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Therapeutic Angiogenesis in Critical Limb Ischemia

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Abstract

Critical limb ischemia (CLI) is a severe form of peripheral artery disease associated with high morbidity and mortality. The primary therapeutic goals in treating CLI are to reduce the risk of adverse cardiovascular events, relieve ischemic pain, heal ulcers, prevent major amputation, and improve quality of life (QoL) and survival. These goals may be achieved by medical therapy, endovascular intervention, open surgery, or amputation and require a multidisciplinary approach including pain management, wound care, risk factors reduction, and treatment of comorbidities. No-option patients are potential candidates for the novel angiogenic therapies. The application of genetic, molecular, and cellular-based modalities, the so-called therapeutic angiogenesis, in the treatment of arterial obstructive diseases has not shown consistent efficacy. This article summarizes the current status related to the management of patients with CLI and discusses the current findings of the emerging modalities for therapeutic angiogenesis.

Keywords

critical limb ischemia; medical therapy; surgical therapy; endovascular therapy; therapeutic angiogenesis; gene therapy; cell therapy

Introduction

Critical limb ischemia (CLI) is a clinical syndrome in which patients with occlusive arterial disease of the legs experience chronic ischemic rest pain, ulcer, or gangrene.[1] This syndrome is a severe form of peripheral arterial disease (PAD) and is associated with grave prognosis, with 1-year mortality exceeding 25% and about 30% to 50% major limb amputation at 1 year from diagnosis.[1] Patients with CLI have multiple comorbidities, with diabetes and smoking being the most important, adding to the high rates of death and the considerable economic burden.[2] The number of patients with PAD having CLI is estimated at 1% to 3% and the annual number is estimated to be around 160,000 in the United States.[3] The traditional standard of therapy for CLI is bypass surgery, but recent advances in the

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coordinated team approach and interventional endovascular technology changed the rates of limb salvage and potentially the prognosis of patients with CLI, with a paradigm shift in the treatment of CLI.[4-6] In a significant proportion of patients with CLI, the distribution and diffuseness of arterial occlusions reduce the success of surgical and interventional techniques. Furthermore, it has been shown that patients with chronic kidney disease and diabetes who develop CLI have worse clinical outcome.[7,8] It is such a group of patients, often referred to as no-option patients, who are the target of angiogenic techniques.

Diverse methods were reported for angiogenic therapies over the last 2 decades. The initial approach to induce angiogenesis in ischemic organs included injection of plasmid DNA encoding for vascular endothelial growth factor (VEGF) and other growth factors or angiogenic proteins such as basic fibroblast growth factor (FGF).[9] More recently, in a phase II trial, hypoxia-inducible factor 1alpha (HIF-1α), a transcription factor, was used as a plasmid in patients with severe claudication as an angiogenic therapy.[10] With the understanding that a single growth factor may not be sufficient for induction of angiogenesis, cell therapy emerged as the leading experimental approach for the induction of angiogenesis. In the current review, we will discuss the medical, endovascular, and surgical management options in CLI and summarize the reports on angiogenic therapies in CLI while assessing future developments in this field.

Current Therapies for CLI (Medical, Surgical, and Endovascular)

Medical Therapy

The main goals for the treatment of CLI are to control pain, heal ischemic ulcers, improve functional class and QoL, prevent amputations, and prolong survival.

Prompt arterial revascularization is the optimal treatment and the central part in the management of patients with CLI. However, as for all the patients with PAD, there is an important place for evaluation and prevention of cardiovascular disease, in order to improve the prognosis and prevent further complications. A multidisciplinary approach to establish treatment goals in PAD is recommended by the recent guidelines.[1,11,12] Hankey and colleagues reviewed the evidence in the literature concerning medical treatments of PAD.[13] Patient management should include lifestyle modification. Smoking is an important contributor to PAD and its complications, and smokers with PAD should be advised to quit and participate in smoking cessation programs.[14] Exercise training improves symptoms in patients with PAD, increases walking distance, and exercise capacity.[15,16] However, patients with advanced CLI such as Fontaine class IV-V may not be able to participate in a supervised exercise training program.

The incidence of coronary events and stroke is high in patients with PAD. Antiplatelet therapy, with either aspirin or clopidogrel, is advocated in guidelines to reduce the secondary vascular events and death in patients with vascular diseases, including PAD.[17,18] Moreover, adjuvant aspirin therapy has shown benefit in patients with previous lower extremity revascularization and is recommended to improve patency rate after lower extremity bypass graft.[19] In patients with isolated asymptomatic PAD without involvement of other vascular beds, it remains uncertain whether aspirin therapy is beneficial in preventing future events. In a recent meta-analysis of 18 trials that included 5269 patients with PAD, aspirin therapy alone or in combination with dipyridamole resulted in a 12% reduction in cardiovascular events (primary end point), but this reduction did not reach statistical significance.[20] Likewise, the Japanese Primary Prevention of Atherosclerosis with aspirin in Diabetes (JPAD) trial and the Scottish prevention of progression of arterial disease and diabetes (POPADAD) trial showed no benefit in the reduction of primary cardiovascular events with aspirin therapy.[21,22] Finally, the aspirin for asymptomatic atherosclerosis (AAA)

trial randomized 3350 asymptomatic patients with isolated PAD to receive aspirin or placebo and there was no benefit with aspirin in the reduction of fatal or nonfatal coronary and stroke events or revascularization.[23]

Other prevention measures are blood pressure control, especially in diabetic patients, in which regulation of glucose levels with HBA1c <7% should be maintained.[24] Diabetes is an independent risk factor for complications and amputations in patients with CLI.[25]

Statins have a central role in the prevention and treatment of cardiovascular diseases, including PAD that is considered as coronary heart disease risk equivalent. Serum low-density lipo-protein (LDL) cholesterol levels should be reduced to <100 mg/dL in patients with PAD, even without coronary artery disease.[26] Statins may also have positive effect on intermittent claudication and even survival in severe PAD.[27] The Heart Protection Study (HPS) demonstrates the benefits of cholesterol-lowering statin therapy in high-risk patients with PAD, regardless of their presenting cholesterol levels, showing significant reduction in the first major vascular events and that in the peripheral vascular events, with large absolute benefits seen in the patients with PAD because of their high vascular risk.[28] The evidence of the effectiveness of various therapies to prevent cardiovascular events is not established sufficiently in the high-risk PAD population with CLI. For example, in the PREVENT III cohort, among the pharmacological therapies, only statins were associated with improved survival in patients with CLI.[29] Nonetheless, possible mechanisms of statins in improving endothelial cell condition and endothelial progenitor cell (EPC) availability have been postulated and include increasing nitric oxide (NO) synthesis.[30] Furthermore, statins help in downregulation of the vasoconstrictor response, by reducing the synthesis of endothelin 1 (ET-1) and the vascular response to angiotensin 2 (AT-2).[30] Statins have also been shown in animals to induce the expression of growth factors that may result in pro-angiogenic effects.[31] However, ambivalent reports on the effects of statins on angiogenesis were reported in different studies.[32,33] Evidence also shows that statins increase functional activity of EPCs and promote EPCs proliferation, migration, and differentiation, independent of their lipid-lowering effects.[34]

Several drugs in clinical studies and meta-analyses have shown to improve walking distance in patients with PAD and intermittent claudication. The improvement was generally mild to moderate in those trials. Among those drugs were the phos-phodiesterase inhibitors cilostazol and pentoxifylline and naftidrofuryl, a 5-hydroxytryptamine antagonist, which showed to significantly increase pain-free walking distance (PFWD) and QoL, and propionyl-l-carnitine that is currently being evaluated.[1,12]

Patients with PAD unsuitable for revascularization or in whom revascularization attempts have failed were treated in several studies by prostanoids, with conflicting results. The prostacyclin analogs are arterial vasodilators that promote angiogenesis and have antiplatelets and anti-inflammatory properties.[35] A meta-analysis showed significant beneficial effect of PGE1 therapy over placebo, concerning pain relief, ulcer healing, and survival.[36] On the contrary, a recent Cochrane database systematic review did not show conclusive evidence of the long-term effectiveness and safety of prostanoids in patients with CLI.[37]

For patients with PAD who are not candidates for vascular reconstruction, several therapeutic options are available. Spinal cord stimulation was evaluated in inoperable patients with CLI, with some efficacy concerning pain relief and reduced amputation rate.[38,39] Intermittent pneumatic compression of the lower ischemic limb is also suggested for the treatment of CLI, with the evidence of increased arterial flow, improved pain, and walking distance.[40-42] Hyperbaric oxygen therapy was evaluated mainly in diabetic ulcers,

with evidence of reduced risk for major amputations and improved wound healing. However, in chronic wounds associated with other pathologies there is no evidence of efficacy, and economic considerations should be taken into account.[43] Multidisciplinary approach, including wound therapy, local surgical debridement, shoe adaptation, pain control, treatment of infections, and initiation of rehabilitation therapy, is also fundamental to patient care.

Surgical and Endovascular Therapies

Patients with CLI, who do not undergo revascularization, often progress to amputation. Options for revascularization include surgery, endovascular interventions, and combinations of both. The guidelines for revascularization management of patients with CLI are based on the Inter-Society Consensus for the management of PAD (TASC II), and categorizes arterial lesions as type A, B, C, or D based on the anatomical location and morphology of the lesion and the overall success rate of treating the lesion either surgically or endovascularly.[1] Simply stated, type A lesions are simple lesions while type D lesions are complex lesions. Types B and C lesions are intermediate lesions. Endovascular therapy is the treatment of choice for type A lesions and is the preferred treatment for type B lesions. Surgery is the treatment of choice for type D lesions and is the preferred treatment of choice for low-risk patients with type C lesions. In general, surgical therapy may be indicated in younger patients with longer life expectancy, and less comorbidities, and in complex lesions that have limited endovascular treatment options. Alternatively, endovascular therapy is a more suitable treatment option for elderly patients with comorbidities.[44]

In the recent years, a significant increase in the use of endovascular procedures have been reported in patients with CLI and angioplasty has become a first-line therapy for patients with CLI in many vascular centers.[45,46] Although the bypass versus angioplasty in severe ischemia of the leg (BASIL) trial found that endovascular revascularization is associated with lower morbidity, decreased hospitalization length, but less durability compared with surgical options,[47] surgical bypass remains the gold standard for revascularization in patients with CLI who are fit for surgery. Specifically, the BASIL trial was a prospective, randomized trial comparing outcomes of surgery versus angioplasty as the first therapeutic strategy in patients with severe limb ischemia due to infrainguinal disease. Surgery-first strategy was associated with higher morbidity, including periprocedural myocardial infarction and stroke and longer hospital stay than angioplasty-first strategy. Angioplasty-treated patients had more immediate technical failures and higher rates of reinterventions than surgery. The 1-month mortality rate was similar between the groups; however, 2-year survival was better in the surgical-first strategy, in a post hoc analysis.[47] Based on these results, the updated American College of Cardiology/American Heart Association guidelines for the management of PAD recommend endovascular therapy as the initial procedure for select patients who have an estimated life expectancy of ≤2 years, or those who do not have autologous vein available as a conduit, and bypass surgery as the initial procedure in those patients with an estimated life expectancy of ≥2 years and who have autologous vein available as conduit.[11]

However, based upon the category of a particular lesion, a patient with CLI might benefit from a bypass surgery-first or angioplasty-first approach, the so-called hybrid revascularization procedures. This combination approach to achieve complete revascularization was shown to result in less extensive procedures with decreased risks of perioperative complications, especially in older patients with comorbidities.[45,48] Current and future use of drug-eluting stents and bioabsorbable stents may shift the therapeutic pendulum to a more intravascular intervention in the coming years.

Gene-Based Therapy Clinical Trials for CLI

Administration of growth factors to patients with PAD increases the concentration of angiogenic factors in the lower ischemic limb, aiming to improve endothelial cell proliferation, migration, and blood vessel formation in the ischemic limb.[49] Angiogenic growth factors are transmitted by different methods of delivery, by recombinant proteins or by viral vector or by a plasmid with a gene that encodes for the angiogenic protein.[50] Studies performed in the animal models have shown that the administration of angiogenic growth factors can augment perfusion and formation of new blood vessels.[51-53] In the past two decades, numerous small-scale trials using several different growth factors have demonstrated angiogenic potential in humans with CLI. Among them are the (VEGF), (FGF), hepatocyte growth factor (HGF) families, and the transcription factor, hypoxia inducible factor 1 (HIF-1). We will review the development and outcomes of human clinical studies of angiogenic gene therapy for the treatment of CLI. Table 1 summarizes the characteristics and main findings of the placebo-controlled, phase II/III gene therapy clinical trials in CLI.

VEGF Trials

The VEGF family regulates vascular growth, increases vascular permeability, and mediates angiogenesis and tissue repair.[63] Several members of the VEGF family have been identified.[64] The VEGF-A is a strong inducer of vascular permeability, has high angiogenic potency, and thus has been the main VEGF used in clinical trials. The VEGF-A has 4 isoforms from a single gene and are named according to the number of amino acids (121, 165, 189, and 206). Yla-Herttuala and colleagues have reviewed the biology and clinical applications of VEGF.[65]

Isner et al published in 1996 the first report on the administration of plasmid DNA encoding human VEGF165 via balloon angioplasty for the treatment of PAD.[66] Later, the same group administered VEGF165 intramuscularly (IM) to patients with limb ischemia.[67] Since then, several groups evaluated the use of VEGF-A isoforms for the treatment of PAD. Shyu et al confirmed safety and beneficial effects of VEGF165 in a non-controlled study of 21 patients with CLI, showing improved ulcer healing, rest pain, and ankle–brachial index (ABI) measures.[68] Kusumanto et al published in 2006 a phase II, double-blind, placebo-controlled study in 54 diabetic patients with CLI. Patients were randomized to receive 2 intramuscular (IM) injections of VEGF165 or placebo.[54] The primary end point of amputation rate was not significantly different between the groups, while the secondary end points of skin ulcer healing and homodynamic improvement were statistically better in the VEGF165-treated group (14 patients improved with therapy while only 3 improved in the placebo group; $P = .0003$).

The VEGF transfer with adenoviral vector has also been reported. Makinen and colleagues conducted a phase II randomized, placebo-controlled, double-blind study evaluating local intra-arterial (IA) catheter-mediated AdVEGF165 gene therapy after percutaneous transluminal angioplasty.[55] Antiadenoviral antibodies were increased in 61% of the patients. After 3 months, angiography showed increased vascularity in the VEGF-treated groups. Moreover, there was a significant improvement (compared to the baseline) in the Rutherford class and ABI, but this improvement was not different than that observed in the placebo arm. No major gene transfer–related side effects were detected between the study groups.

The Regional Angiogenesis with Vascular Endothelial Growth Factor (RAVE) was a randomized controlled study evaluating IM injection of adenoviral gene transfer (AdVEGF121) for the treatment of PAD.[56] After several small open-label phase I trials demonstrating feasibility and safety of AdVEGF121 gene transfer in patients with PAD,[69,70]

the RAVE trial was a phase II, double-blind, placebo-controlled trial, randomizing 105 patients with unilateral, exercise-limiting intermittent claudication to receive low or high dose of AdVEGF121 or placebo, by 20 IM injections in a single session. The primary end point (change in peak walking time) did not differ between the low dose, high dose, and placebo, nor did the secondary end points at 12 and 26 weeks of follow-up. In addition, AdVEGF121 administration was associated with increased peripheral edema (which may indicate its potential bioactivity). Possible explanations of these negative results might have been the single session of therapy indicating limited duration of expression,[71] the selection of patients with unilateral PAD, and the lower transfection efficiency with adenovirus in the skeletal muscle in comparison to the myocardium. Additional and potentially more relevant explanation is that the use of a single gene cannot initiate and maintain the complex biological process of angiogenesis. It is worth mentioning that there are differences between the VEGF isoforms, as VEGF121 lacks the heparin-binding domain that is responsible for the adherence of VEGF to matrix proteins, and therefore has a short tissue half-life, whereas the VEGF165 has longer tissue retention and thus permits a more complete process of angiogenesis.

Fibroblast Growth Factor Trials

Fibroblast growth factor modulates proliferation and migration of several cell types such as endothelial cells, smooth muscle cells, and fibroblasts.[72] Of the FGF family, the acidic FGF-1 is a potent mitogen that induces angiogenesis in vivo. Comerota et al in a phase I trial presented in 2002 examined, for the first time in humans, the safety and tolerability of multiple doses of nonviral DNA plasmid encoding for FGF-1 (NV1FGF) administered to 51 patients with CLI.[73] The NV1FGF was well tolerated and showed improvement in pain and ulcer healing, although the study had no control group.

In a small study evaluating patients with CLI several days before planned amputation, NV1FGF gene transfer and expression was demonstrated in the skeletal muscles of patients with CLI, indicating that even severely ischemic muscles display the potential for response to NV1FGF-mediated therapeutic angiogenesis.[74] Circulating levels of NV1FGF sequences were shown to decrease within days after injection, and FGF-1 expression was shown to be limited to injection sites that support the concept of multiple-site injection for therapeutic use.

The TALISMAN-201 investigation was a multinational, phase II, double-blind, placebo-controlled study evaluating the efficacy and safety of IM NV1FGF injection in 125 patients with CLI.[57] In contrast to the earlier randomized trials with growth factors, this trial included multiple administrations of the angiogenic agent. Patients were treated in 4 sessions, 2 weeks apart, each session with 8 IM injections to the calf and thigh muscles. Improvements in ulcer healing were similar in the NV1FGF and the placebo groups. However, the use of NV1FGF significantly reduced (by 2-fold) the risk of amputations, and there was a trend for reduced risk of death with the use of NV1FGF (HR 0.460; $P = .105$). The incidence of adverse event was high and similar between the groups.

In light of the TALISMAN trial, a similar phase III trial named TAMARIS was conducted.[58] This large double-blind, randomized, placebo-controlled study included 525 patients with CLI from 30 countries, randomly assigned to either NV1FGF or placebo. Patients were treated in 4 sessions, 2 weeks apart, each session with 8 IM injections. The primary end point of time to major amputation or death was not different between the treatment groups, as well as secondary end points in the subgroups of treated patients. These outcomes did not confirm the results of the phase II TALISMAN study. The authors concluded that the absence of benefit might indicate challenges to define the optimum dose, vector, route, and duration of growth factor administration, and the need for multiple

interventions. Trials of gene therapy in CLI are prone to difficulties and angiogenesis may not occur in the ischemic tissues due to inhibiting factors that are not well described. These factors were not part of the many experiments suggesting efficacy, which were carried out in young and healthy animals.[75]

Although the therapeutic angiogenesis with recombinant FGF-2 for intermittent claudication (TRAFFIC) was not a CLI trial, its importance comes from the fact that it was a phase II, double-blind, placebo-controlled study that randomized 190 patients with moderate-to-severe intermittent claudication and abnormal ABI, to bilateral IA infusion of placebo or 2 doses (repeat infusion at day 30) of recombinant FGF-2.[59] Primary end point was change in the peak walking time at 90 days that was increased by 0.60 minutes with placebo, by 1.77 minutes with single dose, and by 1.54 minutes with double dose. Pairwise comparison showed a significant difference between placebo and single dose ($P = .026$) but placebo and double dose did not differ much ($P = .45$). In both the treatment groups, there was significant improvement in ABI at 90 days of follow-up, which did not persist at 180 days. Significant side effects were not reported.

Hepatocyte Growth Factor Trials

Hepatocyte growth factor is an angiogenic protein that regulates multiple genes involved in the angiogenic process. Hepatocyte growth factor is downregulated in the limbs of patients with CLI. Previous trials demonstrated the ability of HGF to induce collateral vessel formation and improve limb perfusion in animal models of PAD.[76]

In a small open-label study, Morishita et al were the first to describe preliminary results in humans related to the safety of IM injection of naked HGF plasmid in 6 patients with CLI.[77] They also recently demonstrated efficacy in an open-label study of 22 patients with CLI treated with 2 sessions of IM injections of HGF plasmid, showing increase in the ABI and reduction in the ulcer size and rest pain.[78]

Powell et al assessed the safety of IM injections of 2 to 3 doses of HGF plasmid into the limbs of 104 patients with CLI (HGF-STAT trial). In their phase I/II placebo-controlled, multiple-dose regimens trial, presented in 2008, they showed that this therapy is safe and well tolerated.[60] There was no difference in the rate of malignancy, infections, or vascular disorders. Concerns about stimulation of systemic angiogenesis remote from the injection site, including proliferative retinopathy or growth of occult tumors were relived as none of these unwanted effects was observed. Moreover, in contrast to the VEGF gene therapy, the HGF gene transfer did not induce limb edema. Considering the efficacy, there was significant increase in the transcutaneous partial pressure of oxygen ($TcPO_2$) at 6 months, in the high-dose-treated patients, but no difference in the end points of pain relief, wound healing, or amputations. Based on these results, they conducted a randomized placebo-controlled, phase II follow-up study (HGF-0205 trial) to further define the safety and potential efficacy of the proven high-dose HGF gene therapy identified in the previous trial and to evaluate a novel patient-specific gene delivery technique utilizing ultrasound-guided injections into the muscle surrounding occluded vessels.[61] Their results, presented in 2010, showed significant increase in the ABI and improved rest pain measures, and a trend of better wound healing compared to placebo. There was no difference in major amputations, mortality, and adverse events.

A second phase II multicenter, randomized, placebo-control trial evaluating the efficacy and safety of the HGF gene therapy in 44 patients was conducted in Japan. Primary end points of reduction of rest pain and ulcer size were achieved when compared to placebo, as well as improved quality-of-life measures with no major safety problems. The authors concluded that HGF is safe and effective for CLI gene therapy.[62] The same group also reported in a

small open-label trial the efficacy and safety of the HGF gene therapy for CLI in patients with thromboangiitis obliterans.[79] Recently, Henry et al presented a small phase I study, examining the safety of IM injection of a novel nonviral plasmid DNA expressing 2 isoforms of human HGF (VM202), appearing to be safe and well tolerated in patients with CLI, with encouraging clinical results.[80] A phase II, double-blind, randomized placebo-controlled, multicenter study to assess the safety and efficacy of VM202 in patients with CLI is currently recruiting patients (clinicaltrials.gov NCT01064440).

Hypoxia-Inducible Factor 1 Trials

Hypoxia-inducible factor is a transcriptional factor that functions as a regulator of oxygen homeostasis and regulates angiogenesis by modulating the expression of multiple genes, including genes regulating vascular growth such as VEGF.[81,82] In 2007, Rajagopalan and colleagues reported a phase I dose-escalation, multicenter trial in 38 patients with PAD and CLI using single treatment of IM-injected adenoviral construct of the transcriptional activator HIF-1α (Ad2/HIF-1α /VP16 or HIF-1α).[83] There were more flu-like symptoms in the treated arm, but no serious adverse events attributed to the study treatment. Evidence of pain relief and ulcer healing was observed. Recently, Creager et al assessed IM administration of recombinant adenoviral vector encoding active HIF-1α transcription factor in 289 patients with PAD having claudication, showing no significant differences in the claudication onset time, ABI, or quality-of-life (QoL) measurements between the placebo and the HIF-1α groups.[10] It should be emphasized that this trial was conducted in patients with intermittent claudication, and not in patients with CLI. Additional clinical trials are needed to evaluate the safety and efficacy of active forms of HIF-1α in CLI.

Despite promising results in animal studies and part of the open-labeled and early phase clinical trials, the placebo-controlled human trials of therapeutic angiogenesis by gene therapy in CLI have had limited and inconsistent results, and the larger randomized trials failed to demonstrate significant efficacy in the main clinical end points.

Although there are potential risks of gene therapy such as stimulation of tumor vascularity, neoplasm growth, and exacerbation of retinopathy, the various trials showed no safety concerns, with no increase in the mortality and the atherothrombotic or malignant adverse events.

There are several possible explanations that were raised in the recent years, to the disparity in clinical results, and their implication to future study design.[1,9,84,85]

A wide difference exists between the models of healthy animals and the patients enrolled in the clinical trials, which can explain the less favorable response to angiogenic therapies in human studies. The patients with CLI are heterogeneous population with a high prevalence of severe comorbidities, such as diabetes and smoking and therefore a more variable outcome. In such a population, a larger number of patients may be needed in order to prove efficacy.

The patients enrolled in the CLI clinical trials had an advanced stage of disease; mostly patients in whom endovascular or surgical therapeutic options have been already utilized or who were excluded due to the high risk of procedures. This end-stage disease phase and the clinical comorbidities such as diabetes are variables that may influence the ability of gene therapy to induce therapeutic angiogenesis. In addition, growth factor concentrations in ischemic tissues may not reach sufficient levels or not persist long enough in order to influence therapeutic angiogenesis. Plasmid DNA vector has a shorter expression duration and tissue transfection efficiency than adenoviral gene expression. Viral vectors also have limitations such as eliciting inflammatory and immune responses. Short half-life of growth

factors, insufficient doses, short time for gene expression, and compromised delivery routes, are all factors that might have had negative influence on the clinical study results. Moreover, the use of a single growth factor that may not be sufficient to restore blood flow to ischemic tissues, and the significant placebo effect of interventional therapies in sick populations, might have contributed to the variance in results.

Finally, there is some variability in the choice of end points in the various gene therapy trials in CLI. Subjective measures may have variations between patients with different comorbidities and disease stages. Hence, there should be more measurable, consistent, and limb-specific end points in the clinical trials in order to better assess the effects of therapeutic angiogenesis in patients with CLI.

Cell-Based Therapy Clinical Trials for CLI

The understanding of the mechanism of neovascularization in adults has continued to evolve over the years. Previously, the process was thought to occur solely through angiogenesis (the sprouting of new vessels from preexisting ones). But there has been a paradigm shift influenced by the fact that attempts to stimulate therapeutic angiogenesis using a single gene or growth factor proved to be suboptimal. The concept of vasculogenesis, the so-called postnatal angiogenesis (the de novo development of new vessels from endothelial progenitor cells with the eventual transformation to mature endothelial cells, vascular smooth muscle cells and pericytes) came to be redefined with the discovery of bone marrow–derived progenitor cells.[86] Evidence from animal studies shows that transplantation of progenitor and stem cells in ischemic tissue results in positive outcomes in the models of ischemia.[87,88] Subsequently, as will be discussed below, several phase I/II human clinical trials were conducted to evaluate the safety and efficacy of stem and progenitor cells as therapeutic modalities in the treatment of CLI. Some of the cell sources under clinical investigation include EPCs, bone marrow mononuclear cells (BM-MNCs), mesenchymal stem cells (MSCs), as well as the use of stem cell–stimulating cytokines such as granulocyte macrophage–colony-stimulating factor (GM-CSF). Other potential cell sources under study include adipose-derived stem cells (ADSCs), human embryonic stem cells (hESCs), and induced pluripotent stem cells (iPSCs). Table 2 is a summary of the results of recent clinical trials using cell therapy in the treatment of CLI.

The EPC trials

Bone marrow–derived EPCs were described more than a decade ago and since then several publications reported evidence that EPCs have positive role in stimulating postnatal angiogenesis. In general terms, EPCs are referred to as peripheral blood or BM-MNCs that are capable of adhering to matrix molecules, such as fibronectin and express dual acetylated LDL and ulex europaeus agglutinin 1 lectin.[96] The mechanism of action of EPCs in promoting vasculogenesis is thought to occur by 2 means: by homing and direct incorporation into the existing vasculature, facilitating the growth of new capillaries, and by paracrine effects (secreting cytokines and other proangiogenic growth factors), thus stimulating resident endothelial cells to proliferate within the vascular wall.[97,98] At least 4 origins of EPCs have been described: hematopeiotic stem cells, myeloid cells (that differentiate into endothelial cells); circulating progenitor cells (side population cells); and circulating mature endothelial cells.[99] However, the exact definition of EPCs still remains controversial, given that hematopeiotic stem cells express cell surface receptors, such as CD34, CD133, VEGF-2 (KDR/Flk-1), and other mixed population of cells during their various stages of development.[100] The different definitions stem from the fact that there has been no definitive assay to determine the exact molecular characteristic of either immature or mature EPCs. Moreover, there are data showing that true EPCs do not express CD133 receptors, thus proving a potential way to discriminate them from mature endothelial

cells.[101,102] There are other distinguishing characteristics between EPCs and mature endothelial cells (ECs): the EPCs are able to proliferate in vivo and form new vessels, while the ECs in the circulation, are committed, nondividing, and are depleted with time. Their potential for angiogenesis is still under study.

Circulating EPCs are rare, especially in patients with multiple comorbidities and risk factors.[103] Therefore, most clinical studies have attempted to increase the number of EPCs by either mobilization from the bone marrow, using cytokines such as GM-CSF, or by ex vivo expansion and eventual transplantation to the area of ischemia. For example, the Stimulation of ARTeriogenesis using subcutaneous application of GM-CSF as a new treatment for peripheral vascular disease (START) study, even though not a CLI trial, investigated the utility of GM-CSF administered subcutaneously in the treatment of patients with moderate-to-severe PAD. The results of the study were negative and did not show any benefit in the GM-CSF-treated group versus placebo group. In fact, there was a placebo effect observed in the control group; after days 14 and 90, there was no difference in the walking distance between the 2 groups, and walking time as well as ABI was similar in both the groups. However, microcirculation as measured by Laser Doppler perfusion imager was decreased in the control group, with no difference observed in the GM-CSF-treated group.[104] Following these results, more focus has been redirected to the use of cellular components of enriched EPC populations in the treatment of CLI. As a result, several ischemia models have demonstrated positive results when using cultured and expanded human EPCs that are then transplanted into immunodeficient animal models of ischemia.[87,105,106]

Recently, some studies have translated the therapeutic potential of EPCs seen in the animal models of ischemic disease into human clinical trials utilizing population of EPCs believed to be either CD34+ or CD133+ enriched. For example, in the EPOCH-CLI trial, a phase I/II study, Kawamoto et al investigated the safety and efficacy of autologous IM transplantation of GM-CSF mobilized CD34+ cells in patients with intractable CLI (due to either atherosclerosis or thromboangiitisobliterans).[89] The CD34+ cells were mobilized from the bone marrow to the peripheral circulation and then isolated by apheresis as EPC-enriched CD34+ populations. A total of 17 patients were enrolled for an escalated dose of CD34+ EPCs with no placebo arm (10^5/kg, n = 6, 5×10^5/kg, n = 8, 10^6/kg, n = 3). The primary end point at 12 weeks was the efficacy score as measured by change in toe brachial index (TBI), Wong-Baker FACES pain rating scale, and total walking distance (TWD) compared to the baseline. The secondary end points for safety were adverse events, and the secondary end points for efficacy were Rutherford Score, Wong-Baker FACES pain rating scale, ulcer healing, TWD, PFWD, TBI, ABI, and $TcPO_2$. All the patients benefited based on the primary and secondary end points, and there was no difference in dose escalation between the groups. In addition, there were no severe adverse events, indicating beneficial effects of EPCs transplantation in the treatment of CLI. However, this is not a controlled randomized trial, and a larger study is needed to offset the possibility of the placebo effect.

In a similar phase I trial, Burt et al evaluated the feasibility of autologous IM transplantation of peripherally derived CD133+ EPCs in patients with intractable CLI.[90] In all, 9 patients were entered into the trial (7 with atherosclerosis, 1 with thromboangiitis obliterans, and 1 with thromboembolitic disease). Mobilization was achieved by the administration of GM-CSF; then CD133+-enriched population of EPCs was isolated from the peripheral circulation, expanded, and then transplanted intramuscularly into the affected leg. The primary end point was amputation-free survival at 12 months. Secondary end points were relief of rest pain, new collateral vessel formation as measured by magnetic resonance angiogram (MRA) or angiogram, TWD, oxygen consumption (VO_2), ABI, summary performance score, and QoL. Follow-up was at days 2 and 5, then at 1, 3, 6, and 12 months

posttreatment. of the 9 patients, 7 survived limb amputation after 12 months. In these 7 patients, there was improvement in QoL and summary performance score at 3 and 6 months but not at 12 months. There was improvement in PFWD and exercise capacity at 12 months. Again, larger randomized trials are needed for better clarity of the efficacy of EPCs in the treatment of CLI and especially head-to-head comparison of the various populations of cells.

The BM-MNCs Trials

The BM-MNCs are derived from the bone marrow and are separated by density centrifugation from the unfractionated, crude bone marrow.[107] The BMCs also act as a reservoir of progenitor cells at several levels of maturity and multipotency and are a source of endothelial precursor cells such as hematopoietic stem cells, mesenchymal stem cells, hemangioblasts, and EPCs. These capabilities have made BMCs an attractive source of cells for therapeutic angiogenesis in the treatment of ischemic diseases. Results from the animal models indicate that transplantation of BMCs improves neovascularization in the ischemic hind limb. These findings led to the first set of human clinical trials utilizing BM-MNCs transplantation in the treatment of CLI.[108]

Recently, in a phase II, randomized, double-blinded, placebo-controlled pilot trial named PROVASA (Intraarterial Progenitor Cell Transplantation of Bone Marrow Mononuclear cells for Induction of Neovascularization in Patients With Peripheral Artery Occlusive Disease), a group of investigators hypothesized that IA infusion of BM-MNCs is associated with improved limb perfusion as measured by ABI as the primary end point, and with reduction of ischemic rest pain and improved ulcer healing as the secondary end point.[91] This study is unique in that it utilizes a novel method called “randomized start” clinical trial design. Using this method, 40 patients were randomized in a 1:1 fashion to receive either IA infusion of autologous BM-MNCs or placebo and at the end of 3 months; the placebo group was crossed over to receive active treatment of the first dose of BM-MNCs, while the active group received a second dose of BM-MNCs. A comparison between the 2 groups was then made at 6 months’ follow-up and patients with residual ulcers (Rutherford class 5) received extra therapy (third dose for the original active treatment group and second dose for the original placebo group). This clinical trial design allows for the assessment of whether repeated doses with autologous BM-MNCs is beneficial when compared to a single dose as has been done in many other studies. The PROVASA trial showed that IA cell injections are safe and feasible and resulted in significant improvement in the secondary end point but not in the primary end point. The authors reason that the choice of ABI as a primary end point measure was a poor selection because the change in ABI did not correlate with the improvement in ulcer healing or rest pain. Moreover, patients with Rutherford class 6 CLI performed poorly, most likely secondary to the severity of their disease and other patient-specific factors such as BMCs function.

An interim analysis for the results of the study “Use of Tissue Repair Cells (TRCs-Autologous Bone Marrow Cells) in Patients with Peripheral Artery Disease to Treat Critical Limb Ischemia” (RESTORE-CLI) was recently reported.[92] RESTORE-CLI is a phase II, prospective, randomized, double-blinded, multicenter trial comparing the safety and efficacy of IM injection of expanded autologous bone marrow cells (“Tissue Repair Cells” or TRCs) in patients with intractable CLI when compared to placebo. The original target was to enroll a total of 150 patients into the trial. However, only 86 patients were enrolled and out of the 86 patients, 33 completed the full duration of follow-up (12 months) and 13 were able to complete up to 6 months of follow-up. Both groups (n = 46) were unblinded and entered for interim analysis (TRCs = 32, control =14). The treatment group was injected with 136 ± 41 $\times 10^6$ of TRCs in 20 sites in the ischemic leg. Similarly, the control group received injections of electrolyte solution to the ischemic limb. Follow-up visits were on day 0 (bone marrow or placebo injection), days 3 and 7, and months 3, 6, 9, and 12. The primary end

point was safety, as measured by adverse events and serious adverse events, and the secondary end points were clinical efficacy and included major amputation-free survival and time to first occurrence of treatment failure (defined as major amputation, death, de novo gangrene, or doubling of wound size) as well as major amputation rate and measurements of wound healing. There was no significant difference in the primary end point outcomes between the 2 groups. However, there was significant improvement in the secondary end points in the TRCs-treated group when compared to the control group; there was a significant increase in the time to treatment failure ($P = .0053$) and amputation-free survival in patients receiving TRCs therapy ($P = .038$). In addition, there was evidence of improved wound healing in the TRC-treated group when compared to the controls at 12 months. This study is the first published placebo-controlled autologous stem cell trial using enriched and expanded BM-MNCs for the treatment of intractable CLI. Hard clinical primary end points such as amputation-free survival were used rather than surrogates such as ABI, TBI, $TcPO_2$, and so on. A larger phase III study is needed to validate the preliminary findings.

In a nonrandomized study, Idei et al evaluated the long-term clinical outcomes after autologous BM-MNCs transplantation in patients with CLI. Specifically, they compared outcomes in patients with atherosclerosis as the cause of CLI (n = 25) versus thromboangiitis obliterans (TAO; n = 26). Totally, 46 patients were included as control (no BM-MNCs transplantation), with 30 having atherosclerosis and 16 having TAO; the median follow-up period was 4.8 years.[93] The primary end points were major amputation-free survival rate and cumulative survival rate. The secondary end points were changes in ABI and $TcPO_2$. The 4-year major amputation-free survival rate after BM-MNCs transplantation was 48% in the atherosclerosis group and 95% in the TAO group, and 0% in the control atherosclerosis group and 6%in the control TAO group. The 4-year cumulative survival rate after BM-MNCs transplantation was 76% in the atherosclerosis group and 100% in the TAO group, and 67% in the control atherosclerosis group and 100% in the control TAO group. There was a sustained improvement in ABI and $TcPO_2$ 1 month to 3 years posttreatment in patients with TAO. However, the opposite was observed in patients with atherosclerosis with improvement in ABI and $TcPO_2$ only seen at 1 month posttreatment. Although these findings are interesting, this study was nonrandomized and is subject to selection bias; patients with TAO in the treatment group were instructed to stop smoking before BM-MNCs could be administered, while the control group was not prohibited from smoking. Also, it seems that patients in the treatment group had less severe disease (rest pain and nonhealing ulcers) when compared to the control group (being considered for major amputation). Moreover, patients with TAO are often young (<50 years old) with less atherosclerosis risk factors other than smoking, have better progenitor and bone marrow function, and generally do better with smoking cessation. These factors render the interpretation of these results challenging; these results could be validated and reproduced in a well-randomized and blinded study.

The MSC Trials

The MSCs are also derived from the bone marrow and are not hematopoietic in nature. The MSCs have unique capabilities and can differentiate into other cell types, namely myoblasts, osteoblasts, chondrocytes, and adipocytes.[109] Currently, no specific marker for MSCs has been identified. However, it is known that MSCs express mainly CD90, CD105, CD44, and Stro-1 and cellular adhesion molecules such as VCAM-1 and ICAM-1 but not hematopoietic markers such as CD34, CD14, CD45, or CD11.[110] The MSCs attract interest as a source of cells for the treatment of ischemic diseases due to several reasons: (1) they are relatively easy to isolate from the bone marrow and expand ex vivo, (2) they are nonimmunogenic and are ideal for allogeneic transplantation without the need for immunosuppression, (3) MSCs can be modified by a transgene so as to deliver specific gene/genes required for

neovascularization, and (4) they can be delivered systemically or locally without adverse effects.[111,112]

Mechanistic-wise, MSCs are thought to influence neovascularization via their release of proangiogenic cytokines that may have paracrine or autocrine tissue effects, resulting in the stimulation of resident ECs to migrate, differentiate, and proliferate in situ. Several animal models of ischemia have demonstrated benefits post-MSCs transplantation.[113,114] Human clinical trials with MSCs dates back to 1995 when the first experimental work was performed, and in 2002 the first infusion of allogeneic MSCs for the treatment of osteogenesi-simperfecta took place.[115,116] Several phase I/II cardiovascular-related trials utilizing MSCs-based therapy have shown promising results.[117,118]

Unlike other promising cardiovascular applications, there is limited data regarding the application of MSCs as a sole cellular agent in the treatment of CLI. The current available studies have used combination cell therapy: EPCs and MSCs or MNCs and MSCs approach. This approach stems from the evidence that the process of angiogenesis may need more than 1 cytokine or cell type to optimize new vessel formation. The MSCs in combination with the other cell types may offer a comprehensive and robust design for the treatment of CLI. For example, in 2 (phases I and II) nonrandomized trials, Lasala et al investigated the utility of administering a mixed population of autologous MSCs and MNCs in the treatment of CLI. In the phase I trial, 10 diabetic patients with Fontaine stages 4B to 4 of CLI were enrolled to receive intra-arterially MNCs and MSCs.[94] The primary end points were safety and feasibility, and the secondary end points were improvement in ischemic rest pain, exercise tolerance, ABI, $TcPO_2$, and collateralization as measured by digital subtraction angiography and nuclear perfusion scintigraphy. After 10 ± 2 months of follow-up, there was benefit in the primary and secondary end points. In the phase II study, 26 patients with CLI (Rutherford class 4 to 6) having multiple comorbidities were enrolled.[95] The IM (gastrocnemius) infusion of a combination cell product (MNCs and MSCs) was administered in the most ischemic leg and placebo in the less ischemic contralateral leg. The primary end points were safety and efficacy with similar secondary end points as in the phase I trial. Again, the authors reported benefit in both the end points after 4 months of clinical follow-up. Due to the limited nature of these trials, no concrete conclusion can be made from these results. Nonetheless, it could be of interest to add outcomes such as major amputation-free survival and a longer duration of follow-up in a future randomized trial.

Conclusions

Patients with CLI require immediate attention and intensive multidisciplinary treatment to achieve limb salvage, pain relief, and improvement in QoL and functionality. Local and medical therapy will suffice in the minority of patients. Endovascular interventions emerge as the treatment of choice in many patients and surgery is considered as an important option. In a significant number of patients these therapeutic options are not available due to the extensity and distribution of the occlusive disease. Additional group of patients who are not amenable to conventional therapies are those with comorbidities or those who exhausted conventional therapies. Gene therapy with a single-gene delivery failed so far to produce significant therapeutic angiogenesis. Currently, different cell types and methods of delivering these cells are being tested to alleviate ischemia in poor-option patients with CLI. Mixed results of these experiments should encourage researchers in the field to continue to pursue cell therapy in patients with CLI.

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Table 1

Selected Phase II/III Clinical Trials of Gene-Based Therapy for Critical Limb Ischemia

Study Name/ Author	Year of Publication	Treatment and Vector	No. of Patients (Active/ Placebo)	Treatment Route and Dose	End Points
Kusumanto et al.[54]	2006	VEGF165; plasmid	27/27	2 sessions, 4 weeks apart; IM injection (2000 μg each session)	= Amputation; rest pain.; ↑ improvement in skin ulcer; ↑ ABI, TBI
Makinen et al.[55]	2002	VEGF165; plasmid and adenovirus	35/19	18 patients: 2×10^{10} pfu VEGF-Ad; 17 patients: VEGF plasmid/ liposome (2000μg/2000μl); IA infusion	↑ Increased vascularity (DSA); Rutherford class, ABI = improvement, but similar to control
RAVE study[56]	2003	VEGF121; adenovirus	72/33	Low dose 4×10^9 pu; High dose 4×10^{10} pu; 20× IM injections in single session. (unilateral PAD)	= Change in peak walking time; = ABI; QoL measures; ↑ peripheral edema
Talisman-201 study[57]	2008	FGF-1; plasmid	59/66	4 sessions, 2 weeks apart; IM injections × 8 of 0.5 mg (each session)	↓ Amputation rate; = ulcer healing
Tamaris study[58]	2011	FGF-1; plasmid	259/266	4 sessions, 2 weeks apart; IM injections × 8 of 0.5mg (each session)	= Major amputation; = death
Traffic study[59]	2002	FGF-2; recombinant	127/63	rFGF2 30 μg/kg = single dose; rFGF2 30 ug/kg ×2 = double dose (day 1 + 30).; IA infusion	↑ peak walking time at 90 days.; = QoL measures.= ABI
HGF-STAT Study[60]	2008	HGF; plasmid	78/26	Low dose:0.4 mg (×3 sessions); mid-dose: 4mg (×2 sessions); High dose:4 mg (×3sessions); IM injections ×8 each session	↑$TcPO_2$ at 6 months in high dose.; = amputation; death; Ulcer size; wound healing; ABI, TBI
HGF-0205 Study[61]	2010	HGF; plasmid	21/6	3 sessions, 2 weeks apart; × 8 IM injections of 0.5 mg (each session)	= Wound healing; amputation; ↑ TBI increase in 6 months; ↑ rest pain improvement (VAS)
Shigematsu et al.[62]	2010	HGF; plasmid	27/13	2 sessions, 4 weeks apart; ×8 IM injections of 0.5 mg (each session).	↑ Reduction in ulcer size.; = rest pain; ABI; ↑ QoL measures

Abbreviations: ABI, ankle–brachial index; DSA, digital subtraction angiography; IA, intra-arterial; IM intramuscular; TBI, toe–brachial index; $TcPO_2$, transcutaneous oxygen pressure; QoL, quality of life; VAS, visual analog scale; VEGF, vascular endothelial growth factor; FGF, fibroblast growth factor; HGF, hepatocyte growth factor; pfu, plaque-forming units; pu, particle units; rFGF, recombinant fibroblast growth factor.

Table 2

A summary of Recent Clinical Trials of Cell-Based Therapy for Critical Limb Ischemia

Trial Name/Authors (Phase)	Cell Type	No. Patients (T/C)	Delivery	Mean Follow-up Period	Results[a]
EPOCH-CLI (I/IIa) 2009[89]	EPCs (CD34)	17 (17/0)	IM	12 weeks	Improvement in exercise capacity (TWD, PFWD), ulcer healing, and perfusion measurements (ABI, TBI, and $TcPO_2$)
Burt et al. (I) 2010[90]	EPCs (CD34/CD133)	9 (9/0)	IM	12 months	Improvement in amputation-free survival, exercise capacity, pain relief, collateral formation, perfusion, and QoL
PROVASA (II) 2011[91]	BM-MNCs	40 (19/21)	IA	3 months	Positive ulcer healing, negative limb perfusion, and ABI
RESTORE-CLI (II) 2011[92]	BM-MNCs	46 (32/14)	IM	12 months	Improvement in amputation-free survival and ulcer healing
Idei et al. (I) 2011[93]	BM-MNCs	97 (51/46)	IM	4. 8 years	Improvement in amputation-free survival, cummulative survival, and perfusion measurements
Lasala et al. (I) 2010[94]	MNCs + MSCs	10 (10/0)	IA	10 months	Improvement in exercise capacity, perfusion measurements, collateral formation and QoL
Lasala et al. (II) 2011[95]	MNCs + MSCs	26 (26 severe CLI legs/26 less severe contralateral CLI legs)	IM	4 months	Improvement in exercise capacity, perfusion measurements, collateral formation and QoL

Abbreviations: ABI, ankle–brachial index; BM-MNCs, bone marrow mononuclear cells; EPCs, endothelial progenitor cells; IA, intra-arterial IM, intramuscular; MSCs, mesenchymal stem cells; PFWD, pain-free walking distance; QoL, quality of life; TBI, toe–brachial index; T/C, treatment/control; $TcPO_2$, transcutaneous oxygen pressure; TWD, total walking distance; CLI, critical limb ischemia.

[a]Indicates the defined primary or secondary end points.