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STATE-OF-THE-ART PAPER

Vascular Endothelial Growth Factors

Biology and Current Status of Clinical Applications in Cardiovascular Medicine

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Members of the vascular endothelial growth factor (VEGF) family are among the most powerful modulators of vascular biology. They regulate vasculogenesis, angiogenesis, and vascular maintenance during embryogenesis and in adults. Because of their profound effects on blood vessels, VEGFs have received much attention regarding their potential therapeutic use in cardiovascular medicine, especially for therapeutic vascular growth in myocardial and peripheral ischemia. However, completed randomized controlled VEGF trials have not provided convincing evidence of clinical efficacy. On the other hand, recent preclinical proangiogenic VEGF studies have given insight, and anti-VEGF studies have shown that the disturbance of vascular homeostasis by blocking VEGF-A may lead to endothelial dysfunction and adverse vascular effects. Excess VEGF-A may contribute to neovascularization of atherosclerotic lesions but, currently, there is no evidence that transient overexpression by gene transfer could lead to plaque destabilization. Here, we review the biology and effects of VEGFs as well as the current status of clinical applications and future perspectives of the therapeutic use of VEGFs in cardiovascular medicine. (J Am Coll Cardiol 2007;49:1015–26) © 2007 by the American College of Cardiology Foundation

Therapeutic vascular growth is a new concept for the treatment of ischemic vascular diseases. It involves stimulation of angiogenesis, arteriogenesis, and lymphangiogenesis (1). Angiogenesis means sprouting, bridging, intussusception, and/or enlargement of capillaries from the pre-existing ones, whereas arteriogenesis is the in situ enlargement and growth of muscular collateral vessels from pre-existing arteriolar anastomoses. Lymphangiogenesis, which will not be covered in this review, means the generation of new lymphatic vessels from the pre-existing ones. The members of the vascular endothelial growth factor (VEGF) family regulate all types of vascular growth. Various strategies also have been presented for the use of postnatal vasculogenesis, i.e., de novo differentiation and formation of vascular structures from endothelial progenitor cells and other stem cells, which could be augmented with VEGFs. Thus far, clinical trials using VEGFs delivered as recombinant protein or via gene transfer for the treatment of myocardial or peripheral ischemia have not shown convincing efficacy. In this review, we summarize the biology of VEGFs, preclinical and clinical advances, and discuss why VEGF therapy has not yet fulfilled the expectations. In addition to cardiovascular biology, VEGFs also play very important roles in tumor angiogenesis, retinal neovascularization, age-related macular degeneration, rheumatoid arthritis, and psoriasis, and the reader is referred to recent reviews covering these areas of VEGF biology (2,3).

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The Biology of VEGF Family

The first member and the master regulator of angiogenesis and vascular permeability, VEGF-A (also called VEGF), was cloned in 1989 based on the pioneering studies of an unknown angiogenesis and vascular permeability factor in the 1970s and 1980s (4–6). After the discovery of VEGF-A, 4 other members in the human VEGF family have been identified: VEGF-B, VEGF-C (also called VEGF-2), VEGF-D, and placental growth factor (PlGF) (7–10). In addition to these VEGFs, viral VEGF homologs (collectively called VEGF-E) and snake venom VEGFs (called VEGF-F) have been found (11,12). The properties of different VEGF family members are shown in Table 1. The downstream signals of VEGFs in the vascular endothelium are mediated by 3 tyrosine kinase-signaling receptors (VEGF receptor [VEGFR]-1, -2, and -3) as shown in Figure 1.

Hypoxia increases VEGF-A expression via up-regulation of hypoxia-inducible factor-1α, e.g., in acute human skeletal muscle ischemia (13,14). Many growth factors and cytokines, such as platelet-derived growth factor-BB, PlGF, transform-

Abbreviations and Acronyms

CAD = coronary artery disease
CCS = Canadian Cardiovascular Society
CLI = critical limb ischemia
EC = endothelial cell
NO = nitric oxide
PAD = peripheral arterial disease
PlGF = placental growth factor
pu = particle units
SMC = smooth muscle cell
VEGF = vascular endothelial growth factor
VEGFR = VEGF receptor

ing growth factor-β, insulin-like growth factor-1, fibroblast growth factors, hepatocyte growth factor, tumor necrosis factor-α, and interleukin-1 up-regulate VEGF-A expression (2,15). VEGF-A is one of the strongest known inducers of vascular permeability, which occurs only within minutes after exposure to VEGF-A, probably as the result of efficient simultaneous nitric oxide (NO) and prostacyclin production (16,17). Because of its major role in vascular biology and because of its high angiogenic potency, VEGF-A has been the prime candidate for therapeutic applications in vivo (1).

The role of PlGF and VEGF-B in vascular biology has remained elusive. PlGF and VEGF-B are only weak mitogens for endothelial cells (ECs) in vitro, and they do not significantly induce acute vascular permeability (8,16). In vivo, long-lasting exposure to PlGF or VEGF-B promotes angiogenesis, vascular permeability, and may potentiate VEGF-induced vascular growth, likely via indirect mechanisms that are still poorly understood (18–21). The VEGF-C and -D form another VEGF subfamily. They both bind to VEGFR-2 and -3, and they are synthesized as large precursor forms that are proteolytically processed, e.g., by plasmin into mature forms (indicated by ΔNΔC) comprising the central VEGF homology domain with much higher affinity toward VEGFR-2 (9,10). Thus, the long forms are mainly lymphangiogenic, whereas the mature short forms also are angiogenic and promote vascular permeability via NO-mediated mechanisms (22). The viral VEGF homologs and snake-venom VEGFs are relatively specific VEGFR-2 ligands and thus have considerable angiogenic potency (11,12).

VEGF in Blood Vessel Homeostasis and Atherosclerosis

Both VEGF-A and -C are required for embryonic development of blood and lymphatic vasculature, respectively (23,24) whereas PlGF, VEGF-B, and VEGF-D appear dispensable for vascular development (25,26). In adults, VEGF-A is expressed in virtually all vascularized tissues, especially in fenestrated and sinusoidal blood vessels in endocrine and secretory organs as well as in large blood vessels, skeletal muscle, and myocardium, suggesting that low physiological VEGF-A levels are needed for the maintenance of general vascular homeostasis (27). On the other hand, VEGF-A up-regulation is important in active physiological angiogenesis processes, such as corpus luteum development and exercise-induced angiogenesis in skeletal muscle, as well as in tissue regeneration after injury and ischemia (2,13). The essential role of VEGF-A in vascular homeostasis is highlighted by the consequences of systemic VEGF-A inhibition in vivo by soluble VEGFR-1, e.g., in preeclampsia (28). Clinical trials using the anti–VEGF-A antibody bevacizumab (Avastin; Genentech Inc., South San Francisco, California) for cancer treatment have indicated that up to 5% of the treated patients may have an increased risk of thromboembolic complications, including unstable angina, myocardial infarction, stroke, and deep vein thrombosis in addition to the more common side effects of hypertension and proteinuria (29). Because of the continuously increasing use of systemic anti-VEGF therapy for cancer, studies addressing the risks of this novel treatment would be important, especially in patients with previous cardiovascular morbidity or multiple risk factors.

The contribution of VEGF-A or other members of the VEGF family to atherogenesis has gained a lot of attention in the recent years but has still remained unclear. The progressive expression of VEGF-A in activated ECs, macrophages and differentiated smooth muscle cells (SMCs) in atherosclerotic lesions and in-stent restenosis presenting neovascularization suggests that VEGF-A might have a role in atherogenesis and plaque instability via proinflammatory and angiogenic mechanisms (30–33). This hypothesis has been supported by experiments using cholesterol-fed ApoE/ApoB100 double knockout mice and cholesterol-fed rabbits (34,35). The roles of other VEGFs in atherogenesis have been less studied (33).

Recently, the contribution of VEGFs to atherogenesis has been challenged. First, systemic adenoviral gene transfer of VEGF-A, -B, -C, or -D, as well as recombinant VEGF-A administration, did not alter plaque area or macrophage influx in LDLR/ApoB48 double knockout mice (36). Second, periadventitial and intra-arterial gene transfer of VEGF-A, -C, and -D has inhibited neointimal growth in many animal studies (37–41). Third, there has been no evidence of increased atherogenesis in clinical trials using VEGF-A protein or gene transfer (42–47). In fact, VEGF-A polymorphism causing higher VEGF-A expression recently was found to be associated with a lower risk of coronary artery disease in an epidemiological study (48).

Taken together, it appears that the effects of VEGF-A are strongly dependent on its local concentration (Fig. 2). Low physiological amounts of VEGF-A seem to be required for blood vascular homeostasis, EC survival, and production of NO and prostacyclin, resulting in vasodilatation, antithrombosis, and suppression of SMC proliferation, i.e., are vasculoprotective as hypothesized previously (49). Much higher concentrations are required for angiogenic and vasculogenic effects (Fig. 2). In ischemia and vascular injury, VEGF-A up-regulation restores sufficient perfusion and the integrity of the endothelium. VEGF-A, or other members in the family, do not appear to have a role in the initiation of atherogenesis. The progressive increase in VEGF-A expression during atherogenesis is likely secondary to hypoxia and inflammation in growing lesions. However, at the

Table 1 The Properties of Different VEGFs

Ligand	Isoforms	Receptor	Solubility	Source in Adults	Biological Activities	Phenotype of Knockout Mouse
VEGF (VEGF-A)	VEGF-A_{121}, VEGF-A_{165} VEGF-A_{189}, VEGF-A_{206} (also VEGF-$A_{138/145/162/165b}$ have been described)	VEGFR-1 and -R-2, $VEGF_{165}$ binds to neuropilin-1 and -2, $VEGF_{145}$ neuropilin-2	$VEGF_{121}$ soluble, longer forms bind to heparan sulfates with increasing affinity	Almost all vascularized tissues, especially fenestrated and sinusoidal endothelium, up-regulated by ischemia (via HIF-1α)	Vasculogenesis, angiogenesis, vascular homeostasis, vascular permeability and recruitment of bone marrow-derived cells	Loss of even single VEGF allele leads to embryonic lethality due to impaired vasculogenesis and angiogenesis
PlGF	$PlGF_{131}$ (PlGF-1), $PlGF_{152}$ (PlGF-2), $PlGF_{203}$ (PlGF-3)	VEGFR-1, $PlGF_{152}$ binds to neuropilin-1 and -2	$PlGF_{131}$ and $PlGF_{203}$ soluble, $PlGF_{152}$ binds to heparan sultates	Placenta, thyroid, lung, and goiter	Angiogenesis, monocyte migration, recruitment of bone marrow-derived cells, up-regulation of VEGF-A	Almost-normal phenotype and fertile with minor defects in vascular growth in pathological conditions
VEGF-B	VEGF-B_{167} and VEGF-B_{186}	VEGFR-1 and neuropilin-1	VEGF-B_{167} binds to heparan sulfates, $VEGF_{186}$ soluble	Heart, skeletal muscle, and vascular smooth muscle cells	Angiogenesis, recruitment of bone marrow-derived cells	Almost-normal phenotype with minor possible defects: reduced heart size, prolonged PQ-time, impaired recovery from ischemia
VEGF-C (VEGF-2)	Unprocessed and proteolytically processed ($\Delta N\Delta C$) forms	VEGFR-2, -R-3, and neuropilin-2, processing increases receptor affinity	Soluble	Neuroendocrine organs, lung, heart, kidney, and vascular smooth muscle cells	Development of lymphatics and lymphangiogenesis, angiogenesis	Lethal because of impaired development of lymphatics
VEGF-D	Unprocessed and proteolytically processed ($\Delta N\Delta C$) forms	VEGFR-2 and VEGFR-3, processing increases receptor affinity	Soluble	Neuroendocrine organs, lung, heart, skeletal muscle, intestine, and vascular smooth muscle cells	Lymphangiogenesis and angiogenesis	Normal
VEGF-E	—	VEGFR-2 and neuropilin-1	Soluble	Virus-derived	Angiogenesis	—
VEGF-F	—	VEGFR-2	Binds to heparan sulfates	Snake venom	Angiogenesis and vascular permeability	—

HIF = hypoxia-inducible factor; PlGF = placental growth factor; VEGF = vascular endothelial growth factor.

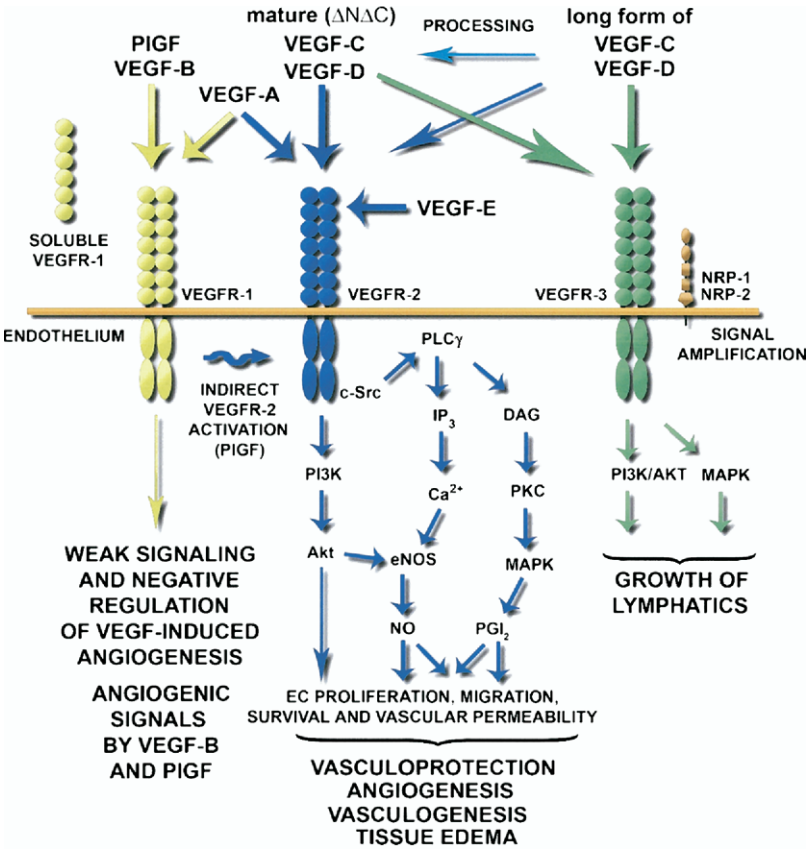

Figure 1 **Binding of VEGF Family Members to 3 High-Affinity Receptors on Endothelium and Downstream Signaling Cascades**

The roles of vascular endothelial growth factor receptor (VEGFR)-2 and -3 as principal regulators of blood and lymphatic vessel effects of vascular endothelial growth factors (VEGFs) are well established, but the biology of VEGFR-1 is still poorly understood. VEGFR-1 appears to mediate ligand-specific actions. It also exists as a soluble decoy receptor and acts as a negative regulator of angiogenesis. However, VEGFR-1 activation by placental growth factor (PlGF) and VEGF-B stimulates vascular growth and may result in the mobilization of EPCs in vivo. VEGFR-1 also mediates monocyte chemotaxis. Neuropilin-1 and -2 are co-receptors for some VEGFs and amplify intracellular signals. Affinity to heparan sulfates and neuropilin co-receptors modulate the biological activities of different VEGFs. VEGF-C and -D are proteolytically processed into mature forms that also effectively bind to VEGFR-2. DAG = diacylglycerol; eNOS = endothelial nitric oxide synthase; EPC = endothelial precursor cell; IP_3 = inositol trisphosphate; MAPK = mitogen-activated protein kinase; NO = nitric oxide; NRP = neuropilin; PGI_2 = prostacyclin; PKC = protein kinase C; PLCγ = phospholipase gamma.

moment the possibility that a long-term high-level VEGF-A expression in advanced atherosclerotic lesions could contribute to plaque neovascularization and plaque vulnerability cannot be excluded even though direct evidence of this cascade is currently lacking. In contrast, transient systemic production of VEGFs via adenoviral gene transfer or recombinant protein therapy seems not to be sufficient to promote progression of atherosclerosis in mice or humans.

Insights From Preclinical VEGF Studies

The efforts to use VEGFs in vascular medicine have been based on their vasculoprotective and angiogenic properties (49). Consistently, early and some more recent experimental studies showed that gene transfers of VEGF-A, -C, and -D prevent restenosis after arterial injury in several animal models (37–41). However, the lack of beneficial clinical effect of VEGF-A gene transfer on restenosis (45) suggests that the most suitable target for gene or protein therapy with VEGFs might be the treatment of myocardial or peripheral ischemia.

In addition to the receptor binding profiles, it appears that 2 important variables define the angiogenic efficacy of VEGFs. First, the local growth factor concentration is the key determinant of the degree of vascular growth (50), also demonstrated by a clear dose-response with the number of applied viral particles in gene therapy experiments (15,51,52). Second, the volume per injection and the number of injections in addition to the matrix-binding properties of the growth factor determine the spread of the angiogenic effects (22,52). Intramuscular gene transfer route yields manifold higher gene transfer efficiency and angiogenesis in the target tissue with less ectopic gene expression than intra-arterially injected adenovirus (15). Whereas intramuscular injections are feasible in peripheral muscles, the heart is obviously a more difficult target. Currently, the most sophisticated way of transducing the myocardium is the use

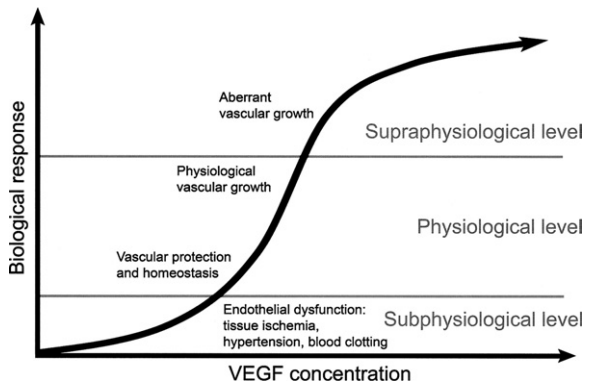

Figure 2 **The Biological Response of VEGF-A in Adults Is Dependent on its Local Tissue Concentration**

Lack of sufficient vascular endothelial growth factor (VEGF)-A results in endothelial dysfunction via diminished nitric oxide and prostacyclin production. Physiological levels maintain vascular homeostasis and protection while higher levels induce physiological vascular growth with sprouting angiogenesis and moderate capillary enlargement. Very high VEGF-A levels promote aberrant vascular growth, i.e., the formation of blood lacunae and glomeruloid bodies as well as significant tissue edema.

of percutaneous catheter injection systems such as NOGA (Biosense Webster, Markham, Ontario, Canada), which allows transmural gene transfer in the heart (52,53). Recombinant VEGF protein or naked plasmid-mediated VEGF gene transfer are not likely efficient enough to induce relevant biological effects and therefore viral vectors should be preferred (44,47,52).

During extensive preclinical studies in large animal models in pigs and rabbits (15,22,52,54,55), which better mimic human situation than experiments using mice and rats, we found that preexisting capillaries were dramatically enlarged via NO-dependent proliferation of ECs and SMCs only a few days after adenoviral (Ad)VEGF-A_{165} and AdVEGF-$D^{\Delta N \Delta C}$ gene transfer, resulting in supraphysiological muscle perfusion (Fig. 3). In the process of "capillary arterialization," preexisting capillaries enlarge, strengthen their wall by SMC hypertrophy due to increased blood flow (leading to the formation of arteriole-like vessels that significantly reduce peripheral resistance), and may even function as shunts (55). Similar observations also have been found to occur with VEGF-A overexpression by other groups (56,57). Furthermore, in the quail chorioallantoic membrane the vessel density increased maximally at lower VEGF-A concentrations, but that vessel diameter increased maximally at higher VEGF concentrations (58). Blood lacunae may be formed by a very high dose of AdVEGF-A_{165} (55). Importantly, local blood flow also appears to modulate the vascular growth response by AdVEGF-A into one that best serves the muscle's needs, e.g., leading to more sprouting angiogenesis in ischemic muscles (55). Secondary to the capillary enlargement, both normal arteries and collateral arteries as well as veins undergo com-

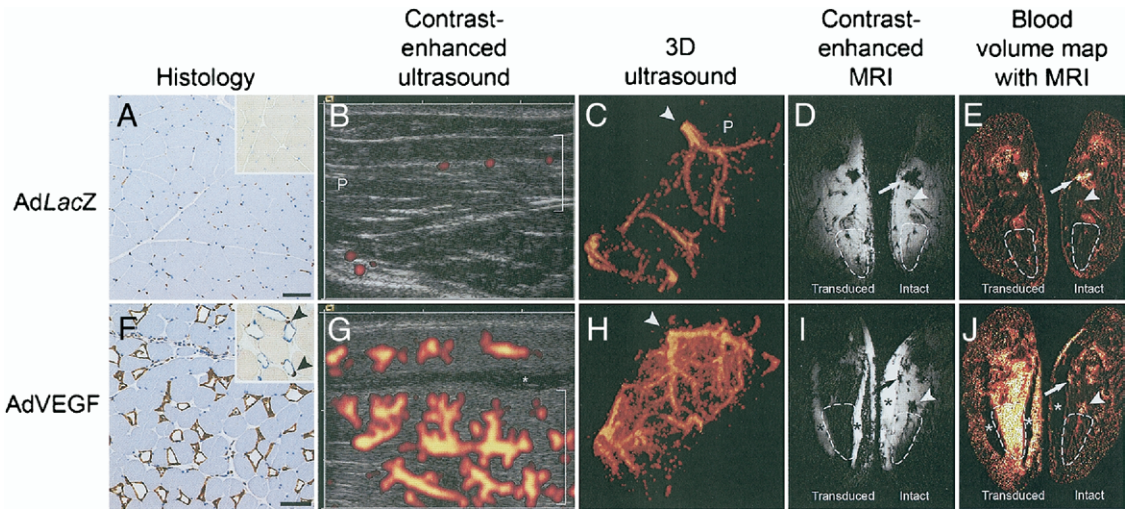

Figure 3 **Adenoviral AdVEGF-A_{165} Gene Transfer Promotes Efficient Angiogenesis and an Acute Increase In Tissue Perfusion**

(A and F) CD31 immunostainings of normal rabbit skeletal muscle transduced intramuscularly with AdLacZ or adenoviral vascular endothelial growth factor (AdVEGF)-A at 6 days earlier demonstrate abundant enlargement of preexisting capillaries after AdVEGF-A gene transfer. **(Insets)** CD31 **(blue)** + BrDU **(brown)** double stainings show that capillary enlargement with AdVEGF-A occurs through cell proliferation **(black arrowheads)**. Scale bar = 50 μ m. **(B and G)** Longitudinal contrast-enhanced ultrasound imaging of rabbit thighs shows that perfusion is increased up to 27-fold 6 days after AdVEGF-A gene transfer in the target muscle **(inside brackets)**. **(C and H)** 3-dimensional (3D) reconstructions of the ultrasound data show the increase in blood flow in the whole vascular tree, including large vessels. **(D and I)** Transversal mid-thigh T_2*-weighted magnetic resonance imaging (MRI) using a superparamagnetic contrast agent (Resovist), which causes intensive signal loss in the AdVEGF-A transduced semimembranosus muscle (outlined with **dashed lines**) as the result of high contrast concentration, i.e., blood volume. **(E and J)** Blood volume MRI maps visualize the difference in blood volume between AdVEGF-A and AdLacZ-treated muscles. Muscle edema is obvious after AdVEGF-A gene transfer both in ultrasound and MRI. P = proximal end of the semimembranosus muscle; **asterisks** = free fluid in between muscles after AdVEGF-A gene transfer; **white arrowheads** = profound femoral artery; **arrows** = superficial femoral artery. The figure, excluding the insets, has been modified from Rissanen et al. (55) with the permission of Lippincott Williams & Wilkins.

pensatory growth as the result of high blood flow (15,22,54–56). Bone marrow-derived cells do not incorporate in growing vessels induced by VEGF-A as previously proposed but may support angiogenesis via paracrine mechanisms (56,59,60). Other VEGFs than VEGF-A and -$D^{\Delta N \Delta C}$ also have significant potential as vascular therapeutics due to their angiogenic (VEGFR-2) or lymphangiogenic (VEGFR-3) properties (1,11,24). Interestingly, VEGFR-1 ligands VEGF-B and PlGF are also angiogenic both in ischemic and nonischemic tissues (18–21).

The most important side effect of successful angiogenic gene transfer with adenoviral VEGFR-2 ligands VEGF-A and -D have is transient tissue edema, which can cause accumulation of fluid in between skeletal muscle compartments and in the pericardium (22,51,52,55,61). Edema is dose-dependent, correlates with the mean capillary size, coincides with the peak perfusion increase after adenoviral gene transfer, and has not caused significant tissue damage or release of myocardial markers in large animal models. However, excess edema especially in the heart and in the calf can potentially be hazardous and should be avoided (62). A "cocktail" gene transfer of other growth factors, such as Ang-1, platelet-derived growth factor, or fibroblast growth factors in combination with VEGFs, could be used but currently there is no convincing data on this approach in clinically relevant models and overexpression of PDGFs or FGFs may even generate unwanted tissue fibrosis. Strong corticosteroids, such as dexamethasone, counteract increased VEGFR-2-mediated vascular permeability but unfortunately also inhibit angiogenesis (P. Korpisalo et al., unpublished data, 2007). One possibility would be the simultaneous stimulation of lymphatic vessel growth by using VEGFR-3 ligands to remove extravasated plasma proteins.

Recently, constructs expressing genomic VEGF-A with an emphasis on the long isoforms or VEGF-inducing transcription factors have been reported to be effective in hindlimb ischemia models (63–65). However, because tissue edema results from the combined effect of increased capillary pressure, perfusion and compromised integrity of capillary wall due to cell proliferation in addition to the direct vascular permeability effects of VEGFs, a complete avoidance of this side effect may be difficult, if not impossible. Although the angiogenic boost provided by AdVEGFs may be sufficient, e.g., when used as an adjuvant therapy with conventional revascularization procedures, more optimal vascular growth may be achieved with a more moderate but long-lasting expression that may result in the stabilization of growing vessels and diminished tissue edema (57,66). A sophisticated possibility would be the use of vector constructs with regulated promoters.

Clinical VEGF Trials

Peripheral arterial disease. Despite advances in by-pass grafting and multimodal percutaneous interventions, approximately one fifth of patients with chronic critical limb ischemic (CLI) cannot be treated by any conventional approach because of the severity and extent of the disease or due to a poor general status. This leaves a significant number of patients with peripheral arterial disease (PAD) without any effective treatment. Thus, local proangiogenic therapy might offer a new treatment option for these patients.

Early nonrandomized, uncontrolled trials. Patients in the first VEGF trials were not suitable for surgical or endovascular revascularization or they had failed other treatment options and were at high risk of amputation. The use of VEGF-A gene therapy initially was tested using naked plasmid DNA delivery and was reported to result in the resolution of rest pain, increased collateral vasculature, and an increase in ankle brachial pressure index (67,68). The safety and feasibility of intra-arterial gene delivery to human peripheral arteries with adenovirus was also tested using titers from 1×10^8 to 3×10^{10} plaque-forming units (42). Intramuscular injections of AdVEGF-A_{121} with titers from $4 \times 10^{8.5}$ to 4×10^{10} particle units (pu) were shown to be well tolerated and improved endothelial function and flow reserve at 30 days (69). A small uncontrolled study using naked VEGF-A_{165} plasmid DNA showed more than 80% improvement in rest pain (70).

Randomized, controlled trials. Only a couple of randomized, placebo-controlled VEGF trials have been reported on patients with intermittent claudication or CLI (Table 2). In a randomized controlled phase II trial (n = 54) of catheter-mediated VEGF-A_{165} gene delivery using plasmid-liposomes or adenoviruses, AdVEGF-A–treated patients showed improved vascularity on digital subtraction angiography at 3 months, but there were no differences between the treatment and placebo groups in terms of clinical outcomes (43). The RAVE (Regional Angiogenesis With Vascular Endothelial Growth Factor in Peripheral Arterial Disease) trial was a phase II randomized, placebo-controlled, double-blind study testing the safety and efficacy of intramuscularly administered AdVEGF-A_{121} in 105 patients with PAD (46). The patients were randomized to receive a low (4×10^9 pu) or high (4×10^{10} pu) dose of AdVEGF-A_{121} or placebo. No significant differences were found between the groups in the change in peak walking time at 3 months or in secondary end points, which were ankle brachial pressure index and quality of life measures. No severe side effects were noted, although transient edema was associated with intramuscular AdVEGF-A_{121} gene transfer. In a double-blind, placebo-controlled study of naked plasmid VEGF in 54 adult diabetic patients with CLI, there was no difference in the primary end point of the amputation rate at 100 days but significant improvements were achieved in secondary endpoints (ABI and clinical condition) (71). Currently, the phase II randomized, double-blind, placebo-controlled WALK trial is ongoing addressing the efficacy of adenoviral hypoxia-inducible

Table 2 Phase II/III Clinical Trials Addressing the Efficacy of Therapeutic Angiogenesis With VEGF Protein or Gene Therapy

Trial	Therapeutic Agent	Administration	Control Treatment	Target Disease	n	Primary End Point	Results*	Reference
VIVA trial	Recombinant VEGF-A_{165} protein	Intracoronary infusion followed by IV infusions	Vehicle	CAD	178	ETT at 2 months	Negative	44
VEGF Peripheral Vascular Disease trial	AdVEGF-A_{165} or plasmid/liposome VEGF-A_{165}	Intra-arterial injection at the angioplasty site	Ringer's lactate	PAD	54	Increased vascularity in angiography at 3 months	Positive	43
KAT trial	AdVEGF-A_{165} or plasmid/liposome VEGF-A_{165}	Intra-coronary injection at the angioplasty site	Ringer's lactate	CAD	103	Improved myocardial perfusion at 6 months	Positive (adenovirus group only)	45
REVASC trial	AdVEGF-A_{121}	Intramyocardial injection via mini-thoracotomy	Best medical treatment	CAD	67	Time to 1-mm ST-segment depression on ETT at 26 weeks	Positive	80
RAVE trial	AdVEGF-A_{121}	Intramuscular injections	Vehicle (no virus)	PAD	105	Peak walking time at 12 weeks	Negative	46
Euroinject One trial	Naked VEGF-A_{165} plasmid	Percutaneous intramyocardial injections	Placebo plasmid	CAD	74	Improved myocardial perfusion at 3 months	Negative	47
Groningen trial	Naked VEGF-A_{165} plasmid	Intramuscular injections	Saline	PAD	54	Decrease in amputation rate	Negative (secondary end points positive)	71
GENASIS trial	Naked VEGF-C plasmid	Percutaneous intramyocardial injections	Vehicle	CAD	295 (404 planned)	ETT at 3 months	Negative at interim analysis, stopped	Unpublished
NORTHERN trial	AdVEGF-A_{121}	Percutaneous intramyocardial injections	Vehicle	CAD	120 (planned)	Change in myocardial perfusion in stress/rest at 12 weeks	Ongoing	Unpublished
NOVA trial	AdVEGF-A_{121}	Percutaneous intramyocardial injections	Vehicle	CAD	129 (planned)	ETT at 26 weeks	Stopped	Unpublished

*Efficacy measured as the study protocol-defined primary or secondary end point.

Ad = adenovirus; CAD = coronary artery disease; ETT = exercise tolerance testing; IV = intravenous; PAD = peripheral arterial disease; VEGF = vascular endothelial growth factor.

factor-1α on peak walking time at 6 months in approximately 300 patients with PAD.

Coronary artery disease (CAD). Approximately 6.8 million people in U.S. suffer from CAD, and a steadily increasing number of patients fall into the category in which current revascularization techniques cannot be applied any more. Thus, novel treatments such as therapeutic angiogenesis might offer new hope also for approximately 100,000 patients in the U.S. only.

Early nonrandomized, uncontrolled trials. Small phase I trials testing VEGF-A_{165} recombinant protein as an intracoronary infusion or naked VEGF-A_{165} plasmid as intramyocardial injections via throracotomy "no-option" patients with CAD reported significant increases in exercise capacity, improved contractile function, rest myocardial perfusion, and angiographic collaterals (72–75). In a trial using naked VEGF-C (VEGF-2) plasmid DNA injected percutaneously into ischemic myocardium of 6 patients with the NOGA catheter improved clinical status, alleviated angina, and reduced the area of ischemic myocardium (76). Recently, 2-year results of an uncontrolled dose-escalation study of 30 patients with Canadian Cardiovascular Society (CCS) class III or IV angina treated with intramyocardial injection of naked VEGF-C (VEGF-2) plasmid DNA via thoracotomy were published (77). In the end of the follow-up, 88.5 % of the patients were reported to have CCS class I or II angina pectoris and only 11% of the patients had CCS class III angina pectoris.

Randomized, controlled trials. There are a few completed phase II/III trials using VEGFs in patients with CAD (Table 2). In a phase II VIVA trial, a total of 178 patients were treated either with a low or high dose of recombinant VEGF-A_{165} protein (a 10-min intracoronary infusion followed by a 4-h intravenous infusion at day 3, 6, and 9) or placebo. No significant effect was found in the primary end point, which was treadmill exercise capacity at 4 months (44). However, the high dose of VEGF-A resulted in a significant improvement on angina class as compared with placebo.

The study of Laitinen et al. (78) was the first randomized, placebo-controlled trial investigating the safety and feasibility of plasmid/liposome VEGF-A_{165} gene transfer in patients with stable CAD undergoing percutaneous coronary intervention (n = 15). Gene transfer was found to be safe and well tolerated. The KAT (Kuopio Angioplasty phase II Trial) study aimed to investigate the efficacy of VEGF-A_{165} gene therapy on restenosis rate and myocardial perfusion as given during PCI (45). A total of 103 patients were randomized to receive plasmid/liposome VEGF-A_{165}, AdVEGF-A_{165}, or placebo at the site of coronary angioplasty as a 10-min injection administered with a Dispatch catheter (SCIMED Life Systems Inc., Maple Grove, Minnesota) before stenting. There was no difference in the restenosis rate (6%) between the study groups but myocardial perfusion showed a significant improvement in the AdVEGF-A_{165} group at 6 months.

Losordo et al. (79) enrolled 19 no-option patients randomized to percutaneous intramyocardial NOGA-delivery of placebo or 3 different doses of naked VEGF-C plasmid with a crossover design. A significant reduction in CCS angina class in patients receiving VEGF-C plasmid after a 12-week follow-up was reported. In the Euroinject One study, a total of 80 no-option CCS angina class III to IV patients were assigned to intramyocardial injections of naked plasmid VEGF-A_{165} or placebo with the NOGA catheter (47). The injections were targeted to the area of myocardium with a perfusion defect detected with SPECT. After a 3-month follow-up, no significant differences were found between the groups in CCS class or in the size of perfusion defect at rest or during exercise. However, local wall motion as assessed by ventriculography or local linear shortening mapping with NOGA were better in the plasmid VEGF-A_{165}–treated group than in the placebo group.

In the phase II REVASC (Randomized Evaluation of VEGF for Angiogenesis in Severe Coronary disease) trial, no-option patients with CAD (n = 67) with CCS class II to IV angina were randomized to either continue best standard medical therapy or to receive AdVEGF$_{121}$ (4×10^{10} pu) into the myocardium via thoracotomy (80). The time to 1-mm ST-segment depression during exercise test was significantly improved at 26 weeks but not at 12 weeks in patients who received AdVEGF$_{121}$ compared with control patients. Secondary end points, CCS class, and total exercise tolerance also were improved at 12 weeks. However, a significant contribution of a placebo effect in these results cannot be ruled out because of thoracotomy. The phase II GENASIS trial was originally planned to enroll 404 no-option CAD patients with CCS III to IV angina for addressing the efficacy of percutaneous, intramyocardial Stiletto catheter (Boston Scientific Corporation, Natick, Massachusetts)-mediated naked VEGF-C (VEGF-2) plasmid gene transfer on exercise tolerance time at 3 months. However, this trial was recently prematurely stopped after 295 patients because of problems related to the catheter and high likelihood of the lack of efficacy on the primary end point. Currently, the phase II/III NORTHERN trial (NOGA Angiogenesis Revascularization Therapy: Evaluation by RadioNuclide Imaging) (planned n = 120) is ongoing to test the efficacy of intramyocardial NOGA-mediated delivery of AdVEGF-A_{121} in no-option CAD patients. The NOVA (NOGA Delivery of VEGF for Angina) trial with very similar design was recently stopped (Table 2).

Safety of VEGF therapy. An important end point in all VEGF trials has been safety. Stimulation of angiogenesis raises theoretical concerns, especially in relation to tumor growth, retinal neovascularization, hemorrhage from fragile new vessels, enhanced atherogenesis, hypotension, edema and inflammatory responses (1). So far, no evidence of increased tumorigenesis, neovascularization in nontarget organs, vascular malformations, increased atherogenesis, or plaque destabilization has been observed in clinical trials

Table 3 Troubleshooting of Clinical Therapeutic Angiogenesis Using VEGF

Problem	Reason	Potential Solutions
• Lack of clear clinical efficacy in randomized controlled trials with VEGF recombinant protein or gene therapy at 2 to 6 months in no-option PAD and CAD patients	• Short half-life of VEGF recombinant protein • Low gene transfer efficiency with naked plasmid or intraarterial gene transfer route • Too low adenoviral dose with i.m. route • Difficult or unresponsive patient population	• Intramuscular gene transfer route • Optimized viral dose • Wider spread of angiogenesis using sufficient volume and number of injections • Use of VEGF gene transfer as adjuvant therapy in combination with conventional revascularization procedures
• No definitive evidence of increased tissue perfusion at 2 to 6 months in clinical VEGF gene therapy trials despite large acute increases in animal models	• Too low gene transfer efficiency • Regression of immature vessels within 2 weeks after transient adenoviral gene transfer	• Perfusion measurement 5 to 6 days after AdVEGF gene transfer to show "proof-of-principle" of increased perfusion in humans • Use of AAV, lentiviruses, gutless adenoviruses, or other vectors that produce long-term VEGF expression
• Angiogenesis-associated tissue edema	• VEGF increases vascular permeability directly • Increased perfusion, capillary pressure, and cell proliferation in vascular wall cause additional plasma protein extravasation	• Optimal viral dose • Vessel maturation via long-term VEGF expression via AAV • Regulation of transgene expression • Growth factor combinations

AAV = adeno-associated virus; i.m. = intramuscular/intramyocardial; other abbreviations as in Table 2.

using VEGF-A protein or gene transfer (42–47). Excess vascular structures also diminish in animal experiments after extinction of VEGF expression (55). High doses of AdVEGFs cause tissue edema and pericardial fluid accumulation in animal experiments. Consistently, peripheral edema was also found to be associated to the $AdVEGF_{121}$ therapy in the RAVE trial (46). Recently, pericardial effusions were reported to occur in 1.37% of patients after percutaneous intramyocardial VEGF-C plasmid gene transfer in the GENASIS-trial, most likely related to Stiletto catheter-mediated injections. Mild transient fever and development of antiadenovirus antibodies have been reported after the intra-arterial administration of adenoviral vectors (43,45). In summary, according to current experience from clinical studies, treatment with VEGFs has been well tolerated, and no VEGF-related serious adverse effects have been reported.

Insights From Clinical Trials and Future Perspectives

Placebo effect. All published uncontrolled gene or protein VEGF therapy trials have reported positive results. On the contrary, none of the randomized, controlled trials have shown clinically relevant differences between the treated and the placebo groups. Also, controlled trials using other growth factors or cytokines, such as recombinant FGF-2 or granulocyte macrophage colony-stimulating factor protein or intracoronary AdFGF-4 have not been able to report convincing improvements in clinical outcomes (81–85). Thus, as a result of the strong placebo effect, randomized, placebo-controlled trials are necessary to assess the efficacy of angiogenic therapies. In this respect, no exceptions can be made, even if most patients treated with this new technology have severe CAD or PAD, full medication and are no-option patients for other revascularization treatments. In addition to VEGF and other growth factors, cell-based therapy for tissue ischemia has recently gained much attention (56,59,60). Currently, the 2 randomized controlled clinical trials using the whole mononuclear cell fraction (containing <0.1% endothelial progenitor cells) to treat myocardial infarction with bone marrow-derived cells did not show a significant improvement in the left ventricular function (86,87). The reason(s) why randomized controlled clinical trials for therapeutic angiogenesis have not shown any therapeutic benefits is still somewhat unclear. Some reasons for the negative results and potential solutions are shown in Table 3 and discussed herein.

Patient selection. It may be possible that therapeutic angiogenesis just does not work as well in elderly patients as in young animals. The main problem in ischemic tissue may not only be the availability of angiogenic growth factors (13) but also defects in the responsiveness to angiogenic stimuli due to severe atherosclerosis. However, because natural collateral vessel formation can rescue ischemic myocardium and lower limbs even in the elderly patients, the concept of therapeutic angiogenesis seems valid but technical or pharmacological shortcomings in the current treatment approaches may have caused the failures (49).

Standard medical therapy and current revascularization methods improve prognosis, relieve symptoms and can be applied to most patients with CAD and PAD. Thus, no-option patients remain as the most likely target group for VEGF trials. A novel approach would be the combination of conventional revascularization procedures with adjuvant VEGF gene therapy, which might represent the next step in the development of clinically relevant proangiogenic treatment. Recent findings on the role of blood flow as the modulator of vascular growth support this kind of novel concept in which peripheral angiogenesis could improve the "run-off" of grafts and possibly lead to better outcomes (55,88). Importantly, in this setting also other than no-option patients would become eligible for VEGF trials

which might increase the likelihood of more positive clinical outcomes.

Gene-delivery methods. The most likely explanation for the negative clinical results is that growth factor concentration in human tissues has not reached sufficient levels and/or has not persisted long enough for triggering relevant vascular growth. This, in turn, can result from several factors, such as the short half-life of recombinant growth factors, insufficient dose of adenovirus, too short a time for gene expression or compromised delivery route such as intra-arterial injection. Intra-arterially injected material (gene transfer vector or cells) is less effective (15), unlikely reaches areas with severely impaired perfusion i.e. areas with the greatest need of therapeutic effect but instead results in a considerable systemic spread of the injected agent. Pericardial delivery is also unlikely to result in an efficient transmyocardial transduction. Intramuscular and intramyocardial injections are currently most effective and the latter can be performed percutaneously without the need of thoracotomy by using the intracardiac catheter systems.

In the Euroinject One and GENASIS trials, intramyocardial injections of naked VEGF-A_{165} or -C plasmid have likely yielded too-low transfection efficiency (47). This result is also in line with our experimental results in pigs, in which the intramyocardial NOGA catheter-mediated injection of naked VEGF-A or -$D^{\Delta N \Delta C}$ plasmid did not induce significant protein production or any vascular effects (52). In the RAVE trial, the adenoviral VEGF-A_{121} dose was approximately 2 logs less per kilogram than what has been used in the recent preclinical experiments showing very high angiogenic efficacy in addition to approximately 2 logs lower biological efficacy of $VEGF_{121}$ than that of $VEGF_{165}$ (2,22,46,55). Despite large initial perfusion increases at around a week after $AdVEGF_{165}$ injections, it may be that vectors such as adeno-associated virus or lentivirus capable of long-term gene expression are needed to achieve clinically relevant vascular growth.

End points. Before gene therapy can become an established method for revascularization, it must demonstrate its capability to relieve symptoms and reduce adverse end points or mortality/amputations in patients with CAD and PAD. Because an improvement in mortality is difficult to achieve, surrogate end points such as exercise capacity and tissue perfusion often are used. Exercise capacity or walking time/distance are very relevant clinical end points, but have the disadvantage that in the end-stage CAD and PAD patients they are very subjective measures and show great intraindividual variation. Another end point used in previous trials, the size of perfusion defect at rest or during exercise assessed with single-photon emission computed tomography (SPECT), also has its limitations. Instead, the hybrid SPECT or PET and computed tomography (SPECT/CT or PET/CT) and novel magnetic resonance imaging techniques might be used for the assessment of myocardial perfusion and function. Importantly, most of the small molecular contrast media such as iodine X-ray contrast agents and gadolinium extravasate from angiogenic, hyperpermeable vessels (22,55). On the contrary, novel contrast-enhanced ultrasound techniques using large 3-μm microbubbles provide reliable measurement of tissue perfusion during angiogenesis (55).

Follow-up time. The follow-up time needs to be long enough to reveal long-term benefits and potential side-effects of the VEGF therapy. On the other hand, atherosclerosis is a progressive disease and to avoid the effects of this confounding factor it can be recommended that the primary end point should be evaluated at 3 to 6 months after the VEGF therapy. However, a short time point of 5 to 6 days after adenoviral gene transfer also should be evaluated to document the peak increase in myocardial or skeletal muscle perfusion as well as maximal tissue edema according to the transient gene expression kinetics of adenovirus (51,55). To assess potential long-term effects of the therapy, follow-up for 3 years is recommended.

Conclusions

The promising preclinical results obtained in animal experiments with VEGF therapy have not yet been translated into clinical success. However, VEGFs have tremendous potential as vascular therapeutics and, therefore, the optimization of the indications of the therapy and the ongoing developments in gene delivery techniques are expected to lead to the generation of novel treatment for ischemic cardiovascular disease.

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