Human Studies of Angiogenic Gene Therapy

Rajesh Gupta, Jörn Tongers, Douglas W. Losordo

Abstract: Despite significant advances in medical, interventional, and surgical therapy for coronary and peripheral arterial disease, the burden of these illnesses remains high. To address this unmet need, the science of therapeutic angiogenesis has been evolving for almost two decades. Early preclinical studies and phase I clinical trials achieved promising results with growth factors administered as recombinant proteins or as single-agent gene therapies, and data accumulated through 10 years of clinical trials indicate that gene therapy has an acceptable safety profile. However, more rigorous phase II and phase III clinical trials have failed to unequivocally demonstrate that angiogenic agents are beneficial under the conditions and in the patients studied to date. Investigators have worked to understand the biology of the vascular system and to incorporate their findings into new treatments for patients with ischemic disease. Recent gene- and cell-therapy trials have demonstrated the bioactivity of several new agents and treatment strategies. Collectively, these observations have renewed interest in the mechanisms of angiogenesis and deepened our understanding of the complexity of vascular regeneration. Gene therapy that incorporates multiple growth factors, approaches that combine cell and gene therapy, and the administration of “master switch” agents that activate numerous downstream pathways are among the credible and plausible steps forward. In this review, we examine the clinical development of angiogenic gene therapy, summarize several of the lessons learned during the conduct of these trials, and suggest how this prior experience may guide the conduct of future preclinical investigations and clinical trials. (Circ Res. 2009;105:724-736.)

Key Words: gene therapy ■ angiogenesis ■ clinical trials

The concept of reconstituting the microvasculature as part of a strategy for treating ischemic tissue (ie, therapeutic angiogenesis) evolved soon after pioneering work by Folkman and colleagues documented the existence of angiogenic growth factors. Recognition that therapies designed to repair the microcirculation may enhance cardiovascular function induced a paradigm shift in treatment strategies for acute and chronic ischemia. The new microvascular strategies were first investigated through a series of preclinical studies that evaluated recombinant growth factors such as basic fibroblast growth factor and vascular endothelial growth factor (VEGF) in models of myocardial and lower-extremity ischemia; however, maintaining adequate levels of the recombinant protein in the target zone was technically challenging and
considered prohibitively expensive. Accordingly, subsequent experiments evaluated the potential use of gene therapy for therapeutic angiogenesis.6,7 The first in-human studies of gene therapy for treatment of peripheral and coronary artery disease (PAD and CAD, respectively) were reported in the late 1990s.8–10 Since these initial reports, much has been learned about the mechanism of new blood vessel formation, and the safety of angiogenic gene therapy has been supported by substantial evidence.11

In this review, we focus on clinical studies of angiogenic gene therapy for treatment of ischemic cardiovascular disease and emphasize the findings from controlled trials (Table 1). We discuss the results from these trials, as well as the lessons learned and the persistent challenges associated with many of these agents. We also speculate about how our new knowledge may be used to develop novel therapeutic strategies and to guide the conduct of future preclinical and clinical investigations. For a comprehensive review of individual growth factors, their biological mechanisms, activity, and preclinical investigations, the reader is encouraged to consult additional resources.12

### Human Trials of Cardiovascular Gene Therapy

#### Vascular Endothelial Growth Factor

Vascular endothelial growth factor (VEGF) is a potent regulator of endothelial cell survival, proliferation, and migration. VEGF mediates angiogenesis during embryonic development,13,14 postembryonic growth,15 and tissue repair,16–18 and pathological angiogenesis in tumors and ocular disease.19 There are 5 known members of the VEGF superfamily: VEGF-A (also called VEGF or VEGF-1), VEGF-B, VEGF-C (VEGF-2), VEGF-D, and placental growth factor. VEGF-A was the first member identified and is the best characterized. Variations in premRNA splicing produce several isoforms of VEGF-A (eg, VEGF121, VEGF165, VEGF189, VEGF206) with different biological properties. For a more comprehensive review of VEGF biological activity, the reader is encouraged to consult any of several excellent resources.20,21

In 1996, Isner et al published the first case report of administration of phVEGF165 (plasmid DNA encoding human VEGF165) via balloon angioplasty for treatment of PAD.8 The same group subsequently reported their initial experiences with direct intramyocardial injection of the same plasmid in “no-option” patients with intractable angina.22 Since these initial in-human studies of cardiovascular gene therapy, several groups have evaluated the use of other VEGF-A isoforms, as well as VEGF-C, for treatment of PAD and CAD.

#### Ischemic Peripheral Arterial Disease

Initial attempts to deliver gene therapy involved the application of plasmid DNA to hydrogel-coated balloon catheters for intraarterial administration. Later, preclinical studies indicated that intramuscular delivery could successfully achieve transgene expression.7 Baumgartner et al reported the results of pilot clinical studies in which VEGF165 plasmid was injected into the limb muscle of patients with resting pain or nonhealing ulcers.23 In an accompanying editorial, Folkman remarked: “Anatomic and functional efficacy was demonstrated by . . . improved hemodynamic measurements and angiographic evaluation, reduced pain, increased healing of ischemic ulcers, limb salvage, and immunohistochemical evidence of proliferating endothelial cells in tissue specimens.”24

Makinen et al compared intravascular delivery of plasmids and adenoviruses encoding VEGF165 in patients with PAD manifesting as claudication and critical limb ischemia (CLI).25 Fifty-four patients with symptomatic PAD amenable to percutaneous transluminal angioplasty were randomized to receive adenoviral VEGF165, plasmid liposome VEGF165, or Ringer’s lactate placebo, all administered via intra-arterial infusion following percutaneous transluminal angioplasty. Both VEGF165 treatments appeared to be safe and were associated with significant increases in vascularity; clinical improvements were also noted in VEGF165-treated patients but did not differ significantly from those observed in patients administered placebo.

The RAVE (Regional Angiogenesis with Vascular Endothelial Growth Factor) trial was the first randomized, double-blind, placebo-controlled study of intramuscular adenoviral gene transfer for the treatment of PAD.26 Patients (n=105) with unilateral, exercise-limiting claudication were randomized to receive direct intramuscular injections of low-dose adenoviral VEGF121 (AdVEGF121), high-dose AdVEGF121, or placebo. Twelve and 26 weeks after treatment, the authors found no significant differences between groups in the primary or secondary efficacy end points, and AdVEGF121 therapy was associated with dose-dependent peripheral edema. This latter finding is notable, because one of the known effects of VEGF is increasing vessel permeability.

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**Non-standard Abbreviations and Acronyms**

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>AAV</td>
<td>adenovirus-associated virus</td>
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<tr>
<td>ABI</td>
<td>ankle–brachial index</td>
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<tr>
<td>AdVEGF121</td>
<td>adenoviral vector encoding VEGF121 isoform</td>
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<tr>
<td>Ad5FGF4</td>
<td>adenoviral vector encoding FGF4</td>
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<tr>
<td>CAD</td>
<td>coronary artery disease</td>
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<td>CCS</td>
<td>Canadian Cardiovascular Society</td>
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<tr>
<td>CLI</td>
<td>critical limb ischemia</td>
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<td>Del-1</td>
<td>developmentally regulated endothelial locus</td>
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<td>EMM</td>
<td>electromechanical mapping</td>
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<tr>
<td>ETT</td>
<td>exercise treadmill test</td>
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<tr>
<td>FGF</td>
<td>fibroblast growth factor</td>
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<tr>
<td>G-CSF</td>
<td>granulocyte colony-stimulating factor</td>
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<td>HGF</td>
<td>hepatocyte growth factor</td>
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<tr>
<td>HIF-1α</td>
<td>hypoxia-inducible factor-1α</td>
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<tr>
<td>NV1FGF</td>
<td>plasmid encoding FGF1</td>
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<tr>
<td>PAD</td>
<td>peripheral artery disease</td>
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<tr>
<td>SPECT</td>
<td>single-photon emission–computed tomography</td>
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<tr>
<td>TcPO2</td>
<td>transcutaneous oxygen tension</td>
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Table 1. Controlled Trials of Angiogenic Gene Therapy

<table>
<thead>
<tr>
<th>Trial Acronym, Author, Reference</th>
<th>Treatment(s)</th>
<th>Route of Administration</th>
<th>Patients (n), Active/Placebo</th>
<th>Patient Characteristics</th>
<th>Follow Up</th>
<th>Primary End Point(s)</th>
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<tr>
<td>Phase I/II</td>
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<tr>
<td>VEGF</td>
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<tr>
<td>Losordo et al35</td>
<td>Plasmid VEGF2</td>
<td>Percutaneous intramyocardial injection using EMM</td>
<td>12/7 CAD, NR, RSA, CCS 3–4</td>
<td>12 wk</td>
<td>Change in CCS class and exercise tolerance</td>
<td></td>
</tr>
<tr>
<td>Mäkinen et al26</td>
<td>Adenoviral and plasmid liposome VEGF165</td>
<td>Percutaneous intraarterial infusion following PTA</td>
<td>18/17/19 VEGF-ad/VEGF P-L/placebo</td>
<td>PAD suitable for PTA</td>
<td>3 mo</td>
<td>DSA</td>
</tr>
<tr>
<td>RAVE</td>
<td>Rajagopalan et al26</td>
<td>Adenoviral VEGF121</td>
<td>Intramuscular injection</td>
<td>40/32/33 high dose/low dose/placebo</td>
<td>PAD, exercise-limiting claudication</td>
<td>12 wk</td>
</tr>
<tr>
<td>KAT</td>
<td>Hedman et al29</td>
<td>Adenoviral and plasmid liposome VEGF165</td>
<td>Intracoronary infusion after PCI</td>
<td>37/28/38 VEGF-ad/VEGF-P-L/placebo</td>
<td>CAD, CCS 2/3, PCI</td>
<td>6 mo</td>
</tr>
<tr>
<td>EUROWINJECT ONE</td>
<td>Kastrup et al30</td>
<td>Plasmid VEGF165</td>
<td>Intracoronary infusion using EMM</td>
<td>40/40 CAD, NR, RSA, CCS 3–4</td>
<td>3 mo</td>
<td>Myocardial perfusion, wall motion by NOGA mapping and LVgram, CCS class</td>
</tr>
<tr>
<td>REVASC</td>
<td>Stewart et al34</td>
<td>Adenoviral VEGF121</td>
<td>Intramyocardial injection via minithoracotomy</td>
<td>32/35 CAD, NR, RSA, CCS 2–4</td>
<td>26 weeks</td>
<td>ETT time to 1-mm ST depression</td>
</tr>
<tr>
<td>Ripa et al32</td>
<td>Plasmid VEGF165 and G-CSF</td>
<td>Percutaneous intramyocardial injection of VEGF165 using EMM and subcutaneous G-CSF injection</td>
<td>16/16/16 VEGF165 + G-CSF/VEGF165/placebo</td>
<td>CAD, NR, RSA, CCS 3–4</td>
<td>3 mo</td>
<td>Change in SPECT perfusion defects</td>
</tr>
<tr>
<td>Kusumanto et al27</td>
<td>Plasmid VEGF165</td>
<td>Intramuscular injection</td>
<td>27/27 PAD, DM, CLI, NR</td>
<td>100 days</td>
<td>Amputations</td>
<td></td>
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<tr>
<td>FGF</td>
<td></td>
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<tr>
<td>AGENT</td>
<td>Grines et al42</td>
<td>Ad5-FGF4</td>
<td>Intracoronary infusion</td>
<td>60/19 CAD, CCS 2–3</td>
<td>12 wk</td>
<td>Safety and ETT time</td>
</tr>
<tr>
<td>AGENT-2</td>
<td>Grines et al44</td>
<td>Ad5-FGF4</td>
<td>Intracoronary infusion</td>
<td>35/17 CAD, NR, RSA, CCS 2–4</td>
<td>12 mo</td>
<td>Change in perfusion defect on adenosine SPECT at 8 weeks</td>
</tr>
<tr>
<td>TALISMAN 201</td>
<td>Nikol et al41</td>
<td>FGF-1 plasmid</td>
<td>Intramuscular injection</td>
<td>51/56 PAD, CLI, NR</td>
<td>1 yr</td>
<td>Ischemic ulcer healing</td>
</tr>
<tr>
<td>HIF</td>
<td>Rajagopalan et al40</td>
<td>Ad2/HIF-1α/VP16</td>
<td>Intramuscular injection</td>
<td>34/7 PAD, CLI, NR</td>
<td>1 yr</td>
<td>Safety</td>
</tr>
<tr>
<td>DELTA-1</td>
<td>Grossman et al61</td>
<td>Del-1 plasmid</td>
<td>Intramuscular injection</td>
<td>52/53 PAD, PWT of 1–10 on ETT</td>
<td>180 days</td>
<td>PWT at 90 days</td>
</tr>
<tr>
<td>HGF</td>
<td>Powell et al60</td>
<td>HGF plasmid</td>
<td>Intramuscular injection</td>
<td>27/25/26/26 high dose/middle dose/low dose/placebo</td>
<td>PAD, CLI, NR and TcPO2 &lt;40 mm Hg or toe pressure &lt;50 mm Hg or ankle pressure &lt;70 mm Hg</td>
<td>12 mo</td>
</tr>
</tbody>
</table>

(Continued)
suggesting that there was evidence for bioactivity following AdVEGF121 administration. This raises several interesting questions not only about this study but about attempts at therapeutic angiogenesis in general. First, the lack of a validated tool to quantify blood flow in the extremities is a challenge for all clinical trials in patients with PAD. For “proof of concept” in therapeutic angiogenesis, measurement of improved perfusion would be sufficient, but thus far, trials have had to rely on observations of changes in clinical end points. Given the muscle mass in the legs and the “geography,” ie, the distance over which perfusion must be modified, it may be unrealistic to expect that measurable clinical benefit will ensue following the limited application of angiogenic therapies that is required while safety data are being accumulated in early-phase studies.

Kusumanto et al performed a randomized, double-blind, placebo-controlled study of plasmid-encoded VEGF165 in diabetic patients with PAD and CLI.27 Fifty-four patients were randomized to receive 2 direct intramuscular injections of VEGF165 or placebo, the first on day 0 and another on day 28, and followed for 100 days. Compared with patients in the placebo group, VEGF165-treated patients experienced fewer amputations (3 patients versus 6 patients, P=NS) more skin ulcer healing (7 versus 0, P=0.01), and more hemodynamic improvement (7 versus 1, P=0.05). Overall, there were 14 subjects in the active treatment group who experienced clinical improvement versus three in the control group (P=0.003).

The differences between VEGF isoforms also deserve comment. VEGF121 lacks the heparin-binding domain that is thought to be responsible for adherence of VEGF to matrix proteins. It is therefore possible that locally expressed VEGF121 has a short tissue half life and, consequently, induces only the first step in angiogenesis (ie, the loosening of adherens junctions between endothelial cells, which results in edema), whereas the longer tissue retention of VEGF165 may permit execution of the full paradigm of angiogenesis.

**Ischemic Coronary Artery Disease**

**VEGF165**

The first investigation to use VEGF165 plasmid for myocardial gene therapy was an open-label study in which the plasmid was administered by epicardial injection after minithoracotomy in five patients with intractable angina.22 This study provided early evidence of safety and observational data regarding symptoms and perfusion. In a subsequent study, Laitinen et al demonstrated that a less invasive method of VEGF165 plasmid administration, liposome-mediated transfection via intracoronary infusion, was both safe and feasible.28

These pilot studies were followed by the phase II KAT (Kuopio Angiogenesis Trial) investigation. The objectives of this study were to assess the safety of intracoronary VEGF165 gene therapy when administered at the time of angioplasty and stenting, and to evaluate whether VEGF165 gene therapy prevented restenosis and improved myocardial perfusion. Patients (n=103) with symptomatic CAD who were amenable to percutaneous revascularization were randomized to receive intracoronary infusions of adenoviral VEGF165, plasmid liposome VEGF165, or placebo at the time of percutaneous coronary intervention.29 VEGF165 gene therapy during percutaneous coronary intervention appeared to be safe, but the investigators found no difference among groups in the primary end points of minimal luminal diameter and percent diameter stenosis (measured by quantitative coronary angiography) 6 months after treatment. Thus, VEGF165 gene therapy did not decrease the rate of restenosis; however, myocardial perfusion (measured by adenosine single-photon-emission computed tomography [SPECT]) at month 6 was significantly greater in patients administered adenoviral VEGF165 than in the plasmid–liposome–VEGF165 and placebo-treatment groups. Thus, this trial provided evidence for angiogenesis following intracoronary administration of AdVEGF.

The Euroinject One trial was a randomized, double-blind, placebo-controlled trial of naked plasmid VEGF165 in patients with symptomatic CAD who were not candidates for revascularization surgery.30 Eighty patients were randomized to receive direct injections of VEGF165 plasmid or a placebo plasmid into ischemic myocardial tissue, which was identified via electromechanical mapping (EMM) and SPECT. The plasmid was administered via endocardial injection using the NOGA system. Three months later, myocardial stress perfusion defects and Canadian Cardiovascular Society (CCS) angina class did not differ between treatment groups, but regional wall motion scores (measured by EMM) and left ventricular function were improved in the VEGF165-treated group.
patients. After the study was completed, a post hoc analysis using an alternative method for interpreting EMM and SPECT perfusion data revealed some evidence of improved perfusion in the injected area of VEGF165-treated patients.\textsuperscript{31} As in other gene therapy trials, a significant placebo effect was observed; both subjective end points (eg, CCS class) and objective end points (eg, myocardial stress perfusion) improved in some placebo-treated patients.

The discordance among the multiple end points measured in this phase II study is worth putting into context. The two largest trials of VEGF165 gene therapy yielded evidence of improved perfusion in treated versus control subjects. Some of the analyses were performed after unblinding, which is unacceptable for phase III data, but can be appropriate in phase II studies where the goal is to gain an understanding of the evidence and measurement methods for detecting bioactivity.

Ripa\textsuperscript{et al} performed a pilot study of combined VEGF165 gene therapy and stem cell mobilization in patients with CAD who were symptomatic but were not candidates for revascularization.\textsuperscript{32} Sixteen patients received intramyocardial injections of VEGF165 plasmid followed one week later by administration of granulocyte colony-stimulating factor (G-CSF) to mobilize progenitor cells from the bone marrow; the historical control groups consisted of 16 VEGF plasmid–treated patients and 16 control plasmid–treated patients from the Euroinject One trial. The number of circulating progenitor cells (identified via CD34 expression) increased significantly after G-CSF treatment, but there was no improvement in the primary end point of change in myocardial stress perfusion. The authors speculated that the homing of mobilized stem cells to the infarcted area may have been inadequate and suggested that cotransfer of a plasmid encoding stromal cell–derived factor 1, a progenitor cell–homing factor, may improve patient outcomes. Other explanations for a lack of benefit must be considered, however, including the fact that SPECT scanning has not been validated for documenting benefit must be considered, however, including the fact that SPECT scanning has not been validated for documenting evidence of improved perfusion in treated versus control subjects. Some of the analyses were performed after unblinding, which is unacceptable for phase III data, but can be appropriate in phase II studies where the goal is to gain an understanding of the evidence and measurement methods for detecting bioactivity.

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VEGF121

Rosengart\textsuperscript{et al} were the first to administer intramyocardial gene therapy with an adenoviral vector encoding VEGF121 (ie, the 121-aa isofrom of VEGF-A). The vector was administered to six individuals as sole therapy and to 15 individuals as an adjunct to bypass surgery.\textsuperscript{33} This pilot study provided initial feasibility data as well as observational evidence of bioactivity.

The protocol for the REVASC (Randomized Evaluation of VEGF for Angiogenesis) trial\textsuperscript{34} was designed to address some possible flaws in methodology that could have contributed to the disappointing results observed in many earlier investigations. Investigators were concerned that plasmid DNA may not be efficiently transfected and expressed, and that intracoronary or intravenous administration may not deliver sufficient amounts of the vector to the target tissue. Thus, adenoviral VEGF121 (AdVEGF121) was administered via direct intramyocardial injection during a minithoracotomy to patients with symptomatic CAD who were not candidates for revascularization. Patients were randomized to continue maximal medical therapy with or without AdVEGF121 treatment, but the study was not blinded because AdVEGF121 was administered surgically. Exercise time to 1-mm ST depression (the primary end point) did not differ significantly between groups 12 weeks after treatment but was significantly greater in the VEGF121 group than in the control group at week 26. Despite this promising objective finding, further development of this therapy in refractory angina has not occurred.

**VEGF-C**

In 2002, a randomized, double-blind, placebo-controlled, phase I/IIa pilot trial evaluated the administration of naked plasmid VEGF-C to patients with chronic myocardial ischemia who were not candidates for conventional revascularization.\textsuperscript{35} The plasmid was delivered intramyocardially via a percutaneous catheter, and the injection sites were selected with EMM. Twelve weeks after treatment, improvement in CCS angina class was significantly greater in patients administered the VEGF-C plasmid than in placebo-treated patients.

A subsequent phase Ib trial of VEGF-C plasmid gene therapy was undertaken with a planned enrollment of 404 subjects; patients would be randomized to one of four treatment groups: control (administration of saline diluent), 20 \(\mu\)g of VEGF-C, 200 \(\mu\)g of VEGF-C, or 800 \(\mu\)g of VEGF-C. In the initial phase I/IIa VEGF-C study, treatment was administered with the NOGA injection system, whereas this phase Ib study used the Stiletto catheter. The phase Ib study was halted early because of catheter-related complications, and the data have not been published.

**Fibroblast Growth Factor**

There are presently 22 known fibroblast growth factor (FGF) ligands that are involved in angiogenesis,\textsuperscript{36} embryonic development,\textsuperscript{37} and other processes. Although named for their ability to induce fibroblast proliferation,\textsuperscript{38} FGFs are mitogens for several other cell types (eg, endothelial cells, smooth muscle cells), and have been shown to influence cardiomyocyte survival and hypertrophy.\textsuperscript{39} FGF1 (also called acidic FGF) and FGF2 (basic FGF) were the first FGFs identified. FGF1 and FGF4 have been studied in human cardiovascular gene therapy trials.

**FGF1**

Comerota\textsuperscript{et al} published the first clinical study of FGF1 gene therapy for treatment of CLI.\textsuperscript{40} Although the study was open-label, patients with advanced PAD experienced improvements in wound healing, pain, and transcutaneous oxygen pressure after intramuscular injection of naked plasmid FGF1 (NV1FGF). Subsequently, Nikol\textsuperscript{et al} reported the results from the TALISMAN 201 (Therapeutic Angiogenesis with Intramuscular NV1FGF Improves Amputation-Free Survival in Patients with Critical Limb Ischemia) trial, a phase II clinical trial in patients with CLI.\textsuperscript{41} Patients were randomized to receive intramuscular injections of NV1FGF (n=59) or placebo (n=66). Twenty-five weeks after treatment, the
proportion of patients randomized to NV1FGF (19.6%) and placebo (14.3%) who met the primary efficacy end point (ulcer healing) did not differ significantly; however, the amputation rate was significantly lower in the NV1FGF-treatment group (37.3%) than among placebo-treated patients (55.4%) (hazard ratio = 0.498; \( P = 0.015 \)) and there was a trend toward lower mortality among NV1FGF-treated patients. Amputation and death are the primary end points in a subsequent, ongoing, phase III study of NV1FGF in patients with CLI.

**FGF4**

FGF4 gene therapy was evaluated in the AGENT (Angiogenic Gene Therapy) trials\(^{42–44} \); therapy was administered via a replication-defective adenovirus containing the human FGF4 gene (Ad5FGF4). The initial AGENT study was a phase I/IIa safety investigation with 79 patients; the results provided evidence that Ad5FGF4 had an acceptable safety profile and was associated with a trend toward an antiischemic effect. The AGENT-2 study enrolled 52 patients with symptomatic CAD who were not candidates for revascularization surgery. The primary end point, change in size of the ischemic defect as assessed by adenosine SPECT, did not differ significantly between groups 12 weeks after treatment; however, in a subsequent analysis that excluded one outlier in the placebo group, the size of the ischemic defect improved significantly more in the Ad5FGF4-treatment group than in placebo-treated patients.

The AGENT-3 and AGENT-4 studies were carried out simultaneously. Both were randomized, double-blind, placebo-controlled trials of Ad5FGF4 therapy for treatment of patients with symptomatic CAD; AGENT-3 enrolled patients who did not require immediate revascularization, whereas the patients in AGENT-4 were not candidates for surgical revascularization. The trials were halted early because an interim analysis indicated that a significant difference in the primary efficacy end point (change from baseline in exercise treadmill test [ETT] time at 12 weeks) was unlikely. A subsequent publication reported a pooled analysis of the results from both trials, which confirmed that there was no significant difference between groups in the primary end point; however, post hoc analyses revealed that ETT time significantly improved with Ad5FGF4 treatment in female patients and in subjects \( \geq 65 \) years old with class 3 or class 4 angina. The authors speculated that the (apparently) divergent treatment responses of men and women could be explained by corresponding differences in the biology of CAD. Evidence from previous reports indicates that, compared to men with symptomatic CAD, symptomatic women have less severe epicardial coronary stenoses but greater microvascular dysfunction\(^{45–48} \); thus, Ad5FGF4 gene therapy may be more effective for angina caused by coronary microvascular dysfunction than by epicardial stenoses. A randomized, controlled trial of Ad5FGF4 gene therapy in women (the AWARE study) was subsequently initiated, but enrollment has been discontinued, apparently attributable to slow recruitment.

**Hypoxia-Inducible Factor-1α**

The transcription factor hypoxia-inducible factor (HIF)-1α regulates both physiological and pathological angiogenesis by modulating the expression of multiple downstream targets and represents a prototypical “master-switch” agent. As such, strategies that target HIF-1α or other components of the oxygen-sensing cellular apparatus for treatment of ischemic disease have been intensively studied.\(^49\) Rajagopalan et al conducted a phase I, dose-identification trial in 38 patients with PAD and CLI to test the safety of a modified, constitutively active form of HIF-1α when delivered intramuscularly as an adenoviral vector.\(^49\) No adverse events were attributed to the study treatment, and evidence of pain relief and ulcer healing was observed, although the small size of the study precluded an efficacy evaluation. Enrollment in the WALK study (ClinicalTrials.gov Identifier: NCT00117650), a larger (\( n = 289 \)), randomized, controlled trial in patients with severe, intermittent claudication, has been completed. The initial results were presented in March 2009 and revealed no significant difference in ETT time (the primary study end point) between the active-treatment and control groups. These findings have yet to be published.

**Hepatocyte Growth Factor**

Hepatocyte growth factor (HGF) is a potent mitogen for a wide variety of cells and has angiogenic, antiapoptotic, and antifibrotic properties.\(^51–54\) Serum levels of HGF, but not VEGF, are elevated in CAD patients with collaterals, and elevated HGF levels are associated with better prognoses in patients with acute coronary syndromes.\(^55\) Morishita and colleagues conducted preclinical investigations and initial human safety studies of naked HGF gene therapy,\(^57,58\) which subsequently led to the phase II, HGF-STAT trial. Patients (\( n = 104 \)) with CLI were randomized to treatment with placebo or 1 of 3 doses of HGF plasmid.\(^59\) Serious adverse events occurred in approximately 60% of patients but were evenly distributed among all treatment groups, including placebo; no safety concerns were attributed to HGF plasmid therapy. For patients in the intent-to-treat population, improvement in transcutaneous oxygen tension (TcPO\(_2\)) was similar in all four treatment groups; however, when patients who exhibited a 15 mm Hg increase in TcPO\(_2\) before treatment initiation were excluded, TcPO\(_2\) was significantly more improved in patients who received the highest dose of HGF plasmid than in patients administered placebo or the lower HGF plasmid doses. These findings underscore the challenges encountered when surrogate end points are used to evaluate patients with severe PAD; TcPO\(_2\), ankle–brachial index (ABI), toe–brachial index, and laser Doppler assessments are useful for disease diagnosis and population studies, but they have not been reliable measures of response to medical treatment in patients with severe PAD. It is not yet known whether the results of the HGF-STAT study will lead to future studies.

A second HGF vector has also recently been tested in early-phase clinical trials. VM202 contains a genomic cDNA hybrid of the human HGF gene, HGF-X7, which can express multiple isoforms of HGF through alternative splicing. A pilot safety and dose escalating study (ClinicalTrials.gov Identifier: NCT00696124) has been completed in twelve subjects with CLI, and results presented in March 2009...
provided initial evidence of safety and bioactivity. A phase II study is planned.

**Developmentally Regulated Endothelial Locus**

Developmentally regulated endothelial locus (Del-1) is an extracellular matrix protein expressed during both embryonic development and ischemia; it induces angiogenesis indirectly by interacting with integrins. Del-1 plasmid gene therapy was evaluated for treatment of PAD in the phase-IIa DELTA (Del-1 for Therapeutic Angiogenesis) trial: 105 patients with PAD and peak ETTS of 1 to 10 minutes were randomized to receive intramuscular injections of Del-1 plasmid with poloxamer 188 (to enhance transfection) or poloxamer 188 alone. Ninety days after treatment, no significant difference between groups was observed in the primary efficacy end point, peak ETT, or in several secondary efficacy parameters; patients in both groups improved, which underscores the substantial placebo effect often present in studies of novel therapeutics in patients with advanced ischemic conditions. No significant safety issues were associated with Del-1 plasmid therapy; however, further development of this therapeutic appears to have been halted.

**Lessons Learned**

Fifteen years have elapsed since the earliest reports of angiogenic gene therapy in humans. Despite ample preclinical evidence demonstrating the bioactivity of transplanted genes and several early clinical trials indicating that gene therapy is safe, feasible, and potentially efficacious, randomized controlled clinical trials have not consistently produced conclusive evidence of benefit. Thus, to continue developing this promising treatment approach, we must critically evaluate trial results and protocols to identify factors that may have impaired the effectiveness of therapy or confounded data interpretation.

**Limitations of Preclinical Models**

The differences between animal models and the patients enrolled in clinical trials for cardiovascular therapeutics cannot be understated. The animals used in preclinical studies are typically young and healthy, whereas patients are typically older with multiple comorbidities. Clinical and epidemiological studies indicate that age is a powerful predictor of advanced disease and adverse outcomes in humans, similarly, aged animals are less likely to recover from vascular and ischemic injury. This impairment appears to evolve, at least in part, from deficiencies in the recruitment of angiogenic cells and growth-factor expression. Comorbid conditions can also impede the response to ischemia, and adenovirus transfection is many-fold less efficient in humans than in mice. Thus, the effectiveness of gene therapy may be impaired not only by species-, age-, and health-related differences between animal models and clinical populations, but also by biological differences that retard gene expression. Furthermore, the inability to precisely quantify gene expression impairs trial design (because precise “dosing” is not possible), and the inability to document transgene expression raises the possibility that vector failure may lead to incorrect conclusions about the active agent.

For these reasons, preclinical experiments conducted in relatively young and healthy animals are useful as “proof-of-principle” investigations and for demonstrating bioactivity; however, successful therapeutic development in the field of therapeutic angiogenesis would be aided by several innovations. One possibility is the development of more predictive preclinical models (eg, aged, atherosclerotic, or diabetic animals). Although these models may not necessarily mimic human disease, they could provide a more stringent test of therapeutic potential; a negative aspect would be the associated cost. Another innovation that could help propel the field forward would be the development of accurate surrogate endpoints/biomarkers for angiogenesis. For example, a method to quantify absolute blood flow, represented as a continuous variable that could be measured repeatedly at low risk, could enable proof of concept studies in humans with relatively small numbers of patients.

**Dose and Duration of Therapy**

The optimal dose, duration, and timing of angiogenic gene therapy have yet to be identified. Preclinical data in nonischemic animals suggest that angiogenesis may need to be induced for weeks or months before the newly formed capillaries mature and no longer require growth-factor stimulation; however, it is not known whether prolonged angiogenic stimulation is necessary in the setting of ischemia. Plasmid DNA is expressed for only a few days after administration, and adenoviral gene expression typically endures for just a few weeks; thus, clinical studies that attempt to treat end-stage ischemic disease with one (or a few) dose(s) of gene therapy may be limited by inadequate duration of exposure to the angiogenic agent. Accordingly, as safety data continue to accumulate regarding these approaches, a more robust exploration of dosing strategies may need to be considered.

**Delivery Route and Vector Selection**

Previous studies suggest that intracoronary and (especially) peripheral administration of gene therapy deliver inadequate amounts of the vector to the target site. Peripheral delivery can also lead to off-target gene expression, and rapid coronary blood flow can cause vector “washout” after intracoronary infusion. Both techniques are limited by suboptimal vector permeability in the endothelium, which may be partially surmounted by increasing the perfusion pressure during intracoronary delivery. As an alternative, intramuscular injection offers the possibility of more efficient delivery into focal areas of ischemic muscle. In peripheral vascular disease, intramuscular gene administration is a simple procedure. For cardiac disease, percutaneous endomyocardial injection appears to be effective, and newer technologies, such as ultrasound-mediated administration of plasmid DNA coupled to lipid microbubbles, retrograde coronary sinus infusion, or transcoronary delivery are being evaluated in preclinical studies.

Vector selection may represent another determinant of patient response to gene therapy. The ideal vector would combine low immunogenicity and a satisfactory safety profile with high transfection efficiency and transgene expression for
specific periods of time. The vectors most commonly used in clinical trials have been plasmid DNA and adenoviruses. To date, plasmid DNA has an apparently unblemished safety profile, likely contributed to by the fact that the transgene does not integrate into the host genome, and from the lack of detectable plasmid levels within weeks of administration. On the other hand, the bioactivity of naked DNA is compromised by the low level and limited duration of transgene expression. This may be due in part to the fact that native bacterial DNA containing CpG dinucleotides can elicit an immune response via activation of Toll-like receptors. Methylation of CpG motifs has been associated with more durable transgene expression. In contrast to plasmid DNA vectors, adenoviral vectors have higher transfection efficiency in preclinical models and a longer period of transgene expression, but are more likely to induce an immune response, and these vectors may be ineffective in patients who developed antibodies to adenoviruses during a previous upper respiratory infection. Adeno-associated viruses (AAVs) vary by serotype, but certain serotypes appear to limit the immune response, sustain transgene expression, and display some degree of cardiac selectivity. However, early experiences with AAVs found lower levels and shorter durations of gene expression than originally anticipated. Although AAVs are largely episomal, rare integration into the host genome does occur. Integration into the host genome raises important safety concerns, specifically the possibility of insertional mutagenesis with subsequent malignant transformation. Likewise, most forms of lentiviral-retroviral vectors integrate into the host genome and also raise the concern of malignant transformation. Some investigators have developed non-integrating versions of these vectors. For a more comprehensive discussion of vector selection, the reader is encouraged to consult excellent reviews by Jazwa et al and Lyon et al.

**End Point Selection**

The studies performed to date have lacked the number of patients needed to quantify the potential benefit of gene therapy with unambiguous parameters such as mortality or limb salvage. Instead, researchers have sought to identify other parameters and surrogate end points that can provide objective evidence of bioactivity and clinical improvement. Functional end points such as the duration of exercise before angina onset may be subjective, highly variable in individual patients, and vulnerable to the placebo effect. For example, improvement in exercise duration among patients who received percutaneous transluminal coronary angioplasty in a randomized, open-label trial was approximately 96 seconds, whereas improvement among patients with severe angina who were treated with placebo in certain trials of angiogenesis and laser myocardial revascularization averaged 93 seconds at the same time point (6 months).

Many placebo-controlled studies provide evidence of symptomatic relief but inconclusive results with standard metrics such as SPECT and ABI. This disparity suggests the possibility that the common surrogate end points used to identify and monitor disease in major conduit arteries may be inappropriate for evaluating angiogenic gene therapy targetting the microcirculation in ischemic muscle. In addition, gene therapy may augment microcirculatory function in a segmental manner that does not correspond to the distribution of individual vessels, further challenging available imaging modalities. SPECT imaging analyses measure the relative blood flow in different regions of the myocardium; however, absolute blood flow measurements, which can be obtained via positron emission tomography or cardiac magnetic resonance perfusion imaging (CMR-PI) may be more appropriate for evaluating gene therapy. Additional preclinical and clinical work is necessary to validate the use of positron emission tomography and CMR-PI in studies of therapeutic angiogenesis and to identify other appropriate methods for evaluating bioactivity.

**Patient Populations**

Nearly all clinical trials of gene therapy for cardiovascular disease have enrolled “end-stage” or “no-option” patients, because the greater risk-to-benefit ratio associated with new treatments is considered more acceptable for patients who have exhausted all other therapeutic options. However, patients with advanced cardiovascular disease often have endured decades of systemic deterioration, so a single dose or short course of therapy may not lead to measurable improvement, even if the treatment is beneficial. Furthermore, end-stage disease is generally progressive, so local improvements in the treated region may be obscured by continued deterioration in other areas and by the development or progression of other diseases. Efficacy assessments are also complicated by the desire of the patient to improve and by the more intensive care and follow up involved in clinical trials, which likely contribute to the considerable improvement observed in some placebo-treated patients. The prominent placebo effect noted in multiple angiogenic gene therapy trials underscores the absolute necessity of randomized, placebo-controlled trials for efficacy assessments.

Angiogenic therapy may also be more effective for preserving function at an early disease stage than for restoring function in more compromised patients. Most trials of new cancer therapies enroll patients with “end-stage” disease; however, open-label investigations are sometimes performed at an earlier disease stage for assessment of bioactivity and safety. As evidence attesting to the safety of cardiovascular gene therapy continues to accumulate, researchers may now consider performing trials in healthier patients. Gene therapy could also be tested as an adjunctive treatment to conventional therapy; for example, patients undergoing surgical or percutaneous revascularization could be randomized to receive concomitant active or placebo gene therapy. Both approaches would enable enrollment of less-compromised patients who may be more amenable to demonstrable therapeutic benefit.

**Safety**

More than 1000 patients have been enrolled in placebo-controlled trials of angiogenic gene therapy that span more than a decade, and thus far no adverse safety signals have been detected (Table 2). Reports of cancer, retinopathy, or other diseases that may be driven by vascular growth have
Table 2. Safety Data From Controlled Trials of Angiogenic Gene Therapy

<table>
<thead>
<tr>
<th>Trial Acronym, Author, Reference</th>
<th>Treatment(s)</th>
<th>Patients (n), Active/Placebo</th>
<th>Death (n)</th>
<th>SAE (n)</th>
<th>Side Effects</th>
<th>Retinopathy</th>
<th>Cancer</th>
</tr>
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<tr>
<td><strong>Phase I/II</strong></td>
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<td>VEGF</td>
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</tr>
<tr>
<td>Losordo et al&lt;sup&gt;35&lt;/sup&gt;</td>
<td>Plasmid VEGF&lt;sub&gt;2&lt;/sub&gt;</td>
<td>12/7</td>
<td>None</td>
<td>2/3</td>
<td>None</td>
<td>NA</td>
<td>NA</td>
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<tr>
<td>Mäkinen et al&lt;sup&gt;25&lt;/sup&gt;</td>
<td>Adenoviral and plasmid liposome VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>18/17/19/1/1/1 unrelated</td>
<td>2/0/0</td>
<td></td>
<td>↑ Anti-Adv Ab in VEGF-ad group; ↑ CRP, ↓ platelets in both active treatment groups at 2 days</td>
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<tr>
<td><strong>RAVE</strong></td>
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<tr>
<td>Rajagopalan et al&lt;sup&gt;26&lt;/sup&gt;</td>
<td>Adenoviral VEGF&lt;sub&gt;121&lt;/sub&gt;</td>
<td>40/32/33 high dose/low dose/placebo</td>
<td>0/0/1</td>
<td>NA (adverse events even except edema)</td>
<td>↑ Edema in treated limb in high dose group</td>
<td>0/2/1 unrelated</td>
<td>2/0/1 unrelated</td>
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<tr>
<td><strong>KAT</strong></td>
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<td></td>
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<tr>
<td>Hedman et al&lt;sup&gt;29&lt;/sup&gt;</td>
<td>Adenoviral and plasmid liposome VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>37/28/38 VEGF-adv/VEGF&lt;sub&gt;165&lt;/sub&gt;-P-L/placebo</td>
<td>1/0/0 unrelated</td>
<td>3/2/6</td>
<td>↑ Fever, CRP, and anti-Adv Ab with VEGF-adv treatment</td>
<td>NA</td>
<td>0/2/0</td>
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<tr>
<td><strong>EUROINJECT ONE</strong></td>
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<tr>
<td>Kastrup et al&lt;sup&gt;30&lt;/sup&gt;</td>
<td>Plasmid VEGF&lt;sub&gt;165&lt;/sub&gt;</td>
<td>40/40</td>
<td>0/1</td>
<td>10/12 unrelated</td>
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<td>None</td>
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<td></td>
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<td>Stewart et al&lt;sup&gt;34&lt;/sup&gt;</td>
<td>Adenoviral VEGF&lt;sub&gt;121&lt;/sub&gt;</td>
<td>32/35</td>
<td>2/1</td>
<td>11/11 Early procedure-related adverse events</td>
<td>1/0</td>
<td>1/0</td>
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<tr>
<td><strong>Ripa et al&lt;sup&gt;32&lt;/sup&gt;</strong></td>
<td>Plasmid VEGF&lt;sub&gt;165&lt;/sub&gt; and G-CSF</td>
<td>16/16/16 VEGF&lt;sub&gt;165&lt;/sub&gt;+G-CSF/VEGF&lt;sub&gt;165&lt;/sub&gt;/placebo</td>
<td>0/0/1</td>
<td>3/3/5</td>
<td>None</td>
<td>NA</td>
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<td>27/27</td>
<td>2/2 unrelated</td>
<td>19/14 unrelated</td>
<td>None</td>
<td>None</td>
<td>None</td>
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<td><strong>FGF</strong></td>
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<td><strong>AGENT</strong></td>
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</tr>
<tr>
<td>Grines et al&lt;sup&gt;42&lt;/sup&gt;</td>
<td>Ad5-FGF4</td>
<td>60/19</td>
<td>1/0</td>
<td>15/4</td>
<td>↑ Fever with high dose ad5-FGF4 treatment; One patient with ↑ LFTs</td>
<td>NA</td>
<td>2/0/0 unrelated</td>
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<td><strong>AGENT-2</strong></td>
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<td>Grines et al&lt;sup&gt;44&lt;/sup&gt;</td>
<td>Ad5-FGF4</td>
<td>35/17</td>
<td>0/0</td>
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<td>None</td>
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<td>6/13</td>
<td>40/49</td>
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<td>1/0</td>
<td>3/3</td>
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<td><strong>HIF</strong></td>
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<tr>
<td>Rajagopalan et al&lt;sup&gt;39&lt;/sup&gt;</td>
<td>Ad2/HIF-1&lt;sub&gt;cr&lt;/sub&gt;/VP16</td>
<td>34/7</td>
<td>3/2 unrelated</td>
<td>18/4 unrelated</td>
<td>Flu-like symptoms</td>
<td>None</td>
<td>None</td>
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<tr>
<td><strong>Del-1</strong></td>
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<tr>
<td>Grossman et al&lt;sup&gt;51&lt;/sup&gt;</td>
<td>Del-1 plasmid</td>
<td>52/53</td>
<td>1/2 unrelated</td>
<td>11/9 unrelated</td>
<td>None</td>
<td>None</td>
<td>1/1</td>
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<td><strong>HGF</strong></td>
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<td><strong>HGF-STAT</strong></td>
<td>HGF plasmid</td>
<td>27/25/26/26 high dose/middle dose/low dose/placebo</td>
<td>2/1/2/1 unrelated</td>
<td>19/12/16/15 unrelated</td>
<td>None</td>
<td>0/0/0/1</td>
<td>0/1/2/2</td>
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</tbody>
</table>

(Continued)
been equally distributed in active-treatment and placebo groups. A definitive analysis will require more prolonged follow up and additional patient-years of experience. In the meantime, researchers must remain vigilant for signs of pathological angiogenesis.

Future Directions

As the populations of developed nations age, the number of patients with chronic angina, ischemic cardiomyopathy, claudication, and CLI will increase. Thus, angiogenic gene therapy and other unconventional approaches are needed to lessen the burden of ischemic disease and to enhance quality of life in this ever-expanding patient group. The challenges encountered during trial design are not unique to this field, and sporadic progression from preclinical, proof-of-concept studies to clinical use is the rule rather than the exception during therapeutic development.99,100 The first antiangiogenic tumor drug was approved approximately 30 years after Folkman proposed targeting angiogenesis for treatment of cancer.1 The concept of therapeutic angiogenesis has been pursued for approximately half as long, and successful navigation through phase II and III clinical trials will require an iterative exchange between clinical and preclinical investigators. In addition, the definition of successful angiogenic gene therapy may also need to be reconsidered. Traditionally, therapies for treatment of cardiovascular disorders must demonstrate improvements in morbidity or mortality; however, patients may consider diminished quality, rather than quantity, of life to be the primary burden of advanced cardiovascular disease. Avoiding hospitalization and other manifestations of progressive disease could also be appropriate goals for angiogenic gene therapy.

The genesis, growth, and maintenance of the neovascularization occurs through complex interactions and crosstalk between mechanisms involved in vasculogenesis, angiogenesis, and arteriogenesis.101,102 As our understanding of these mechanisms becomes more refined, it seems likely that combinations of angiogenic factors, or single factors (eg, HIF-1α, sonic hedgehog) that activate numerous angiogenic pathways, will be targeted for research. Cell therapy, combinations of cell therapy and angiogenic factors (eg, via administration of genetically modified stem cells), and the use of biomaterials to enhance the microenvironment are other promising strategies for ischemic tissue repair.

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D.W.L. is currently a consultant to AccelRX and Viromed.

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Rajesh Gupta, Jörn Tongers and Douglas W. Losordo

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