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STATE-OF-THE-ART REVIEW

Therapeutic Angiogenesis for Peripheral Artery Disease

Lessons Learned in Translational Science

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SUMMARY

Peripheral arterial disease (PAD) is a major health care problem. There have been limited advances in medical therapies, and a huge burden of symptomatic patients with intermittent claudication and critical limb ischemia who have limited treatment options. Angiogenesis is the growth and proliferation of blood vessels from existing vasculature. For approximately 2 decades, "therapeutic angiogenesis" has been studied as an investigational approach to treat patients with symptomatic PAD. Despite literally hundreds of positive preclinical studies, results from human clinical studies thus far have been disappointing. Here we present an overview of where the field of therapeutic angiogenesis stands today and examine lessons learned from previously conducted clinical trials. The objective is not to second-guess past efforts but to place the lessons in perspective to allow for trial success in the future to improve agent development, trial design, and ultimately, clinical outcomes for new therapeutics for PAD. (J Am Coll Cardiol Basic Trans Science 2017;2:503–12)

In the spring of 2016, AnGes (a biotechnology company based in Japan) announced the termination of the multinational phase III AGILITY (Efficacy and Safety of AMG0001 in Subjects With Critical Limb Ischemia) trial (NCT02144610) of the hepatocyte growth factor (HGF) plasmid for critical limb ischemia (CLI) (1). This announcement may well end gene therapy trials for therapeutic angiogenesis for peripheral arterial disease (PAD). In <20 years, this field of investigation moved from being one of the most promising, with more than a dozen different agents having been developed and tested, to a (complete) failure? What are the lessons that can be learned from the translational science in PAD, and how could it relate to other fields of clinical investigation in the future?

Angiogenesis is defined as the growth and proliferation of blood vessels from pre-existing vascular structures. Therapeutic angiogenesis emerged as a promising strategy to treat symptomatic PAD, starting with work pioneered by the late Dr. Jeffery M. Isner and colleagues, who used intra-arterial plasmid-encoded vascular endothelial growth factor (VEGF) to treat a 70-year-old female with midfoot 3-vessel runoff occlusion and digit gangrene (2). This open-label study demonstrated an increase in collateral vessels by angiography, although the patient required below the knee amputation 5 months later. The group performed additional open-label studies, and what followed were trials (open-label and placebo-controlled) performed in thousands of patients (**Table 1** contains a partial list of the studies). Overall,

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ABBREVIATIONS AND ACRONYMS

ABI = ankle-brachial index

BM-MNC = bone marrow mononuclear cells

CLI = critical limb ischemia

FGF = fibroblast growth factor

HGF = hepatocyte growth factor

IM = intramuscular

PAD = peripheral arterial disease

PB-MNC = peripheral blood mononuclear cells

TAO = thromboangitis obliterans

VEGF = vascular endothelial growth factor

the results of these studies are disappointing, and they did not lead to advances in patient-directed therapies.

The first question that could be asked, "Was PAD the right area to pursue for therapeutic angiogenesis?" The answer is an easy yes. PAD, in which systemic atherosclerosis limits blood flow to the leg(s), affects approximately 8.5 million people in the United States and is on the rise due to the aging of our population: worldwide incidence of PAD increased by 23.5% between 2000 and 2010 (3,4). In patients over 50 years of age, 40% to 50% will present with atypical leg symptoms, 10% to 35% with classic intermittent claudication, and 1% to 2% with threatened limb (5,6). Medical therapy with statins, angiotensin-converting enzyme inhibitors, angiotensin receptor blockers, and antiplatelet agents have been shown to reduce morbidity and mortality related to cardiovascular events, but the effects of these agents on leg symptoms or disease progression are largely absent, with only rare positive reports (7). Today, only supervised walking programs (with limited availability) and cilostazol (approved in 1999) have shown symptomatic benefit with improved peak walking time (PWT). The main treatment for lifestyle-limiting claudication and CLI has been revascularization, either surgical or endovascular. However, even with advances in surgical and endovascular technology, 20% to 40% of patients with CLI are not anatomically amenable to revascularization or have failed revascularization (8–10). The growing clinical burden coupled with the unmet clinical need supported investigation into this area. **Tables 2 and 3** provide the overview for designing early- and late-stage clinical trials, respectively.

Beyond representing an unmet clinical need in PAD, the leg was easily assessable for "agent" administration (either by an intravascular or a percutaneous approach), and this is certainly true in contrast to the heart. Easy access allowed for the exploration of different routes of delivery, multiple courses of treatment, and most importantly, allowed for randomized controlled trials. Despite these advantages, in reality, different delivery strategies were not well explored in early clinical trials. This approach may also have led to a false sense of security, because although delivery to the human leg is far easier than the heart, the human leg is vastly different in scale from the mouse leg, which was the site of early agent development. Also, although accessibility allowed easy imaging of the leg, imaging methods, such as perfusion imaging of the leg lag behind the heart. Because the goal of therapeutic angiogenesis was to improve blood flow to the ischemic muscles and soft tissue of the distal leg, the lack of direct imaging tools, in retrospect, was a major limitation in early agent development.

Human studies of therapeutic angiogenesis agents in PAD were conducted with protein, modified and unmodified plasmid deoxyribonucleic acid (DNA), and replication-deficient adenoviral vectors, and for some factors, the same agent was delivered in multiple ways. Protein therapy, which involves introduction of recombinant protein to the target site to promote angiogenesis, fell out of favor quite early at least in part due to the disappointing results in trials in coronary artery disease, and the short protein half-life was thought to limit target exposure to angiogenic stimulus (11). The disadvantages were obvious, but the advantages of knowing pharmacokinetics and pharmacodynamics of recombinant proteins were clearly overlooked. Gene therapy sought to deliver an agent by plasmid nucleic acid or a virus vector/plasmid, and had the potential advantage of sustaining production of the angiogenic agent compared with protein therapy. Was this "potential benefit" really explored in detail, or were many of the limitations of gene therapy superficially addressed or even ignored? First, for many of the angiogenic agents it was difficult, or even not possible, to measure gene expression in the target tissue. In a trial that used a replication-deficient adenovirus expressing hypoxia-inducible factor 1-α, there was no evidence for enhanced gene expression of targets downstream of the transcription factor. The most studied vectors for gene delivery have been adenovirus gene delivery and plasmid gene delivery systems, both of which had the potential for vector-limiting toxicity (12). Significant concerns about the safety of adenovirus gene delivery were raised after the death of a patient with ornithine transcarbamylase deficiency who received 4×10^{13} particles of the virus intravascularly, which triggered significant immune response, systemic inflammatory response syndrome, disseminated intravascular coagulation, and eventual death (13,14). Additionally, in phase I and II cancer trials, the maximal tolerated dose was 2.5×10^{13} particles secondary to hypotension and cardiac output suppression (13). Evidence supports the theory of immune response activation by plasmid DNA. It has been noted that there are greater amounts of unmethylated CpG motifs in plasmids than in eukaryotic cell DNA, which is thought to interact with Toll-like receptors and activate an immune response (15). Also, although a comprehensive review of preclinical studies is beyond the scope of this review, it is

TABLE 1 A Summary of Human PAD Therapeutic Angiogenesis Clinical Trials

First Author/Trial	Phase Year	Disease	Treatment	Subject (n) Treatment/Control	Findings
Baumgarter et al.	Phase I 1998	CLI	Intramuscular phVEGF165	9/0	Improved perfusion by angiography, MRA and ABI Trend toward improved ulcer healing
Lazarous et al.	Phase I 2000	IC	Intra-arterial FGF-2	13/6	Trial tested safety, not efficacy Calf blood flow increased at 1 and 6 months
Comerota et al.	Phase I 2002	CLI	Intramuscular NV1FGF	51/0	Decreased pain Improved ABI, ulcer healing, TcPO2
Rajagopalan et al.	Phase I 2002	IC	Intramuscular AdVEGF121	15/3	Trend toward improved ABI and PWT
TRAFFIC	Phase II 2002	IC	Intra-arterial recombinant FGF	116/58	Improved PWT Evidence of early ABI improvement
Makinen et al.	Phase II 2002	IC + CLI	Intra-arterial Ad and P/LVEGF165	35/19	Improved ABI and Rutherford class, but NS vs control group
TACT	Phase II 2002	CLI	Intramuscular BM-MNC	25 unilateral 22 bilateral	Improved ABI, TcO2 Improved rest and pain free walking Evidence of increased collateral vessels
Shyu et al.	Phase I 2003	CLI	Intramuscular phVEGF165	21/0	Improved perfusion by MRA and ABI Improved ulcer healing Rest pain relieved
Mohler et al.	Phase I 2003	CLI	Intramuscular AdVEGF121	13/2	Safety analysis only, not efficacy
RAVE	Phase II 2003	IC	Intramuscular AdVEGF121	71/30	No change in exercise capacity or quality of life
Higashi et al.	Phase I 2004	CLI	Intramuscular BM-MNC	7/0	Improved ABI, pain free walking, TcPO2 Increased vasodilation dependent leg blood flow by plethymography
START	Phase II 2005	IC	Subcutaneous GM-CSF	19/17	No difference in pain free walking, ABI
Kusumanto et al.	Phase II 2006	CLI + DM	Intramuscular phVEGF165	27/27	Improved ulcer healing No difference in amputations, ABI, rest pain or quality of life
Rajagopalan et al.	Phase I 2007	CLI	Intramuscular Ad2/HIF1 /VP16	21/7	Safety only, no hypothesis testing between groups
Bartsch et al.	Phase II 2007	CLI	Intra-arterial + Intramuscular BM-MNC	13/12	Improved pain free walking, ABI Increased oxygen saturation and both rest and peak blood flow
OPTIPEC	Phase I 2008	CLI	Intramuscular BM-MNC	3/0	Evidence of endothelial cell proliferation in distal amputated limb
Van Tergeren et al.	Phase II 2008	CLI	Intra-arterial + Intramuscular vs Intramuscular alone BM-MNC	12/15	Both groups improved pain free walking and ABI
HGF STAT	Phase II 2008	CLI	Intramuscular HGF plasmid	56/23	TcPO2 increased in high dose No difference in TBI, ABI, wound healing
TALISMAN 201	Phase II 2008	CLI	Intramuscular FGF-1 plasmid	59/66	Improved rest pain, Rutherford class, quality of life, amputation risk No difference in ulcer healing
Shigematso et al.	Phase II 2010	CLI	Intramuscular Plasmid HGF	27/13	Improved Rutherford class Decreased ulcer size Improved quality of life No difference in rest pain, ABI, limb salvage
Shigematsu et al	Phase III 2010	CLI	Intramuscular Plasmid HGF	27/13	Improved rest pain, ulcer size, and quality of life No difference in ABI or amputation
Morishita et al.	Phase I 2011	CLI	Intramuscular HGF plasmid	15/0	Proven safety Improved ABI, rest pain, ulcer size and PWT TcPO2 not changed
Creager et al.	Phase II 2011	IC	Intramuscular Ad2/HIF-1α/VP16	213/76	No difference in PWT, pain free walking, quality of life or ABI
PROVASA	Phase II 2011	CLI	Intra-arterial BM-MNC	19/21	Improved ulcer size, rest pain No difference in ABI, amputation, death
TAMARIS	Phase III 2011	CLI	Intramuscular NVFGF-1	259/256	No difference in amputation or death
JUVENTAS	Phase II 2015	CLI	Intra-arterial BM-MNC	81/79	No difference in amputation, death, ABI, ulcer size, quality of life, rest pain, TcPO2

ABI = ankle-brachial index; ad = adenovirus; BM-MNC = bone marrow mononuclear cells; FGF = fibroblast growth factor; GM-CSF = granulocyte mononuclear colony-stimulating factor; HGF = hepatocyte growth factor; HIF1α = hypoxia inducible factor 1 alpha; MRA = magnetic resonance angiography; NS = not significant; NV1 = non-viral 1; ph = plasmid human; PWT = peak walking time; TcPO2 = transcutaneous partial pressure of oxygen; VEGF = vascular endothelial growth factor; VP16 = herpes simplex virus VP1 transactivator.

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TABLE 2 Acceptable Endpoints for Labeling/Approval of Agents for Therapeutic Angiogenesis

Intermittent claudication
Accepted • ↑ Peak walk time (FDA) • ↑ Claudication onset time (EMA) Possible/probable • ↑ Peak VO_2 • ↑ 6-min walk time
Critical limb ischemia
Accepted • ↓ Major amputations • ↓ Mortality Probable • ↑ Complete (not partial) ulcer healing • ↓ Major adverse leg events
Asymptomatic PAD
No trials looking at this group have yet been conducted.
Likely: overall survival/cardiovascular mortality
PAD-specific quality of life measures
Evidence of long-term safety

EMA = European Medicines Agency; FDA = Food and Drug Administration; PAD = peripheral arterial disease; VO_2 = oxygen uptake.

interesting to note this example: preclinical PAD studies used doses of 2 to 4 mg, and in the corresponding human studies, 0.4 mg naked plasmid HGF was delivered to assess for allergic reaction followed by 4 injections of 0.5 mg at 2 weeks and 4 injections of 0.5 mg at 4 weeks, for a total of 4 mg (16). Should one really have expected 1:1 dosing moving from the small mouse to human? This is just one of several questions outlined in Table 4 that deserve consideration in gene therapy trials.

TABLE 3 Proof of Concept Endpoints Within Safety Studies to Establish Confidence for Late-Phase PAD Trials

Intermittent claudication

Accepted
• ↑ Peak walk time (FDA)
• ↑ Claudication onset time (EMA)
Explored
• ↑ Peak VO_2
• ↑ 6-min walk time
• Calf muscle perfusion
  • MRA
  • Plethysmography
  • ABI (not recommended)

Critical limb ischemia
Accepted • ↑ TcPO2 • ↑ Toe pressure • Partial or Complete ulcer healing Explored • Improved laser Doppler perfusion • Improved MR perfusion • Muscle biopsy for angiogenesis
Asymptomatic PAD
None tested

ABI = ankle-brachial index; MR = magnetic resonance; MRA = magnetic resonance angiography; TcPO2 = transcutaneous oximetry; other abbreviations as in Table 2.

TABLE 4 Questions to Be Answered for Successful Gene Therapy

Gene(s) delivered and to what extent are the answers below generalizable across all agents
Vector for gene delivery
Quantity and duration of treatment
Single vs. interval multiple doses (time of intervals?)
Method of delivery (IV, IA, IM)
Location of delivery (for IA and IM, proximal to occlusion, at occlusion, distal to occlusion)
Patient selection (IC, CLI, no-option CLI?)
Measurement of successful gene transfer
Quantifying clinically significant response

CLI = critical limb ischemia; IA = intra-arterial; IC = intermittent claudication; IM = intramuscular; IV = intravenous.

For the purposes of this review, clinical trial results will be organized around the growth factor products that have bene studied most extensively, which include VEGF, fibroblast growth factor (FGF), and HGF.

VEGF CLINICAL TRIALS

VEGF remains the most widely studied family of angiogenic growth factors since it was first identified in 1983 by Senger et al. (17). After several promising preclinical trials, as well as the results published by Isner et al. (1) in their patient with CLI, several phase II clinical trials were performed. No trial was more emblematic of the VEGF trial results as that by Rajagopalan et al. (18). This trial studied intramuscular (IM) injections of a replication-deficient adenovirus encoding the 121-amino-acid isoform of vascular endothelial growth factor ($AdVEGF_{121}$) in a randomized controlled trial of patients, including 105 patients with intermittent claudication and unilateral PAD. Patients received low-dose $AdVEGF_{121}$ of 4×10^9, high-dose $AdVEGF_{121}$ of 4×10^{10}, or placebo as 20 IM injections to the index leg in 20 sessions. The highest dose was thought to be close to the maximum tolerable dose for humans, and was determined from phase I safety data (18). At 12 weeks, there was no difference in the primary endpoint of change in peak walking time (PWT), nor was there a significant difference in change in ankle-brachial index (ABI) or quality of life measures. In addition, $AdVEGF_{121}$ administration was associated with increased peripheral edema, which was more frequent at the higher dose (19). Kusumanto et al. (20) studied IM plasmid vascular endothelial growth factor ($phVEGF_{165}$) in 54 patients with diabetes and CLI in a randomized-controlled trial, and although there was no difference in the primary endpoint of amputation rate at 100 days or secondary endpoints of quality of life, rest pain, and ABI, there was a significant

difference in improved ulcer healing ($p = 0.01$) in VEGF versus placebo. $VEGF_{121}$ and $VEGF_{165}$ differ in their relative ability to bind to the extracellular matrix, and yet differences between these isoforms were never systematically explored. Also, it is generally assumed that the lack of VEGF in the muscle of patients in PAD is not the limiting problem, and therefore, the problem in the lack of VEGF activation is likely downstream of the VEGF receptor (21). Given that the change in VEGF following intramuscular gene transfer in humans was likely small, it would have been ideal to quantify the change in VEGF protein in the muscle after IM injections and compare it with what was found in preclinical models. Also, our understanding of the complexity of the biology of the VEGF receptor-ligand system is continuing to evolve. Human studies drove some of the preclinical studies in VEGF, but it is yet to be determined whether these will translate into clinical reality (22).

FGF CLINICAL TRIALS

FGF is a family of heparin-binding angiogenic growth factors that have been shown to regulate vascular development and various cellular pathways, as well as play a significant role in angiogenesis (23). In particular, FGF1 (aka acidic-FGF) and FGF2 (basic FGF) have been studied in promoting angiogenesis in PAD (24,25). After encouraging results of phase I trials, the European randomized-controlled, phase II, late-stage trial, TALISMAN 201 (Therapeutic Angiogenesis Leg Ischemia Study for the Management of Arteriopathy and Non-Healing Ulcer), was conducted in 125 patients who were determined not to be candidates for revascularization. Patients underwent 4 cycles of 8 IM injections of plasmid-based NV1FGF versus placebo over 45 days. This approach used a modified plasmid backbone that had the potential to increase gene expression compared to unmodified plasmid. After 25 weeks, there was no significant difference in the primary endpoint of ulcer healing or the secondary endpoint of ABI. There was, however, a significant reduction in all amputations (hazard ratio: 0.498; $p = 0.015$) and major amputations (hazard ratio: 0.371; $p = 0.015$) at 12 months (26). This prompted the larger, phase III, randomized controlled TAMARIS (Gene therapy for critical limb ischemia) trial using NV1FGF, which sought to examine labeling indications (**Table 3**), but showed no significant difference in the primary endpoint of time to major amputation or death from any cause in 525 patients with CLI (20). There was no signal in almost any of the secondary endpoints in the trial. After these disappointing results, there have been no further human trials with NV1FGF. Should we rely on secondary endpoints, even if they appear compelling, when the primary measures and those consistent with the mechanism of action of the drug were negative?

FGF is the 1 growth factor that was studied by protein delivery. In 1 study, intra-arterial FGF was delivered proximal to the occlusion in patients with intermittent claudication. Over 30 days, subjects received intra-arterial FGF twice, FGF once and placebo once, or placebo twice. The primary endpoint was PWT at 90 days. PWT increased by 0.60 min in placebo-only patients, by 1.77 min in single-dose FGF, and by 1.54 min in 2-dose FGF (difference between groups by analysis of variance: $p = 0.075$). In the secondary intention-to-treat analysis, the difference was $p = 0.034$ (27). Although the regimen was not likely to be clinically practical, this study provided the most convincing evidence that therapeutic angiogenesis could be achieved in humans (28).

HGF CLINICAL TRIALS

HGF, a mitogenic protein that works through tyrosine phosphorylation of the c-Met receptor, has drawn significant attention because it has been shown to have the unique capability of promoting angiogenesis without causing inflammation (29,30). After encouraging results from preclinical and phase I trials, a phase II randomized controlled trial, HGF-STAT (Study to Assess the Safety of Intramuscular Injection of Hepatocyte Growth Factor Plasmid to Improve Limb Perfusion in Patients With Critical Limb Ischemia) (31), was conducted in patients with CLI. Patients received placebo or low-, middle-, or high-dose HGF plasmid IM injections. A total of 86% of all patients in the trial had adverse events, although there were no significant differences in events across the groups that could not be attributed to CLI or comorbid conditions. There was a significant increase in transcutaneous oxygen pressure (TcPO2) in the high-dose group, but no difference in the other secondary endpoints of ABI, toe-brachial index, pain relief, wound healing, or major amputation (31). Shigematsu et al. (32) completed a phase III randomized-controlled trial using naked plasmid-encoding human HGF in 44 patients with CLI. Placebo or plasmid HGF was delivered by IM injection at 0 and 28 days. At 12 weeks, the primary endpoint of improvement of rest pain without ulcers or reduction in ulcer size was significant ($p = 0.014$). There was also an improvement in the secondary endpoint of quality of life, but no difference in ABI or amputation (32). In a randomized controlled study by Powell et al. (33), 27 subjects with ulcers secondary to CLI were

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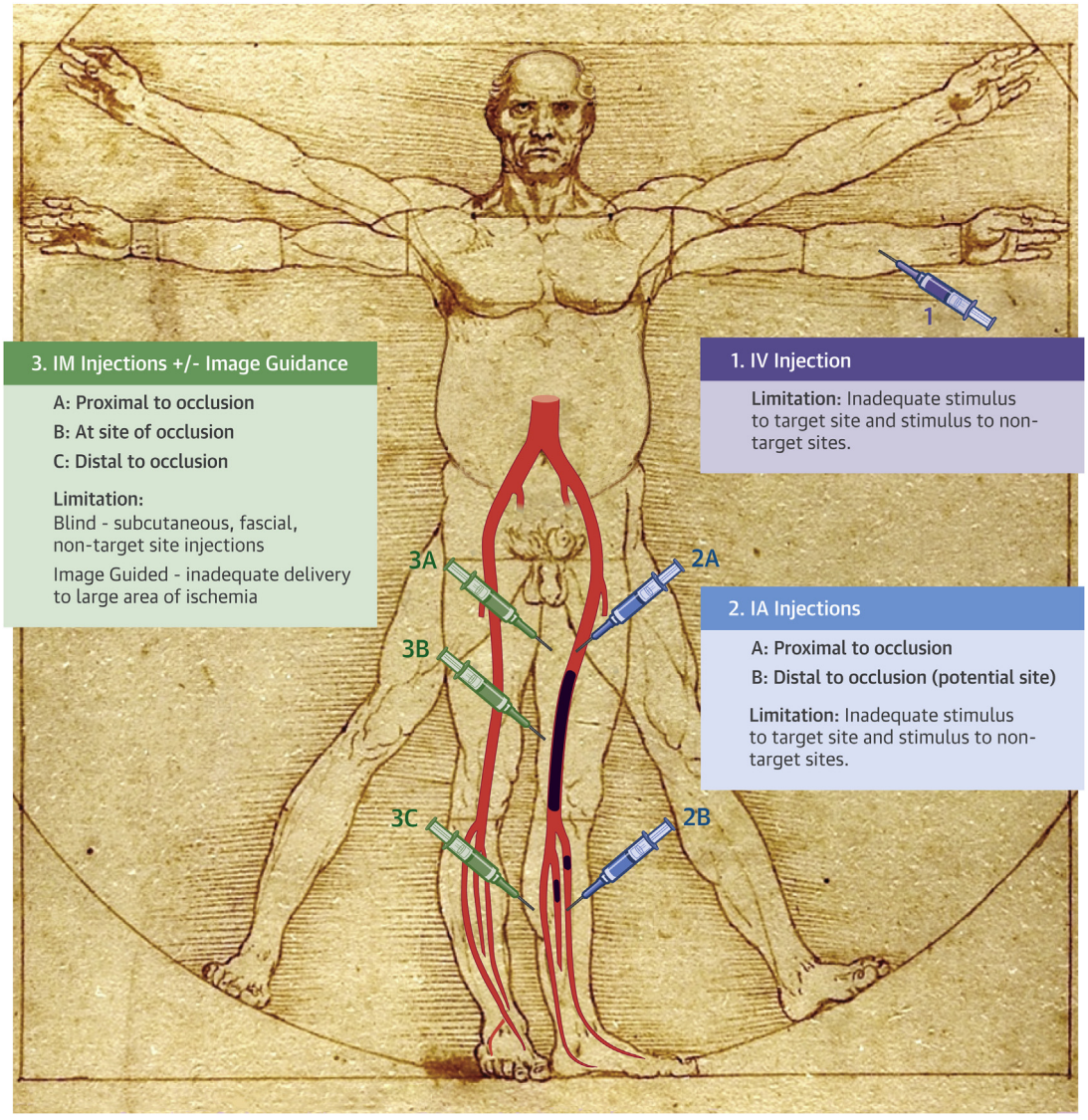
CENTRAL ILLUSTRATION **Potential Patient for Gene Therapy and Routes of Delivery**



Iyer, S.R. et al. J Am Coll Cardiol Basic Trans Science. 2017;2(5):503–12.

IA = intra-arterial; IM = intramuscular; IV = intravenous.

randomized in a 3:1 ratio to IM injections on days 0, 14, and 28 to ultrasound-guided 4 mg plasmid HGF or placebo. There were 21 patients in the plasmid HGF group and 6 in the placebo group. At 12 months, wounds had healed in 31% of patients in the HGF group and 0% in the placebo group, although this result was not statistically significant ($p = 0.28$). There was a significant increase in toe-brachial index and in pain assessment as per visual analogue score. There was no significant in amputation-free survival or mortality (33). These results have sparked interest for a larger phase III trial that has been terminated. In totality, the HGF package of studies was impressive, although there was no bioactivity of effects that could be measured. Problems in the complexity of patient selection often lead to slow enrollment rates, which were the stated reason for the termination of this and other CLI trials (34). Certainly, to be practical, the therapeutic signal must exceed the inherent noise in the clinical manifestations of the disease.

Cell therapy trials have become an attractive study alternative for promoting angiogenesis, because

strategies of specific growth factor angiogenic stimuli have failed to show consistently positive results in treating PAD. Cell therapy has the theoretical advantage of being a more durable and efficient angiogenic treatment by regulating multiple factors: producing an array of cytokines, having a paracrine angiogenic effect in ischemic tissue, and/or differentiating into supporting cells or endothelial cells. The first major reported human clinical cell therapy trial for PAD was the TACT (Therapeutic Angiogenesis using Cell Transplantation) study, in which IM injections of bone marrow mononuclear cells (BM-MNC) were compared with placebo and with peripheral blood mononuclear cells (PB-MNC) in CLI patients who were not candidates for revascularization. Group A consisted of 25 patients with unilateral limb ischemia who were given IM BM-MNC to the ischemic limb and IM saline to the other limb. Group B consisted of 22 patients with bilateral limb ischemia who were randomized to IM BM-MNC or IM PB-MNC. Limbs injected with BM-MNC showed improved ABI, TcPO2, rest pain, and PWT compared with limbs injected with placebo or PB-MNC (35). These encouraging results led to the phase II randomized-controlled PROVASA (Intraarterial administration of bone marrow mononuclear cells in patients with critical limb ischemia) trial, in which 40 patients with CLI received either intra-arterial BM-MNC or placebo, and after 3 months, both groups were treated with BM-MNC. There was no difference in the primary endpoint of ABI at 3 or 6 months. There were significant improvements in the secondary endpoints of ulcer healing and reduced rest pain, but no difference in limb salvage (36). The largest randomized controlled trial published to date using cell therapy is the JUVENTAS (Rejuvenation endothelial progenitor cells via transcutaneous intra-arterial supplementation) trial, in which 160 patients with CLI received 3 separate intra-arterial infusions of BM-MNC versus placebo, at 3-week intervals, into the common femoral artery of the involved limb. There were no significant differences in the primary endpoint of 6-month major amputation rate or the secondary endpoints of quality of life, rest pain, ABI, or TcPO2 (37).

BARRIERS TO SUCCESS OF CLINICAL TRIALS

The results in late-stage clinical trials, especially when looking at clinical outcomes such as limb salvage and amputation, have been underwhelming. There are several factors that are likely responsible for these disappointing results.

PRECLINICAL MODELS. There are a number of differences between preclinical animal models and patients with PAD and particularly CLI. The endogenous response to hind limb ischemia in wild-type mouse models, even with complete inflow occlusion, is robust: often normal or near-normal perfusion is present by 2 to 3 weeks in many but not all inbred mouse strains (38). The endothelium and endothelial function is normal in wild-type mice and is known to be abnormal in patients with PAD (39,40). In addition to this, preclinical animal models tend to be younger, healthier, and with less comorbidities, all of which likely contribute to the less robust angiogenic response in patients with PAD (34,41,42). Despite these limitations, in general, what is bad for humans with PAD (i.e., diabetes) is also bad for mice with experimental PAD (43). What is clear is that preclinical studies should be conducted in a background where efficacy is difficult, not easy, to achieve.

PATIENT SELECTION. Therapeutic angiogenesis has been studied in patients with intermittent claudication and CLI. The trials that included patients with intermittent claudication have particularly disappointing results (12,19,44,45). CLI trials mainly have been restricted to patients who cannot be revascularized, otherwise known as no-option CLI, and a significant amount of neovascularization and increased collateral blood flow is expected to be required to see clinical improvement in these patients (46). The selected CLI patients have shown to have a less robust angiogenic response, as they often are older, have more comorbidities, and have failed previous revascularization. In addition, standardization of the definition of no-option CLI may be needed, as technological advances have made endovascular interventions possible in more and more patients. Also, it has been shown that although patency rates of tibioperoneal angioplasty are low (22% to 92%), limb salvage rates are encouraging (50% to 92%) (47,48). In several cell therapy trials, patients with thromboangitis obliterans (TAO) were included in addition to PAD patients. Patients with TAO had better results in general compared with those with PAD; however, it is difficult to attribute these findings to cell therapy alone and not at least in part to smoking cessation (49). In the TACT trial, amputation-free survival was 60% in PAD patients and 91% in TAO patients (34). In the PROVASA trial, 100% of patients with TAO had improved rest pain or ulcer healing, whereas only 56% of patients with PAD had improvement (36). Finally, clinical event rates of amputation are declining in CLI patients, which will make sample size estimates challenging for future studies that plan to use this hard endpoint.

MEASURING THERAPEUTIC EFFECT. One of the main barriers to gene therapies is the inability to show and

measure successful gene transfer. This limits the ability to evaluate vectors of therapy, method of delivery, and dosing and duration of therapies. In a study by Creager et al. (12), 40 patients were randomized to IM ad2/HIF-1α/VP16 versus placebo. In this trial, there were no differences in plasma VEGF levels (even of $VEGF_{121}$), which is freely soluble or endothelial progenitor cells. Even when there is success of vector delivery via plasmid or adenovirus-mediated gene transfer, transfection efficiency estimates are only in the single digit percent of cells (50). In the OPTITEC (Optimization of Progenitor Endothelial Cells in the Treatment of Critical Leg Ischemia) trial, amputated limbs were evaluated after IM BM-MNC. The locus of endothelial cell proliferation was distal and not at the site of injection, calling into question whether a hypoxic response rather than treatment of BM-MNC was responsible for this finding (51). Therefore, biomarkers to quantitate successful gene transfer as well as tracking of EPCs would be of significant value in allowing assessment of gene transfer approaches.

ROUTE OF DELIVERY. The inability to quantify the success of the various therapeutic angiogenesis therapies has hindered the ability to find the most effective route of delivery. There are limitations that should be considered with each route available (**Central Illustration**). Systemic delivery can lead to nontarget effects and washout prior to reaching target sites. Intra-arterial delivery, although more localized, can also preferentially lead to nontarget effects in areas of greater perfusion. IM injection may result in more targeted delivery, but may be limited to the injection site and not extend into large areas of ischemic muscle. Also, blind injections may result in delivery into the fascia or subcutaneous tissue instead of the skeletal muscle (52). In addition, when specifically considering intra-arterial and IM injections, delivery could be proximal to the site, at the site, or distal to the site of stenosis (in the area of ischemia). The optimal site of delivery is unknown. Finally, one must consider how the results of the questions for one agent apply to a different agent.

FUTURE DIRECTIONS

After the encouraging results from preclinical animal trials and the first human gene therapy study by Isner et al. (1) in 1996, there has been significant excitement in the field of therapeutic angiogenesis. The results in human clinical trials have thus far been underwhelming, although interest in the field continues as PAD and particularly CLI remain a significant issue for many patients and providers. Recently, the results from the phase III multicenter MOBILE (MarrowStim PAD Kit for the Treatment of Critical Limb Ischemia in Subjects with Severe Peripheral Arterial Disease) trial in patients with no-option CLI were presented at Vascular Interventional Advances 2016. A total of 152 patients were randomized in a 3:1 fashion to Marrowstim (Biomet Biologics, Warsaw, Indiana), the patient's own concentrated bone marrow aspirate containing mesenchymal stem cells, or placebo. Patient received 40 blind IM injections to the limb in 1 procedure. There was a trend, but no statistically significant difference, in amputation-free survival. When a pre-specified group of patients, including those with diabetes and with tissue loss, were excluded, there was a significant difference in amputation-free survival in the Marrowstim versus placebo patients (86.2% vs. 66.7%; $p = 0.018$). Results are yet to be published at this time. These results are promising and bring into question whether there is a certain subset of patients with CLI that will derive the most benefit from therapeutic angiogenesis therapies. Also, yet to be investigated is gene or cell therapy as an adjuvant to surgical or interventional procedures to improve blood flow in patients. The trend has been for trials to focus on patients with no-option CLI, likely secondary to ethical issues of the risk-benefit ratio of such therapies. There is possibly a certain stage of PAD, or particularly CLI, where patients would still have a robust response to gene and/or cell therapies; in these patients, in adjunct to improved inflow through surgical or interventional procedures, a clinical benefit could be seen. In addition to this, more efficient and more durable vectors for gene delivery are being investigated. As research in the field continues, adeno-associated viruses have emerged as an exciting new vector for efficient and potent gene transfer. The possibility even exists that some adeno-associated virus serotypes can efficiently target ischemic muscle following systemic delivery (53). Advances in noninvasive vascular imaging may soon, if not already, allow for reliable assessment of muscle perfusion in patients with PAD. This has the potential to allow for more focused early human trials. Ultimately, the combination of new agents, new vectors, and new ways to evaluate early human studies will be needed to overcome the translation from mouse to man.

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