Therapeutic Neovascularization – Quo Vadis?

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CONTENTS

PREFACE

Therapeutic Angiogenesis – quo Vadis? This was the question left after several clinical trials probing the clinical applicability of a tried and proven experimental concept yielded mixed results. Patients reported relief from symptoms, at times in the placebo group as well. Nevertheless this achievement may be viewed as major success in a painful no-option situation. More objective endpoints were rarely met with pro-angiogenic growth factor protein application. As Jens Kastrup illustrates, this data set blunted some of the hopes associated with the concept of new vessel formation, a situation not profoundly changed with the advent of adenoviral based gene therapy. In great detail, Petra Korpisalo, Tuomas Rissanen and Seppo Ylä-Herttuala scrutinize the strengths and weaknesses of this widely used vector system.

One of the potential factors causing the bench-to-bedside gap within the therapeutic angiogenesis concept is the difference between an otherwise healthy lab animal (even though a large one) and a patient population with various comorbidities confounding the principles of angiogenesis. Vadim Tchaikovski and Johannes Waltenberger illustrate the multiple dysfunctional elements in angiogenic signalling of diabetic patients. In real world coronary artery disease patients treated by percutaneous coronary interventions, Rohit Khurana and Michael Simons point to the problem of endothelial activation which is helpful for luminal endothelial regeneration, however, at the expense of advential neovascularization and increased neointima formation.

As the status quo in the therapeutic neovascularization field suggests, there is room for improvement. Mark Post, Richard Cornelussen and Frits Prinzen recapitulate the current molecular concepts of cardioprotection and explore the value of pre- and postconditioning for the (post)ischemic heart. One obstacle to patient treatment is targeting of therapeutic agents towards the region of ischemia. In the setting of severe arterial disease, using the venous system might be advantegous, as Peter Boekstegers and Christian Kupatt suggest. Even if expressed in the ischemic region, one factor might be less effective than a family of growth factors, i.e. driven by the same transcription factor. Karen Vincent and Ralph Kelly followed the integrative approach overexpressing a constitutively active HIF 1α/VP16 construct. In order to make therapeutic neovascularization last, Andrea Banfi, Philipp Fueglistaler and Roberto Gianni-Barrera focus on the unresolved issue of vessel maturation and provides stunning evidence for a successful partnership of VEGFs and PDGFs. Beyond the vascular tool box, Serena Zacchigna, Carmen Ruiz

de Almodovar, Peggy Lafuste and Peter Carmeliet draw parallels between vascular and neuronal networks and provide novel therapeutic options. As a surprise candidates for induction of neovascularization, the cathelicidins as antimicrobiologic peptids were identified recently. Robert Bals and Rembert Koczulla summarize their experience with LL37, a human peptide of this family.

A separate collection of evolutionary concepts of neovascularization is dedicated to cell based approaches, which are at times more integral, at times more selective and regulated than mono- or bimolecular interventions, since instead of a factor a whole factory in principle capable of adapting to the environments demands is offered as therapeutic principle. An array of different adult and embryonic cell-based approaches is investigated to date, as Mathias Lamparter and Antonis Hatzopoulos point out, usually offering paracrine software rather than vasculo-specific hardware (building blocks). Olivier Feron traces the role of eNOS and its microenvironmental partner, caveolin-1, in the context of adult vasculogenesis, from mobiliazation of EPCs from bone marrow niches towards their recruitment to the ischemic musculature. Wulf Ito scrutinizes the role of monocytes/macrophages and resident vascular precursor cells for the induction of a neovasculatory response.

Reviewing the whole body of work, we can't deny the impression that the concept of therapeutic neovascularization is far from exhaustion. Instead, a variety of substantial improvements, at times break-throughs, at the conceptional level as well as at the delivery and vector level are currently being evolved. Therefore, this volume is presenting some of the most impressive steps towards a vital future of biological induction of new vessels. We are confident that this fascinating collection of experienced perspectives will offer fresh insights allowing to refine our understanding and therapeutic approaches of therapeutic neovascularization. Indeed, it is our conviction that scientific modifications of a fundamentally sound concept will enable its applicability in the not so distant future

Munich, December 2006

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CHAPTER 1

CLINICAL VASCULAR GROWTH FACTOR THERAPY FOR NEOVASCULARIZATION IN PATIENTS WITH CORONARY ARTERY DISEASE

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Abstract: Several vascular growth factors have the potential to induce angiogenesis in ischemic tissue. However, only vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF) have been tested in clinical studies of patients with coronary artery disease. Several small and unblinded studies with either recombinant growth factor proteins or genes encoding the growth factors have been performed in patients with severe CAD and results have been encouraging, demonstrating both clinical improvement and evidence of angiogenesis. However, a few larger double-blind randomised placebo-controlled studies have not been able to confirm the initial high efficacy of the growth factor therapy. Ongoing clinical trials with increased gene dose will demonstrate whether the used methodologies and genes are effective. In future trials one have to consider whether improved transfection vectors, combination of genes and stem cells or gene transfected cells will enhance the efficacy of the treatments. The conducted clinical studies with growth factor therapies have all been without any gene related adverse events, which supports the initiation of more large scaled clinical trials to evaluate whether vascular growth factor therapy either as a gene or recombinant slow-release protein formulation therapy could be a new treatment modality to patients with severe coronary artery disease, which cannot be treated with conventional revascularization

Keywords: gene therapy, Vascular Growth Factors, angiogenesis, ischemic heart, review, VEGF, FGF, stem cell

Abbreviations: Ad.: Adenovirus; CABG: Coronary by-pass grafting; FGF: Fibroblast growth factor; i.c: Intra-coronary; i.m.: Intramyocardial; i.v.: Intra-venous; M.c.: Sustained release heparin-alginate FGF2 microcapsules; MRI: Magnetic Resonance Imaging investigation; PCI: Percutaneous coronary intervention; Pl.: Plasmid; SPECT: Single Photon Emission Computerized Tomography; VEGF: Vascular endothelial growth factor

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Within the last two centuries, the development of and progress in modern cardiovascular drug therapies and mechanical revascularization with balloon angioplasty and coronary artery by-pass surgery has improved the prognosis for patients with both acute and chronic myocardial ischemia. However, there is still a large amount of patients with severe coronary artery disease, which cannot be treated satisfactorily with conventional therapies. This has lead to an extensive research to find new treatment modalities. The intensive research within the field of molecular biology has discovered several families of proteins with an angiogenic potential [1]. However, only a few of these vascular growth factors have moved from preclinical animal studies into clinical trials. The vascular endothelial growth factors and the fibroblast growth factors both have the potential to induce therapeutic angiogenesis, i.e. growth of new vessels, in human myocardium and they have both been tested in patients with coronary artery disease.

This review focus on the results obtained by clinical therapeutic angiogenesis with vascular growth factors and the perspective for this treatment in the future in patients with severe ischemic coronary artery disease.

1. ANGIOGENESIS AND ARTERIOGENESIS

Neovascularization, the formation of new blood vessels, is inherent in vascular tissue, and it can be induced by trauma, ischemia, inflammation or tumour growth [1]. The creation of new blood vessels is dependent on a complicated interaction between local produced cytokines and cell derived from the tissue area and the blood circulation [1].

However, the vascular growth factors are of crucial importance for the neovascularization, which can be divided into three processes: angiogenesis, vasculogenesis and arteriogenesis. *Angiogenesis* is the formation of new capillaries by sprouding from the existing capillary net, probably from the postcapillary venules [2]; *arteriogenesis* is the transformation of pre-existing arterioles/collaterales into small muscular arteries and/or de novo formation of new vessels with a tunica media [3, 4]; and *vasculogenesis* is the formation of new vessels from multipotent endothelial stem cells [1, 5–7]. Angiogenesis, arteriogenesis and vasculogenesis are functional connected phenomenon's, which cannot be separated. Formation of new capillaries (angiogenesis) without simultaneously formation of larger arteries for supplying the capillaries is without any meaning.

Angiogenesis in the tissue can be initiated by local production and liberation of vascular growth factors. Many different vascular growth factors have now been discovered, which can induce angiogenesis by stimulation of growth and migration of endothelial cells [1]. The vascular growth factors are polypeptides, initially isolated in studies of tumour growth. These proteins are responsible for normal as well as pathological vessel growth. For therapeutic treatment in myocardial ischemia, the most used proteins have been members of the fibroblast growth factor (FGF) family and the vascular endothelial growth factor (VEGF) family. FGF induce vascular growth by binding to receptors at the surface of the endothelial

cells. However, receptors for FGF are also located on other cell types, e.g. fibroblasts. VEGF binds to receptors, which mainly are located on the endothelial cells. Presently, VEGF-A and VEGF-C are the two factors with the greatest clinical impact in the adult man. VEGF-C is important for growth of lymphatics, while VEGF-A is of importance for angiogenesis. VEGF-A can be divided into five isoformes with 121, 145, 165, 189, and 206 amino acids. Both VEGF and FGF have in animal studies induced angiogenesis and arteriogenesis with formation of new capillaries and arteries in ischemic myocardium [1, 8, 9].

2. VASCULAR GROWTH FACTOR THERAPY IN CARDIAC DISEASE

Several clinical angiogenesis trials have been conducted in patients with coronary artery disease. The investigators have either used synthesized recombinant vascular growth factor proteins or the genes encoding these proteins. Both methods have advantages and disadvantages. The optimal growth factor therapy to induce angiogenesis in ischemic myocardium can be defined as a therapy with 1) local accumulation of or stimulation of the production of growth factors, 2) to a certain therapeutic concentration, 3) for a certain appropriate period, 4) in an isolated ischemic tissue area, and 5) with a minimal overflow of growth factors into non-ischemic tissues far away from the tissue area of interest. The used growth factor formulations and the methods used for application of the treatment in clinical trials have fulfilled these demands to a varying degree.

2.1. Trials Using Recombinant Vascular Growth Factor Protein Therapy

Different delivery modalities have been applied in clinical studies using recombinant growth factor therapies (Table 1). In most studies the growth factors have either been injected directly into the myocardium after a thoracotomy, into the coronary artery supplying the ischemic myocardium, administered intravenously systemically, or by a combination of these treatments [10–19]. The goal has been to reach a sufficient stimulation of the ischemic myocardium without any or minimal systemic side effects.

1. Recombinant fibroblast growth factor (FGF) protein therapy

In the initial safety and efficacy studies recombinant FGF2 protein was delivered to the myocardium either as direct myocardial injections or as slow-release formulations in microcapsules during CABG in the tissue areas, where it was impossible to perform surgical revascularization (10–12) (Table 1). Schumacher et al. (10) investigated in a double-blind 1:1 placebo controlled design recombinant FGF2 protein treatment in 40 patients with three vessels disease. The patients all had both a proximal left anterior descending artery (LAD) stenosis, which could be bridged by an internal mammary artery graft (IMA) bypass and a distal LAD stenosis, which could not be revascularized. After bypass grafting of all treatable arteries, several injections were given intramyocardially,

Table 1. Treatment with recombinant vascular growth factor proteins for myocardial angiogenesis in chronic ischemic heart disease

	Recombinant vascular growth factors protein trials					
	Growth factor	n	Administration	Randomized	Double-blind	Treatment effect
Schumacher et al. 1998[10]	FGF2 10 μg pr kg body-weight	40	Thoracotomy + i.m.	20:20(placebo)	Yes	Improved perfusion on angiography
Sellke et al. 1998[11]	FGF2 10 μg (4), 100 μg (4)	8	Thoracotomy + m.c.	8:0(controls)	No	Tendency to improved perfusion on SPECT
Laham et al. 1999[12]	FGF2 10 μg (8), 100 μg (8)	24	Thoracotomy + m.c.	8:8:8 (placebo)	Yes	Improved perfusion on SPECT with 100 μg
Laham et al. 2000[14]	FGF2 0.33–48 μg pr kg body-weight	52	I.c.	52:0(controls)	No	Improved wall motion and ischemic area with MRI**
Unger et al. 2000[15]	FGF2 3–30 μg pr kg body-weight	25	I.c.	17:8 (placebo)	No	No improvement in exercise**
Simons et al. 2002[16]	FGF2 0.3–30 μg pr kg body-weight	337	I.c.	82:84:85:86(placebo)	Yes	Identical improvement in end-points in all groups
Hendel et al. 2000[17]	VEGF-A_{165} 0.005–0.167 μg pr kg body-weight	14	I.c.	14:0(controls)	No	Tendency to improved perfusion on SPECT
Henry et. 2003[18]	VEGF-A_{165} 0.34 or 1.0 μg pr kg body-weight	178	I.c. + i.v.	56:59:63 (placebo)	Yes	Identical improvement in end-points in all groups

* Published as an abstract

** Data pooled for all patients

+ Treatment effect, − No treatment effect

I.m. = Intramyocardial, I.c. = Intracoronary,

I.v. = Intravenous. M.c. = Sustained release heparin-alginate FGF2 microcapsules

MRI – Magnetic Resonance Imaging investigation

SPECT – Single Photon Emission Computerized Tomography

10 microgram/kg FGF2 or saline, close and distally to the IMA anastomosis alongside the LAD as far as the lower end of the distal anastomosis.

Angiographic control 12 weeks after treatment suggested more capillaries and more contrast accumulation in these areas in the recombinant FGF2 treated patients compared with the control group. With the same surgical approach in an identical group of patients, Laham et al. (12) implanted epicardially sustained-release heparin-alginate microcapsules with recombinant FGF2 during CABG in a double-blind, placebo controlled dose-titrating trial in 24 patients. Three groups, with 8 patients in each, received either 10 microgram FGF2, 100 microgram FGF2 or saline. The FGF2 was released slowly within 4–6 weeks. There was 2 operative death and 3 Q-wave myocardial infarctions. At the 3 months follow-up the nuclear single photon emission computerized tomography (SPECT) disclosed a reduction in defect size in the group treated with 100 microgram in comparison to no improvement in the two other treatment groups. The long-time follow-up, 32 months, of the 22 surviving patients found no difference in CCS class between the two FGF2 groups, but both groups had more freedom from recurrent angina compared to placebo treated. When combining the two FGF2 groups, SPECT demonstrated less reversible or fixed perfusions defects in these patients compared to controls. Importantly, no long-term side-effects to the FGF2 therapy was registered.

It is well known from PCI studies of occluded or subtotal occluded vessels that the collaterals supplying the ischemic myocardium disappear within minutes after opening of the vessel. Identical changes are seen after grafting of occluded vessels. This re-distribution of blood flow also influences the interpretation of perfusion scans. It is, therefore, very difficult in this type of studies with vascular growth factor treatment simultaneously with CABG to evaluate and separate the effect of the coronary by-pass grafting and the recombinant FGF2 treatment on improvement or changes in perfusion scans. Moreover, it is impossible to test whether an improvement in symptoms is due to the by-pass grafting or the growth factor treatment. In another study, fifty-two patients that were suboptimal candidates for conventional revascularization were treated with intracoronary infusion of increasing dosis of recombinant FGF2 (14). The infusions were generally well tolerated, although hypotension occurred in some patients at the highest dose. There were 3 deaths and 4 Q-wave myocardial infarctions in the follow-up period unrelated to the FGF2 dose used. At the two months follow-up, the patients had less angina, improved exercise capacity and reduced ischemic territory at MRI perfusion imaging. These data were supported by a small placebo controlled, dose escalating safety study [15]. Intracoronary infusion of recombinant FGF2 was performed in 17 patients and a placebo infusion in 8 patients, all with angiographic significant coronary stenosis. There were only few side-effects such as mild hypotension, slight transient trombocytopenia and proteinuria. These results suggest that intracoronary treatment with recombinant FGF2 is safe and may have a clinical beneficial effect.

Simons et al [16] tested this hypothesis in the FIRST-trial, a double-blind dose-escalating placebo controlled phase II study, with 337 patients with three different intracoronary dosages of recombinant FGF2 (0.3, 3 and 30 microgram/kg) versus placebo (16). All four groups had an increase in primary endpoint, exercise tolerance test, after 90 days, without any difference between groups. Moreover, there was no improvement in nuclear myocardial perfusion scans in the groups. There was a significant reduction in clinical angina in the 3 microgram/kg group at 90 days follow-up, but not at 180 days in any of the treated groups.

2. Recombinant vascular endothelial growth factor (VEGF) protein therapy
Two small phase I safety and feasibility trials using either intra-venous or intra-coronary delivery of recombinant VEGF-A_{165} treatment in patients with severe coronary artery disease demonstrated an increase in exercise capacity without any safety issues [17] (Table 1). The resting nuclear myocardial perfusion scans indicated a VEGF-A_{165} treatment effect. However, no effect was demonstrated on stress scans using exercise, dobutamine, or dipyridamole stimulation tests (18).

Henry et al [18] then conducted the Vascular endothelial growth factor (VEGF) in Ischemia for Vascular Angiogenesis - VIVA trial [18]. It was a double-blind, placebo-controlled, phase II trial designed to evaluate the safety, efficacy, pharmacokinetics of combined intracoronary and intravenous infusions of recombinant human VEGF for angiogenesis. A total of 178 patients with coronary artery disease were treated with two intracoronary recombinant VEGF-A_{165} or placebo infusions each for 10 minutes (placebo, 17 or 50 nanogram VEGF-A_{165} /kg/min) followed by 4 hours intravenous infusion of the randomised drug (placebo, 17 or 50 nanogram VEGF/kg/min) on day 3, 6, and 9. The chosen treatment regimes were safe, however no improvement was discovered in the primary endpoint – treadmill exercise performance in any of the groups.

2.1.1. Conclusions on recombinant vascular growth factor protein trials

It was a surprising that neither of the two larger controlled trials with recombinant FGF2 or VEGF-A_{165} protein therapy could detect any clinical or objective improvement in the patients with moderate to severe coronary artery disease [16, 18], when comparing with previous published animal and unblinded clinical trials. An explanation could be, that when recombinant FGF2 is administered i.c., only 3–5% of the dose is recovered in the myocardium, and only 0.5 % of the dose after an i.v. administration [19]. Therefore, the long-time epicardial sustained-release heparin-alginate microcapsules principle is of potential great interest and importance, due to the prolonged delivery of the growth factors locally, but limited by its invasive approach [12]. The used treatment regimes with the chosen doses seemed to be safe. It has been suggested that a higher dose potentially could had improved the clinical out-come. However, it has to be documented that increasing the dose improves the clinical endpoints without increasing the side-effects of the treatment.

As in many angiogenesis trials a large improvement was also discovered in the placebo group in these two large scaled, well designed and well-conducted phase II studies with recombinant FGF2 and VEGF-A_{165} [16, 18]. The exercise

capacity and the symptoms improved by the same amount in the active treated and the placebo group. It emphasizes the importance of having both subjective and objective end-point in the trials. Identical results were discovered in the in the larger randomised placebo-controlled double-blind TRAFFIC trial with intra-arterial FGF2 injections in patients with intermittent claudication [20]. Peak walking time increase 90 days after treatment, but disappeared after 180 days. However, this effect was probably a random finding, since treatment with the same dose FGF2 at day 1 and 30 was without any clinical effect. Also the RAVE trial [21] a randomised placebo-controlled double-blind study with AdVEGF-121intramuscular injections in patients with peripheral artery disease could not detect any clinical improvements. Another explanation for the negative results could be, that a single intracoronary and intravenous dose of recombinant FGF2 and VEGF-A_{165} is unable to induce formation of collaterals.

The conflicting results emphasise the importance of conducting well-designed placebo-controlled phase II studies to clarify an eventually beneficial effect of treatment with recombinant vascular growth factor proteins.

2.2. Trials Using Genes Encoding Vascular Growth Factor

In patients with coronary artery disease, studies have evaluated the angiogenic potential of genes encoding VEGF and FGF. The study population has in almost all studies been patients with severe coronary artery disease, which could not be treated optimal with conventional revascularization therapies.

The principle of gene therapy is that a gene encoding the vascular growth factor is delivered to the cells as a cDNA (complementary DNA) formulation, which then is transcribed into the nucleus of the cell (transfection). The vascular growth factors are then produced locally for a longer period, hereby having a more steady biological effect [1]. The DNA vector has in clinical trials been delivered to the myocardium by direct intracoronary injections or direct intra-myocardial injections during by-pass surgery or using a more a-traumatic percutaneous method. By using injections directly into the ischemic myocardium, the side effects caused by increased systemic levels of growth factors in non-ischemic tissue out-side the treatment area are few.

The gene encoding for a vascular growth factor can be transfected to a tissue by three different formulations; As a naked plasmid-DNA, as a liposome plasmid-DNA complex or by the use of different viral vectors (retrovirus, adeno-associated virus or adenovirus). Transfection with plasmid-DNA alone or in a liposom complex is very simple, but the efficacy is low. Less than 1 % of the plasmid DNA is entering the cells. The retrovirus is entering the cells by specific receptors on the cell surface. The retrovirus can only transfect proliferating cells, which is a limitation of the method in ischemic heart disease, since only a few cells are in a proliferative phase in the myocardium. Moreover, the retrovirus RNA-genom is integrated into the host DNA, where it persists in the host genom in the daughter cells during the following cell proliferations. This integration can be a limitation, if the aim is to initiate an expression of the gene for only a shorter period. Adenovirus is also using specific

cell surface receptors to enter the cells, but the gene activation is independent of cell proliferation, since it not is integrated in the host genom. However, the adenovirus induces immunological and inflammatory reactions, which can reduce the period of gene activity and inhibit a later re-administration of adenovirus. In the recent years much interest has been put into the adeno-associated virus, which might be a better vector since it induce less immunological reaction.

The gene transcription occurs in the nucleus and initiates the production of vascular growth factors in the cytoplasma. In opposition to the transfection with retrovirus, the plasmid-, adeno-associated- or adenovirus-DNA are not incorporated into the host genom. Therefore, when using plasmid/adenovirus-DNA, the growth factors will only be produced in a short period of maybe 4 weeks, then the genes are metabolised and removed from the cells.

Both the plasmid and the adeno-virus formulations have been used for transfection of the myocardial cells with gene coding for VEGF or FGF in clinical therapeutic studies in patients with coronary artery disease. The genes were initially delivered to the myocardium either by directly intracoronary infusions or by direct intramyocardial injections during CABG or with a thoracotomy alone. However, with the development of the percutaneous delivery systems as the NOVA system (Biosense Webster, Cordis, Warren, US) the trend is now to use the less traumatic percutaneous method.

2.2.1. Trials with intracoronary delivery of vascular growth factor genes

The first published study of intracoronary VEGF gene transfer was a small phase I safety and efficacy trials. Laitinen [22] (Table 2) found that it was safe to perform intracoronary infusion for 10 minute of 1.000 μgram plasmid-VEGF-A_{165} in 10 patients treated with PCI. Hedman et al [23] compared intracoronary injections of plasmid/liposome-VEGF-A_{165} and Ad-VEGF-A_{165} treatment in patients undergoing elective PCI for coronary artery stenosis. They found that the myocardial perfusion improved significantly in the Ad-VEGF-A_{165} treated patients but the improvements in the plasmid/liposome-VEGF-A_{165} and control patients were not significant. However, there was no difference in improvements between the three groups. Therefore, the changes discovered in the Ad-VEGF-A_{165} treated patients could either be due to the gene therapy alone, the revascularization therapy alone or the combined therapy.

Grines et al have performed several studies using adenovirus for gene transfer of FGF4 in patients with coronary artery disease [24, 25]. In opposition to most other growth factor trials, these patients had less coronary artery disease and angina pectoris, and they were all by the core angiography laboratory judged to be suitable candidates for angioplasty or by-pass surgery. In the AGENT-1 trial the patients were in a randomised, double-blind placebo-controlled trial treated in a ratio 3:1 with 6 ascending doses from 3.3×10^8–10^{11} particle unit (pu)/patient [24]. The ad5-FGF4 virus was infusion into all major patent coronary arteries that could be engaged. The median calculated extraction rate across the coronary circulation was 87%. The infusion was generally well tolerated, but a majority of patients had a rise

Table 2. Intracoronary treatment with genes encoding for vascular growth factors for myocardial angiogenesis in chronic ischemic heart disease

	Growth factors – Gene					
	Growth factor	n	Administration	Randomized	Double-blind	Treatment effect
Laitinen et al 2000[22]	Pl.-VEGF-A_{165} 1, 000 μg	15	I.c.	10:5(placebo)	No	Not measured
Hedmann et al 2003[23] KAT study	Pl.-VEGF-A_{165} 2, 000 μg Ad.- VEGF-A_{165}	103	PCI with stent followed by I.c.	28:37:38 (placebo)	Yes	Improved perfusion on SPECT with Ad.-VEGF-A_{165}
Grines et al. 2002[24] Agent-1	Ad-FGF4	69	I.c.	9:9:9:22:11:19 (placebo)	Yes	(Tendency to improved exercise time in subgroup)
Grines et al. 2003[25] Agent-2	Ad-FGF4	52	I.c.	35:17 (placebo)	Yes	Improved perfusion on SPECT
Grines et al. 2004 Agent-3 and -4	Ad-FGF4	456 250	I.c.		Yes	Closed January 2004 due to low efficacy in interim analysis

Pl. – plasmid, Ad. – adenovirus.
+ Treatment effect, − No treatment effect
I.m. = Intramyocardial, I.c. = Intracoronary, I.v. = Intravenous.
SPECT – Single Photon Emission Computerized Tomography

in neutralizing antibodies to the adenovirus. There was no significant improvement in primary end-point exercise capacity, neither for the individual doses nor for the polled data. In a subgroup analyse, an improvement was discovered in patients with baseline exercise time ≤ 10 min.

In the following double-blind placebo-controlled study AGENT-2 [25] they authors used 10^{10} ad5-FGF4 virus particle unit (pu)/patient and demonstrated improved myocardial perfusion compared to the placebo group. Based on these encouraging results they initiated the two larger multi-centre studies AGENT-3 and-4, aiming to include a total of 456 and 250 patients, respectively. However, these two trials were both stopped January 2004 after an interim analysis demonstrating insufficient evidence of efficacy. Hopefully, the results, from these two studier, will be published to increase the knowledge about efficacy and side-effects for the planning of future clinical gene therapy trials.

2.2.2. *Trials with direct intramyocardial delivery of vascular growth factor genes*

The majority of clinical VEGF gene therapy trials have used directly intramyocardial injection of the gene either during coronary by-pass surgery or by using a percutaneous delivery method [26]. The studies included patients with chronic stable angina due to angiographically documented coronary artery disease, which could not be treated with conventional therapy.

In an open study, Symes et al [27] (Table 3) injected via a mini-thoracotomy two different doses of plasmid VEGF-A_{165} (125 microgram (n = 10) and 250 microgram (n = 10)) intramyocardially into the antero-lateral region of the left ventricle with the patients in general anaesthesia without any problems. No side-effects were registered. Nuclear myocardial perfusion scans demonstrated an improvement 60 days after treatment. Using identical surgical set-up for gene transfer, Sylvén et al [28, 29] treated 6 patients with intramyocardial injections of 250 micrograms plasmid VEGF-A_{165}. An improvement was registered in echocardiographic myocardial tissue Doppler velocity and in clinical status. Myocardial perfusion improved on SPECT in 3 of the 6 patients. One patient had a perioperative myocardial infarction. The results from 12 months follow-up in these patients demonstrated persistent improvement in clinical status and in echocardiographic evaluation [29]. No long-term side-effects to the gene therapy was reported.

The invasive thoracotomy approach was also used in two trials testing the safety and efficacy of adenovirus transfection with the $VEGF_{121}$ gene [30, 31] (Table 3). In the first study $Ad_{GV}VEGF_{121}$ was injected intramyocardial in an area with reversible ischemia either as an adjunct to conventional CABG (n = 15) or as a sole therapy via a minithoracotomy (n = 6) [30]. Five different vector doses were used in the first patient group (4×10^9–4×10^{10} particle unit (pu)/patient), while the second group was treated with 4×10^9 pu/patient. No evidence of systemic or cardiac related adverse effects was reported, and no adenovirus was detected in peripheral blood samples. Only a slight increase

Table 3. Direct intramyocardial delivery of genes encoding for vascular growth factors for myocardial angiogenesis in chronic ischemic heart disease

	Growth factors – Gene					
	Growth factor	n	Administration	Randomized	Double-blind	Treatment effect
Symes et al. 1999[27]	Pl.-VEGF-A_{165} 125 μg (10) : 250 μg (10)	20	Thoracotomy + i.m.	10:10:0 (controls)	No	Improved perfusion on SPECT and angiography
Vale et al. 2000[32]	Pl.-VEGF-A_{165} 250 μg (5) : 500 μg (8)	13	Thoracotomy + i.m.	13:0 (controls)	No	Improved perfusion on SPECT and NOGA
Sylvén et al. 2001[28]	Pl.-VEGF-A_{165} 250 μg (4) : 1, 000 μg (2)	6	Thoracotomy + i.m.	6:0 (controls)	No	Improved perfusion on SPECT and improved symptoms
Tio et al 2004[34]	Pl.-VEGF-A_{165} 500 μg	10	Percutaneously	10:12 DMR*: 13 (controls)		Improved perfusion on PET with VEGF
Kastrup et al 2005[35] EUROINJECT trial	Pl.-VEGF-A_{165} 500 μg	80	Percutaneously	40:40 (placebo)	Yes	Improved wall motion
Ripa et al 2006[50]	Pl.-VEGF-A_{165} 500 μg followed by 10 μgram/kg body weight G-CSF for 6 days	48	Percutaneously	16 VEGF + G-CSF:16 VEGF:16 (placebo)	No	No improvement in perfusion on SPECT
Rosengart et al. 1999[30]	Ad.-VEGF-A_{121}	21	Thoracotomy + i.m.	21:0 (controls)	No	Tendency to improved wall motion and perfusion on SPECT and angiography
Stewart et al 2006[31] REVASC trial	Ad.-VEGF-A_{121}	67	Thoracotomy + i.m.	32:35 (controls)	No	Increased exercise time
Vale et al. 2001[32]	Pl.-VEGF2 200 μg	6	Percutaneously	3:3 (controls)**	No	Improved perfusion on SPECT and NOGA. Improved symptoms
Losordo et al. 2002[33]	Pl.-VEGF-2 200 μg (6) : 800 μg (6) : 2, 000 μg (1)	19	Percutaneously	12:7 (placebo)	No	Increased exercise time and improved symptoms

Pl. – plasmid, Ad. – adenovirus.
+ Treatment effect, − No treatment effect
I.m. = Intramyocardial, I.c. = Intracoronary, I.v. = Intravenous.
* Direct myocardial revascularization (DMR) with laser therapy
** The controls crossed over to active treatment 90 days after inclusion in trial.
SPECT – Single Photon Emission Computerized Tomography

in plasma VEGF could be detected on day three. This is in opposition to the studies using plasmid formulations, where all patients had a persistent increase for 2–3 weeks. This might be explained by the development of neutralizing antibodies against the virus in all patients. However, it was possible to detect a reduction in angina pectoris and a trend towards improved myocardial perfusion evaluated by angiography. The REVASC study randomised patients to either maximum medical therapy (n = 35) versus AdVEGF121 (4×10^{10} particle unit (pu)) (n = 32) administered by direct intramyocardial injections via a minithoracotomy [31]. Patients treated with AdVEGF121 had significant improved exercise time and reduced symptoms compared to controls. However, there was no effect on myocardial perfusion measured by SPECT. There was some procedure related adverse events in the thoracotomy group, but there was no overall significant difference in adverse events between the groups. Recently, a new method (NOGA, Biosense Webster, Cordis, Warren, US) for evaluation of the function of left ventricle and for percutaneously delivery of substances to the myocardium has been applied in some studies [26]. This method creates a three-dimensional electromechanical map of the left ventricle by a mapping catheter introduced into the left ventricle via a femoral arterial puncture (Figure 1a). It is then possible to guide an injection catheter to the area of the myocardium with reduced perfusion, but living myocytes "Electro-mechanical mismatch" and perform the injections of the genes (Figure 1b). Studies utilizing the minimal invasive catheter delivery system may avoid the complications of thoracotomy and allow for a rigorous blinded design.

This NOGA method was initially used in two minor safety and efficacy studies to evaluate the effect of gene therapy with plasmid VEGF-2. [32, 33] (Table 3). In the first study, plasmid VEGF-A_{121} (250 microgram) was injected directly into the ischemic myocardium in six patients without any complications [32]. The patients improved clinically with less angina pectoris and nitroglycerin consumption. In addition, both the electromechanical and nuclear perfusion studies demonstrated reduced ischemic myocardium. Based on these results a phase I/II placebo-controlled, double-blind, dose escalating trial was performed [33]. The investigators used percutaneous delivery into the myocardium of placebo (saline) or plasmid VEGF-2 (20 micrograms, n = 9, or 800 microgram, n = 9, or 2.000 microgram, n = 1) in a randomised design (2 (VEGF-2):1 (placebo)). The study was scheduled to include nine patients in each dose treatment group. However, after inclusion of 19 patients, the trial was interrupted by the FDA in the wake of the death of an 18-years-old subject enrolled in an unrelated study of ornithine transcarbaamylase deficiency involving adenoviral GTx. Accordingly, data for the used doses of VEGF-A_{121} were pooled to improve statistical power. At 12 weeks follow-up, angina pectoris was significantly reduced in all active treated patients compared to placebo treated. There was a non-significant trend towards improvement in treadmill exercise time and Seattle Angina Pectoris Questionnaire.

Tio et al. 2004 [34] compared VEGF-A gene therapy with direct myocardial revascularization (DMR) with laser therapy. The myocardial perfusion was

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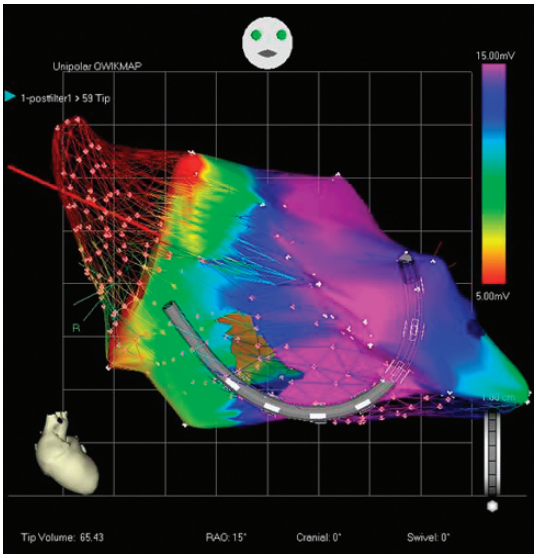

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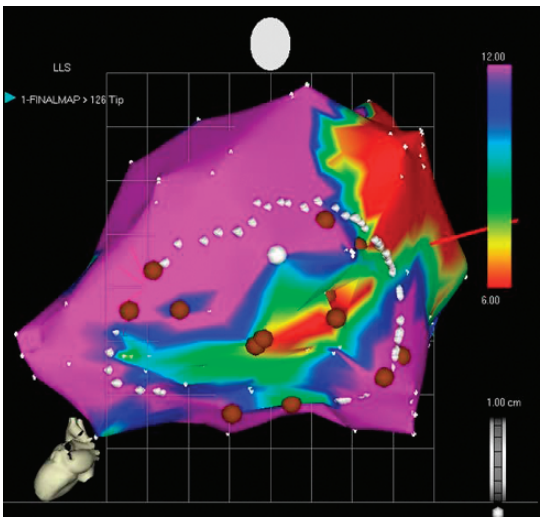

Figure 1. (a) Mapping of the left ventricle with the NOGA XP system (Biosense Webster, Cordis, Warren, US). (b) Left ventricle NOGA map with injection of genes encoding VEGF-A into ischemic myocardium indicated by brown dots

improved in the VEGF group in the regional ischemic area, but not compared to DMR and controls. The later two groups had no changes in myocardial perfusion measured by PET. The randomized double-blind placebo-controlled EUROINJECT -1 trial was the first larger gene therapy study using the percutaneous delivery technique [35, 36]. Forty patients received ten injections of plasmid

VEGF-A_{165} (total dose 0.5 mg) and 40 patients received ten placebo plasmid injections in an ischemic region of the left ventricle. The plasmids were delivered via the percutaneous route, using the percutaneous NOGA catheter system (Biosense Webster, Cordis, Warren, US). Myocardial perfusion improved ($P < 0.02$) following VEGF gene transfer in 44%, was unchanged in 41% and impaired in 15% of

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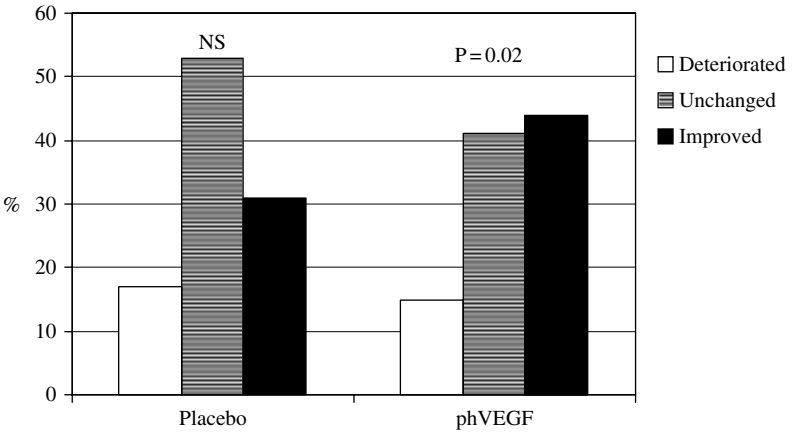

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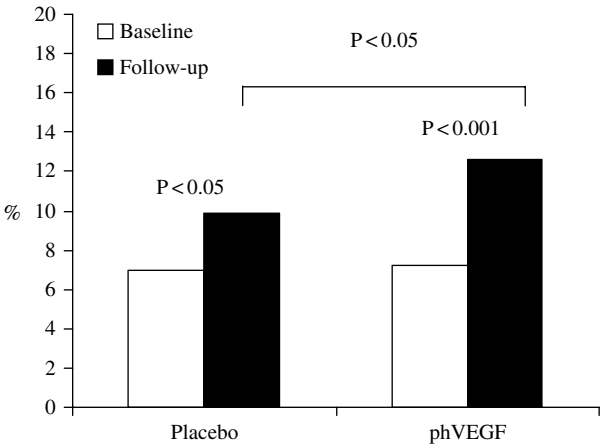

Figure 2. Results from the Euroinject One gene therapy trial with VEGF-$_{A165}$ plasmid injection or placebo into ischemic myocardium in patients with chronic refractory myocardial ischemia. (a) Changes in myocardial perfusion measured with SPECT in placebo and VEGF-$_{A165}$ plasmid treated patients. (b) Changes in local contractility in ischemic myocardium measured with NOGA in placebo and VEGF-$_{A165}$ plasmid treated patients

the patients (Figure 2a). Although, there was no statistical change in myocardial perfusion in the placebo group, the improvement in the phVEGF-A_{165} group was not statistically different compared to placebo. Measured with the NOGA method the local linear shortening of the treated region increased significantly in the follow-up period in both groups (from 7.0 ± 1.1 to $12.6 \pm 0.9\%$, $P < 0.001$ with VEGF and from 7.2 ± 1.0 to $9.9 \pm 0.9\%$, $P = 0.05$ with placebo) (Figure 2b). The follow-up local linear shortening was significantly higher in patients with VEGF gene transfer in comparison to those on placebo ($P = 0.05$) A significant reduction in angina pectoris attacks and nitroglycerine consumption was seen in the phVEGF-A_{165} treated group but not in the placebo group. However, only the nitroglycerine consumption was reduced significantly in the VEGF gene transfer group compared to the placebo group. An improvement in CCS and a tendency to improved exercise capacity was seen in both treatment groups.

Haematopoietic stem cells from the bone marrow have the potential to induce vasculogenesis in animals with an acute myocardial infarction. [37, 38] Recent human studies indicate that mononuclear cell solutions aspirated from the bone marrow can induce vasculogenesis both in acute and chronic myocardial ischemia. [39–45] However, it remains unknown, whether the vasculogenesis is induced by the few (2–3%) stem cells within the mononuclear cells suspension [43] or by cytokines released from the leucocytes. It has been demonstrated, that treatment with Granulocyte Colony Stimulating Factor (G-CSF) in order to mobilize stem cells from the bone marrow does not induce vasculogenesis in patients with chronic myocardial ischemia [46, 47] or following acute myocardial infarction. [48] Animal studies suggest, that a combination of treatment with VEGF-A gene transfer followed by G-CSF mobilization of stem cells might be superior to either of the therapies. [49].

In a recent published trial Ripa et al [50] has combined the VEGF-A gene transfer and G-CSF stem cell mobilization treatment in patients with stable chronic myocardial ischemia. The authors treated prospectively treated 16 patients with severe chronic coronary artery disease and no option for further revascularization with open-label VEGF-A_{165} gene transfer followed by G-CSF treatment. Patients were treated with direct intramyocardial injections of the VEGF-A_{165} plasmid followed one week later by in-hospital daily subcutaneous injection of $10\,\mu$gram/kg body weight G-CSF (Neupogen®) for six days. The treatment was compared with two control groups; I) 16 patients treated with VEGF gene transfer alone and II) 16 patients treated with placebo gene injections. The combined VEGF-A_{165} and G-CSF treated group could not demonstrate any changes in myocardial perfusion at rest and stress between baseline and follow-up, and they had identical summed difference perfusion scores. Left ventricular end-diastolic and end-systolic volumes, and ejection fraction showed not significant difference in any of the three groups from baseline to follow-up, and there were no differences between changes in these parameters between groups. In addition, regional wall thickening and motion were unchanged from baseline to follow-up in the group treated with VEGF-A_{165} and G-CSF.

3. SAFETY

None of the gene therapy studies have demonstrated any serious adverse events due to the delivered genes. Isner et al. [51] in 2001 reviewed the performed growth factor protein and gene studies. In accordance with later published studies, they authors could not detect any increased appearance of death, development of new neoplasm, or retinopathy in diabetics and non-diabetics.

There has, however, been demonstrated a few serious events caused by the delivery methods mainly using the thoracotomy, but also a few with the percutaneous direct intracoronary delivery of the genes. The thoracotomy-induced events were identical to adverse events seen during normal coronary by-pass grafting procedures. A few fever reactions have also been seen when using adenovirus as the vector. Therefore, there has until now not been found any serious adverse events to the given recombinant growth factor proteins or gene therapy, whether it was VEGF-A or FGF. However, safety issues should still have a high priority in future trials to avoid unexpected side-effects of the gene therapy.

4. DISCUSSION

The reasons for the disappointing discrepancy between the results in the early phase I/II studies using recombinant protein formulations of the growth factors have been discussed extensively. The combination of simultaneous revascularization with both coronary by-pass grafting and growth factor treatment, which have been used in many of the early phase I trials, makes it very difficult to evaluate the contributing effect of a recombinant growth factor treatment alone. Some of the included patients in the phase II studies had only minor coronary artery disease. The route of administration may not have been appropriate to obtain prolonged angiogenic stimulation in the ischemic tissue area. The use of exercise capacity as a primary endpoint may not be useful due to great intrapersonal variation. However, important safety data have been collected in these studies. It can be concluded, that with the used delivery methods, the use of intracoronary or intravenous delivering of recombinant VEGF-A or FGF growth factor proteins therapy seems not to be the right treatment to induce angiogenesis in ischemic myocardium. However, the use of other formulations and delivery in capsules might completely change the efficacy of these treatment regimes.

Several small and uncontrolled clinical studies have indicated that growth factor gene transfer might have the potential to improve myocardial perfusion. In spite of that, the Euroinject one Trial, the first larger double-blind placebo-controlled study could not demonstrated any improvement in myocardial perfusion after VEGF-A_{165} gene transfer compared to placebo in patients with severe coronary artery disease. [35, 36] However, the local contractility in the ischemic area was improved in the VEGF-A treated compared to the placebo patients. Since the study demonstrated a significant improvement in myocardial perfusion within the VEGF-A_{165} group, the study might have been underpowered.

For safety reasons only small doses of VEGF-A genes have been used in the initial trials, without any safety concerns. Larger doses of genes might therefore be of importance to induce a measurable improvement in myocardial perfusion and clinical parameters. Treatment regimes with larger VEGF doses are presently investigated in larger clinical trials.

The discrepancy between animal research and clinical studies in patients might be related to the definition of chronic ischemia. In animal studies chronic ischemia normally include components of both acute and subacute ischemia as well. Most animal studies induce chronic myocardial ischemia using an ameroid constrictor around the circumflex or anterior descendent artery. Four to five weeks later the myocardium is often called chronic ischemic myocardium. However, the intracellular milieu is probably not equivalent to patients' myocardium suffering from chronic ischemia for several years. In patients with acute myocardial infarction, plasma concentrations of the vascular growth factors VEGF and FGF, and the stem cell homing factor SDF-1 increase gradually above control levels with maximum approximately 3 weeks after the infarction. This could indicate that it takes some time to initiate the transcription of the genes for the cytokine production. [52]

Furthermore, transfection of cells with the VEGF gene after intramyocardial injection is probably similar in chronic human or pig ischemic myocardium. However, the transcription of the transferred VEGF gene and thus the induced VEGF production might be different within the human cells after prolonged ischemia and in animal cells after short term experimental ischemia.

Recently, it has been speculated if the VEGF and FGF production is already increased within chronic ischemic human myocardium, thus attempts to further stimulate angiogenesis via an additional VEGF or FGF gene stimulation would potentially be without effect. However, it has recently been studied in biopsies from human chronic ischemic myocardium. Wang et al [53] found identical quantities of VEGF mRNA in chronic ischemic myocardium compared to non-ischemic normal perfused myocardium in the same patient. Thus, it seems that VEGF-A_{165} or FGF gene therapy can potentially increase the local production of the growth factors and hereby stimulating the growth of new blood vessels.

It is now evident, that adult stem cells from the bone marrow can participate in the development of new blood vessels in the myocardium. Whether autologous stem cells from the bone marrow have a place in the treatment of acute and chronic myocardial ischemia is presently investigated in clinical trials.

Animal studies have suggested that the combination of gene transfer for VEGF-A_{165} and G-CSF mobilization of stem cells from the bone marrow could induce angiogenesis more effective than gene therapy alone. [49] However, two clinical studies have demonstrated, that neither G-CSF mobilization of stem cells from the bone marrow alone nor VEGF-A_{165} gene therapy followed by G-CSF stem cell therapy did improve myocardial perfusion or symptoms. [46, 50] Therefore, these two clinical studies in patients suffering from severe, chronic coronary artery disease could not confirm the hypothesis, that the combination therapy would increase local production of VEGF and the number of circulation endothelial progenitor

cells homing into the ischemic myocardium, suggested by experimental animal studies. [37, 38]

SDF-1 has been found essential for stem cell mobilization/homing after arterial injury. In a recent study, it has been demonstrated that SDF-1 gene transfer increased the homing of bone marrow derived stem cells in infarcted myocardium but not in normally perfused myocardium and induced both vasculogenesis and angiogenesis. [54, 55] Moreover, blockade of VEGF production prevented all such SDF-1 effects. [55] It has been found that there is no difference between the SDF-1 mRNA levels in normally perfused and chronic ischemic human myocardium.[53] Therefore, the missing effect of combined gene therapy and stem cell mobilization might be due to a low SDF-1 level in the chronic ischemic tissue resulting in poor engraftment of stem cells despite an increased number of circulating stem cells as seen during G-CSF treatment.

Recent animal data indicate, that transfection of stem cell or skeletal myoblasts with VEGF gene before transplantation of the cells to ischemic myocardium may improve the survival of the transplanted cells and reduce the infarct size compared to un-transfected cells or VEGF gene alone. [56, 57]

In conclusion, clinical trials in patients with ischemic heart disease with either recombinant growth factors or genes encoding vascular growth factors have not been able to mimic the encouraging results from animal studies. However, important safety data have been generated in the studies, demonstrating no gene related adverse events. To improve the efficacy of the gene growth factor therapy one have to consider larger dose, different vectors and gene delivery methods, combinations of more than one growth factor, or combined stem cell and gene therapy. The ongoing and future larger scaled double-blind placebo-controlled studies with genes encoding for the vascular growth factors will indicate the potential role of vascular growth factor treatment in patients with ischemic heart disease.

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