Chapter 2

Therapeutic Angiogenesis with Vascular Endothelial Growth Factor in Peripheral and Coronary Artery Disease: A Review

Yoka H. Kusumanto¹, Geke A.P. Hoppers¹, Nanno H. Mulder¹, Rene A. Tio²

¹Department of Medical Oncology
²Department of Cardiology
University Medical Center Groningen, The Netherlands

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INTRODUCTION

DESPITE major advances in medical treatment and intervention surgery, coronary artery disease and peripheral vascular disease continue to be the major causes of death and disability in the Western world. Efforts in the treatment of arterial disease have recently focussed on therapeutic angiogenesis as a new treatment strategy to stimulate new collateral blood vessel formation in patients requiring alternative revascularization.

Different processes may contribute to the growth of new blood vessels. In the adult new vessels predominantly develop via angiogenesis. Angiogenesis refers to the sprouting of new vessels into an avascular tissue. It involves the proliferation of endothelial cells, resulting in the formation of capillaries lacking a developed tunica media. Arteriogenesis is the process responsible for the development of collaterals from pre-existing arterioles. These pre-existing arterioles need to grow in diameter to allow relevant blood flow. Involved in this process is the proliferation of endothelial cells and peri-endothelial mural cells, including smooth muscle cells and pericytes. The peri-endothelial cells form a fully developed tunica media. Vasculogenesis involves the differentiation of pluripotent endothelial precursor cells into endothelial cells that assemble into primitive blood vessels. Vasculogenesis is the primary process responsible for the formation of vasculature during embryonic development. Vasculogenesis may also have an as yet undefined contribution in vessel formation in the adult. It is increasingly accepted that vessel formation through the process of arteriogenesis is the preferred process in angiogenic responses as it involves the formation of fully functional mature vessels.

Therapeutic angiogenesis is the controlled stimulation of new blood vessel formation by the introduction of angiogenic growth factors, or the genes encoding for them, in order to reduce detrimental tissue effects caused by ischemia. Many angiogenic growth factors have been identified. Their complete molecular structures have been clarified, and their genes have been cloned and expressed through recombinant technologies.

At present Vascular Endothelial Growth Factor (VEGF) has been the most widely used agent in experimental and human clinical trials. New blood vessel formation is extremely dose dependent in the case of VEGF. During embryonic development the loss of only one VEGF allele results in embryonic lethality due to severe abnormalities in the cardiovascular system. Considering this extremely dose dependent effect of VEGF on vessel development, it is conceivable that profound stimulation of new blood vessel formation could be expected in adult tissue with even small changes in the local concentration of VEGF. Subsequently research focussed on VEGF as a therapeutic agent to induce blood vessel growth in the treatment of ischemic tissue disease. Promising animal studies established the proof of concept of therapeutic angiogenesis with VEGF. Although VEGF has been introduced already in human clinical trials, the full spectrum of VEGF’s biological effects is not yet fully known. Therefore this review will focus on pre-clinical animal and human clinical trials with VEGF, in particular VEGF-A and C, in order to review insights obtained from these experiences that point to VEGF's critical position as a potent inducer of collateral vessel development.
Vascular Endothelial Growth Factor: a pleiotropic cytokine

VEGF-A is a member of a family of closely related growth factors. These include recently identified growth factors such as VEGF-B, C, D, E and placenta growth factor (PLGF).22-24 This review will be restricted to the VEGF members that have already reached first experimental and clinical application in therapeutic angiogenesis, i.e. VEGF-A and VEGF-C.

VEGF-A was initially recognized as permeability enhancing factor of blood vessels, and mitogenic factor for endothelial cells.25-27 VEGF-A regulates multiple important biological functions of endothelial cells. It induces an increased production of vaso-active mediators regulating vaso-relaxation, inhibition of thrombosis and neo-intimal vascular smooth muscle cell proliferation.28,29 In embryonic development VEGF-A has been demonstrated to be crucial for blood vessel development.18,19 In adults VEGF-A is generally accepted as a key mediator of physiological as well as pathological angiogenesis.30

Alternative splicing of human VEGF-A mRNA from a single gene results in at least five different isoforms: 121, 145, 165, 189 and 206. The short isoforms, 121, 145 and 165, lack an exon 6, which encode for a heparin-binding domain. Consequently, the shorter isoforms are not bound to the extra-cellular matrix but diffuse freely. In contrast to the longer isoforms, which are, heparin-binding proteins, which remain sequestered upon secretion. In in-vitro studies, VEGF165 is the most bioactive isoform.24,31 In endothelial cells the 121 and 145 are also biologically active. VEGF 189 stimulates endothelial proliferation after proteolytic cleavage by urokinase.32

Hypoxia is an important stimulus for VEGF-A expression. VEGF-A expression involves an increased rate of gene transcription, mediated by the transcription factor Hypoxia-Inducible Factor1, and by enhancement of the VEGF-A mRNA stability.33,34 VEGF-A expression is also regulated by various cytokines or growth factors such as bFGF, TGFβ interleukin-1β.35-37

The biological effects of VEGF-A are mediated by two receptor tyrosine kinases Flt-1 (also referred to as VEGF-R1), and or KDR/Flk-1 (or VEGF-R2).23,24 Almost all of the biologically relevant VEGF-A signaling appears to involve mainly the KDR receptor.24 Stimulation by ligand of receptor dimerization and transphosphorylation results in increased nitric oxide production and increased expression of endothelial nitric oxide synthase involved in subsequent proliferation and migration of endothelial cells.28,29 The role of the Flt-1 receptor in postnatal angiogenesis is not yet fully elucidated. The Flt-1 receptor has been identified on monocytes and vascular smooth muscle cells. It mediates monocyte migration and tissue factor expression by monocytes.38,39 In vascular smooth muscle cells the Flt-1 receptor mediates expression of metalloproteinase by VEGF.40

The other member of the VEGF family that has reached the first phase of clinical evaluation is VEGF-C. VEGF-C was isolated as ligand and specific activator of the Flt-4 receptor (also referred to as VEGFR-3), a recently identified receptor in lymphatic endothelium.41 In addition it stimulates autophosphorylation of the KDR receptor which is in agreement with the mitogenic and chemotactic responses of endothelial cells in response to VEGF-C shown in vitro.41-43
ANIMAL STUDIES

The proof of concept that therapeutic angiogenesis could be achieved by VEGF-A was demonstrated in the early 1990s with the administration of the recombinant protein using a rabbit ischemic hind limb model and the myocardial ischemia model. These studies showed VEGF-A’s potential to induce beneficial vessel formation. After these initial studies with the recombinant VEGF protein, research relatively soon focussed on gene therapy as severe cardiodepressant side effects were observed after intra-coronary administration, indicating a potential adverse effect after systemic administration of the VEGF protein. The results of the studies with the recombinant protein indicated that sustained VEGF administration is needed in order to achieve local beneficial effects. However study results were not conclusive with regard to the route and dose of administration. Gene therapy could potentially provide a means to achieve local delivery of VEGF-A and minimize the risk of systemic effects. In addition, gene therapy could also potentially address the considerable expenses associated with the production of the recombinant protein.

Animal studies using intra-muscular gene transfer with the naked plasmid DNA

Intramuscular gene transfer with naked plasmid DNA represents a potential means to induce therapeutic angiogenesis. It obviates potential immunological responses associated with gene transfer with viral vectors. In addition, it addresses the technical limitations of intra-arterial gene transfer. This is especially true in peripheral arterial disease in which case the extensive and diffusely diseased vessels often cannot be accessed by conventional percutaneous sites for arterial puncture. Importantly the secreted nature of the VEGF-A protein could be exploited to overcoming the low transfection efficiency that is inherent to the use of naked plasmid DNA. The VEGF-A gene includes a secretion signal sequence, which allows the VEGF-A protein to be secreted naturally by intact cells. Through the paracrine effects of the secreted gene product biologically meaningful effects could be achieved, even with a relatively small number of cells transfected. Therefore shortly after the initial intra-arterial gene transfer experiments the technique of intra-muscular gene transfer with naked plasmid DNA was investigated.

Tsurumi et al was the first to demonstrate in vivo beneficial neovascularization in the rabbit hind limb ischemia model with the administration of naked plasmid DNA encoding VEGF165 (phVEGF 165) under the direction of the cytomegalovirus (CMV) promoter. Anatomic and physiological evidence of augmented vascularization was demonstrated by angiography and histological analysis which was accompanied by improved hemodynamic deficits in the treated animals compared to the control animals. Only 2% of skeletal muscle myocytes were transfected by this technique.

In the setting of myocardial ischemia a study by Tio et al in pigs confirmed the beneficial effects of therapeutic angiogenesis using the naked plasmid DNA. Importantly safety issues
of intramyocardial delivery were addressed. No change in heart rate, blood pressure or malignant ventricular arrhythmia’s was reported. Although a thoracotomy was required for gene delivery, intramyocardial delivery of phVEGF$_{165}$ was shown to be a safe and potential therapeutic treatment strategy for myocardial ischemia.

One study tested the hypothesis that VEGF-C promotes angiogenesis. In the ischemia hindlimb model rabbits received either a plasmid DNA encoding for VEGF-C or the recombinant human VEGF-C protein intra arterially. VEGF-C treated animals showed improved vascularity compared to controls. This study demonstrated that VEGF-C in vivo stimulates angiogenesis in the setting of tissue ischaemia.$^{56}$

Several important principles were exposed by these animal studies. Augmented vascularization was restricted to the ischemic limb. RT-PCR and immunohistochemistry also documented transgene expression at the site of ischemia. The upregulation of the VEGF-A receptors under hypoxic conditions is considered responsible for this site-specific effect. Furthermore studies have demonstrated that in the absence of ischemia-induced upregulation of the KDR/Flk-1 receptor no angiogenesis occurred in response to transient overexpression of VEGF-A.$^{57,58}$ Another important principle is the transient gene expression. Gene expression did not exceed a period of 30 days.$^{54}$ Nevertheless, this period appeared to be sufficient to achieve a considerable angiogenic response.

With regard to the nature of VEGF induced neovascularization, several animal studies have provided evidence that therapeutic angiogenesis with VEGF-A augments formation of collateral vessels at the level of arterioles rather than capillary formation. It has been shown that proliferation was typically seen not only in endothelial cells but also in smooth muscle cells in the larger, so called midzone arteries, as was shown with bromodeoxyuridine labelling.$^{59}$ In addition Sundberg et al have shown that the fate of VEGF-A (adenovirus directed) induced new vessels is the formation of glomeruloid bodies from pre-existing microvessels which enlarge by endothelial cell, pericyte and smooth muscle cell proliferation and subsequent macrophages participation. Events appeared in direct relationship to the level of VEGF-A expression by the infected host cells. Striking in this study is the demonstration that on single administration VEGF-A is able to orchestrate the development of semistable vascular structures.$^{60}$

**Animal studies: conclusions**

Despite the low transfection efficiency which is inherent to the use of naked plasmid DNA and despite the transient gene expression sufficient new blood vessel formation in ischemic tissue could be achieved using phVEGF$_{165}$. This is due to the fact that VEGF is a secreted protein and the context of ischemic disease. Intra-muscular delivery of the naked plasmid DNA encoding for VEGF$_{165}$ may thus represent an alternative treatment for patients with extensive tissue ischemic in whom primary vascular reconstruction procedures are not feasible or have previously failed.
Clinical studies using the recombinant protein

The promising findings from animal studies were translated in case studies and phase I and II clinical trials. The phase I and II studies using the recombinant VEGF-A protein indicated sufficient treatment benefit in ischemic heart disease. The VIVA study, a placebo-controlled phase II study, however, did not confirm the early results. In this trial patients with ischemic heart disease (n=178) received placebo or a low or high dose of the recombinant VEGF-A protein (rhVEGF-A) administered intra-coronary followed by an intravenous infusion. The drug was well tolerated and appeared to be safe. Unfortunately, the interim results led to the premature termination of the study because both placebo and treated patients showed an improvement in the clinical status and the treadmill test which was not significantly different. The authors concluded that the results indicate a prominent placebo effect. Long-term follow-up revealed a trend for improvement in exercise time at 120-day follow-up in the high dose VEGF treated patients compared to controls. The VIVA study emphasizes the need for a large, placebo-controlled study. Although safe, clinical benefit with this dose an route of administration of the recombinant protein remains unproven. Gene therapy provides a potential means to achieve sustained and local delivery of VEGF-A.

Clinical studies using gene therapy

Initial studies on gene therapy as a means to achieve beneficial therapeutic angiogenesis were performed in patients with peripheral artery disease. Transfer of 2 mg phVEGF165 was administered either by intra-arterial or intra-muscular gene-transfer. These procedures demonstrated safety and feasibility. However, although efficacy claims were implied, these reports involved individual cases or a small number of cases. A larger study, also conducted in peripheral artery disease substantially extended the preliminary observations. PhVEGF165 under the control of a CMV promotor was delivered direct intra-muscular in patients with critical limb ischemia (non-healing ulcers and rest pain). Newly visible collateral vessels on angiography was demonstrated which was accompanied with significant hemodynamic improvement. In a tissue specimen derived from one amputee endothelial cell proliferation was seen, which is a remarkable finding as endothelial cell proliferation is almost absent in normal arteries. After one to three weeks post gene transfer a transient peak in VEGF-A serum blood levels was documented in 7 of the 9 patients. The authors were appropriately cautious because a control group was not included. However this study can be regarded as a pioneering study which prepared the way for intramyocardial VEGF-A gene therapy of the human heart. In a recent study in patients with critical limb ischemia i.m. delivery of phVEGF165 also improved clinical and electro-physiological deficits of chronic ischemic neuropathy. The first study in ischemic heart disease study provided evidence that intra-myocardial gene delivery of naked plasmid DNA encoding for VEGF165 is a safe procedure with potential
therapeutic effects.\textsuperscript{69} Five patients with inoperable multi-vessel coronary artery disease and severe refractory angina pectoris were treated. VEGF-A gene therapy was performed as sole therapeutic intervention without parallel conventional surgical revascularization during a minimally invasive thoracotomy (500 µg phVEGF\textsubscript{165}). There were no major peri-operative complications. All patients experienced a significant reduction of angina.

Another study in myocardial ischemic proved that administration of a replication-deficient adenovirus vector expressing the VEGF\textsubscript{121} isoform as an adjunct to CABG or as sole therapy is well tolerated.\textsuperscript{70} Also in this phase I study all patients reported improvement of angina class after gene therapy.

In the above mentioned studies a mini-thoracotomy was required to expose the myocardium for intra-muscular injections. These studies have shown that the procedure is well tolerated in patients with advanced myocardial ischaemia.\textsuperscript{69-71} However a mini-thoracotomy procedure limits the feasibility of repeat administrations as some morbidity is inherently associated with surgical intervention, especially in patients with severe coronary disease. This issue was recently addressed in two studies conducted by Vale et al. In these studies catheter-based gene transfer was evaluated for feasibility and safety in patients with chronic myocardial ischaemia.\textsuperscript{72,73} Electromechanical mapping with the NOGA system revealed reduced ischemic in the treated patients. Myocardial perfusion in rest and after stress improved (SPECT-sestamibi) 90 days after gene-transfer as compared to the control procedure.

In addition in a study performed by Tio et al catheter-based intra-muscular delivery of phVEGF\textsubscript{165} was investigated.\textsuperscript{74} Preliminary results indicated again that catheter-based delivery is safe and feasible. Using a mini-thoracotomy or a catheter base approach, a phase I dose-escalating study with direct intra-muscular injection of the naked plasmid DNA encoding for VEGF-C was performed in 55 patients. Both approaches proved to achieve similar results to phVEGF\textsubscript{165} gene therapy (Isner, paper presented at the American College of Cardiology meeting, Anaheim, CA, 2000). The use of catheter based interventions clearly facilitates randomized controlled studies in the future for gene therapy for myocardial angiogenesis. Currently, randomized placebo controlled phase III studies for intra-muscular gene transfer with VEGF-C and phVEGF\textsubscript{165} in critical limb ischemia and myocardial ischemia are ongoing.\textsuperscript{75,76} Definitive conclusions of efficacy will have to await final results.

\textbf{Clinical studies: safety aspects}

All treatment strategies designed to enhance local angiogenesis carry the theoretical risk of potential unwanted angiogenesis at a site remote from the target or injected site, such as growth of a previously existing tumor or retinopathy. Data from published studies so far have shown that development of neoplasms does not exceed expectation in these kinds of population.\textsuperscript{77} The same accounts for the risk of developing retinal angiogenesis. No retinopathic changes to proliferative disease have occurred as assessed by direct ophthalmoscopic examination in both the diabetics (n=44) as well as in non-diabetic subjects (n=85).\textsuperscript{77}
A theoretical concern is the development of plaque formation as VEGF may be involved in plaque angiogenesis.\textsuperscript{78,79} This issue will have to be resolved by evaluation of neo-intimal thickening in ongoing and future human trials with VEGF gene transfer.

The principal side effect documented in clinical studies published so far is peripheral limb edema and telangiectesia. Clinically apparent peripheral edema was observed in nearly one half of the patients treated for critical limb ischaemia.\textsuperscript{80} Life- or limb threatening edema has not been reported in these patients. Lower extremity edema was self-limiting or exceptionally required brief treatment with oral diuretics. Transient telangiectesia has been reported in a single report in a distal limb after arterial gene transfer.\textsuperscript{58}

**FUTURE PERSPECTIVES**

Supported by recent animal studies a new concept has emerged that angiogenic factors must be used in a co-ordinated manner in order to form fully functional vessels with a fully developed tunica media.\textsuperscript{81-85} This could be translated in the administration of combination of genes, such as the combination of VEGF-A and bFGF or VEGF-A and the angiopoietins.\textsuperscript{86-88} Although in the new concept of vessel formation VEGF-A maintains its position as the critical driver of vessel formation it may initiate leaky vessels, and combination of VEGF with other angiogenic growth factors may lead to the development of mature vessels.

Another important issue is how to maintain and sustain therapeutically induced vessels. There may be circumstances in which it is indicated to re-administer the gene if further or recurrent ischemia develops. Whereas in critical limb ischemia intra-muscular gene delivery is a non-invasive procedure, in myocardial ischemia it requires an invasive intervention. This may be overcome by the use of gene constructs and promotors which can be turned on or off on demand. For example in the future hypoxia inducible promotors and "DNA integrating vectors" could be administered which restrict gene expression to periods of recurrent ischemia. Especially in myocardial ischemia it is important to develop gene transfer techniques requiring minimal invasiveness.

Finally, identification of factors that attenuate angiogenic responses, such as hypercholesterolemia and diabetes may have implications for developing new treatment strategies.\textsuperscript{92-94} Stratification should also account for these factors in future studies.
CONCLUSIONS

Presently, early clinical information suggests beneficial effects from VEGF gene therapy, with minimal side effects. Data will mature in ongoing phase III studies within a few years. Future improvements in this treatment modality include optimization of transfer techniques, and of the angiogenic product.
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