Published in final edited form as:
*Circ Res*. 2013 April 26; 112(9): . doi:10.1161/CIRCRESAHA.113.300565.

# Cell Therapy of Peripheral Arterial Disease:

## From Experimental Findings to Clinical Trials

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## Abstract

The age-adjusted prevalence of peripheral arterial disease in the US population was estimated to approach 12% in 1985, and as the population ages, the overall population having peripheral arterial disease is predicted to rise. The clinical consequences of occlusive peripheral arterial disease include intermittent claudication, that is, pain with walking, and critical limb ischemia (CLI), which includes pain at rest and loss of tissue integrity in the distal limbs, which may ultimately lead to amputation of a portion of the lower extremity. The risk factors for CLI are similar to those linked to coronary artery disease and include advanced age, smoking, diabetes mellitus, hyperlipidemia, and hypertension. The worldwide incidence of CLI was estimated to be 500 to 1000 cases per million people per year in 1991. The prognosis is poor for CLI subjects with advanced limb disease. One study of >400 such subjects in the United Kingdom found that 25% required amputation and 20% (including some subjects who had required amputation) died within 1 year. In the United States, ≈280 lower-limb amputations for ischemic disease are performed per million people each year. The first objective in treating CLI is to increase blood circulation to the affected limb. Theoretically, increased blood flow could be achieved by increasing the number of vessels that supply the ischemic tissue with blood. The use of pharmacological agents to induce new blood vessel growth for the treatment or prevention of pathological clinical conditions has been called therapeutic angiogenesis. Since the identification of the endothelial progenitor cell in 1997 by Asahara and Isner, the field of cell-based therapies for peripheral arterial disease has been in a state of continuous evolution. Here, we review the current state of that field.

### Keywords

ischemia; peripheral arterial disease; progenitor cell; stem cell; vasculogenesis

The term peripheral arterial disease (PAD) is used most commonly to refer to ischemia of the limbs secondary to atherosclerotic occlusion. This is a highly prevalent and debilitating condition, estimated to affect >25 million patients in Europe and North America alone.[1–5] The exact incidence of amputation attributable to PAD is difficult to ascertain. Jones et al recently examined Medicare data and found that overall rates in this population seem to be decreasing slightly but remain significant.[6] PAD is graded by symptom severity and degree of tissue ischemia, commonly with the Rutherford classification.[7–9] Traditionally, the



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This manuscript was sent to Joseph Wu, Consulting Editor, for review by expert referees, editorial decision, and final disposition.

**Disclosures**
None.

treatment of PAD has centered on smoking cessation, exercise to promote collateral blood flow/improve functional performance, pharmaceutical vasodilatation to optimize microvascular reserve, and percutaneous or surgical revascularization to restore blood flow to at-risk tissue.[10–12]

All too often, however, chronic ischemia in the peripheral vascular beds follows an aggressive clinical course, culminating in critical limb ischemia (CLI) and irreversible tissue loss.[13–18,19,20] Occlusive vasculitis, although less common, can take a similarly aggressive clinical course. The definition of CLI as a subset of PAD has evolved over the years, with the most recent definition involving chronic ischemic rest pain or tissue loss with objective findings of arterial occlusion (≈1% of patients with PAD, Rutherford class 4–6).[21,22] Patients with CLI are estimated to face >30% 1-year amputation rates and >20% 1-year mortality,[22] although these rates may be decreasing and exhibit a certain degree of geographic variability.[6] Of those who undergo amputation, ≈30% will require repeat surgery in the same or contralateral leg >2 years.[22] For patients who fail surgery, there are few traditional options left. As expected with this debilitating disease, psychological testing of CLI subjects has shown their quality of life indices to be similar to that for terminally ill cancer patients.[13] In addition to the profound physical and psychological problems associated with CLI, treatment for this condition is costly; the annual total hospital and surgical costs for a CLI subject has been estimated to be $47,000.[14–23]

Such progression in no-option patients reflects a burden of chronic ischemia that exceeds tissue capacity for simple diffusion of oxygen and nutrients from peri-ischemic territories, as well as for endogenous remodeling. Novel treatment strategies in advanced PAD have focused on restoring this balance in favor of tissue survival using exogenous molecular and cellular agents to promote regeneration of diseased microvasculature.[24–27] To meet the myriad hemodynamic and metabolic needs of the human body, our circulatory system must accommodate a diversity of endothelial phenotypes and vascular functions.[28] Healthy capillary beds tailor themselves to meet the unique needs of their end organs, taking on increased glomerular tuft volume after partial nephrectomy, for example, or greater capillary density after intense exercise.[29,30] Maintenance of such tissue-responsive vasculature includes homeostatic mechanisms in response to end-organ distress and depends on dynamic genetic and molecular signaling from local tissues and some degree of cellular plasticity throughout adulthood.[31] Dysfunction in the vascular bed, including attrition of the microvasculature or the inability to vasodilate to accommodate the need for increased blood flow, merits analysis at the molecular, cellular, and clinical levels, with potential therapies targeted toward the same.

Early studies in vascular regeneration used angiogenic proteins such as vascular endothelial growth factor (VEGF) and fibroblastic growth factor and cell-mobilizing cytokines such as granulocyte-colony stimulating factors and granulocyte macrophage–colony stimulating factors (G-CSF/GM-CSF) to promote vasculogenesis.[32–34] Although preclinical and early-stage clinical results were promising, single-dose protein therapy did not achieve a lasting clinical effect, perhaps secondary to the short half-life of the proteins administered. Gene therapies using naked plasmid DNA–encoding angiogenic factors were developed to improve duration of transgene expression over direct protein injection.[35,36] Although these plasmids are minimally immunogenic, they typically result in low gene transfer into target cell nuclei and remain episomal with a duration of transgene expression that is still short. Adenoviral vectors encoding VEGF and fibroblastic growth factor showed improved transduction and gene transcription attributable to uptake by both replicating and nonreplicating host cells, but these vectors also remained episomal.[37–40] Design of retroviral vectors to allow integration into host DNA for lifelong expression came at the expense of moderately decreased gene transduction (retroviral uptake by replicating cells only). All

told, the strategy of augmenting expression of a single growth factor has thus far failed to deliver significant clinical improvement, although studies are ongoing, applying the learning of earlier studies to dosimetry, vector, and transgene selection.

With the pending results of molecular therapy trials and the evolving knowledge of the potential of progenitor and stem cells as agents of tissue repair, the field of regenerative medicine has moved toward the use of cell-based approaches for therapeutic neovascularization.

## Vascular Stem Cell Biology

Of the 3 primary embryonic germ layers, the mesoderm (middle layer, source of mesenchyme) gives rise to the primitive circulatory system at ≈3 weeks of development, presumably because the embryo becomes too large to be sustained by vitelline/placental nutrient diffusion alone.[41,42] This mesoderm is induced via fibroblastic growth factor to form blood islands comprising hemangioblasts, the common precursor to both blood cells and blood vessels.[43] Hemangioblasts located centrally within these islands differentiate into hematopoietic stem cells, some of which migrate into the fetal liver and ultimately take up residence in the bone marrow (BM).[41] Peripherally located island hemangioblasts differentiate into angioblasts, which join together into solid plexuses and form intracellular vacuoles via liquefaction.[42] These vacuoles ultimately represent the endovascular lumen, and the surrounding spindle-shaped cells become known as endothelium.[42] This formation of de novo blood vessels from angioblasts is called vasculogenesis.

Angiogenesis, in contrast, refers to the migration and proliferation of differentiated endothelial cells to allow sprouting of new capillary branches from existing vessel walls. Arteriogenesis refers to the remodeling of endothelium, smooth muscle, and extracellular matrix to allow formation of larger, bridging collaterals between existing arterial networks. In 1997, it was suggested that vasculogenesis is not limited to the embryo, when selected circulating mononuclear cells (MNC) were seen to differentiate into endothelium-like cells in vitro and to incorporate into neovasculature in murine and rabbit models of ischemia.[44] Since that time, the exact identity and therapeutic potential of endothelial progenitor cells (EPCs) have been heavily investigated in both preclinical and clinical studies.

EPCs can be imprecisely defined as any cell that retains endothelial potential in the developmental pathway from hemangioblast to fully differentiated endothelial cell. Not surprisingly, hematopoietic stem cells and EPCs have been noted to share a variety of cell-surface antigen markers, namely CD34, CD133, and VEGF receptor-2.[45–48] As EPCs mature toward endothelial cells, CD133 expression wanes, whereas adhesion molecule expression is upregulated, and endothelial functions such as low-density lipoprotein uptake and nitric oxide synthesis are gained.[28,49] These and other cell-surface features have been used to isolate EPCs in the scientific pursuit of therapeutic vasculogenesis.

The landmark study by Asahara et al[44] classified EPC lineage on the basis of CD34 expression and found that $CD34^+$ cells selected from peripheral MNCs comprised 1% of the total MNC population, but represented >60% of spindle-shaped endothelial-like cells in vitro, resembling those found in the periphery of hemangioblast blood islands. Of note, however, coculture of $CD34^+$ and the remaining $CD34^-$ MNCs resulted in greater proliferation and differentiation of endothelial cells than with $CD34^+$ alone, indicating that this marker taken in isolation may not identify all cellular elements involved in vasculogenesis.

An alternative method of EPC isolation is the culture of selected/unselected peripheral blood MNCs in specialized media, with subsequent isolation of early- and late-outgrowth colony-

forming units. It has been suggested that use of late-outgrowth colony-forming units (allowing ≈2–3 weeks of culture) selects cells with greater proliferative capacity.[50] Late EPCs have also been shown to better organize and incorporate into vessel-like tubules.[51] Culture of $CD133^+$ EPC-derived colony-forming unit endothelial cells and nonadhesive peripheral blood MNC–derived colony-forming unit Hill cells has also been described.[45,52,53]

Although fluorescence-activated cell sorting and plated cell culture remain the most commonly used EPC isolation methods, novel identifiers of stem cells such as intracellular enzymes shared by multiple progenitor lines are being developed. For example, aldehyde dehydrogenase–expressing cells have been isolated and tested alongside more traditionally defined EPCs.[54] To date, however, no single marker or combination of markers identifies a pure EPC population.

A variety of EPC sources have been used and include BM, peripheral blood, unselected mesenchyme, adipose tissue, and umbilical cord blood, among others.[55–64] In 2006, murine fibroblasts were dedifferentiated into stem cells with the capacity to form all 3 germ layers.[65] The scientific and therapeutic potential of such induced pleuripotent cells and the capacity for transdifferentiation of resident adult stem cells are being actively investigated.[66–71]

## EPC Mobilization

Regardless of the isolation method used, the collection of EPCs for therapeutic purposes presents certain challenges. Given the shared embryological origin of hematopoietic stem cells and angioblasts and the ultimate migration of early hematopoietic stem cells to the BM, the marrow is felt to be a major reservoir of cells, retaining some hemangioblastic properties. In addition to directly harvesting cells from this source, a variety of mobilization techniques have been used to increase EPC concentration in the peripheral blood. G-CSF and GM-CSF remain the most widely used cytokines for this purpose. Preclinical and clinical studies demonstrate a dose-dependent increase in circulating EPCs using G-CSF and GM-CSF.[72,73] G-CSF–mobilized CD34 cells expanded in vitro retained proliferative markers and the ability to form vascular structures in vivo in murine models of ischemia.[74] CXC chemokine receptor-4 antagonists have also been shown to mobilize EPCs in resting conditions via direct blockade of CXC chemokine receptor-4: stromal cell–derived factor-1 binding and in the setting of ischemia via increased matrix metallopeptidase 9 signaling in the BM.[75,76]

One clinical trial comparing conventional PAD therapy with BM transplantation or subcutaneous G-CSF found similar improvements in symptoms and ankle-brachial index (ABI) among both treatment arms at 1-month follow-up.[77] ABI was not significantly improved in trials of GM-CSF versus placebo, although 1 study did report improvement in total and pain-free walking time.[78,79] Differences among GM-CSF study outcomes may be attributed to differences in patient selection, because those with improved functional capacity had less severe peripheral vascular disease at baseline and were instructed to exercise to claudication in addition to cytokine therapy. Angiogenic growth factors such as VEGF, fibroblastic growth factors, hypoxia-inducible factors, and stromal cell–derived factors have also been shown to recruit EPCs.[80–82] Novel mobilization techniques using traditionally nonangiogenic ligands with cell-surface receptors that are expressed on EPCs are also being explored. Parathyroid hormone, for example, has been shown to exert synergistic effects on circulating progenitor cell numbers and tissue perfusion when administered in combination with G-CSF.[83] The biphasic effects of statins on angiogenesis and the dose-dependent mobilization of EPCs with statins remain to be reconciled in terms of the application of statin therapy in the setting of angiogenesis-dependent diseases.[84,85]

Mechanisms governing the homing, incorporation, survival, and differentiation of EPCs, once they have been successfully mobilized, remain incompletely characterized.

In addition to mobilization of EPCs to enhance their therapeutic potential, genetic engineering of cells has been used. It has been proposed that concurrent expression of angiogenic growth factors with EPC transplantation may improve stem cell–mediated outcomes by overcoming age- and vascular disease–mediated deficiencies in EPC number, function, and angiogenic cytokine production. The use of EPC transduction provides the added benefit of circumventing immunogenic viral vectors with short-lived episomal transgene expression. Preclinical models suggest improved transplanted cell survival and incorporation with the use of VEGF-expressing EPCs.[86,87] This success hinges on optimal duration and distribution of transgene expression.[88] From such data, an intriguing concept emerges: the use of transduced EPCs to modulate neovascular stability and maturation by providing a more physiological balance of multiple angiogenic cytokines and matrix components in time and space than would be possible with either molecular or cell therapies alone.[89–92] It is conceivable that the timing and sequence of multiple gene expression could be preprogrammed using molecular mechanisms to optimize cell-based neovascularization of ischemic tissues.[93,94] Encoding of vascular permeability factors may additionally aid in improved systemic vector delivery at lower vector doses.[95] Biomaterials, ranging from simple collagen to algae-based matrices to specifically designed nanostructures, are being developed to provide further biochemical and mechanical support to transplanted cells in their host environment.[96–98]

Finally, the interaction of transplanted EPCs with the local environment, that is, vasculogenesis mediated by the paracrine effects of cell and molecular agents, must also be considered. It has long been known that endogenous hematopoietic cells shed surface microparticles and exosomes, which are capable of mediating measureable effects even after source-cell depletion.[99–101] Similarly, EPC-depleted media have been shown to sustain endothelial cells in vitro and to recruit EPCs to mitigate ischemia in murine models to an extent comparable to that of EPC transplantation itself.[102,103] In an elegant experiment using peripheral MNC transplantation, it was suggested that the degree of vasculogenesis correlated with the ability of the host tissue to synthesize angiogenic cytokines, not with the synthesis of cytokines by transplanted cells themselves.[104] This suggests that the vasculogenic effects of transplanted stem cells arise largely from paracrine signals to local tissues. These paracrine mechanisms may prove useful in vascular beds, where traditional regenerative medicine has not borne fruit. For example, studies comparing conditioned media with stem cell therapy in models of ischemic stroke and pulmonary vascular disease suggest superior protection against progenitor cell death and adverse vessel remodeling with conditioned media administration.[105,106] For angiogenic agents that exert their effects primarily through paracrine mechanisms, short transgene expression may suffice, and intermediate goals of harvesting sizable progenitor cell numbers and achieving prolonged on-site stem cell survival and incorporation may become less crucial. Toward this goal, 3-dimensional models are being developed to aid in prediction of ideal growth factor concentrations and extracellular matrix interactions.[107]

To date, no single cell-surface marker has been found to specifically identify all EPCs, and no single cell source, culture, or selection method, dosing regimen, or route of administration has been clearly proven superior, making the ongoing study of therapeutic vasculogenesis quite heterogeneous in research design and outcome measurement.

## Clinical Stem Cell Studies in Peripheral Vascular Disease

Tables 1 through 4 summarize the clinical trials of cell therapy for PAD. Many clinical trials have investigated the safety and efficacy of EPCs in the treatment of PAD. These studies have ranged from case reports to small, randomized, placebo-controlled trials, usually involving Rutherford 4 to 6 (CLI) patients with atherosclerotic or vasculitic disease. Studies that include patients with less severe ischemia (Rutherford class 1–3) run the risk of overestimating stem cell efficacy as applied to its target population of no-option PAD. However, scientific insights gained from such trials remain valuable.

The majority of trials to date have used either BM-derived or peripheral MNCs, although other cell types have also been tested.[108] BM harvests are usually processed by density gradient centrifugation (Ficoll method) or plasmapheresis, both of which require access to certified good clinical practice facilities.[109] Newer bedside centrifugation systems have been developed to circumvent this resource-and labor-intensive process.[110] Peripheral EPCs are usually mobilized using several doses of subcutaneous G-CSF, and harvests are similarly processed via plasmapheresis to enrich target cell content.

EPCs isolated by these methods can be administered either whole (unselected) or after further selection for cell-surface antigens. Usual routes of delivery are direct intramuscular and intra-arterial injection.

The first major clinical trial of stem cell therapy in peripheral vascular disease was the Therapeutic Angiogenesis using Cell Transplantation study, demonstrating safety and providing evidence for bioactivity of unselected BM-MNCs injected intramuscularly into the ischemic limbs of patients with CLI. BM-MNC therapy resulted in improved rest pain, ABI, transcutaneous oxygen pressure, and pain-free walking distance at 24-week follow-up compared with placebo (saline injection). BM-MNC therapy similarly outperformed intramuscular injection of nonmobilized peripheral MNCs, suggesting EPC number or concentration in the latter may be insufficient for therapeutic effect.[111] Improvements in leg pain, walking distance, and ulcer size were maintained at 2-year follow-up.[112] Intramuscular BM-MNC implantation has been shown to increase acetylcholine-mediated endothelium-dependent blood flow for up to 4 weeks, suggesting that endothelial dysfunction may be reversible by stem cell therapy in patients with severe atherosclerotic CLI.[113] Pathological analysis of amputated tissue suggested functional neocapillarization distal to the site of intramuscular BM-MNC treatment that was not seen in age- and sex-matched controls who required amputation.[114] Subsequent trials of unselected BM-MNCs delivered intramuscularly in patients with CLI vasculitis mirrored improvements in rest pain and ulcer size at 24 weeks and in ABI and ulcer size at 2-year follow-up.[115,116]

Head-to-head comparisons of different cell preparations are scarce and have yielded inconclusive results. One randomized, controlled trial of 150 patients with CLI receiving intramuscular injections of G-CSF–mobilized peripheral MNCs versus BM-MNCs reported greater improvements in ABI and rest pain with peripheral MNCs but no difference in pain-free walking distance, ulcer healing, or amputation rates between the 2 groups.[117] A small open-label study in patients with CLI vasculitis reported notable improvement in rest pain with both therapies at 1-month follow-up and greater improvement in blood flow by intra-arterial digital subtraction angiography with BM-MNC treatment in 1 patient.[118] Further clinical trials are needed to identify optimal cell source and processing.

Additionally, the benefit of intramuscular versus intra-arterial cell delivery remains unclear. It has been hypothesized that intramuscular delivery results in a transient cell depot within ischemic tissue, allowing local paracrine activity and some degree of cell incorporation into the neovasculature. In contrast, intra-arterial therapy is thought to direct stem cells to viable

peri-ischemic zones, with enough oxygen and nutrient content to support cell functions. One clinical trial of unselected BM-MNCs delivered intra-arterially demonstrated a similar degree of improvement in ABI compared with previous trials of intramuscular BM-MNC administration (an ABI increase of ≈0.1 points), as well as 2-fold improvement in capillary density and 2- to 10-fold improvement in pain-free walking distance.[119] An intra-arterial study in patients with diabetes mellitus suggested much greater ABI improvements, nearing 0.4 points at 1-year follow-up, as well as marked improvement in wound healing and blood flow by intra-arterial digital subtraction angiography despite the use of lower-dose cell therapy ($10^6$ rather than $10^9$ BM-MNCs).[120] The only multicenter, randomized trial of intra-arterial BM–MNC therapy in patients with CLI to date (the Intraarterial Progenitor Cell Transplantation of Bone Marrow Mononuclear Cells for Induction of Neovascularization in Patients With Peripheral Arterial Occlusive Disease study) showed dose-dependent improvement in wound healing and significant reductions in rest pain compared with placebo, despite a lack of improvement in ABI or limb salvage rates.[121] This study used cell concentrates on the order of only $10^6$ cells and demonstrated that the use of multiple cell treatments was associated with greater clinical gains. Finally, on the basis of available data, concurrent intramuscular and intra-arterial administration of unselected BM-MNCs seems to result in a magnitude of benefit similar to that of either therapy alone.[122–125]

G-CSF–mobilized peripheral MNCs have been also been investigated in PAD. Both intramuscular injection and intra-arterial injection of such unselected mobilized peripheral cells have been shown to result in a >0.1-point improvement in ABI and 2-fold increase in maximum walking distance in small clinical series.[126–129] Although an earlier trial showed improvement in soft end points with the use of G-CSF–mobilized selected $CD34^+$ cells administered intramuscularly in CLI, cell yield was lower than expected in this study (as low as $10^5$ cells).[130]

Our recent data suggest a dose-dependent improvement in freedom from amputation with G-CSF–mobilized selected CD34 cells administered via intramuscular injection (autologous cell therapy-34 CLI trial).[131] In this 28-patient double-blind study, patients were randomized to receive either $10^5$ (n=7) or $10^6$ (n=9) autologous $CD34^+$ cells per 1 kg body weight via intramuscular injection or placebo, consisting of the cell diluent alone (n=12). In the combined cell–treatment groups, the incidence of any amputation trended strongly in favor of cell-treated subjects ($P$=0.054); however, a larger sample size is clearly needed in future studies.

One unique approach to G-CSF–mobilized therapy involved the creation of tibial fenestrations to allow unselected BM cells to directly mobilize into ischemic lower extremities. Although administration of G-CSF in this study increased peripheral EPC concentration, efficacy of this method requires further investigation.[108] In 1 trial, G-CSF administration following intramuscular BM-MNC transplantation did not result in added clinical benefit despite likely peripheral mobilization.[77]

Differences have been noted in efficacy of stem cell therapy based on the pathogenesis of PAD. Two-year survival rates and 1-year amputation rates are notably worse in atherosclerotic than vasculitic PAD.[132] PROVASA investigators and others have noted lesser overall therapeutic benefit in patients with advanced atherosclerotic CLI compared with patients with vasculitic CLI.[121,133] Moreover, these benefits are less likely to be sustained over the long term in patients with atherosclerotic PAD compared with patients with vasculitic PAD.[134] Such observations call into question the health of autologous stem cells in the setting of advanced age and chronic cardiovascular disease.

Indeed, traditional cardiovascular risk factors associated with peripheral vascular disease have been associated with decreased circulating progenitor cell number and function. A number of studies report dysfunction of endogenous EPCs in the setting of hypertension, dyslipidemia, smoking, and diabetes mellitus.[119,135–141] For example, EPC recruitment in response to tissue hypoxia may be impaired in the setting of diabetes mellitus; even the allogenic injection of healthy EPCs into diabetic mice improved, but did not normalize, ischemic tissue survival to the level seen in nondiabetic mice.[142] Additionally, patients with diabetes mellitus can exhibit several-fold-greater intimal plaque neovascularization by histology and microscopy, a process that has previously been associated with greater plaque instability and cardiovascular events.[143] Despite these challenges, therapeutic benefit can be derived using autologous strategies.[144,145] Reversal of risk factors and modulation of cellular microenvironments, for example, through smoking cessation or use of hyperoxygenation, can overcome intrinsic deficiencies of autologous EPCs.[146–148] In 1 study, healthy EPCs injected into diabetic mice resulted in improved allograft function and donor cell recruitment only when transplanted in combination with therapies to promote synergism with local trophic pathways.[149] Attention must be given, therefore, to the application of EPCs in advanced cardiovascular states, with special effort to optimize the metabolic and mechanical context of cell therapy.

Finally, a challenge for the field of cell-based therapies and for all therapies that target the microcirculation is the limitation of available surrogate end points for the prediction of major clinical outcomes. Although the traditional end points such as ABI, toe-brachial index, or transcutaneous oxygen-saturation are reliable indicators of prognosis in population studies, they have failed to consistently provide useful metrics for the development of microvascular therapies. Future studies will need to develop new algorithms using existing technologies or identify novel methods for reliably quantifying tissue perfusion that will permit the design of early-phase studies to evaluate dose in modest-sized study populations.

## Context of Cell Therapy

Oxidative stress, hormonal milieu, ischemic conditioning, and shear stress have all been shown to affect stem cell efficacy. Adjuvant therapies to gainfully modulate these conditions in vivo are being investigated. For example, although low levels of reactive oxygen species act constructively as signaling molecules, higher levels can lead to increased stem cell senescence.[150,151] This identifies a potential target for optimizing EPC function. Preclinical studies in healthy, diabetic, and dyslipidemic mice showed that intravenous and oral antioxidant therapy synergistically improved neovascularization when given in combination with BM-MNC therapy (but not as a stand-alone therapy).[141,152,153] A clinical case-control trial of oral L-arginine and antioxidants after intra-arterial BM–MNC transplantation in patients with CLI resulted in significantly improved amputation rates at 1-year follow-up, but this was in comparison with standard medical therapy alone.[119] Incremental benefit of antioxidant therapy over stem cell transplant in patients has yet to be quantified.

Similarly, estradiol has been postulated to exert positive effects on endothelial remodeling after injury. Accelerated re-endothelialization and decreased apoptosis/neointimal formation were demonstrated in preclinical models of ovariectomized mice.[154,155] Although these mechanisms may also contribute to the protective cardiovascular effects of estrogen observed in premenopausal women, the larger clinical context of hormone therapy must be taken into account before the study or application of this preclinical signal in humans.

Multiscale computational models have predicted that exercise may improve vasculogenesis by increasing angiogenic factor concentrations and gradients toward ischemic tissues.[156] Preclinical models of ischemic mice randomized to exercise exhibited greater histological neovascularization and suffered less neointimal hyperplasia than those that were sedentary.

This effect was attenuated in endothelial nitric-oxide–deficient states. Patients completing a 4-week standardized exercise program had an increase in circulating EPC numbers and a decrease in EPC apoptosis compared with before exercise.[157] The importance of ischemic conditioning through exercise was elegantly demonstrated in a series of 3 prospective, randomized, clinical trials. Nonrevascularized patients with PAD who achieved an increase in serum lactate (a marker of anaerobic metabolism) benefited from >300% increased VEGF concentration and >400% increased EPC mobilization compared with control patients who did not exercise to ischemia.[158]

Shear stress may have a role in improved neovascularization and may mediate some of the benefits seen with exercise. Vessels exposed to high-shear stress after surgical induction of ischemia in animal models display accelerated collateralization (arteriogenesis).[159] The effect of in vivo flow conditions on EPCs has been studied in vitro and is shown to influence EPC alignment and adhesion molecule expression consistent with the endothelial phenotype.[160,161]

## Measuring Neovascularization

Currently, there is no reliable measure of blood flow at the tissue level in ischemic limbs. This is an impediment to therapeutic development because outcome measures such as limb amputation present major challenges for early-phase trial design. A reliable surrogate end point, capable of assessing improvements in blood flow and predicting physiological and clinical improvement, will greatly enhance the development of novel therapies for PAD.

As can be seen in the trials described above, use of stem cell therapy has vast potential for improved clinical outcomes in PAD. Identifying appropriate end points is vital to the progression of therapeutic neovascularization. To this end, many imaging modalities have been developed in addition to existing pathological analyses and hard end points such as mortality, freedom from amputation, and hemodynamic measures of perfusion. These technologies can be used to aid in tracking EPCs in vivo and in understanding correlations of angiographic outcomes with meaningful clinical end points. The following 5 main imaging modalities exist: ultrasound, computed tomography, magnetic resonance, perfusion scintigraphy, and angiography. Test operating characteristics of each these modalities in CLI are being worked out.[162–168]

## Challenges and Future Directions

In summary, the field of progenitor cell therapy for therapeutic neovascularization in PAD is gaining momentum. Scientific inquiry has yielded many promising tools, and a robust movement toward clinical study has uncovered several questions that remain to be fully answered. Effective cell populations, isolation, and processing methods must continue to be refined to gain a deeper understanding of the feature that defines potency. Optimal delivery method, timing, and dosing regimens will be tailored to the disease state and clinical trajectory. Adjunctive therapies to overcome endogenous impairments in EPC health and vascular responsiveness must also be developed, along with molecular and bioengineering tools to advance therapeutic effects of stem cells in time and space. One area that remains to be explored is the possible synergy of macrovascular revascularization with efforts to restore the microcirculation. Finally, trials incorporating valid surrogate measures of success that predict hard end points must be designed to evaluate cell safety, efficacy, and long-term outcomes. With this broad framework in mind, we are confident that basic, translational, and clinical study of therapeutic neovascularization will move steadily toward safe, improved outcomes in PAD by altering the natural history of this progressive disease via vascular repair and regeneration.

## Acknowledgments

**Sources of Funding**

The authors are supported by National Institutes of Health funding.

## Nonstandard Abbreviations and Acronyms

| | |
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| **ABI** | ankle-brachial index |
| **BM** | bone marrow |
| **CLI** | critical limb ischemia |
| **EPC** | endothelial progenitor cell |
| **G-CSF** | granulocyte-colony stimulating factors |
| **GM-CSF** | granulocyte macrophage–colony stimulating factors |
| **MNC** | mononuclear cell |
| **PAD** | peripheral arterial disease |
| **VEGF** | vascular endothelial growth factor |

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**Table 1**

Clinical Trials of Unselected Autologous BM-MNCs Administered Intramuscularly and Intra-Arterially

| Trials | n | Rutherford Class | Cell Processing Method | EPC Concentration | Delivery Method | Follow-up Duration | Outcomes: Bioactivity Parameters Improved |
|---|---|---|---|---|---|---|---|
| Higashi et al[113] | 7 | Class 4–6 atherosclerosis | CS3000-Plus blood-cell separator (Baxter) | $10^9$ (included $10^7$ $CD34^+$) | IM | 4 wk | Significant improvements in $TcPo_2$, pain-free walking time, acetylcholine-mediated endothelium-dependent blood flow with cell therapy compared with baseline. |
| Van Huyen et al[114] | 8 | Class 4–6 | Cobe 2991 device by centrifugation on a Ficoll density gradient | $10^9$ (included $10^6$ $CD34^+$) (1:1, cell tx:age-or sex-matched control) | IM | 1 y | Active angiogenesis distal to injection site by immunohistochemistry (endothelial cell markers: $CD31^+$, $CD34^+$, $vWF^+$) on pathological amputation specimens after cell therapy compared with age- or sex-matched controls. |
| Motukuru et al[115] | 38 | Class 4–6 TAO | Enrichment in cyclic guanosine monophosphate conditions | $10^7$ (included $10^6$ $CD34^+$) | IM | 6 mo | Significant improvements in ABI, $TcPo_2$, ulcer healing with cell therapy compared with baseline. Potential confounder: all patients achieved smoking cessation. Safety: 1 injected cell sample was later found to have strongyloides infestation. |
| Saito et al[116] | 14 | Class 4–6 TAO | CS3000-Plus blood-cell separator (Baxter) | $10^9$ | IM | 24 wk | Significant improvements in rest pain, ulcer healing with cell therapy compared with baseline. Safety: found no proliferative retinopathy, malignant tumor, MI/CVA, hemangioma, or ectopic bone/adipose formation. |
| Cobellis et al[119] | 10 | Class 4–6 atherosclerosis | Purified of particulate matter using Fenwal Bone Marrow kit collection 4R2104, (Fenwal Inc) | $10^9$ | IA | 12 mo | Significant improvements in ABI, capillary density, pain-free walking distance with cell therapy compared with a control group meeting all inclusion criteria. |
| Ruiz-Salmeron et al[120] | 20 | Class 4–6 | Ficoll density gradient (Amersham Pharmacia Bio) | $10^6$ (also included $10^6$ $CD34^+$) | IA | 1 y | Significant improvements in ABI, wound healing, |

| Trials | n | Rutherford Class | Cell Processing Method | EPC Concentration | Delivery Method | Follow-up Duration | Outcomes: Bioactivity Parameters Improved |
|---|---|---|---|---|---|---|---|
| | | | | | | | blood flow at 3 mo (angiographic) with cell therapy compared with baseline.<br>Safety: found no procedure-related complications or local embolic events. |
| Walter et al[121] | 40 | Class 4–6 | Density gradient centrifugation | $10^8$ (included $10^6$ $CD34^+$) dose escalation: single dose or double dose | IA | 6 mo | Significant improvements in ulcer healing, rest pain with cell therapy in this multicenter, double-blinded, randomized-start clinical trial.<br>No significant improvements found in ABI or limb salvage.<br>Safety: reports procedural safety. |
| Bartsch et al[122] | 13 | Class 3–6 (Fontaine 2b) | Ficoll density separation (lymphocyte separation medium, closed automated SEPAX cell separation CS900, BIOSAFE) | $10^7$ | IA+IM | 13 mo | Significant improvements in ABI, pain-free walking distance, capillary venous oxygen saturation, venous occlusion plethysmography, mean reactive hyperemia, peak flow with cell therapy compared with a control group meeting all inclusion criteria. |
| Franz et al[123] | 9 | Class 4–6 (recommended for amputation) | GenesisCS Component Concentrating System; EmCyte Corp) | Not listed | IA+IM | 3 mo | Nonsignificant improvements in ABI, rest pain, ulcer healing, limb salvage with cell therapy compared with baseline. |
| Franz et al[124] | 20 | Class 4–6 | GenesisCS Component Concentrating System; EmCyte Corp) | Not listed | IA+IM | 3 mo | Significant improvements in Rutherford class with cell therapy compared with baseline.<br>Nonsignificant improvements in ABI, rest pain, limb salvage.<br>Safety: found no procedure-related complications. |
| Van Tongeren et al[125] | 27 | No-option CLI | COBE Spectra Apheresis System (Gambro, Sweden) | $10^9$ BM-MNC (included $10^6$ CD34+ cells) | IA+IM 15:IM 12:IA+IM | 12 mo | Significant improvements for both tx arms in ABI, pain score, pain-free walking distance with cell therapy compared with baseline. |

| Trials | n | Rutherford Class | Cell Processing Method | EPC Concentration | Delivery Method | Follow-up Duration | Outcomes: Bioactivity Parameters Improved |
|---|---|---|---|---|---|---|---|
| | | | | | | | No significant difference in limb salvage rates between tx arms.<br>Safety: reports transient heart failure after BM aspiration in 2 patients. Found no embolic/ leukostatic complications of delivery. |

ABI indicates ankle-brachial index; BM, bone marrow; CLI, critical limb ischemia; CVA, cardiovascular accident; EPC, endothelial progenitor cell; IA, intra-arterial; IM, intramuscular; MI, myocardial infarction; MNC, mononuclear cell; TAO, thromboangiitis obliterans; $TcPo_2$, transcutaneous oxygen pressure; tx, treatment; and vWF, von Willebrand factor..

**Table 2**

Clinical Trials of Unselected G-CSF–Mobilized Peripheral Blood-MNCs Administered Intramuscularly or Intra-arterially

| Trials | n | Rutherford Class | Cell Processing Method | EPC Concentration | Delivery Method | Follow-up Duration | Outcomes: Bioactivity Parameters Improved |
|---|---|---|---|---|---|---|---|
| Ishida et al[126] | 6 | Class 4–6 1 patient atherosclerosis 5 patients TAO | AS104 cell separator (Fresenius Medical Care) | $10^{10}$ (included $10^{8}$ $CD34^{+}$) | IM | 24 wk | Significant improvements in ABI at 4 wk, pain scale at 243wk, walking distance t 24 wk with cell therapy compared with baseline. Safety: found no serious adverse events. |
| Lenk et al[169] | 7 | Class 4–6 | Ficoll density gradient centrifugation | $10^{7}$ | IA | 12 wk | Significant improvements in ABI, $TcPo_2$, pain-free walking distance, flow-dependent vasodilation, flow reserve in response to adenosine endothelium-dependent vasodilation with cell therapy compared with baseline. Safety: found no increase in infection/ inflammation nor significant change in clinical/ serological parameters. |
| Lara-Hernandez et al[128] | 28 | Class 4–6 | Apheresis using Cell Separator Machine CS-3000 PLUS; (Bayer) | Not listed (high $CD34^{+}$ and $CD133^{+}$ rates) | IM | 14 mo | Significant improvements in ABI, pain scale, limb salvage with cell therapy compared with baseline. Safety: found no serious adverse events. |
| Huang et al[129] | 28 | Class 4–6 | Version 4 blood-cell separator (Cobe) | $10^{8}$ (≈0.4% $CD34^{+}$) | IM | 3 mo | Significant improvements in ABI, ulcer healing, laser Doppler blood perfusion, angiographic scores, limb salvage with cell therapy compared with randomized control. Safety: found no treatment-related adverse events. |

ABI indicates ankle brachial index; BM, bone marrow; EPC, endothelial progenitor cell; G-CSF, granulocyte-colony stimulating factor; MNC, mononuclear cells; TAO, thromboangiitis obliterans; and $TcPo_2$, transcutaneous oxygen pressure.

**Table 3**

Clinical Trials of Unselected Autologous BM-MNCs Versus Peripheral Blood-MNCs Administered Intramuscularly

| Trials (References) | n | Rutherford Class | Cell Processing Method | EPC Concentration | Delivery Method | Follow-up Duration | Outcomes: Bioactivity Parameters Improved |
|---|---|---|---|---|---|---|---|
| Tateishi-Yuyama et al[111] | 45 | Class 4–6 | CS3000-Plus blood-cell separator (Baxter) | $10^9$ BM- or PB-MNCs (the BM-MNC infusion included $10^7$ $CD34^+$ cells) | IM gastroc | 24 wk | Significant improvements in ABI, $TcPo_2$, rest pain, pain-free walking time with BM-MNC compared with placebo (pilot study), as well as with BM-MNC compared with PB-MNC (randomized control trial). Safety: found no serious adverse events. |
| Matoba et al[112] | 115 | Class 4–6, 74 atherosclerosis 41 TAO | CS3000-Plus blood-cell separator (Baxter) | $10^9$ BM-MNC vs PB-MNCs as control (the BM-MNC infusion included $10^7$ $CD34^+$ cells) | IM | 3 y | Significant improvements in leg pain, walking distance, ulcer size at 2 y with BM-MNC compared with PB-MNC. No significant difference in detected. ABI or $TcPO_2$ Safety: found iliac artery occlusion in 1 patient with TAO. Remaining nonserious adverse events among patients with PAD are tabulated in article. |
| Huang et al[117] | 150 | Any Rutherford class; arteriosclerosis obliterans | Version 4 blood-cell separator (COBE, BCT, CO, ) | $10^9$ G-CSF–mobilized PB-MNCs (includes $10^8$ $C34^+$ cells) vs $10^8$ BM-MNCs (includes $10^7$ $CD34^+$ cells) | IM | 12 wk | Significant improvements in ABI, skin temperature, rest pain with PB-MNC compared with BM-MNC. No significant difference in $TcPO_2$, pain-free walking distance, or amputation rates detected. Safety: found bone pain and lassitude during G-CSF treatment. Found no cell-treatment–related complications. |
| Kamata et al[118] | 6 | Class 4–6 vasculitic | Centrifugation on Ficoll-Hypaque (Axis Shield) | $10^8$ BM-MNC or PB-MNC (included $10^6$ $CD34^+$ cells in each group). Note: PB-MNCs were not mobilized. | IM | 1 mo | Significant improvements in rest pain (both treatment groups) with both BM-MNC and PB-MNC therapy. |

ABI indicates ankle brachial index; BM, bone marrow; EPC, endothelial progenitor cell; G-CSF, granulocyte-colony stimulating factor; gastroc, gastrocnemius muscle; MNC, mononuclear cell; PAD, peripheral arterial disease; PB, peripheral blood; TAO, thromboangiitis obliterans; and $TcPo_2$, transcutaneous oxygen pressure.

**Table 4**

Clinical Trials of Autologous G-CSF–Mobilized Peripheral Blood-CD34$^+$ Cells Administered Intramuscularly

| Trials (References) | n | Rutherford Class | Cell Processing Method | EPC Concentration | Delivery Method | Follow-up Duration | Outcomes: Bioactivity Parameters Improved |
|---|---|---|---|---|---|---|---|
| Kawamoto et al[130] | 17 | Class 4–6 | Magnetic separation of CD34 using a CliniMACS Instrument (purity verified by FACS) | Dose escalation: $10^5$ $5\times10^5$ $10^6$ | IM | 12 wk | Significant improvements in efficacy score (toe-brachial pressure index, pain scale, total walking distance) with cell therapy compared with baseline. No dose-response effect detected. Safety: found no treatment- related serious adverse events. |
| Losordo et al[131] | 28 | Class 4–5 | ISOLEX 300i Magnetic Cell Selection System (Baxter) | Dose escalation: placebo (cell diluent alone) $1\times10^5$ $1\times10^6$ | IM | 12 mo | Nonsignificant improvement in amputation rates at 12 mo ($P$=0.058) with increased-dose cell therapy compared with placebo control. Safety: found no treatment- associated adverse safety signal. |

EPC indicates endothelial progenitor cell; FACS, fluorescence-activated cell sorter; and G-CSF, granulocyte-colony stimulating factors.