The therapeutic agents were well tolerated in both the FIRST and the VIVA trial. Although negative these studies are important as they put into perspective the reliability of uncontrolled clinical trials (Laham et al 2000, Henry et al 2001) [110, 111].

In the AGENT trial 79 patients with stable angina pectoris were randomized to receive dose-escalating intracoronary injection of an Ad vector carrying the FGF-4 gene (Ad5-FGF-4) or placebo [112]. There was no significant difference in exercise treadmill testing (ETT) between the groups, but a protocol-specified analysis showed a significant improvement in Ad5-FGF-4 treated patients with ETT $\leq$ 10 minutes. Building on theses results 52 patients with stable angina CCS II-IV were in the AGENT 2 trial randomized to receive intracoronary injection of the highest dose Ad5-FGF-4 or placebo [113]. The reversible perfusion defect size assessed with adenosine SPECT decreased significantly in the Ad5-FGF-4 group compared to the placebo group after excluding one patient from the placebo group. The REVASC trial randomized 79 patients with advanced angina and no option for conventional revascularization to receive either 30 intramyocardial

injections of Ad vector carrying the VEGF-A_{121} gene (AdVEGF-A_{121}) via a mini-thoracotomy, or continued maximal medical treatment [114]. This study was obviously not possible to blind. The objective measurement of myocardial ischemia was ETT to additional 1 mm ST-segment depression. The AdVEGF-A_{121} treated group had significant improvement in ETT compared to the control group. In the KAT trial 103 patients with 1-2 vessel disease and CCS class II- III were randomized to receive plasmid/liposome-VEGF-A_{165}, Ad-VEGF-A_{165} or placebo via an infusion/perfusion catheter after PCI [115]. There was no difference between groups in the primary end-point of minimal lumen diameter. In the Ad group myocardial perfusion significantly improved from baseline values after 6 months, but not compared to the plasmid or control group.

Trial	Therapeutic agent		n	Endpoint	Results
FIRST [108]	FGF-2 protein	ic	337	ETT at 90 d	Negative
VIVA [109]	VEGF-A_{165} protein	ic+iv	178	ETT at 60 d	Negative
AGENT [112]	Ad5-FGF-4	ic	79	ETT at 4 weeks	Negative (One sub-group positive)
AGENT 2 [113]	Ad5-FGF-4	ic	52	Reversible perfusion defect size assessed with SPECT at	Negative (Positive when 1 patient was excluded from placebo group)
REVASC [114] (Open trial)	Ad-VEGF-A_{121}	im	67	Time to 1 mm ST segment depression on ETT at 26 weeks	Positive
KAT [115]	Ad-VEGF-A_{165} Plasmid/ liposome-VEGF-A_{165}	ic	103	Re-stenosis after PCI	Negative (Improved perfusion at 6 months in the Ad group)

Table 2. *Completed phase II/III clinical trials on myocardial angiogenesis. ETT=exercise treadmill test, ic=intracoronary delivery, im=intramyocardial delivery.*

AIMS OF THE STUDY

The general aim of this thesis was to explore the use of plasmid DNA as a vector for myocardial VEGF-A_{165} gene therapy. Specific aims were:

- To compare protein expression efficiency and angiogenic effect in normoxic with hypoxic myocardium after intramyocardial injection of plasmid encoding VEGF-A_{165}.

- To examine the localization of plasmid gene transfer after catheter-based transendocardial injection.

- To examine time kinetics, dose-response in protein expression and promotor dependence after direct intramyocardial injection of plasmids encoding VEGF-A_{165} or a reporter gene.

- To compare plasmid DNA uptake and gene expression after direct injection into the myocardium with direct injection into skeletal muscle.

- To test the safety and bioactivity of plasmid encoding VEGF-A_{165} after intramyocardial injection in CAD patients without options for further revascularization.

MATERIALS and METHODS

A detailed description of the materials and methods used in this thesis are found in the individual papers I-V.

Paper I-IV

Animals

Male Sprauge-Dawley rats, body-weight ranging between 380-420 g, were housed four in a cage under conditions of 12-hour light/dark periods (paper I, III, IV). Water and chow was provided ad libitum. The rats were sacrificed with carbon dioxide inhalation and tissues of interest were immediately extracted.

Pigs, bodyweight 32-56 kg, were housed and treated in accordance with the institutional guidelines for care of laboratory animals (paper II). They were sacrificed by an overdose of pentobarbital and the hearts were immediately extracted.

Plasmids

The plasmid phVEGF-A_{165} is a eukaryotic expression vector of encoding the 165 amino acid isoform of human cytomegalovirus immediate early (HCMV IE) promoter/enhancer (paper I-III, V). The plasmid pEGFPLuc is a commercial reporter plasmid where the CMV promoter/enhancer drives the expression of a fusion protein of enhanced green fluorescent protein (EGFP) and luciferase (paper III). The plasmids pNGL-CMV driven by a virus promoter, pNGL-EF1α by a human promoter and pNGL-SRα by a hybrid between SV40 early and human T-lymphotropic virus type 1 promotors, are all reporter plasmids encoding for luciferase and with the same vector backbone (paper III). pGTC500 is an expression vector with the luciferase gene, flanked by intron A and IgG2 intron, driven by a CMV promoter/enhancer (paper IV). (Figure 7).

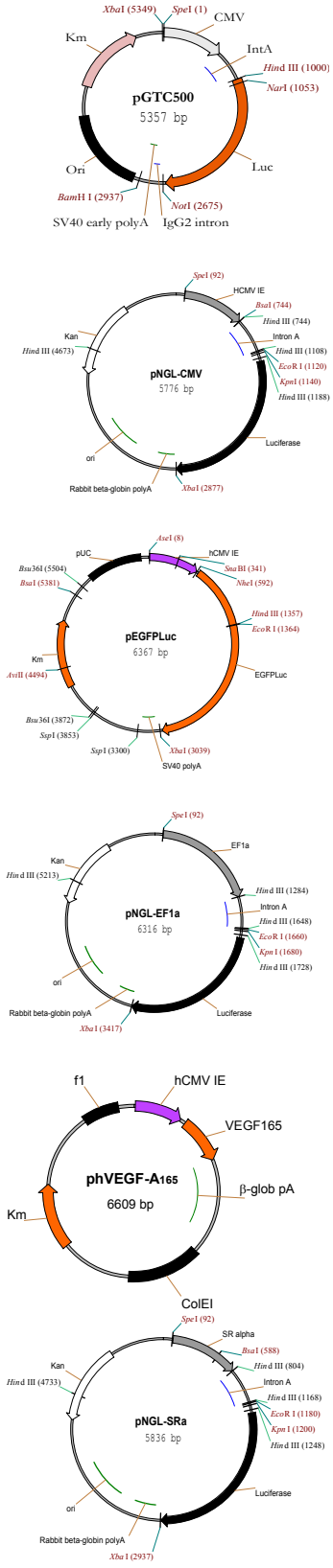


Figure 7. *Plasmids used in paper I-V*

Intramyocardial gene delivery (paper I, III, IV)

Anaesthetised rats were placed in a stable position on their backs, and the posterior left ventricular wall was echo-cardiographically located utilising a 10 MHz probe. With a subthoracic approach, the plasmid solution was there-after under echocardiographic guidance injected directly into the posterior wall. An area of increased echogenicity visible at the needle tip after the plasmid solution was injected was considered an indication of successful intramyocardial gene delivery (Figure 8).

Catheter-based transendocardial gene delivery (paper II)

The NOGA system (Cordis-Webster) was utilized for intramyocardial injection plasmids in anaesthetised pigs. The mapping catheter was positioned in the left ventricle under fluoroscopic control. The first three points of the subsequent three-dimensional map of the left ventricular inner surface (apex, lef+right angles of the base) were located with fluoroscopic guidance. Mapping precision was evaluated in a twelve-segment bulls-eye view. At least three points in each segment and a total of at least 60 points were required precision criteria. After switching to the injection catheter, which basically is a 7 French mapping catheter integrated with a re-tractable 27-gauge needle (Cordis-Webster), plasmid solution was injected at ten spots into a predefined region of the left ventricular wall. Ventricular extra-systoles were regarded as evidence of appropriate needle protrusion into the left ventricular wall, while transient ST-elevation recorded from the catheter was regarded as evidence of successful injectate delivery.

Histologic analysis of VEGF-A$_{165}$, β-galactosidase and EGFP expression

For detection of VEGF-A$_{165}$ expression serial 5µm cryo cross-sections of whole (paper I) or pieces of hearts (paper II) were stained with monoclonal mouse anti-hVEGF-A antibody (Sigma), streptavidin-biotin-horseradish peroxidase complex (Dako) and 3,3'-diamino-benzidine tetra hydrochloride (DAB) (Sigma). VEGF-A expressing cells were counted under a light-microscope with a 10 x objective. Five sections per heart with the highest detected expression efficiency were selected for microvessel analysis in paper I.

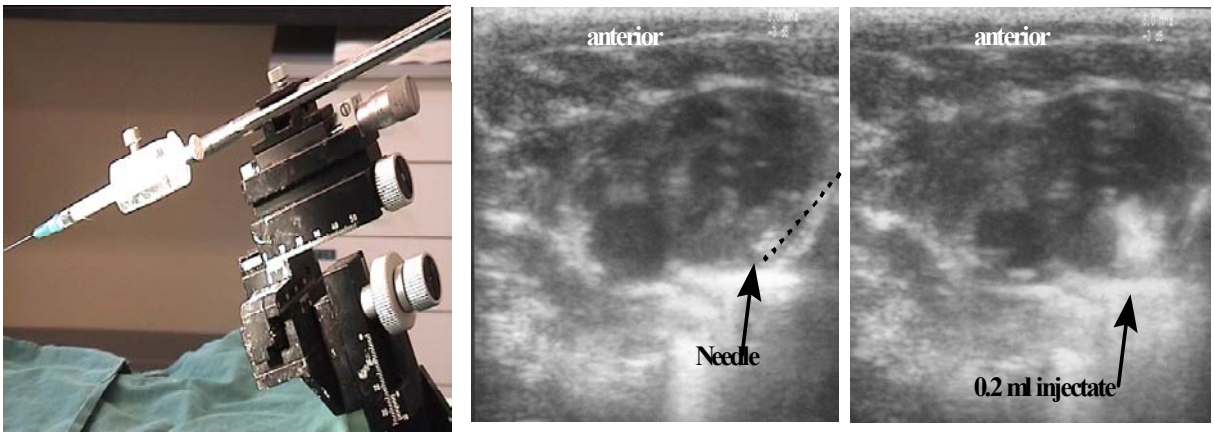


Figure 8. *Intramyocardial injection of plasmid solution in rats. A. Micromanometer. B. Needle visible in the left ventricular wall. C. Visible plasmid injectate.*

Expression of β-galactosidase was macroscopically detected by staining of every second whole slice of the pig heart with Xgal colour substrate (paper II). Five µm sections of adjacent areas were stained with X-gal colour substrate and counterstained with hematoxylin-eosin. Cells expressing β-galactosidase were counted under a light-microscope with a 10 x objective.

For detection of EGFP expression, 5 µm cryo-cross-sections of the heart were directly examined by fluorescence microscopy (paper III).

Analysis of microvessel density (paper I)

Sections adjacent to the ones previously selected were stained with Griffonia Bandeiraea Simplicifolia Isolectin B4 (paper I). Of these, two sections with the highest transfection efficiency were selected from each heart for evaluation of microvessel density. Ten fields of 0,58 mm² each in the transfected area were examined under a light-microscope with a 10 x objective. A Micro Image analysis program (Omega) was utilised for quantification of the microvessel density in the examined fields.

Analysis of VEGF-A protein expression

The hearts were homogenised in 1.2 ml lysis buffer consisting of 20 mM HEPES pH 7.4, 1.5 mM EDTA, 0.5 mM PMSF, 0.5 mM benzamidine and 10 µg/ml trypsin inhibitor (paper III). Protein expression was measured with a VEGF enzyme-linked immunosorbent assay (ELISA) Assay Kit (R&D Systems). Hundred µl supernatant was added to 100 µl assay diluent. Standard curves were prepared according to the directions of the manufacturer.

Analysis of Luciferase activity

Tissues were homogenised in luciferase lysis buffer and chemi-luminescent luciferase activity was measured using a Promega Luciferase Assay Kit (paper III, IV). Twenty µl supernatant was added to 100 µl of luciferase substrate and the relative light units (RLU) were measured over 10 seconds in a single well luminometer. Standard curves were prepared by serial dilutions of purified luciferase in control tissue homogenate.

In vitro gene transfer and analysis of VEGF-A expression (paper III)

Plasmids were transfected into COS-7 cells by polyethyleneimine (PEI) transfection. Twenty-four hours prior to transfection 50,000 cells were seeded in a 6-well plate (Corning Costar). For each well 2.5 µg of plasmid was mixed with PEI in a total volume of 20 µl. After incubation at room temperature for 30 minutes the PEI:plasmid complex was added to the cells. Gene expression was measured by analysing the amount of VEGF-A in the supernatant with ELISA at different time points after transfection.

DNA extraction from tissues (paper IV)

Tissues were directly after removal weighed, cut into small pieces on dry ice and stored in –70ºC until DNA extraction. Total DNA from tissue samples was extracted using QIAGEN DNeasy Kit, essentially according to the manufacturer's specifications (QIAGEN Inc.). Two 19 ml samples of each homogenate (=2x400 mg tissue) were aliquoted into QIAGEN anion-exchange resin tubes and genomic DNA was isolated separately.

Plasmid DNA quantification (paper IV)

Real time quantitative PCR was used for measurement of pGTC500 in tissues (ABI-PRISMA 7700 Sequence Detector, Perkin-Elmer Applied Biosystems Inc, Foster City, CA, USA). Oligonucleotide primers and TaqMan probes were designed for the plasmid specific sequence ColE1 using Primer Express version 1.5 (Perkin-Elmer Applied Biosystems Inc.). All reactions were performed in 96-well MicroAmp Optical plates. Amplification mixes (25 µl) contained the diluted sample DNA, 2 X TaqMan Universal PCR Mastermix, forward and reverse primers and probe for the specific plasmid sequences.

Quantification of plasmid values into µg per extracted tissue was carried out by calibration against a standard curve generated by serial dilution of pGTC500. Samples of the precipitated genomic DNA were analysed for specific plasmid levels in duplicate. Dilution curves of transfected heart muscle homogenates were also performed to ensure that endogenous DNA did not inhibit the quantification of the injected plasmid.

Clinical methods (Paper V)

Patients

Patients with multivessel occlusive coronary artery disease, Canadian Cardiovascular Society (CCS) class III-IV and no possibility of further revascularisation were included in the study. Further inclusion criteria were: perfusion defects extending over > 10% of the anterolateral left ventricle wall, detectable with adenosine single photon emission computerised tomography (SPECT), at least 1 patent vessel visible with coronary angiography and ejection fraction > 30%. Exclusion criteria were unstable angina pectoris during the last 6 months, cancer, chronic inflammatory disease or diabetic retinopathy. Screening for occult tumours was performed before inclusion with chest x-ray, mammography and blood tests for chorioembryonic antigen (CEA) and prostate-specific antigen (PSA).

Study design

Via a left mini-thoracotomy under general anaesthesia, a total of 0.25-1 mg phVEGF-A_{165} was injected directly into the myocardium at four sites in the anterolateral region of the left ventricular wall. The patients were postoperatively observed in the intensive care unit for 48 hours and then in a routine ward until discharge.

Physical examinations, ECG, standard laboratory tests including CEA and PSA tests and exercise tolerance tests were performed prior to, and 2 weeks, 1, 3, 6 and 12 months after the operation. The patients were monitored for signs of congestive heart failure and their angina episodes as well as weekly nitroglycerine consumption were documented. Myocardial function was evaluated prior to and 2 months after gene transfer with adenosine SPECT and tissue velocity imaging (TVI) during dobutamine stress echocardiography and the presence of collaterals with coronary angiography. SPECT scans and angiograms were evaluated by 3 blinded independent observers.

RESULTS

Paper I

Aims

The aim of this study was to morphologically examine the dose-response effect on transfection efficiency and angiogenesis of phVEGF-A_{165} after intramyocardial injection in normoxic and hypoxic rats.

Results

Injection of plasmid solution or saline alone produced a macroscopically detectable scar after 5 days, a finding that was utilised to locate the transfected area in the myocardium. VEGF-A expressing cells were located around the distal part of the injection tract. No VEGF-A expressing cells were detected in the control hearts injected with saline or subjected to hypoxia alone, confirming an absence of cross-reactivity with endogenous rat VEGF with the staining method used. Very few, or no inflammatory cells were detected in the gene transfer region. No angiomas were observed.

The number of VEGF-A expressing cells detected per cross-section in the normoxic hearts increased dose-dependently ($p<0.05$) (Figure 9). A tendency of a higher number of VEGF-A

expressing cells was observed in the hypoxic hearts when compared with normoxic hearts injected with the same dose phVEGF-A_{165} (Figure 9).

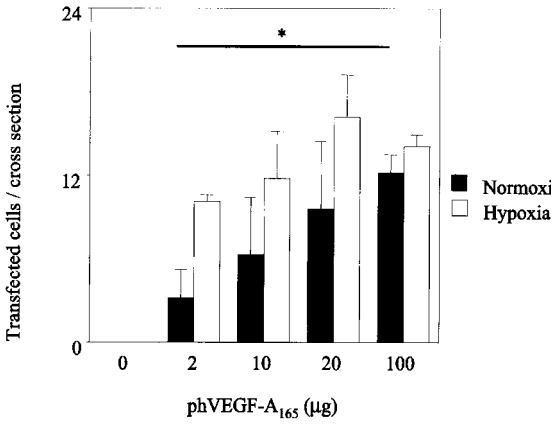


Figure 9. *Dose-response in number of transfected cells in normoxic and hypoxic hearts. A significant increase was detected between 2 and 100 µg phVEGD-A165 in normoxic rats (*p<0.05).*

In the rats exposed to hypoxia alone, an increase in microvessel density was detected after 5 days, peaking day 7 with a 20% ($p<0.05$) increase in microvessel density when compared with normal myocardium. No further increase in microvessel density was detected day 10.

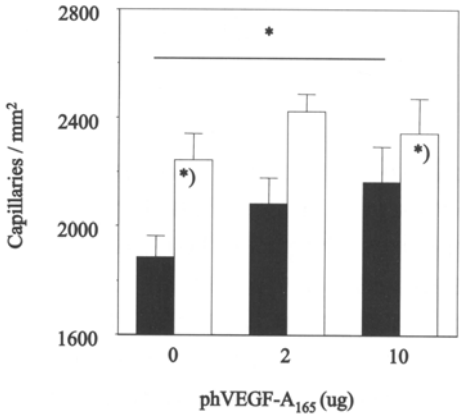


Figure 10*. Microvessel density measured in normoxic and hypoxic hearts after phVEGF-A165 injection. 0 phVEGF-A165 = control hearts injected with saline solution. * = p<0.05 increase between 0 and 10 µg phVEGF-A165 in normoxic hearts. *) = p<0.05 diference between normoxic and hypoxic hearts receiving the same treatment.*

In the normoxic rats intramyocardial injection of phVEGF-A_{165} resulted in a dose-dependent increase in microvessel density up to 10 µg plasmid, at which dose an approximately 10% increase ($p<0.05$) was detected compared to control normoxic rats (Figure 10). A further increase ($p<0.05$) in microvessel density was detected in hypoxic rats treated with 10 µg phVEGF-A_{165} compared to normoxic rats treated with the same dose (Figure 9).

Conclusion

Intramyocardial injection of phVEGF-A_{165} resulted in a dose-dependent increase in the number of transfected cells as well as in an angiogenic effect. Hypoxic conditions tended to enhance both transfection efficiency and angiogenic effect. Acute hypoxia alone stimulated angiogenesis without treatment.

Paper II

Aims

The aim of this study was to characterize macroscopic and microscopic localisation of gene transfection after catheter-based transendocardial injection of plasmid DNA in pigs utilizing the NOGA mapping and injection system.

Results

All animals survived the study. One pig had developed myocardial infarction at harvest, although there were no signs of myocardial infarction during the procedure. As there was no vessel occlusion, catheter-mediated trauma was interpreted as the probable cause of this complication.

Ten gene-expressing spots were obtained from ten injections in the first analysed heart, but in addition, there were granulations and subendocardial superficial hemorrhages in the target area. Consequently, less catheter pressure was

applied in the remaining animals. The following hearts had fewer gene-expressing spots, but also less myocardial trauma. Totally, approximately 60% of the injections made resulted in detectable gene transfer. The criterions for appropriate needle penetration into the myocardium (ventricular extrasystoles) as well as myocardial injectate deposition (transient ST-elevation) were fulfilled in 70-80% of the injections made.

All identified gene-expressing spots were located where expected, i.e. in the target area between the papillary muscles, with a 5x5x5 mm distribution around the distal end of the needle track (Figure 11). The macroscopic β-galactosidase identified spots were located subendocardially (n=12), in the middle of the left ventricle wall (n=15) and subepicardially (n=9).

β-galactosidase and VEGF-A_{165} expression was detected microscopically in sections corresponding to the areas identified macroscopically. Fort-two % of transfected cells expressed both β-galactosidase and VEGF-A_{165}, where as 32% only β-galactosidase and 26% only VEGF-A_{165}.

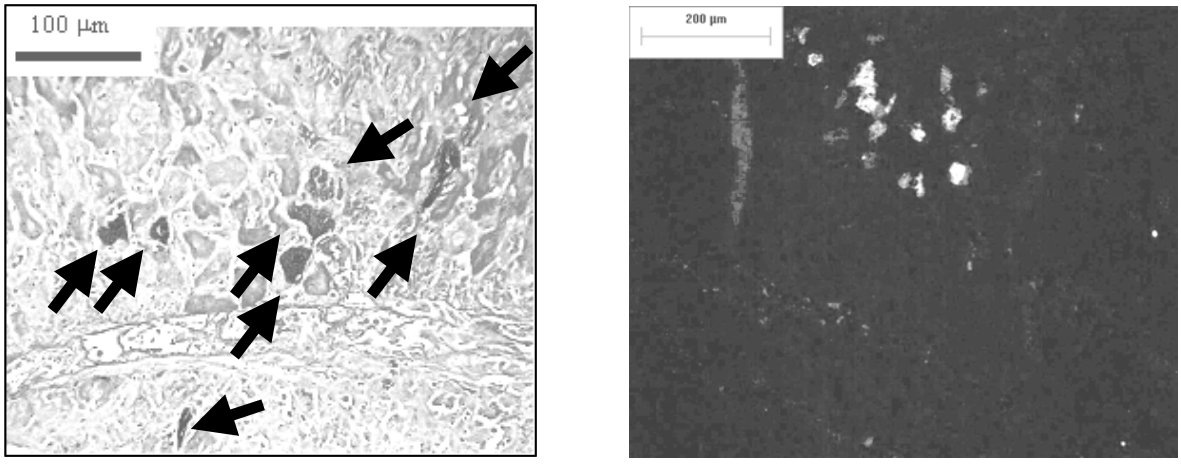
Conclusion

Catheter-based intramyocardial gene delivery is feasible to accomplish with a high degree of local precision in the left ventricular wall.

Paper III

Aims

The aim of this study was to explore the dose-response relationship of protein expression of plasmids encoding a therapeutic or a reporter gene, the time kinetics including a comparison between different promotor/enhancers after direct intramyocardial injection in rats. Target tissue distribution and systemic spread to the liver was also examined.



A. *B.*

Figure 11. *A VEGF-A165 expressing spots and B â-galactosidase expressing spots in pig hearts after catheter-based intramyocardial injection of plasmids.*

Results

EGFP gene expression was restricted to cardiomyocytes in a focal area surrounding the distal part of the needle track. The pEGFPLuc plasmid expression was low and unevenly distributed over 2-4 mm in the longitudinal plane of the left ventricular wall (Figure 12). This area was found to correspond to the area of increased echogenicity surrounding the distal part of the needle visualised after the injection of plasmid solution (Figure 12). The luciferase activity detected in the liver was very low. The highest levels, detected day one, corresponded to less than 1 % of the luciferase activity detected in the heart ($p<0.01$). By day four, there was no detectable luciferase activity in the liver.

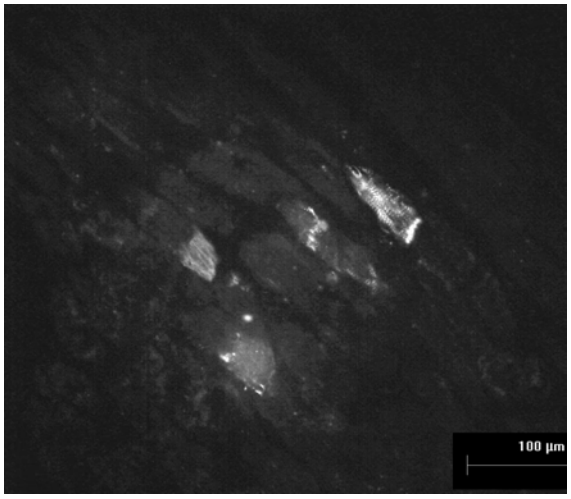


Figure 12. *EGFP expressing cells in rat myocardium.*

In rat hearts the luciferase expression level obtained with 30 µg pEGFPLuc was three-fold higher than the levels obtained with 3 µg ($p=0.0002$), and also significantly higher than levels obtained with 150 µg ($p<0.03$) and 300 µg ($p<0.01$). There was no significant difference in luciferase activity between 150 and 300 µg (figure 11). Similarly, the VEGF-A protein level obtained with 30 µg injected phVEGF$_{165}$ was five–fold higher than the levels obtained with 3 µg ($p<0.02$) and three-fold higher than with 300 µg ($p<0.01$). It was, however, not significantly higher than the level obtained with 150 µg (Figure 13).

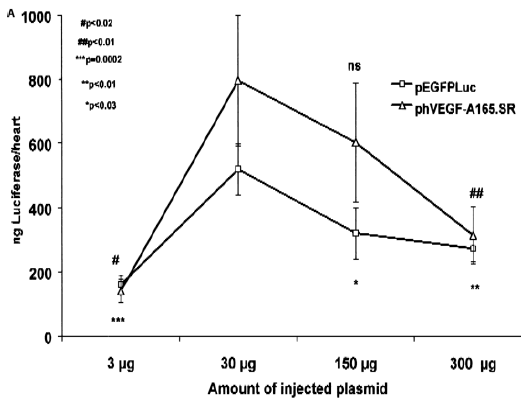


Figure 13. *Dose-response in pEGFPLuc and phVEGF-A165 protein expression.*

The expression of both the luciferase and the VEGF-A$_{165}$ protein had similar time kinetics (Figure 14). Both proteins were detected 8 hours after injection, reaching a peak level by day 1 with a three-fold increase in luciferase expression ($p<0.0001$) and five–fold increase in VEGF-A$_{165}$ expression ($p<0.001$). A plateau level was retained until day 2-3, subsequently rapidly declining to very low VEGF-A$_{165}$ levels by day 3 ($p<0.0005$) and luciferase levels by day 4 ($p<0.0001$). phVEGF-A$_{165}$ expression followed similar kinetics *in vitro* as *in vivo*. VEGF-A levels in COS-7 cultures were highest day 3, followed by a distinct rapid decline day 5.

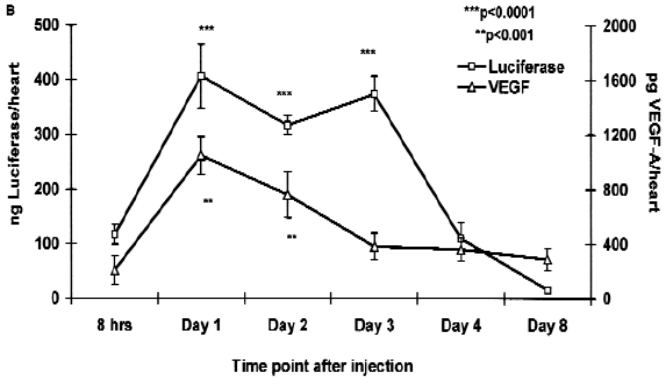


Figure 14. *Time kinetics of pEGFPLuc and phVEGF-A165 protein expression.*

In order to test if the short time course could be attributed to promotor attenuation three different promotor types were compared. The luciferase activity after injection of 30 µg pNGL-CMV, pNGL-EF1α or pNGL-SRα followed similar kinetics as pEGFPLuc. The highest levels were detected day 1, with a rapid decline by day 4. On day 1 pNGL-CMV yielded higher ($p<0.05$) luciferase activity than pNGL-EF1α, whereas no significant difference was observed when compared with pNGL-SRα. There was no significant difference between the plasmids day 4 and 8.

Conclusion

Plasmid DNA transfection was localised to the injection site with minimal detected systemic spread to the liver. The time course of the reporter and the therapeutic gene expression was similar and promotor independent. The dose-response in protein expression was likewise similar for both genes.

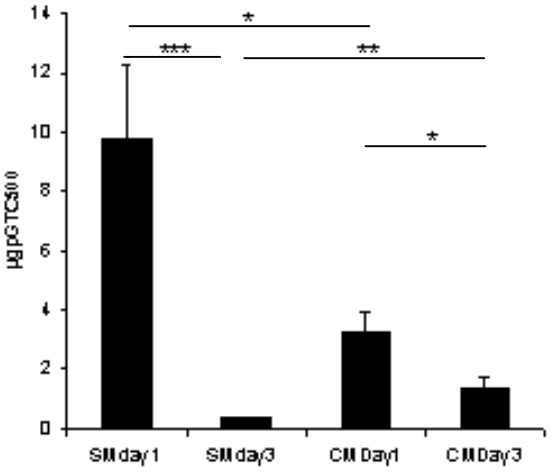


Figure 15. *Measured plasmid levels depicted in mean µg pGTC500/tissue from skeletal (SM) and cardiac muscle (CM) one and three days after direct injection. (n=6 at each time point.)*

A marked difference in transgene expression was observed between cardiac and skeletal muscle. Luciferase activity in cardiac muscle was 40-fold higher than in skeletal muscle day one ($p<0.004$) and 10-fold higher ($p<0.03$) day three post-transfection (Figure 16).

Paper IV

Aims

The aim of this study was to detect differences in plasmid uptake efficiency after direct injection of the same dose plasmid DNA encoding luciferase into the myocardium and into skeletal muscle in rats.

Results

pDNA levels were approximately 3-fold higher in skeletal than in cardiac muscle one day after gene transfer ($p<0.03$). Three days after gene transfer the pDNA levels in skeletal muscle however had abruptly decreased, and were now significantly lower than in cardiac muscle ($p<0.02$) (Figure 15).

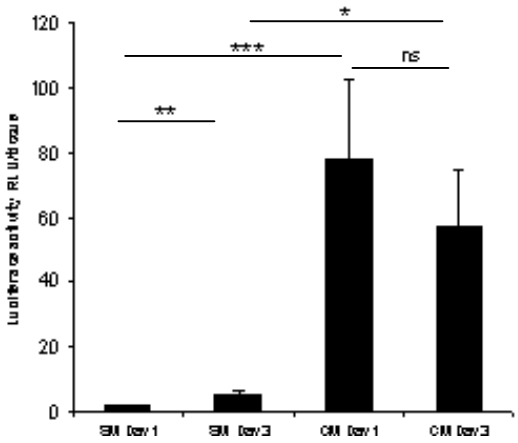


Figure 16. *Luciferase activity detected in skeletal (SM) and cardiac (CM) muscle one and three days after intramuscular and intramyocardial injection of 30 µg pGTC500 (n=5 each time point).*

A comparison of pGTC500 levels and luciferase activity in skeletal and cardiac muscle are depicted in Figure 17. As seen, the behaviour of the same plasmid differed completely in the different tissues.

Conclusion

Transgene expression efficiency is not equivalent to plasmid uptake efficiency after direct pDNA injection in skeletal and cardiac muscles in rats. This finding questions the relevance of indirectly testing plasmid DNA uptake by monitoring transgene expression.

Paper V

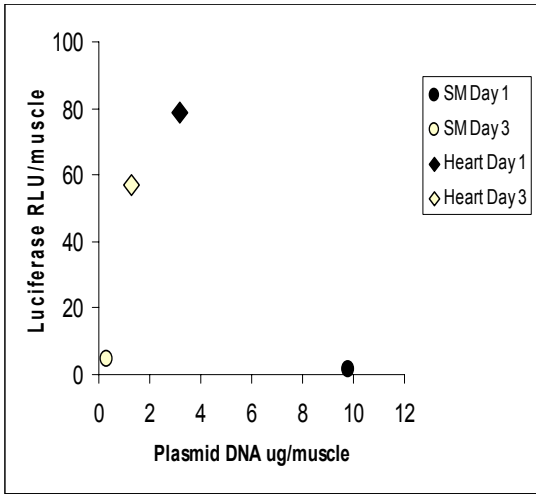
Aims

The aim of this study was to test the safety and bioactivity of intramyocardial injection of phVEGF-A_{165} as sole therapy for patients with end-stage angina pectoris and without any option of further conventional revascularisation procedures. We also wanted to ascertain if the previously reported positive results of a similar trial could be independently reiterated.

Results

The demographics and clinical history of the patients included in this study are described in Table 3. Intramyocardial gene transfer was successful in all patients. There were no perioperative deaths. Release of myocardial markers and new ECG changes were detected in 2 patients. Mean plasma VEGF-A levels increased 2- to 3-fold peaking day 6 after gene transfer and returning to baseline levels by day 30.

Two months after gene transfer, reduced reversible ischaemia in the gene transfer region was detected in 4 of the patients by adenosine SPECT, indicating improved myocardial perfusion (Figure 18).



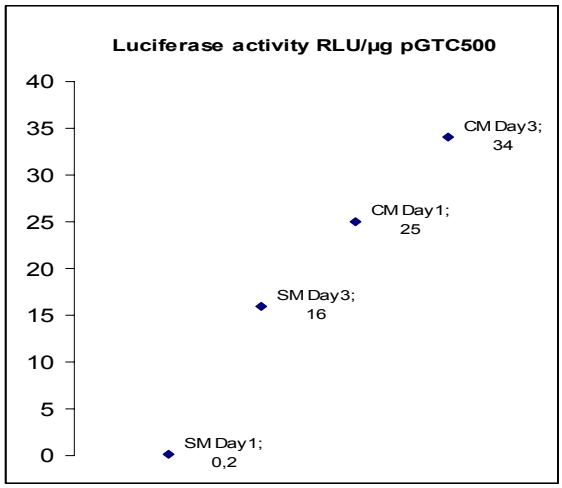


Figure 17. *pDNA levels and luciferase activity are jointly depicted to better visualize the behavior of pGTC500 one and three days after intramyocardial and intramuscular injection. Above: Luciferase activity/skeletal muscle (SM) and cardiac muscle (CM) in relation to µg pGTC500/SM and CM. Below: Luciferase activity/µg pGTC500 detected in SM and CM respectively*

Improved collateral filling was detected in four patients with coronary angiography. Improved myocardial function in the gene transfer region was detected in all patients, with a significant increase ($p<0.016$) in mean TVI value during dobutamine stress echo-cardiography. A tendency of improved exercise tolerance and a later onset of ST-depression were observed. Two patients reported an absence of chest pain during all exercise tests after gene transfer, while the others remained unchanged.

Parametrar	n
Female	2
Male	5
Age, years	mean 68 (56-76)
Prior smokers	3
Diabetes	0
Hypertension	3
Intermittent claudication	2
Prior myocardial infarction	2
Beta blocker	7
Calcium chanel blocker	7
Lipid lowering agent	7
ASA	6
Warfarin	1
Slow release nitroglycerine	7
CABG	7
PCI	4
Ejection fraction, %	mean 49 (40-55)
Collateral source: LIMA-LAD	5
Vein graft → marginal	1

Table 3. *Characteristics of patients included in the trial.*

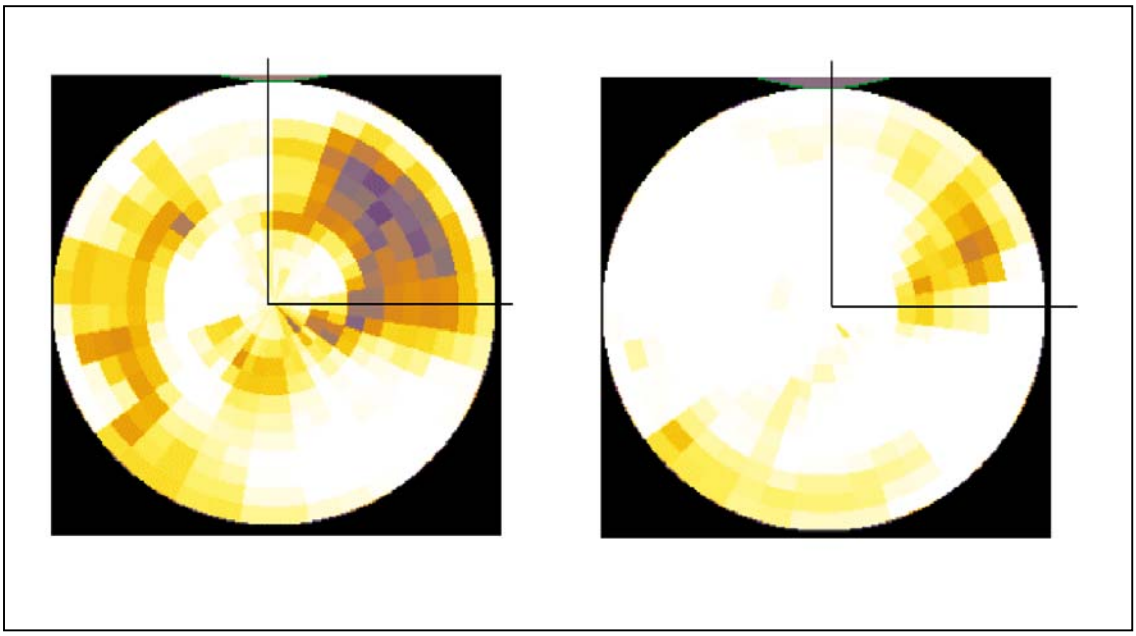


Figure 18. *Polar maps of SPECT scans showing reversible ischaemia before (left) and 2 months after (right) gene transfer in patient six. A marked reduction of reversible ischaemia was detected in the left anterior wall after treatment (upper right quadrants indicated by lines).*

No signs of congestive heart failure or new ECG changes were detected during the 12-month follow-up period. One late death due to a myocardial infarction occurring 7 months after the operation was deemed as unrelated to the gene transfer. A significant subjective improvement was reported after gene transfer. The CCS class had decreased after 2 months ($p<0.01$) and this improvement was retained at 12 months (Figure 19). Intake of nitroglycerine tablets had markedly lessened after 2 months ($p<0.001$) and approached zero at 12 months (Figure 19).

Conclusions

Intramyocardial injection of phVEGF$_{165}$ in patients with refractory angina pectoris is safe and resulted in improved myocardial perfusion and function as well as a long-term symptomatic relief.

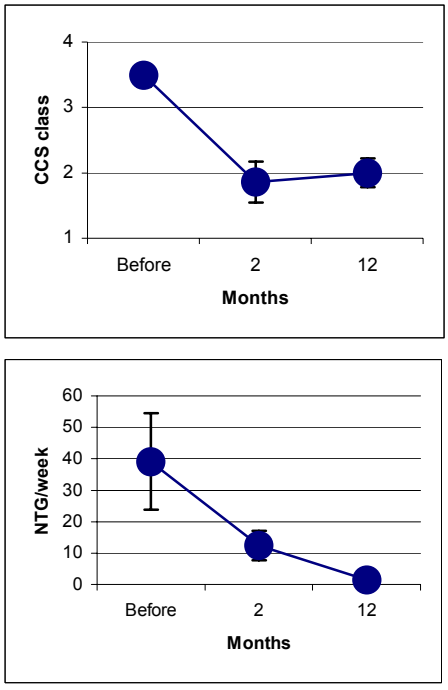


Figure 19. *CCS class (above) and nitroglycerine intake (below) during the 12-month follow up period. Values are mean ± SEM.*

GENERAL DISCUSSION

The challenge of gene therapy is to I) determine which gene is best to use, then to II) deliver the genetic material into the target tissue in III) sufficient quantities for the synthesis of an adequate amount of gene product to VI) elicit the desired therapeutic effect while V) limiting adverse side effects.

Choice of VEGF-A165 as therapeutic agent

As described previously (pp11-13) neovascularisation is an extremely complicated process, involving a multitude of angiogenic and anti-angiogenic factors interacting with each other and numerous cell types. That VEGF-A$_{165}$ is a key regulator of vessel growth is undisputed. VEGF-A$_{165}$ is a highly specific mitogen for vascular endothelial cells, which represent the critical cellular element responsible for new vessel formation [11]. This characteristic makes VEGF-A$_{165}$ an appealing candidate for use in therapeutic angiogenesis. However, is the delivery of a single factor, as VEGF-A$_{165}$, capable of inducing all the complementary activities essential for the development of fully functional vascular network? Dor et al designed a transgenic mouse system with a genetic on-off switch allowing reversible induction of high levels of VEGF-A in the heart and liver in adult mice [116]. VEGF-A if expressed during a sufficient period induced the formation of vessels durable for months after withdrawal of the angiogenic stimulus, providing evidence that VEGF-A alone indeed can promote the entire angiogenic cascade [116].

Choice of plasmid as a gene transfer vector

The use of plasmid DNA for gene transfer has many benefits in comparison with Ad vectors. They are proportionately simpler and cheaper to produce, they are able to transfer genes with less restriction by cDNA size and, most importantly, they do not to the same degree evoke an

immune response [117]. Ad infections are endemic virtually worldwide and specific Ad-antibodies with differing ability to neutralize Ad-vector transduction are detectable in 97% of individuals [118]. This might hamper the clinical use of Ad vectors, especially if repeat administration is considered. The single death so far associated with human gene therapy trials was due to an acute inflammatory response to Ad vectors, albeit in this case given in an exceptionally high dose [119, 120].

Although plasmid DNA transfection efficiency is not as high as Ad vectors, this may be less relevant in strategies where the gene product has a secretory signal sequence, as VEGF-A_{165}, and a paracrine effect on vessel growth [121]. More explicitly, this means that transfer of genes that encode secreted proteins can achieve a biological effect by transfecting a limited amount of cells, if the transfected cells secrete a substantial amount of the gene product. The paracrine effect of the secreted protein might then modulate the bioactivity of a large number of target cells, in this case endothelial cells.

Very few clinical trials have directly compared the therapeutic effect of plasmid DNA and Ad encoding the same gene. Local intraarterial plasmid/liposome-VEGF-A_{165} or Ad-VEGF-A_{165} gene transfer in a double-blind randomised trial including 45 patients with lower limb ischemia resulted in increased vascularity in both groups compared to placebo, with no difference between plasmid and Ad groups [122]. On the other hand, after intracoronary infusion of plasmid/ liposome-VEGF-A_{165} or Ad-VEGF-A_{165} only the Ad group showed improved myocardial perfusion [115]. The last mentioned results can however be interpreted as showing that different delivery routes suite different vectors in different organs.

Gene delivery route

Selection of delivery route suited to the vector used is a key issue for efficacious targeted gene transfer. While intravascular delivery of Ad vectors is possible, circulating DNases rapidly degrade plasmid DNA. Although incorporation of pDNA in liposome complex is protective, its passage through the vessel wall to the myocardium is limited and transgene expression is mainly located in endothelial cells [123]. Ad and plasmid vectors did not differ in efficacy when the target tissue was the coronary artery (i.e. re-stenosis rate) [115]. If the myocardium is the target tissue for gene transfer, direct injection is more appropriate when using plasmid vectors. The large size of pDNA restricts its diffusion within the tissue after direct injection, enabling transfection only of cells surrounding the injection site, which in turn makes this delivery route tissue specific [124, 125]. In accordance, transgene expression was after direct injection of pDNA into the myocardium only detected at the delivery site in paper I-III. The very low level of luciferase activity detected in the liver in paper III indicates that systemic spread after intramyocardial pDNA injection is negligible.

Gene delivery site

It is currently unknown if optimal effect of therapeutic angiogenesis will be achieved by delivering angiogenic cytokines to regions of hypovascularized ischeamic myocardium or to regions of non-ischaemic myocardium from which collaterals will originate. The general opinion has been that nonischaemic tissues may be unresponsive to angiogenic stimuli, while acute ischaemia enhances VEGF-A and VEGFR-2 expression [69, 126] as well as gene transfer efficiency [127]. In paper I, phVEGF-A_{165} transfection efficiency increased in correspondence with a moderately increased angiogenic effect in hypoxic compared with normoxic myocardium. Microvessel density however increased to the same degree in rat hearts exposed to acute hypoxia alone.

Acute ischaemia even in severe CAD is not constant but instead provoked by exertion. The myocardial response to chronically reduced blood flow is

hibernation, a transitory adaptive state where oxygen consumption and contractile function is down-regulated at rest in order to preserve high-energy metabolism [128]. Transgenic mice lacking VEGF-$A_{164 \text{ and } 188}$ isoforms developed myocardial ischemia with signs of hibernation that progressed to cardiac failure, thus providing evidence that insufficient availability of angiogenic growth factors can aggravate the consequence of CAD [129]. The finding that phVEGF-A_{165} transfer evoked an angiogenic response in normoxic myocardium is relevant as it supports the feasibility of rescuing hibernating myocardium by delivery of exogenous angiogenic growth factors during stable CAD conditions, when endogenous angiogenic growth factors are not upregulated [130-132].

In a recent report, pVEGF-A_{165} injected into the ischemic region of the myocardium in a pig model of chronic ischemia did not result in improved myocardial blood flow in the target area [133]. Instead, myocardial blood flow increased in regions adjacent to the pVEGF-A_{165} delivery zone, a spatial delivery-efficacy mismatch [133]. As most angiogenic activity occurred in the ischemic border zone, this may be the optimal delivery site. Such regions are impossible to localize during intramyocardial injection via a mini-thoracotomy. They are however fully feasible to localize with the NOGA system, with which regions of hibernating myocardium can be identified and serially evaluated before and after gene transfer [134].

We achieved a high degree of local precision in the left ventricular wall with catheter-based transendocardial gene delivery in paper II. That the success rate of identified transgene expressing spots was not 100% can be due to different factors. The study design entailed macroscopic assessment of gene expression only in parts of the left ventricle. Hence, it is possible that more gene expressing spots existed than detected. Secondly, although the gene delivery procedure in it self is successful a substantial amount of the injectate can be lost by direct leakage [135]. Thirdly, since the myocardium is highly vascular [136-138], some of the injected pDNA may be lost via venous and lymphatic drainage before transfection.

It is important to test if previously reported amazingly positive effects of a therapeutic agent are possible to reproduce in independent trials. In paper V we therefore used a similar study design as in the trial reported by Symes et al [98]. Intramyocardial gene transfer in humans was at the initiation of our clinical trial (paper V) only possible via a mini-thoracotomy, which limited the delivery site to the anterolateral region of the left ventricle. Although VEGF-A_{165} is a secreted protein, a considerable amount remains sequestered on the cell surface and in the ECM [65]. The highest VEGF-A_{165} levels in the target tissue are consequently likely in the region surrounding the injection site. Acting on this assumption we, in contrast to the previous study [98], only included patients with reversible ischaemia detected in the anterior region. We furthermore deemed it compulsory that the delivery region was consistent in all patients, to minimize variables influencing the evaluation of phVEGF-A_{165} biological effects.

Plasmid dosage

Determining the optimal gene vector dosage is another key issue for achieving successful gene therapy. Robust differences in biological response have to date not been documented, despite several dose-escalating studies performed on both animal models and in clinical trials. A significant dose-response correlation between VEGF-A expressing cells and increase in microvessel density was observed between 2 and 10 µg phVEGF-A_{165} in both normoxic and hypoxic hearts in paper I, but not with the higher doses phVEGF-A_{165}. One limitation with plasmid gene transfer is the high interindividual variation in gene expression. We in paper

III therefore increased the space between delivered plasmid doses. A significant dose-response increase in luciferase and VEGF-A_{165} protein expression was obtained between 15 µg and 30 µg injected plasmid. The higher plasmid doses did however not result in a continued rise. Instead, protein levels after 150 µg were the same as obtained with 30 µg, while they decreased after delivery of 300 µg. Our results indicate that a saturable mechanism, independent of which transgene the plasmid is carrying, is involved in plasmid uptake and/or gene expression. Similar speculations were presented previously [139, 140]. Evaluation of dose efficacy was not an aim in our clinical trial (paper V), but due to a misinterpretation of the original study protocol [98] the first 2 patients received 1.0 mg instead of the pre-intended 0.25 mg phVEGF-A_{165}. Interestingly, no divergence in plasma VEGF-A levels was detected between patients.

Taken together our results in paper I+III implicate the existence of a therapeutic window for the pharmacological use of plasmid vectors. Several questions arise if this is proven true in further studies. One such important question being if the plasmid doses within that therapeutic window are sufficient for promoting functional angiogenesis with one administration or if repeated administration might be necessary to obtain optimal angiogenic effect.

Duration of plasmid gene expression

It is essential that the time course of transgene expression is appropriate for the disease to be treated. Seminal papers by Wolff et al in the early 1990's reported slow gene expression elevation but persistence for weeks or months after direct injection of pDNA into the myocardium or skeletal muscle [141-143], while later studies report differing gene expression duration. In paper III, we obtained rapid but considerably shorter pDNA expression with an abrupt decline four days after intramyocardial injection in rats, independent of which gene the plasmid encoded. The decline was on the other hand not to zero levels and gene expression could maybe have been detected for as long time as in the earliest reports. The relevance of such very low levels is debatable, as they are unlikely to have any biological effect. More important is how to detain stable high-level gene expression after pDNA delivery. Promotor inactivation is a factor determining the duration of gene expression and strong viral promoters, such as the CMV promotor, can be easily attenuated [144, 145]. We however detected no difference in time kinetics between the strong viral CMV promotor, the human EF1α and the hybrid SRα promoters. Vector backbone fragility can also affect the duration. While the vector construct was identical when comparing promoters, the CMV promotor was in total utilized in three vectors with differing backbones in this study. pDNA is prone to be entrapped and rapidly degraded in the cytoplasm, with an apparent half-life reported to be 50-90 minutes [146]. Thus, the most likely explanation to the short gene expression duration in paper III was loss of pDNA from transfected cells.

The short transient transgene expression derived from plasmids or Ad vectors has previously been considered advantageous, as indefinite angiogenic growth factor expression is neither required or desirable for treatment of CAD [147]. However, in a recent report prolonged VEGF-A expression (4 weeks) was obligatory to produce durable vessels [116]. Interestingly, although VEGF-A expression for two weeks alone produced nondurable vessels, two periods of two weeks VEGF-A expression separated by five weeks produced vessels persisting for months [116]. This implies that repeat administration of plasmids encoding VEGF-A_{165} can achieve persistent therapeutic effect.

Behaviour of plasmid in different tissues

Despite the fact that intense gene therapy research is currently ongoing, the pharmacokinetics and pharmacodynamics

of gene transfer vectors are largely unknown [148]. For example it is not known why bFGF protein was ineffective in treating CAD (FIRST trial [108]), but effective in treating peripheral artery disease (TRAFFIC trial [149]). Paradoxically, Ad-VEGF121 was effective in the myocardium (REVASC trial [150]) but instead ineffective in treating peripheral artery disease (RAVE trial [45]).

In previous preclinical reports, intramyocardial injection of pDNA resulted in higher gene expression than when the same pDNA was injected into skeletal muscle [151, 152]. In accordance with a common supposition that gene expression reflects vector uptake we hypothesized that this discrepancy in gene expression most likely was due to lower pDNA uptake in skeletal than in cardiac muscle. To examine the uptake of plasmids in different tissues more closely we therefore in paper IV quantitatively evaluated pDNA levels with RT-PCR after direct injection into respective muscle. Detecting substantially higher luciferase activity in cardiac than in skeletal muscle was anticipated. Finding approximately 3-fold higher pDNA levels in skeletal than in cardiac muscle one day after gene transfer was on the other hand completely unexpected. Preclinical studies on gene transfer have so far only indirectly tested pDNA uptake efficiency by monitoring transgene expression. Our finding that high transgene expression is not concurrent with presence of high gene vector levels question the relevance of using this method for evaluating pDNA uptake. Instead, it emphasizes the need to scrutinize different components of gene transfer and transgene expression by utilizing techniques that are more advanced.

The biotransformation of the delivered gene into the final therapeutic transgene product is a complex process where uptake of the transfer vectors, their ability to utilize intracellular transport mechanisms and innate transcriptional and translational machinery present within the host cell all are important components. It is pertinent to increase our understanding of biotransformation processes to achieve clinically successful gene therapy. Finding that pDNA behaves differently in different tissues in paper IV may contribute to clarify the contradictory results in clinical trials described above.

Measuring therapeutic effect in clinical trials

The goal of therapeutic angiogenesis in CAD patients is both relief of symptoms and improvement of cardiac function by increasing perfusion to the ischemic regions. There is yet no consensus on the best way to evaluate the effect of therapeutic angiogenesis.

Hard end-points such as death, myocardial infarction, or recurrent ischemia necessitating intervention are difficult to use on patients with advanced coronary artery disease as their degree of morbidity and mortality already is high. Two patients referred to our clinical trial dying before inclusion illustrates this point (paper V). The morbidity of concomitant surgery and the inability to enrol patients in a placebo arm limits evaluation of therapeutic effect in studies involving a mini-thoracotomy for intramyocardial gene delivery.

Quality of life measurements and exercise tolerance testing are often used clinical end-points. Significantly improved CCS class with a marked decrease in nitroglycerine intake as well as a tendency of increased exercise tolerance in our trial, match the positive results in other open trails. These measures are however subject to a strong placebo impact, as seen in the VIVA and FIRST trials [108, 109]. Objective end-points are therefore a necessity.

Angiography has long been the golden standard for objectively determining collateral vessel growth, but its use for assessment of neo-vascularization after gene transfer has lately been questioned [147]. Most collateral vessels formed after therapeutic angiogenesis have a diameter less than 200

μm [153, 154], a size beyond the resolution of current angiography [154, 155]. Perfusion imaging with SPECT provides an objective evaluation method, where improved reversible ischaemia after gene transfer is inferential evidence of angiogenesis. The spatial resolution in SPECT however cannot differentiate transmural gradients in blood flow and flow reserve [155]. Hence, the ability to detect subtle flow changes in the left ventricular wall with SPECT is suboptimal. The insensitivity of SPECT may explain why only four of seven patients in paper V had improved perfusion in the target region seen with SPECT, while all patients had improved TVI values in the target region [156]. Rapid development of other non-invasive methods for quantifying the collateral circulation, such as magnetic resonance imaging [157] and contrast echocardiography [158] provide new means for evaluating the effect of therapeutic angiogenesis.

Safety aspects of VEGF-A$_{165}$ gene therapy

The primary apprehension with the pharmacological use of phVEGF-A$_{165}$ for therapeutic myocardial angiogenesis has been the risk of enhancing unwanted angiogenesis at remote sites, such as stimulating occult tumour growth. We therefore repeatedly screened CEA and PSA values in our clinical trial, with solely negative results during the 12-month follow-up period. Negative side effects as hypotension or congestive heart failure were not noted.

The safety profile of completed therapeutic angiogenesis trials has overall been encouraging and the misgivings of negative side effects have yet not been justified. The incidence of cancer has so far been same as or lower than that in the general population of the same age [45, 159]. Likewise, other anticipated risks as worsening of retinopathy or atherosclerosis has not been detected in the clinical trials [45, 159, 160].

CONCLUSIONS

- The expression efficiency of plasmids encoding for hVEGF-A_{165} and the angiogenic effect with increased microvessel density after intramyocardial injection in rats is dose-correlated and tends to be enhanced in hypoxic conditions. Acute hypoxia in itself without treatment enhanced microvessel density.

- Transgene expression was, after catheter-based intramyocardial plasmid delivery in pigs, macroscopically localized only in the target region of the myocardium and microscopically at all levels of the left ventricular wall. Plasmids encoding hVEGF-A_{165} or a reporter gene were expressed to the same degree.

- Intramyocardial injection of plasmid DNA in rats resulted in localised transfection with minimal systemic spread. Transgene expression was transient and independent of which promotor was used. Plasmid protein expression was dose-related, with a possible saturable mechanism limiting the amount of plasmid DNA that can be transfected.

- Plasmid levels were markedly higher in skeletal muscle than in the myocardium one day after intramuscular and intramyocardial injection, but were after three days instead significantly lower than in the myocardium. Reporter gene expression was markedly higher in the myocardium than in skeletal muscle both one and three days after gene delivery, indicating that plasmid gene transfer efficacy differs between tissues.

- Intramyocardial injection of phVEGF-A_{165} via a mini-thoracotomy in patients with end-stage angina pectoris was performed without occurrence of transgene related adverse effects, and resulted in objective signs of improved myocardial perfusion and function as well as long-time subjective improvement.

Future perspectives: The results of the studies behind this thesis suggest that positive therapeutic effects can be achieved in the future with intramyocardial injection of plasmids encoding VEGF-A_{165} for treatment of coronary artery disease. It is feasible to with a catheter-based approach accomplish intramyocardial gene delivery with a high degree of anatomical precision, avoiding the potential risks associated with surgery, thus making it possible to design randomised double-blind studies for a more objective assessment of the therapeutic effect of angiogenic gene therapy. Although later controlled clinical trials on angiogenic gene therapy so far have only partially lived up to the great expectations evoked by the earlier open trials, gene transfer holds great promise for the treatment of cardiovascular diseases. To realize the goal of enhancing neovascularisation with angiogenic gene therapy it is however pertinent to increase our understanding of biotransformation processes involved in gene transfer. The results reported in this thesis emphasize the importance of continued research on the pharmacokinetics and pharmacodynamics of plasmid vectors, as well as improvement and refinement of plasmid constructs in order to amplify their clinical utility.

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References

[1] American Heart Association, in, 2004 americanheart.org/downloadable/heart/1077185395308FS06INT4(ebook).

[2] D. Mukherjee, D. L. Bhatt, M. T. Roe, V. Patel, and S. G. Ellis, Direct myocardial revascularization and angiogenesis--how many patients might be eligible?, Am J Cardiol 84 (1999) 598-600, A598.

[3] J. F. Hansen, Coronary collateral circulation: clinical significance and influence on survival in patients with coronary artery occlusion, Am Heart J 117 (1989) 290-295.

[4] S. Sasayama, and M. Fujita, Recent insights into coronary collateral circulation, Circulation 85 (1992) 1197-1204.

[5] M. Billinger, P. Kloos, F. R. Eberli, S. Windecker, B. Meier, and C. Seiler, Physiologically assessed coronary collateral flow and adverse cardiac ischemic events: a follow-up study in 403 patients with coronary artery disease, J Am Coll Cardiol 40 (2002) 1545-1550.

[6] D. Antoniucci, R. Valenti, G. Moschi, A. Migliorini, M. Trapani, G. M. Santoro, L. Bolognese, G. Cerisano, P. Buonamici, and E. V. Dovellini, Relation between preintervention angiographic evidence of coronary collateral circulation and clinical and angiographic outcomes after primary angioplasty or stenting for acute myocardial infarction, Am J Cardiol 89 (2002) 121-125.

[7] T. Pohl, C. Seiler, M. Billinger, E. Herren, K. Wustmann, H. Mehta, S. Windecker, F. R. Eberli, and B. Meier, Frequency distribution of collateral flow and factors influencing collateral channel development. Functional collateral channel measurement in 450 patients with coronary artery disease, J Am Coll Cardiol 38 (2001) 1872-1878.

[8] M. Hockel, K. Schlenger, S. Doctrow, T. Kissel, and P. Vaupel, Therapeutic angiogenesis, Arch Surg 128 (1993) 423-429.

[9] H. K. Hammond, and M. D. McKirnan, Angiogenic gene therapy for heart disease: a review of animal studies and clinical trials, Cardiovasc Res 49 (2001) 561-567.

[10] M. Azrin, Angiogenesis, protein and gene delivery, Br Med Bull 59 (2001) 211-225.

[11] N. Ferrara, and T. Davis-Smyth, The biology of vascular endothelial growth factor, Endocr Rev 18 (1997) 4-25.

[12] M. Greenblatt, and P. Shubi, Tumor angiogenesis: transfilter diffusion studies in the hamster by the transparent chamber technique, J Natl Cancer Inst 41 (1968) 111-124.

[13] R. L. Ehrmann, and M. Knoth, Choriocarcinoma. Transfilter stimulation of vasoproliferation in the hamster cheek pouch.studied by light and electron microscopy, J Natl Cancer Inst 41 (1968) 1329-1341.

[14] J. Folkman, Tumor angiogenesis: therapeutic implications, N Engl J Med 285 (1971) 1182-1186.

[15] J. Folkman, E. Merler, C. Abernathy, and G. Williams, Isolation of a tumor factor responsible or angiogenesis, J Exp Med 133 (1971) 275-288.

[16] J. Folkman, and M. Klagsbrun, Angiogenic factors, Science 235 (1987) 442-447.

[17] D. Gospodarowicz, Localisation of a fibroblast growth factor and its effect alone and with hydrocortisone on 3T3 cell growth, Nature 249 (1974) 123-127.

[18] T. Maciag, J. Cerundolo, S. Ilsley, P. R. Kelley, and R. Forand, An endothelial cell growth factor from bovine hypothalamus: identification and partial characterization, Proc Natl Acad Sci U S A 76 (1979) 5674-5678.

[19] D. Gospodarowicz, N. Ferrara, L. Schweigerer, and G. Neufeld, Structural characterization and biological functions of fibroblast growth factor, Endocr Rev 8 (1987) 95-114.

[20] J. Slavin, Fibroblast growth factors: at the heart of angiogenesis, Cell Biol Int 19 (1995) 431-444.

[21] K. A. Thomas, M. Rios-Candelore, G. Gimenez-Gallego, J. DiSalvo, C. Bennett, J. Rodkey, and S. Fitzpatrick, Pure brain-derived acidic fibroblast growth factor is a potent angiogenic vascular endothelial cell mitogen with sequence homology to interleukin 1, Proc Natl Acad Sci U S A 82 (1985) 6409-6413.

[22] F. Esch, A. Baird, N. Ling, N. Ueno, F. Hill, L. Denoroy, R. Klepper, D. Gospodarowicz, P. Bohlen, and R. Guillemin, Primary structure of bovine pituitary basic fibroblast growth factor (FGF) and comparison with the amino-terminal sequence of bovine brain acidic FGF, Proc Natl Acad Sci U S A 82 (1985) 6507-6511.

[23] M. Jaye, R. Howk, W. Burgess, G. A. Ricca, I. M. Chiu, M. W. Ravera, S. J. O'Brien, W. S. Modi,

T. Maciag, and W. N. Drohan, Human endothelial cell growth factor: cloning, nucleotide sequence, and chromosome localization, Science 233 (1986) 541-545.

[24] J. A. Abraham, A. Mergia, J. L. Whang, A. Tumolo, J. Friedman, K. A. Hjerrild, D. Gospodarowicz, and J. C. Fiddes, Nucleotide sequence of a bovine clone encoding the angiogenic protein, basic fibroblast growth factor, Science 233 (1986) 545-548.

[25] D. R. Senger, S. J. Galli, A. M. Dvorak, C. A. Perruzzi, V. S. Harvey, and H. F. Dvorak, Tumor cells secrete a vascular permeability factor that promotes accumulation of ascites fluid, Science 219 (1983) 983-985.

[26] N. Ferrara, and W. J. Henzel, Pituitary follicular cells secrete a novel heparin-binding growth factor specific for vascular endothelial cells, Biochem Biophys Res Commun 161 (1989) 851-858.

[27] D. T. Connolly, J. V. Olander, D. Heuvelman, R. Nelson, R. Monsell, N. Siegel, B. L. Haymore, R. Leimgruber, and J. Feder, Human vascular permeability factor. Isolation from U937 cells, J Biol Chem 264 (1989) 20017-20024.

[28] P. J. Keck, S. D. Hauser, G. Krivi, K. Sanzo, T. Warren, J. Feder, and D. T. Connolly, Vascular permeability factor, an endothelial cell mitogen related to PDGF, Science 246 (1989) 1309-1312.

[29] D. W. Leung, G. Cachianes, W. J. Kuang, D. V. Goeddel, and N. Ferrara, Vascular endothelial growth factor is a secreted angiogenic mitogen, Science 246 (1989) 1306-1309.

[30] D. Maglione, V. Guerriero, G. Viglietto, P. Delli-Bovi, and M. G. Persico, Isolation of a human placenta cDNA coding for a protein related to the vascular permeability factor, Proc Natl Acad Sci U S A 88 (1991) 9267-9271.

[31] B. Olofsson, K. Pajusola, A. Kaipainen, G. von Euler, V. Joukov, O. Saksela, A. Orpana, R. F. Pettersson, K. Alitalo, and U. Eriksson, Vascular endothelial growth factor B, a novel growth factor for endothelial cells, Proc Natl Acad Sci U S A 93 (1996) 2576-2581.

[32] V. Joukov, K. Pajusola, A. Kaipainen, D. Chilov, I. Lahtinen, E. Kukk, O. Saksela, N. Kalkkinen, and K. Alitalo, A novel vascular endothelial growth factor, VEGF-C, is a ligand for the Flt4 (VEGFR-3) and KDR (VEGFR-2) receptor tyrosine kinases, Embo J 15 (1996) 290-298.

[33] J. Lee, A. Gray, J. Yuan, S. M. Luoh, H. Avraham, and W. I. Wood, Vascular endothelial growth factor-related protein: a ligand and specific activator of the tyrosine kinase receptor Flt4, Proc Natl Acad Sci U S A 93 (1996) 1988-1992.

[34] M. Orlandini, L. Marconcini, R. Ferruzzi, and S. Oliviero, Identification of a c-fos-induced gene that is related to the platelet-derived growth factor/vascular endothelial growth factor family, Proc Natl Acad Sci U S A 93 (1996) 11675-11680.

[35] D. J. Lyttle, K. M. Fraser, S. B. Fleming, A. A. Mercer, and A. J. Robinson, Homologs of vascular endothelial growth factor are encoded by the poxvirus orf virus, J Virol 68 (1994) 84-92.

[36] D. T. Connolly, D. M. Heuvelman, R. Nelson, J. V. Olander, B. L. Eppley, J. J. Delfino, N. R. Siegel, R. M. Leimgruber, and J. Feder, Tumor vascular permeability factor stimulates endothelial cell growth and angiogenesis, J Clin Invest 84 (1989) 1470-1478.

[37] S. Banai, M. T. Jaklitsch, W. Casscells, M. Shou, S. Shrivastav, R. Correa, S. E. Epstein, and E. F. Unger, Effects of acidic fibroblast growth factor on normal and ischemic myocardium, Circ Res 69 (1991) 76-85.

[38] A. Yanagisawa-Miwa, Y. Uchida, F. Nakamura, T. Tomaru, H. Kido, T. Kamijo, T. Sugimoto, K. Kaji, M. Utsuyama, C. Kurashima, and et al., Salvage of infarcted myocardium by angiogenic action of basic fibroblast growth factor, Science 257 (1992) 1401-1403.

[39] S. Banai, M. T. Jaklitsch, M. Shou, D. F. Lazarous, M. Scheinowitz, S. Biro, S. E. Epstein, and E. F. Unger, Angiogenic-induced enhancement of collateral blood flow to ischemic myocardium by vascular endothelial growth factor in dogs, Circulation 89 (1994) 2183-2189.

[40] S. Takeshita, L. P. Zheng, E. Brogi, M. Kearney, L. Q. Pu, S. Bunting, N. Ferrara, J. F. Symes, and J. M. Isner, Therapeutic angiogenesis. A single intraarterial bolus of vascular endothelial growth factor augments revascularization in a rabbit ischemic hind limb model, J Clin Invest 93 (1994) 662-670.

[41] S. Takeshita, L. Q. Pu, L. A. Stein, A. D. Sniderman, S. Bunting, N. Ferrara, J. M. Isner, and J. F. Symes, Intramuscular administration of vascular endothelial growth factor induces dose-dependent collateral artery augmentation in a rabbit model of chronic limb ischemia, Circulation 90 (1994) II228-234.

[42] Y. Tsurumi, S. Takeshita, D. Chen, M. Kearney, S. T. Rossow, J. Passeri, J. R. Horowitz, J. F. Symes, and J. M. Isner, Direct intramuscular gene transfer of naked DNA encoding vascular endothelial growth factor augments collateral

development and tissue perfusion, Circulation 94 (1996) 3281-3290.

[43] I. Baumgartner, A. Pieczek, O. Manor, R. Blair, M. Kearney, K. Walsh, and J. M. Isner, Constitutive expression of phVEGF165 after intramuscular gene transfer promotes collateral vessel development in patients with critical limb ischemia, Circulation 97 (1998) 1114-1123.

[44] A. H. Hamawy, L. Y. Lee, R. G. Crystal, and T. K. Rosengart, Cardiac angiogenesis and gene therapy: a strategy for myocardial revascularization, Curr Opin Cardiol 14 (1999) 515-522.

[45] S. Yla-Herttuala, and K. Alitalo, Gene transfer as a tool to induce therapeutic vascular growth, Nat Med 9 (2003) 694-701.
[46] J. Folkman, Angiogenesis in cancer, vascular, rheumatoid and other disease, Nat Med 1 (1995) 27-31.

[47] P. Carmeliet, Mechanisms of angiogenesis and arteriogenesis, Nat Med 6 (2000) 389-395.
[48] A. Luttun, and P. Carmeliet, De novo vasculogenesis in the heart, Cardiovasc Res 58 (2003) 378-389.

[49] A. Papapetropoulos, G. Garcia-Cardena, J. A. Madri, and W. C. Sessa, Nitric oxide production contributes to the angiogenic properties of vascular endothelial growth factor in human endothelial cells, J Clin Invest 100 (1997) 3131-3139.

[50] H. F. Dvorak, L. F. Brown, M. Detmar, and A. M. Dvorak, Vascular permeability factor/vascular endothelial growth factor, microvascular hyperpermeability, and angiogenesis, Am J Pathol 146 (1995) 1029-1039.

[51] E. M. Conway, D. Collen, and P. Carmeliet, Molecular mechanisms of blood vessel growth, Cardiovasc Res 49 (2001) 507-521.

[52] W. Risau, Differentiation of endothelium, Faseb J 9 (1995) 926-933.

[53] J. A. Forsythe, B. H. Jiang, N. V. Iyer, F. Agani, S. W. Leung, R. D. Koos, and G. L. Semenza, Activation of vascular endothelial growth factor gene transcription by hypoxia-inducible factor 1, Mol Cell Biol 16 (1996) 4604-4613.

[54] K. P. Claffey, S. C. Shih, A. Mullen, S. Dziennis, J. L. Cusick, K. R. Abrams, S. W. Lee, and M. Detmar, Identification of a human VPF/VEGF 3' untranslated region mediating hypoxia-induced mRNA stability, Mol Biol Cell 9 (1998) 469-481.

[55] N. Van Royen, J. J. Piek, W. Schaper, C. Bode, and I. Buschmann, Arteriogenesis: mechanisms and modulation of collateral artery development, J Nucl Cardiol 8 (2001) 687-693.

[56] W. Schaper, and W. D. Ito, Molecular mechanisms of coronary collateral vessel growth, Circ Res 79 (1996) 911-919.

[57] T. Asahara, T. Murohara, A. Sullivan, M. Silver, R. van der Zee, T. Li, B. Witzenbichler, G. Schatteman, and J. M. Isner, Isolation of putative progenitor endothelial cells for angiogenesis, Science 275 (1997) 964-967.

[58] T. Asahara, H. Masuda, T. Takahashi, C. Kalka, C. Pastore, M. Silver, M. Kearne, M. Magner, and J. M. Isner, Bone marrow origin of endothelial progenitor cells responsible for postnatal vasculogenesis in physiological and pathological neovascularization, Circ Res 85 (1999) 221-228.

[59] T. Takahashi, C. Kalka, H. Masuda, D. Chen, M. Silver, M. Kearney, M. Magner, J. M. Isner, and T. Asahara, Ischemia- and cytokine-induced mobilization of bone marrow-derived endothelial progenitor cells for neovascularization, Nat Med 5 (1999) 434-438.

[60] C. Kalka, H. Masuda, T. Takahashi, W. M. Kalka-Moll, M. Silver, M. Kearney, T. Li, J. M. Isner, and T. Asahara, Transplantation of ex vivo expanded endothelial progenitor cells for therapeutic neovascularization, Proc Natl Acad Sci U S A 97 (2000) 3422-3427.

[61] T. Asahara, T. Takahashi, H. Masuda, C. Kalka, D. Chen, H. Iwaguro, Y. Inai, M. Silver, and J. M. Isner, VEGF contributes to postnatal neovascularization by mobilizing bone marrow-derived endothelial progenitor cells, Embo J 18 (1999) 3964-3972.

[62] E. Tischer, R. Mitchell, T. Hartman, M. Silva, D. Gospodarowicz, J. C. Fiddes, and J. A. Abraham, The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing, J Biol Chem 266 (1991) 11947-11954.

[63] K. A. Houck, N. Ferrara, J. Winer, G. Cachianes, B. Li, and D. W. Leung, The vascular endothelial growth factor family: identification of a fourth molecular species and characterization of alternative splicing of RNA, Mol Endocrinol 5 (1991) 1806-1814.

[64] G. Neufeld, T. Cohen, S. Gengrinovitch, and Z. Poltorak, Vascular endothelial growth factor (VEGF) and its receptors, Faseb J 13 (1999) 9-22.

[65] J. E. Park, G. A. Keller, and N. Ferrara, The vascular endothelial growth factor (VEGF) isoforms: differential deposition into the

subepithelial extracellular matrix and bioactivity of extracellular matrix-bound VEGF, Mol Biol Cell 4 (1993) 1317-1326.

[66] K. A. Houck, D. W. Leung, A. M. Rowland, J. Winer, and N. Ferrara, Dual regulation of vascular endothelial growth factor bioavailability by genetic and proteolytic mechanisms, J Biol Chem 267 (1992) 26031-26037.

[67] N. Ferrara, H. P. Gerber, and J. LeCouter, The biology of VEGF and its receptors, Nat Med 9 (2003) 669-676.

[68] S. Soker, S. Takashima, H. Q. Miao, G. Neufeld, and M. Klagsbrun, Neuropilin-1 is expressed by endothelial and tumor cells as an isoform-specific receptor for vascular endothelial growth factor, Cell 92 (1998) 735-745.

[69] S. Banai, D. Shweiki, A. Pinson, M. Chandra, G. Lazarovici, and E. Keshet, Upregulation of vascular endothelial growth factor expression induced by myocardial ischaemia: implications for coronary angiogenesis, Cardiovasc Res 28 (1994) 1176-1179.

[70] H. P. Gerber, V. Dixit, and N. Ferrara, Vascular endothelial growth factor induces expression of the antiapoptotic proteins Bcl-2 and A1 in vascular endothelial cells, J Biol Chem 273 (1998) 13313-13316.

[71] H. P. Gerber, A. McMurtrey, J. Kowalski, M. Yan, B. A. Keyt, V. Dixit, and N. Ferrara, Vascular endothelial growth factor regulates endothelial cell survival through the phosphatidylinositol 3'-kinase/Akt signal transduction pathway. Requirement for Flk-1/KDR activation, J Biol Chem 273 (1998) 30336-30343.

[72] L. E. Benjamin, D. Golijanin, A. Itin, D. Pode, and E. Keshet, Selective ablation of immature blood vessels in established human tumors follows vascular endothelial growth factor withdrawal, J Clin Invest 103 (1999) 159-165.

[73] F. Yuan, Y. Chen, M. Dellian, N. Safabakhsh, N. Ferrara, and R. K. Jain, Time-dependent vascular regression and permeability changes in established human tumor xenografts induced by an anti-vascular endothelial growth factor/vascular permeability factor antibody, Proc Natl Acad Sci U S A 93 (1996) 14765-14770.

[74] M. Clauss, M. Gerlach, H. Gerlach, J. Brett, F. Wang, P. C. Familletti, Y. C. Pan, J. V. Olander, D. T. Connolly, and D. Stern, Vascular permeability factor: a tumor-derived polypeptide that induces endothelial cell and monocyte procoagulant activity, and promotes monocyte migration, J Exp Med 172 (1990) 1535-1545.

[75] D. I. Gabrilovich, H. L. Chen, K. R. Girgis, H. T. Cunningham, G. M. Meny, S. Nadaf, D. Kavanaugh, and D. P. Carbone, Production of vascular endothelial growth factor by human tumors inhibits the functional maturation of dendritic cells, Nat Med 2 (1996) 1096-1103.

[76] P. Carmeliet, V. Ferreira, G. Breier, S. Pollefeyt, L. Kieckens, M. Gertsenstein, M. Fahrig, A. Vandenhoeck, K. Harpal, C. Eberhardt, C. Declercq, J. Pawling, L. Moons, D. Collen, W. Risau, and A. Nagy, Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele, Nature 380 (1996) 435-439.

[77] N. Ferrara, K. Carver-Moore, H. Chen, M. Dowd, L. Lu, K. S. O'Shea, L. Powell-Braxton, K. J. Hillan, and M. W. Moore, Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene, Nature 380 (1996) 439-442.

[78] F. Shalaby, J. Rossant, T. P. Yamaguchi, M. Gertsenstein, X. F. Wu, M. L. Breitman, and A. C. Schuh, Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice, Nature 376 (1995) 62-66.

[79] G. H. Fong, J. Rossant, M. Gertsenstein, and M. L. Breitman, Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium, Nature 376 (1995) 66-70.

[80] A. Rivard, J. E. Fabre, M. Silver, D. Chen, T. Murohara, M. Kearney, M. Magner, T. Asahara, and J. M. Isner, Age-dependent impairment of angiogenesis, Circulation 99 (1999) 111-120.

[81] A. Rivard, M. Silver, D. Chen, M. Kearney, M. Magner, B. Annex, K. Peters, and J. M. Isner, Rescue of diabetes-related impairment of angiogenesis by intramuscular gene therapy with adeno-VEGF, Am J Pathol 154 (1999) 355-363.

[82] J. Waltenberger, J. Lange, and A. Kranz, Vascular endothelial growth factor-A-induced chemotaxis of monocytes is attenuated in patients with diabetes mellitus: A potential predictor for the individual capacity to develop collaterals, Circulation 102 (2000) 185-190.

[83] T. Couffinhal, M. Silver, M. Kearney, A. Sullivan, B. Witzenbichler, M. Magner, B. Annex, K. Peters, and J. M. Isner, Impaired collateral vessel development associated with reduced expression of vascular endothelial growth factor in ApoE-/- mice, Circulation 99 (1999) 3188-3198.

[84] J. Duan, T. Murohara, H. Ikeda, A. Katoh, S. Shintani, K. Sasaki, H. Kawata, N. Yamamoto, and T. Imaizumi, Hypercholesterolemia inhibits angiogenesis in response to hindlimb ischemia: nitric oxide-dependent mechanism, Circulation 102 (2000) III370-376.

[85] A. Schultz, L. Lavie, I. Hochberg, R. Beyar, T. Stone, K. Skorecki, P. Lavie, A. Roguin, and A. P. Levy, Interindividual heterogeneity in the hypoxic regulation of VEGF: significance for the development of the coronary artery collateral circulation, Circulation 100 (1999) 547-552.

[86] E. Van Belle, A. Rivard, D. Chen, M. Silver, S. Bunting, N. Ferrara, J. F. Symes, C. Bauters, and J. M. Isner, Hypercholesterolemia attenuates angiogenesis but does not preclude augmentation by angiogenic cytokines, Circulation 96 (1997) 2667-2674.

[87] F. D. Ledley, Pharmaceutical approach to somatic gene therapy, Pharm Res 13 (1996) 1595-1614.

[88] D. F. Lazarous, M. Shou, J. A. Stiber, D. M. Dadhania, V. Thirumurti, E. Hodge, and E. F. Unger, Pharmacodynamics of basic fibroblast growth factor: route of administration determines myocardial and systemic distribution, Cardiovasc Res 36 (1997) 78-85.

[89] R. Kornowski, S. Fuchs, M. B. Leon, and S. E. Epstein, Delivery strategies to achieve therapeutic myocardial angiogenesis, Circulation 101 (2000) 454-458.

[90] D. F. Lazarous, M. Scheinowitz, M. Shou, E. Hodge, S. Rajanayagam, S. Hunsberger, W. G. Robison, Jr., J. A. Stiber, R. Correa, S. E. Epstein, and et al., Effects of chronic systemic administration of basic fibroblast growth factor on collateral development in the canine heart, Circulation 91 (1995) 145-153.

[91] R. Yang, G. R. Thomas, S. Bunting, A. Ko, N. Ferrara, B. Keyt, J. Ross, and H. Jin, Effects of vascular endothelial growth factor on hemodynamics and cardiac performance, J Cardiovasc Pharmacol 27 (1996) 838-844.

[92] M. D. Hariawala, J. R. Horowitz, D. Esakof, D. D. Sheriff, D. H. Walter, B. Keyt, J. M. Isner, and J. F. Symes, VEGF improves myocardial blood flow but produces EDRF-mediated hypotension in porcine hearts, J Surg Res 63 (1996) 77-82.

[93] F. J. Giordano, P. Ping, M. D. McKirnan, S. Nozaki, A. N. DeMaria, W. H. Dillmann, O. Mathieu-Costello, and H. K. Hammond, Intracoronary gene transfer of fibroblast growth factor-5 increases blood flow and contractile function in an ischemic region of the heart, Nat Med 2 (1996) 534-539.

[94] R. J. Laham, M. Rezaee, M. Post, X. Xu, and F. W. Sellke, Intrapericardial administration of basic fibroblast growth factor: myocardial and tissue distribution and comparison with intracoronary and intravenous administration, Catheter Cardiovasc Interv 58 (2003) 375-381.

[95] D. Hou, F. Maclaughlin, M. Thiesse, V. R. Panchal, B. C. Bekkers, E. A. Wilson, P. I. Rogers, M. C. Coleman, and K. L. March, Widespread regional myocardial transfection by plasmid encoding Del-1 following retrograde coronary venous delivery, Catheter Cardiovasc Interv 58 (2003) 207-211.

[96] R. J. Laham, F. W. Sellke, E. R. Edelman, J. D. Pearlman, J. A. Ware, D. L. Brown, J. P. Gold, and M. Simons, Local perivascular delivery of basic fibroblast growth factor in patients undergoing coronary bypass surgery: results of a phase I randomized, double-blind, placebo-controlled trial, Circulation 100 (1999) 1865-1871.

[97] M. Ruel, R. J. Laham, J. A. Parker, M. J. Post, J. A. Ware, M. Simons, and F. W. Sellke, Long-term effects of surgical angiogenic therapy with fibroblast growth factor 2 protein, J Thorac Cardiovasc Surg 124 (2002) 28-34.

[98] J. F. Symes, D. W. Losordo, P. R. Vale, K. G. Lathi, D. D. Esakof, M. Mayskiy, and J. M. Isner, Gene therapy with vascular endothelial growth factor for inoperable coronary artery disease, Ann Thorac Surg 68 (1999) 830-836; discussion 836-837.

[99] T. K. Rosengart, L. Y. Lee, S. R. Patel, T. A. Sanborn, M. Parikh, G. W. Bergman, R. Hachamovitch, M. Szulc, P. D. Kligfield, P. M. Okin, R. T. Hahn, R. B. Devereux, M. R. Post, N. R. Hackett, T. Foster, T. M. Grasso, M. L. Lesser, O. W. Isom, and R. G. Crystal, Angiogenesis gene therapy: phase I assessment of direct intramyocardial administration of an adenovirus vector expressing VEGF121 cDNA to individuals with clinically significant severe coronary artery disease, Circulation 100 (1999) 468-474.

[100] R. Kornowski, M. B. Leon, S. Fuchs, Y. Vodovotz, M. A. Flynn, D. A. Gordon, A. Pierre, I. Kovesdi, J. A. Keiser, and S. E. Epstein, Electromagnetic guidance for catheter-based transendocardial injection: a platform for intramyocardial angiogenesis therapy. Results in normal and ischemic porcine models, J Am Coll Cardiol 35 (2000) 1031-1039.

[101] A. H. Baker, Designing gene delivery vectors for cardiovascular gene therapy, Prog Biophys Mol Biol 84 (2004) 279-299.

[102] E. M. Eastman, and R. H. Durland, Manufacturing and quality control of plasmid-based gene expression systems, Adv Drug Deliv Rev 30 (1998) 33-48.

[103] W. J. Kelly, Perspectives on plasmid-based gene therapy: challenges for the product and the

process, Biotechnol Appl Biochem 37 (2003) 219-223.

[104] V. Budker, T. Budker, G. Zhang, V. Subbotin, A. Loomis, and J. A. Wolff, Hypothesis: naked plasmid DNA is taken up by cells in vivo by a receptor-mediated process, J Gene Med 2 (2000) 76-88.

[105] C. M. Wiethoff, and C. R. Middaugh, Barriers to nonviral gene delivery, J Pharm Sci 92 (2003) 203-217.

[106] J. A. Wolff, Naked DNA transport and expression in mammalian cells, Neuromuscul Disord 7 (1997) 314-318.

[107] D. W. Losordo, P. R. Vale, R. C. Hendel, C. E. Milliken, F. D. Fortuin, N. Cummings, R. A. Schatz, T. Asahara, J. M. Isner, and R. E. Kuntz, Phase 1/2 placebo-controlled, double-blind, dose-escalating trial of myocardial vascular endothelial growth factor 2 gene transfer by catheter delivery in patients with chronic myocardial ischemia, Circulation 105 (2002) 2012-2018.

[108] M. Simons, B. H. Annex, R. J. Laham, N. Kleiman, T. Henry, H. Dauerman, J. E. Udelson, E. V. Gervino, M. Pike, M. J. Whitehouse, T. Moon, and N. A. Chronos, Pharmacological treatment of coronary artery disease with recombinant fibroblast growth factor-2: double-blind, randomized, controlled clinical trial, Circulation 105 (2002) 788-793.

[109] T. D. Henry, B. H. Annex, G. R. McKendall, M. A. Azrin, J. J. Lopez, F. J. Giordano, P. K. Shah, J. T. Willerson, R. L. Benza, D. S. Berman, C. M. Gibson, A. Bajamonde, A. C. Rundle, J. Fine, and E. R. McCluskey, The VIVA trial: Vascular endothelial growth factor in Ischemia for Vascular Angiogenesis, Circulation 107 (2003) 1359-1365.

[110] R. J. Laham, N. A. Chronos, M. Pike, M. E. Leimbach, J. E. Udelson, J. D. Pearlman, R. I. Pettigrew, M. J. Whitehouse, C. Yoshizawa, and M. Simons, Intracoronary basic fibroblast growth factor (FGF-2) in patients with severe ischemic heart disease: results of a phase I open-label dose escalation study, J Am Coll Cardiol 36 (2000) 2132-2139.

[111] T. D. Henry, K. Rocha-Singh, J. M. Isner, D. J. Kereiakes, F. J. Giordano, M. Simons, D. W. Losordo, R. C. Hendel, R. O. Bonow, S. M. Eppler, T. F. Zioncheck, E. B. Holmgren, and E. R. McCluskey, Intracoronary administration of recombinant human vascular endothelial growth factor to patients with coronary artery disease, Am Heart J 142 (2001) 872-880.

[112] C. L. Grines, M. W. Watkins, G. Helmer, W. Penny, J. Brinker, J. D. Marmur, A. West, J. J. Rade, P. Marrott, H. K. Hammond, and R. L. Engler, Angiogenic Gene Therapy (AGENT) trial in patients with stable angina pectoris, Circulation 105 (2002) 1291-1297.

[113] C. L. Grines, M. W. Watkins, J. J. Mahmarian, A. E. Iskandrian, J. J. Rade, P. Marrott, C. Pratt, and N. Kleiman, A randomized, double-blind, placebo-controlled trial of Ad5FGF-4 gene therapy and its effect on myocardial perfusion in patients with stable angina, J Am Coll Cardiol 42 (2003) 1339-1347.

[114] D. Stewart, A phase 2, randomized, multicenter, 26-week study to assess the efficacy and safety of BIOBYPASS (AdGVVEGF121.10) delivered through minimally invasive surgery versus maximum medical treatment in patients with severe angina, advanced coronary artery disease, and no option for revascularization., Circulation 106 (2002) 2986-a.

[115] M. Hedman, J. Hartikainen, M. Syvanne, J. Stjernvall, A. Hedman, A. Kivela, E. Vanninen, H. Mussalo, E. Kauppila, S. Simula, O. Narvanen, A. Rantala, K. Peuhkurinen, M. S. Nieminen, M. Laakso, and S. Yla-Herttuala, Safety and feasibility of catheter-based local intracoronary vascular endothelial growth factor gene transfer in the prevention of postangioplasty and in-stent restenosis and in the treatment of chronic myocardial ischemia: phase II results of the Kuopio Angiogenesis Trial (KAT), Circulation 107 (2003) 2677-2683.

[116] Y. Dor, V. Djonov, R. Abramovitch, A. Itin, G. I. Fishman, P. Carmeliet, G. Goelman, and E. Keshet, Conditional switching of VEGF provides new insights into adult neovascularization and pro-angiogenic therapy, Embo J 21 (2002) 1939-1947.

[117] N. Bessis, F. J. GarciaCozar, and M. C. Boissier, Immune responses to gene therapy vectors: influence on vector function and effector mechanisms, Gene Ther 11 Suppl 1 (2004) S10-17.

[118] N. Chirmule, K. Propert, S. Magosin, Y. Qian, R. Qian, and J. Wilson, Immune responses to adenovirus and adeno-associated virus in humans, Gene Ther 6 (1999) 1574-1583.

[119] S. Lehrman, Virus treatment questioned after gene therapy death, Nature 401 (1999) 517-518.
[120] E. Marshall, Gene therapy death prompts review of adenovirus vector, Science 286 (1999) 2244-2245.

121] D. W. Losordo, J. G. Pickering, S. Takeshita, G. Leclerc, D. Gal, L. Weir, M. Kearney, J. Jekanowski, and J. M. Isner, Use of the rabbit ear artery to serially assess foreign protein secretion

after site-specific arterial gene transfer in vivo. Evidence that anatomic identification of successful gene transfer may underestimate the potential magnitude of transgene expression, Circulation 89 (1994) 785-792.

[122] K. Makinen, H. Manninen, M. Hedman, P. Matsi, H. Mussalo, E. Alhava, and S. Yla-Herttuala, Increased vascularity detected by digital subtraction angiography after VEGF gene transfer to human lower limb artery: a randomized, placebo-controlled, double-blinded phase II study, Mol Ther 6 (2002) 127-133.

[123] Y. Liu, L. C. Mounkes, H. D. Liggitt, C. S. Brown, I. Solodin, T. D. Heath, and R. J. Debs, Factors influencing the efficiency of cationic liposome-mediated intravenous gene delivery, Nat Biotechnol 15 (1997) 167-173.

[124] T. Nomura, S. Nakajima, K. Kawabata, F. Yamashita, Y. Takakura, and M. Hashida, Intratumoral pharmacokinetics and in vivo gene expression of naked plasmid DNA and its cationic liposome complexes after direct gene transfer, Cancer Res 57 (1997) 2681-2686.

[125] M. Nishikawa, and M. Hashida, Nonviral approaches satisfying various requirements for effective in vivo gene therapy, Biol Pharm Bull 25 (2002) 275-283.

[126] Y. Hojo, U. Ikeda, Y. Zhu, M. Okada, S. Ueno, H. Arakawa, H. Fujikawa, T. Katsuki, and K. Shimada, Expression of vascular endothelial growth factor in patients with acute myocardial infarction, J Am Coll Cardiol 35 (2000) 968-973.

[127] S. Takeshita, T. Isshiki, and T. Sato, Increased expression of direct gene transfer into skeletal muscles observed after acute ischemic injury in rats, Lab Invest 74 (1996) 1061-1065.

[128] J. A. Fallavollita, B. J. Malm, and J. M. Canty, Jr., Hibernating myocardium retains metabolic and contractile reserve despite regional reductions in flow, function, and oxygen consumption at rest, Circ Res 92 (2003) 48-55.

[129] P. Carmeliet, Y. S. Ng, D. Nuyens, G. Theilmeier, K. Brusselmans, I. Cornelissen, E. Ehler, V. V. Kakkar, I. Stalmans, V. Mattot, J. C. Perriard, M. Dewerchin, W. Flameng, A. Nagy, F. Lupu, L. Moons, D. Collen, P. A. D'Amore, and D. T. Shima, Impaired myocardial angiogenesis and ischemic cardiomyopathy in mice lacking the vascular endothelial growth factor isoforms VEGF164 and VEGF188, Nat Med 5 (1999) 495-502.

[130] K. W. Lee, G. Y. Lip, and A. D. Blann, Plasma angiopoietin-1, angiopoietin-2, angiopoietin receptor tie-2, and vascular endothelial growth factor levels in acute coronary syndromes, Circulation 110 (2004) 2355-2360.

[131] H. Suzuki, M. Murakami, M. Shoji, Y. Iso, T. Kondo, M. Shibata, H. Ezumi, Y. Hamazaki, S. Koba, and T. Katagiri, Hepatocyte growth factor and vascular endothelial growth factor in ischaemic heart disease, Coron Artery Dis 14 (2003) 301-307.

[132] A. Kawamoto, H. Kawata, Y. Akai, Y. Katsuyama, E. Takase, Y. Sasaki, S. Tsujimura, Y. Sakaguchi, M. Iwano, S. Fujimoto, T. Hashimoto, and K. Dohi, Serum levels of VEGF and basic FGF in the subacute phase of myocardial infarction, Int J Cardiol 67 (1998) 47-54.

[133] P. W. Radke, A. Heinl-Green, O. M. Frass, U. Griesenbach, S. Ferrari, D. M. Geddes, and E. W. Alton, Effects of intramyocardial pVEGF165 delivery on regional myocardial blood flow: evidence for a spatial 'delivery-efficacy' mismatch, Gene Ther 11 (2004) 1249-1255.

[134] A. Keck, K. Hertting, Y. Schwartz, R. Kitzing, M. Weber, B. Leisner, C. Franke, E. Bahlmann, C. Schneider, T. Twisselmann, M. Weisbach, R. Kuchler, and K. H. Kuck, Electromechanical mapping for determination of myocardial contractility and viability. A comparison with echocardiography, myocardial single-photon emission computed tomography, and positron emission tomography, J Am Coll Cardiol 40 (2002) 1067-1074; discussion 1075-1068.

[135] P. M. Grossman, Z. Han, M. Palasis, J. J. Barry, and R. J. Lederman, Incomplete retention after direct myocardial injection, Catheter Cardiovasc Interv 55 (2002) 392-397.

[136] G. S. Kassab, C. A. Rider, N. J. Tang, and Y. C. Fung, Morphometry of pig coronary arterial trees, Am J Physiol 265 (1993) H350-365.
[137] G. S. Kassab, and Y. C. Fung, Topology and dimensions of pig coronary capillary network, Am J Physiol 267 (1994) H319-325.

[138] G. S. Kassab, D. H. Lin, and Y. C. Fung, Morphometry of pig coronary venous system, Am J Physiol 267 (1994) H2100-2113.

[139] M. Manthorpe, F. Cornefert-Jensen, J. Hartikka, J. Felgner, A. Rundell, M. Margalith, and V. Dwarki, Gene therapy by intramuscular injection of plasmid DNA: studies on firefly luciferase gene expression in mice, Hum Gene Ther 4 (1993) 419-431.

[140] M. Y. Levy, L. G. Barron, K. B. Meyer, and F. C. Szoka, Jr., Characterization of plasmid DNA transfer into mouse skeletal muscle: evaluation of uptake mechanism, expression and secretion of gene products into blood, Gene Ther 3 (1996) 201-211.

[141] J. A. Wolff, R. W. Malone, P. Williams, W. Chong, G. Acsadi, A. Jani, and P. L. Felgner, Direct gene transfer into mouse muscle in vivo, Science 247 (1990) 1465-1468.

[142] J. A. Wolff, J. J. Ludtke, G. Acsadi, P. Williams, and A. Jani, Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle, Hum Mol Genet 1 (1992) 363-369.

[143] G. Acsadi, S. S. Jiao, A. Jani, D. Duke, P. Williams, W. Chong, and J. A. Wolff, Direct gene transfer and expression into rat heart in vivo, New Biol 3 (1991) 71-81.

[144] L. Qin, Y. Ding, D. R. Pahud, E. Chang, M. J. Imperiale, and J. S. Bromberg, Promoter attenuation in gene therapy: interferon-gamma and tumor necrosis factor-alpha inhibit transgene expression, Hum Gene Ther 8 (1997) 2019-2029.

[145] J. S. Harms, and G. A. Splitter, Interferon-gamma inhibits transgene expression driven by SV40 or CMV promoters but augments expression driven by the mammalian MHC I promoter, Hum Gene Ther 6 (1995) 1291-1297.

[146] D. Lechardeur, K. J. Sohn, M. Haardt, P. B. Joshi, M. Monck, R. W. Graham, B. Beatty, J. Squire, H. O'Brodovich, and G. L. Lukacs, Metabolic instability of plasmid DNA in the cytosol: a potential barrier to gene transfer, Gene Ther 6 (1999) 482-497.

[147] M. Simons, R. O. Bonow, N. A. Chronos, D. J. Cohen, F. J. Giordano, H. K. Hammond, R. J. Laham, W. Li, M. Pike, F. W. Sellke, T. J. Stegmann, J. E. Udelson, and T. K. Rosengart, Clinical trials in coronary angiogenesis: issues, problems, consensus: An expert panel summary, Circulation 102 (2000) E73-86.

[148] S. Pislaru, S. P. Janssens, B. J. Gersh, and R. D. Simari, Defining gene transfer before expecting gene therapy: putting the horse before the cart, Circulation 106 (2002) 631-636.

[149] R. J. Lederman, F. O. Mendelsohn, R. D. Anderson, J. F. Saucedo, A. N. Tenaglia, J. B. Hermiller, W. B. Hillegass, K. Rocha-Singh, T. E. Moon, M. J. Whitehouse, and B. H. Annex, Therapeutic angiogenesis with recombinant fibroblast growth factor-2 for intermittent claudication (the TRAFFIC study): a randomised trial, Lancet 359 (2002) 2053-2058.

[150] N. P. Fam, S. Verma, M. Kutryk, and D. J. Stewart, Clinician guide to angiogenesis, Circulation 108 (2003) 2613-2618.

[151] R. N. Kitsis, P. M. Buttrick, E. M. McNally, M. L. Kaplan, and L. A. Leinwand, Hormonal modulation of a gene injected into rat heart in vivo, Proc Natl Acad Sci U S A 88 (1991) 4138-4142.

[152] R. von Harsdorf, R. J. Schott, Y. T. Shen, S. F. Vatner, V. Mahdavi, and B. Nadal-Ginard, Gene injection into canine myocardium as a useful model for studying gene expression in the heart of large mammals, Circ Res 72 (1993) 688-695.

[153] F. C. White, S. M. Carroll, A. Magnet, and C. M. Bloor, Coronary collateral development in swine after coronary artery occlusion, Circ Res 71 (1992) 1490-1500.

[154] S. Takeshita, T. Isshiki, H. Mori, E. Tanaka, K. Eto, Y. Miyazawa, A. Tanaka, Y. Shinozaki, K. Hyodo, M. Ando, M. Kubota, K. Tanioka, K. Umetani, M. Ochiai, T. Sato, and H. Miyashita, Use of synchrotron radiation microangiography to assess development of small collateral arteries in a rat model of hindlimb ischemia, Circulation 95 (1997) 805-808.

[155] J. D. Pearlman, R. J. Laham, and M. Simons, Coronary angiogenesis: detection in vivo with MR imaging sensitive to collateral neocirculation--preliminary study in pigs, Radiology 214 (2000) 801-807.

[156] S. Altinmakas, B. Dagdeviren, M. Turkmen, M. Gursurer, B. Say, T. Tezel, and B. Ersek, Usefulness of pulse-wave Doppler tissue sampling and dobutamine stress echocardiography for identification of false positive inferior wall defects in SPECT, Jpn Heart J 41 (2000) 141-152.

[157] S. Mankad, R. Khalil, and C. M. Kramer, MRI for the diagnosis of myocardial ischemia and viability, Curr Opin Cardiol 18 (2003) 351-356.

[158] S. F. de Marchi, M. Schwerzmann, M. Fleisch, M. Billinger, B. Meier, and C. Seiler, Quantitative contrast echocardiographic assessment of collateral derived myocardial perfusion during elective coronary angioplasty, Heart 86 (2001) 324-329.

[159] M. Simons, and J. A. Ware, Therapeutic angiogenesis in cardiovascular disease, Nat Rev Drug Discov 2 (2003) 863-871.

[160] J. M. Isner, P. R. Vale, J. F. Symes, and D. W. Losordo, Assessment of risks associated with cardiovascular gene therapy in human subjects, Circ Res 89 (2001) 389-400.

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