Cell therapy in critical limb ischemia: current developments and future progress

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Abstract
Critical limb ischemia (CLI) is a syndrome manifested by ischemic rest pain, non-healing ulcers and tissue loss. CLI patients are at very high risk of amputation and experience poor physical function, leading to severe morbidity and mortality. The fundamental goal for CLI treatment is to relieve ischemic rest pain, heal ulcers, prevent limb loss and improve the quality of life, thereby extending the survival of the patient. Surgical or endovascular revascularization aimed at increasing blood flow is currently available for limb salvage in CLI. However, up to 30% of CLI patients are not suitable for such interventions because of high operative risk or unfavorable vascular anatomy. Therefore exploring new and more effective strategies for revascularization of ischemic limbs is imperative for the establishment of a viable therapeutic alternative. With the emergence of new approaches, this review describes up-to-date progress and developments in cell-based therapy as a novel and promising alternative for CLI treatment. Preliminary clinical data have established the safety, feasibility and efficacy of stem cells, and numerous studies are underway to consolidate this evidence further. However, significant hurdles remain to be addressed before this research can be responsibly translated to the bedside. In particular, we need better understanding of the behavior of cells post-transplantation and to learn how to control their survival and migration proliferation/differentiation in the hostile pathologic environment. Future research should focus on methods of isolation, optimal dosage, appropriate cell type, route of administration, role of tissue-derived factors and supportive endogenous stimulation.

Key Words: angiogenesis, animal studies, cell replacement therapy, clinical trials, critical limb ischemia, peripheral artery disease, stem cells

Introduction
The term critical limb ischemia (CLI) is used for all patients with chronic ischemic rest pain, ulcers or gangrene in limbs attributable to objectively proven peripheral artery disease (PAD). PAD is associated with several clinical conditions, for example hypertension, cardiovascular disease, hyperlipidemia, diabetes, obesity and stroke. CLI is an advanced form of PAD that is responsible for approximately 100 000 amputations/year in the USA and a major cause of morbidity and mortality world-wide (1). Amputation without revascularization continues to be performed for many patients with CLI. Although CLI is primarily a clinic-based diagnosis, it should be confirmed objectively and early in the disease process through use of the ankle–brachial index (ABI), toe–brachial index (TBI), first toe pressure (FTP), toe systolic pressure or transcutaneous partial pressure of oxygen (TcPO₂). Further, computer tomography (CT) and magnetic resonance imaging (MRI) angiography are important non-invasive modalities in assessing the severity of CLI.

Recent studies have reported that revascularization rates in CLI patients are only 40% (2). Such low rates of revascularization are partly because of the advanced disease condition (3). Over the past decade, advances in percutaneous devices and techniques for endovascular revascularization have rapidly emerged as the preferred primary treatment strategy for CLI (4). Adjunctive pharmacotherapy with antithrombotic drugs, statins and beta-blockers is critical to decrease perioperative cardiovascular complications.
in patients undergoing surgical vascular reconstruction and to enhance post-revascularization arterial and graft patency. Recent studies have reported that 90% limb salvage rates can be achieved with endo-vascular therapy in CLI patients (5) and this therapy could be possible where there is a lesion that is amenable to intervention.

Despite improvements in medical therapy for atherosclerosis and associated co-morbidities, as well as advancements in interventional and surgical techniques to improve limb perfusion, CLI continues to carry a major risk of limb amputation (6,7). A significant portion of patients with CLI are considered to have ‘no option’ for revascularization, and no medical intervention has demonstrated success in reducing the need for amputation. The prognosis of chronic CLI is poor, and effective treatments have not been established in patients who are not eligible for the traditional revascularization therapies, such as angioplasty and bypass procedures, because of the inappropriate anatomy of the leg arteries or frequent re-occlusion following revascularization.

Therefore it is necessary to conceptualize and develop therapies that can induce revascularization and remodel the vascular system to prevent further complications of CLI (Figure 1). The initial impetus towards cell-based therapies originated in the 1990s. Prior to that, it was thought that angiogenesis was the only possible mechanism for new vessel formation. Japanese researchers Asahara et al. (8) and Shi et al. (9) demonstrated that there was another mechanism at work, known as vasculogenesis; this mechanism involves the in situ assembly of endothelial progenitors into capillaries, as opposed to the formation of new capillaries from existing vessels (angiogenesis) (10), originally thought to be independent events occurring at different periods of the life cycle. Moreover, it is now clear that both mechanisms occur in the same tissue environment (11,12). This clarification led to investigators proposing that transplantation therapy could deliver progenitor cells to the ischemic tissue, which may be the answer to what was previously an unsolvable problem.

How can cell therapy be a suitable alternative?

Cell-based therapy promises to be a suitable option for mitigating the major problems associated with CLI. Stem cells have been postulated and established to act via enhancing angiogenesis, integrating to form new blood vessels, providing trophic support via cytokine release and modulating inflammation, including reduction of infection (Figure 2).

Cell therapy offers neovascularization, through angiogenic potency, the ability to induce/secrete several trophic factors by themselves, or via interaction with host cells. The most commonly discussed role of stem cells involves the replacement of cells lost as a result of disease or injury by transplanting cells into the affected region (13,14). Further, angiogenic stimulation by stem cells could be induced from their...

Figure 1. Commonly available modes of treatment for CLI. Various strategies of intervention at different stages of disease progression followed by the emergence of cell therapy as a possible strategy to overcome unsuccessful intervention.
secretion of pro-angiogenic, pro-survival growth factors and cytokines (pro-angiogenic paracrine activity of stem/progenitor cells). Physical contact of stem cell-derived mural progenitor cells, namely pericytes (15), capable of stabilizing and maturing capillaries into arterioles may also trigger angiogenesis. In addition to their role in therapeutic neovascularization, vascular derivatives of stem cells could play an essential role in the construction of vascularized biotissues (multilayer cell sheets or patches) (16).

Another possible mechanism of repair mediated by stem cells is the modulation of inflammatory responses. Stem cells can serve as mediators of immunomodulation during inflammation, based on their trophic activities. The infused human mesenchymal stromal cells (hMSC) secrete immunomodulatory agents that deactivate T-cell surveillance and chronic inflammatory processes (17). Further, allogeneic hMSC can not only suppress graft-versus-host disease (GvHD) but also exhibit a profound anti-inflammatory and regenerative capacity in animal disease models. Animal studies using the bleomycin model have shown that administration of hMSC immediately after exposure to bleomycin is accompanied by a significant reduction in inflammation associated with lung disease (18).

These studies reveal that stem cell therapy is effective in attenuating or reversing the pathology associated with inflammation, and indicate a probable mechanism by which interferon (IFN)-γ/interleukin (IL)-1β levels may repress the host inflammatory response through altering systemic cytokines. It has also been shown that mesenchymal stromal cells (MSC) have beneficial effects on experimental sepsis, possibly by paracrine mechanisms, suggesting that cell therapy-mediated immunomodulation may be an effective adjunct for treating sepsis-related morbidity and mortality (19).

Nevertheless, the extension of cell therapy to clinical practice would necessitate the use of different types of stem cells, and selection of the best source of endothelial progenitors to carry out important functions towards repair and regeneration. A summary follows of the key research focused on identifying the most suitable cellular sources or combinations of sources.

**Classification and source of stem cells**

**Embryonic stem cells**

Embryonic stem cells (ESC) have emerged as a potentially unlimited source of pluripotent stem cells with the additional property of being able to differentiate into endothelial progenitors, which can efficiently complete the process of angiogenesis in ischemic tissue (20). During the last decade studies have demonstrated separate development of vascular components derived from mouse, primate and human ESC (21–26). The ability of ESC to generate reproducibly scalable and enriched numbers of functional human endothelial progenitor cell (EPC)
offers a useful experimental opportunity to study mechanisms of CLI.

During the early stages of embryoid body (EB) development, vasculogenesis takes place through a multistep process in which endothelial cell (EC) precursors differentiate, expand and coalesce to form a network of primitive tubules. Researchers have shown that human (h)ESC differentiate into EC and smooth muscle cells (SMC), both via EB formation and stromal cell co-culture (27–30). Vascular progenitor cells from mouse ESC have been differentiated via EB formation and selected by fit-related receptor tyrosine kinase (Flk)-1 expression (31). Subsequent reports have indicated that ESC can spontaneously differentiate into EC (32) or can be obtained from Flk-1- or Stem cell antigen (Sca)-1-positive cell fractions of ESC (33). ESC-derived EC express endothelium-specific genes and proteins, and are found to form mature vascular, capillary-like structures in two-dimensional (2-D) and three-dimensional (3-D) culture systems (34). Another report has shown that platelet/endothelial cell adhesion molecule (PECAM)-positive cells are located mainly in the center of EB during hESC differentiation. After attachment of EB on culture dishes, PECAM-positive cells were collected by mechanical isolation and transplanted into limb muscles of a mouse hind limb ischemia model (35) to demonstrate their therapeutic angiogenesis in severe ischemia. Other groups have generated SMC from mouse ESC and hESC using retinoic acid (36). Another study has shown the generation of hemangioblasts from hESC in a serum-free and animal feeder-free differentiation system. These cells were tripotent and able to form multilayered blood vessels with functional EC and SMC, along with hematopoietic cells (37). Vascular components derived from hESC have been phenotyped and proven to be functional both in vitro and in vivo. In addition, several groups have shown that EC and SMC placed in an ischemic hind limb mouse model re-organize to form vasculature and improve blood flow (35,37,38).

**Induced pluripotent stem cells**

The advent of induced pluripotent stem cells (iPSC) promised a new way to circumvent some of the therapeutic limitations of ESC and adult stem cells. This includes the deep-rooted ethical concerns with ESC and the question of immunogenicity/allograft rejection for both ESC and adult stem cells. For replacement therapy in CLI, iPSC offer an exciting and additional option as these cells are capable of self-renewal and differentiation into three germ layers like ESC. However, the need for genetic manipulation to reprogram cells via ectopic expression of a select group of transcription factors is a major drawback of this technology (Sry-related high-mobility-group box (Sox)-2, Lin-28 homolog (Lin)-28, Cellular-myelocytomatosis viral oncogene homolog (c-Myc), Octamer (Oct)-3/4, Kruppel-like factors (Klf)-4 and Nanog) (39,40), which raises potential concerns of oncogenicity for the recipients. In order to overcome these limitations, researchers have generated iPSC from fibroblasts and liver cells by using non-integrating adenoviruses transiently expressing Oct-4, Sox-2, Klf-4 and c-Myc (41).

Researchers have succeeded in deriving patient-specific iPSC lines, including adenosine deaminase deficiency-related severe combined immunodeficiency (ADA-SCID), Shwachman–Bodian–Diamond syndrome (SBDS), Gaucher disease (GD) type III, Duchenne (DMD) and Becker (BMD) muscular dystrophy, Parkinson disease (PD), Huntington disease (HD), juvenile-onset, type 1 diabetes mellitus (JDM), Down’s syndrome (DS)/trisomy 21 and the carrier state of Lesch–Nyhan syndrome (42). Further studies have even shown the generation of iPSC from an 82-year-old woman diagnosed with a familial form of amyotrophic lateral sclerosis (ALS) (43). Similarly, one can attempt to isolate iPSC from a CLI patient, which can be used effectively without immunologic challenges in an autologous set-up. Researchers have been able to obtain vascular cells from human iPSC (44,45). Transplantation studies using iPSC derivatives in CLI are rare and have yet to be evaluated, as optimization of their differentiation protocols and elimination of adverse effects such as tumor formation require extensive studies. Exploring the fullest potential of iPSC in this fields faces several hurdles yet to be addressed, such as safety issues, differentiation efficiency, scalability, epigenetic modifications, tumorigenicity and overcoming immune rejection. Nonetheless, patient-specific iPSC can be used for the identification of molecules that can correct affected genetic networks contributing to the development of vascular insufficiency. In this direction, Hmadcha et al. have (46) described the optimal and safe use of stem cells, with particular emphasis on prospective interventions to deal with the challenges generated by immune rejection.

**Adult stem cell-derived EPC from postnatal tissues**

EPC derived from adult stem cells have been identified for use in physiologic and pathologic neovascularization and therapeutic angiogenesis. MSC derived from postnatal tissues differentiate into EC, express angiogenic markers and exhibit the functional properties of endothelium (47). Several tissue-specific stem or progenitor cell types, such as bone marrow (BM) MSC, adipose tissue-derived stem cells (ADSC) and umbilical cord or dental pulp stromal cells (DPSC), have been evaluated for their influence
on restoring blood supply and/or muscle function in ischemic limbs.

BM MSC show an increased vascular endothelial growth factor/pigment epithelium-derived factor (VEGF/PEDF) ratio when cultured under Cobalt (II) chloride (CoCl(2))-induced hypoxic conditions, and VEGF/PEDF expression closely correlates with the degree of neovascularization as well as hypoxia-induced pro-angiogenic activity in BM MSC (48). CXC chemokine receptor type-4 (CXCR-4)-positive cell fractions isolated from BM MSC using paramagnetic microbeads expressed EC phenotypes and showed the formation of capillary-like tubes (49). MSC when cultured with glioblastoma-conditioned medium (GCM) showed angiogenic potential and expressed CD151, VE-cadherin, desmin, α-smooth muscle actin, nestin and nerval/glial antigen-2 (NG-2) in a GCM concentration-dependent manner (50); this led to the conclusion that glioblastoma-based differentiation of hMSC into pericyte-like mural cells contributes to neovascularization. BM cells, when treated with microparticles (MP) and Peroxisome proliferator-activated receptor (PPARα), showed an enhanced expression of EC markers such as VE-cadherin, PECAM-1 and Intercellular adhesion molecule (ICAM)-1 (51). These results clearly emphasize the role of PPAR-α in modulating the angiogenic potential of BM-derived cells to promote the angiogenic propensity of both EPC and EC.

The properties of ADSC towards EC plasticity have steered researchers to investigate their application to angiogenesis. In fact, adipose tissue enlargement is the result of adipocyte hypertrophy, and the recruitment and differentiation of regenerative precursors located in the stromal vascular fraction. The development of a capillary network is also required to ensure adipose tissue remodeling. There are several requirements for the development of well-perfused adipose tissue. First, the vascular pattern existing during embryonic development indicates that the formation of capillary convolutions is a specific, decisive phase in the development of fat lobules. Second, extensive vascularization is needed for adipose tissue to function optionally as a metabolic and endocrine tissue. Third, cells of the adipose lineage have been shown to release potent angiogenic factors that are vital for vessel development (52). In the light of such considerations, researchers realized that ADSC have a strong commitment towards neovascularization through the paracrine effects exerted by angiogenic secretary factors, and this also ensures long-term survival when implanted in vivo (53). However, the mechanisms for this action have yet to be fully explained.

An important study has demonstrated that the non-adhesive serum-free culture conditions of the CD34+ human cord blood (CB) fraction provide effective EPC expansion (54). Most recently, researchers have shown that CB contains more EPC and is functional after expansion (55), and that it is possible to culture large numbers of EC from one unit of CB, thus making potential clinical applications possible. Further research has demonstrated that CXC chemokine ligand-8 (CXCL-8) can stimulate the angiogenic activity of umbilical cord blood (UCB)-derived outgrowth endothelial cells (OEC) (56).

The angiogenic fate of DPSC has been demonstrated through VEGF induction (57). This VEGF-induced DPSC maintained EC-like features, displayed focal organization into capillary-like structures when cultured in a 3-D fibrin mesh, and expressed endothelial-specific markers such as Flt-1 and Kinase insert domain receptor (KDR). Another report showed that CD31+ CD146– side population (SP) cells from porcine dental pulp exhibit highly vasculogenic potential in hind limb ischemia (58). These observations indicate the angiogenic potential of DPSC, which may prove to be a remarkable tool for vascularization of ischemic tissues.

BM mononuclear cells (MNC) promote proliferation of endogenous neural stem cells through vascular niches after cerebral infarction and have the potential of angiogenesis via EC proliferation (59). Thus it is evident that there are several types/sources of stem cells that have the propensity for vascular development and could potentially be useful in the management of CLI. Some of these cells have been used in pre-clinical models as well as clinically to treat this condition. Of these, MSC and MNC have been the most widely used, and the results of some of these studies are discussed in this review.

Stem cell-based therapy for CLI

Possible mechanisms underlying the reversal of ischemic conditions

Several mechanisms have been proposed for the cell-mediated effect in ischemic disease. Epidermal growth factor receptor (EGFR) signaling regulates the release of angiogenic factors in MSC. Transplantation of hMSC with transforming growth factor (TGF)-α activates EGFR, VEGF and different intracellular signaling proteins, including the Phosphoinositide-3-kinase/v-akt murine thymoma viral oncogene homolog (PI3K/AKT) and Mitogen-activated protein kinase (MEK/MAPK) pathways (60). VEGF is a key multifunctional cytokine responsible for spontaneous new blood vessel formation during peripheral ischemia by activating the highly specific tyrosine kinase receptors Flt-1 and Flk-1/KDR. Ischemia generates a strong biologic
such factors into MV may be exploited in regenera-
tive angiogenic program by MV. Further, the angiogenic and anti-apoptotic program (64,65). Microvesicles (MV) trigger activation of the PI3K/AKT signaling pathway and eNOS in target EC by enhancing the protein expression and phosphorylation of v-akt murine thymoma viral oncogene homolog (Akt) and eNOS. MV also induce the expression of the anti-apoptotic protein B-cell lymphoma-extra large (Bcl-xL) in target EC (66). Subsequent blockade of the PI3K/AKT signaling pathway and eNOS prevented MV-induced angiogenesis, suggesting a critical role in activation of the endothelial angiogenic program by MV. Further, MV derived from stem cells/precursors are specifically enriched for certain proteins and transcription factors, suggesting that the existence of a cellular mechanism for selective compartmentalization of such factors into MV may be exploited in regenerative medicine. The cell cycle protein p21Cip1 (p21) regulates cell-cycle progression (67) and inhibits apoptosis (68) of mature EC, suggesting a potential role for angiogenesis (69). Likewise, p21 maintains the quiescence of hematopoietic stem cells (70), thus suggesting that p21 is a common precursor for vascular progenitor cells. Further, researchers have shown that decreased p21 protein levels in mice lacking one p21 allele increase the proliferative capacity of mature EC and are still sufficient to prevent EC apoptosis. Haplo-insufficiency of p21 enhances the number and clonal expansion capacity of EPC and augments adult blood vessel formation in vivo (71,72). In contrast, homozygous p21 deficiency in mice sensitizes EC to apoptosis induction and results in increased EC death during neovascularization (73). These studies suggest that protection against apoptosis is essential to ensure that an increase in neovascularization results from accelerated cycling of mature EC or EPC.

**Enhancement of angiogenesis in vivo**

EPC resident within BM and peripheral blood (PB) can contribute to injury and pathology-induced neovascularization (74,75). This finding has raised the possibility that these resident progenitor cells may be used to restore the damaged EPC in ischemic disease or injury. This finding was supported by subsequent studies in animals and humans suggesting the ability of EPC to home to areas with reduced oxygen supply and induce vasculogenesis and angiogenesis (76,77).

In addition, researchers have demonstrated that MV released by various cell types, such as circulating blood cells and cells of the vessel wall during cell activation by agonists and physical or chemical stress, stimulate in vitro proliferation, migration of EPC and endothelial tube formation (78). Moreover, MV are able to trigger angiogenesis by horizontal transfer of mRNA to human microvascular and macrovascular EC (79). So it can be speculated that the appropriate manipulation of MV will be particularly useful for EPC modulation of angiogenesis. It is also possible that sildenafil therapy could augment the production of angiogenic growth factors, which would facilitate ischemia-induced angiogenesis (80). Sildenafil treatment could make ischemic tissues more sensitive to endogenous angiogenic or arteriogenic stimuli during ischemic stimulation of angiogenic activity. An intramuscular (i.m.) injection of granulocyte–colony-stimulating factor (G-CSF) mobilizes PB MNC into ischemic thighs and legs, bringing a number of EPC directly into ischemic foci where the EPC can initiate angiogenesis (81). Thus G-CSF augments the differentiation of marrow cells into EPC of blood vessels, resulting in early recovery of blood flow in the ischemic tissues. Soluble E-selectin is another candidate molecule that increases EPC migration and incorporation of EPC into capillary network formation (82). Platelet-derived MP promote angiogenesis (83), whereas endothelial-derived MP increase capillary-like structure formation via plasminogen generation (84). MP from apoptotic/activated lymphocytes evoke angiogenesis via up-regulation of adhesion and pro-angiogenic factors (85). Thus MP can be considered to be real vectors of biologic messages, such as induction of angiogenesis or differentiation, through which endothelial, platelet or lymphocytic MP can modulate in vitro and in vivo angiogenic properties of EPC and EC. Overall improvement of EPC differentiation may greatly aid reparative neovascularization.

Emerging evidence has revealed that PPAR-α, a member of the nuclear hormone receptor superfamily, improves nitric oxide (NO)-mediated vasodilatation and induces neovascularization through
a VEGF-dependent mechanism (86,87). NO is a critical angiogenic mediator that regulates several aspects of vascular cell function, including proliferation, migration and maturation (88).

Further, bio-artificial matrices serve as engineered delivery vehicles and temporary matrices to support tissue growth and remodeling of the vasculature during ischemic conditions. Polyethylene glycol diacrylate (PEGDA)-based bio-artificial matrices have been shown to promote cell survival and endothelial tube formation in vitro (89). Successful strategies to utilize the endogenous progenitor pool will require in-depth understanding in order to stimulate the generation of new cells, direct their migration to the damaged area and control their differentiation into the appropriate kind of EPC and EC. In addition, proper integration of the EPC and EC into the newly formed capillaries and blood vessels is essential. Based on the above discussion we envisage co-transplantation of cells along with pro-angiogenic factors may offer the best long-term benefits.

Survival and engraftment of transplanted cells
A major obstacle to employing cell-based therapies for ischemic disease is that transplanted cells must survive in an ischemic microenvironment characterized by low oxygen, glucose and pH. Various techniques, such as metabolic reprogramming, hypoxic culture conditions and angiogenic factors, have been adopted to overcome the poor survival and homing of implanted cells. In addition to these techniques, other methods, such as pharmacologic preconditioning, scaffold usage and physical and chemical stimuli, have been shown to improve the performance of transplanted cells. Treatment of BM-derived angiogenic cells (DAC) with dimethylfumarate (DMOG), an α-ketoglutarate antagonist that induces hypoxia-inducible factor (HIF)-1 activity, results in metabolic reprogramming of cells with increased glucose uptake and intracellular pH and a decrease in oxygen consumption, lactate and reactive oxygen species production (90). Another study has shown that VEGF expression in BM cells is maintained in response to HIF-1, and subsequent transplantation of these cells has shown better results compared with untreated cells in CLI patients (91). Hence this study revealed that the implantation of hypoxia-treated cells maintains angiogenic factors in ischemic patients. It is evident that novel strategies need to be developed for the augmentation of survival and propagation of cells after infusion, which will rely on close cooperation and interaction between scientists and clinicians.

Animal models and pre-clinical studies
Researchers have shown promising and exciting results relating to cell therapy for CLI and suggested that these findings could be effectively translated clinically. This necessitates the use of pre-clinical models to validate laboratory investigations. Therefore the next step has been to construct animal models in order to demonstrate the feasibility of these emerging concepts. It was hypothesized that an ideal experimental model would develop atherosclerosis or ischemic disease as in human beings and show tissue reserves such that adequate numbers of autologous cells with a comparable anatomy may develop (92). Not surprisingly, a variety of models, ranging from mice and rabbits to pigs, has been used. Recent innovations include the use of endovascular methods to create an ischemic limb model.

Asahara et al. (8) first showed, more than a decade ago, that PB EPC can develop into EC. They were able to show an increase in capillary density and blood flow in a mouse model. Other workers have shown that EPC are responsible for postnatal neovascularization by mobilization into the circulatory system and formation of blood vessels in ischemic tissue (93). In a recent study, basic FGF-2 and G-CSF were immobilized in fibrin matrices and co-delivered in combination with BM cells (94). This co-delivery system enhanced therapeutic recovery of CLI in Balb/c mice after 8 weeks of treatment, with 87.2% blood flow recovery and a significant increase in capillary formation, compared with either growth factor delivery or BM cell administration alone.

All of these studies have fuelled speculation that EPC could have a significant impact on the neovascularization of an ischemic limb. While most studies have concentrated on the cardiac ischemia model, others (95) have shown that ex vivo human EPC could cause 60% limb salvage in athymic nude mice consequent to an increase in blood flow and enhanced capillary density. Similar results were obtained using UCB EPC and BM MNC (96). Other studies have suggested that both intra-arterial (i.a.) and i.m. injection of BM MNC have similar effects (97); further intravenous administration of BM MSC induces angiogenesis in the ischemic boundary zone after stroke in rats (98). Animal studies have established preliminary evidence on the safety, feasibility and efficacy of several important endpoints using BM-derived stem and progenitor cells as a potential therapeutic option to induce angiogenesis (99). A body of compelling pre-clinical evidence reveals that transplantation of stem/progenitor cells is safe and effective in animal models of CLI; however, these data need to be evaluated for their translational relevance to clinical applications.
Towards clinical application

Several clinical trials have demonstrated that it is safe and feasible to treat CLI using adult stem or progenitor cells. However, most of the pioneering studies used small numbers of subjects and failed to use a randomized design. The first, and understandably the most discussed, was the Therapeutic Angiogenesis using Cell Transplantation (TACT) study (100). This study had an interesting design in that it combined an initial pilot study with a subsequent randomized controlled phase. In the pilot study, 25 patients with unilateral leg ischemia were injected with BM MNC into the gastrocnemius muscle, while the other unaffected limb was used as a control and injected with saline. The patients who qualified for inclusion had chronic limb ischemia, including those with rest pain, with or without non-healing ulcers, were not candidates for non-surgical or surgical revascularization, and were assessed at 4 and 24 weeks. There were significant improvements in Ankle brachial pressure index (ABPI), pain score and TcPO₂ in the treated limbs. In the randomized portion of the study, 22 patients with bilateral ischemia were recruited and randomly allocated to receive BM MNC or PB MNC. The final assessment was done at 24 weeks, by which time two patients had dropped out of the study. Both arms showed improvement in BPI and TcPO₂, although the improvement in the BM MNC group was significantly better. The authors noted that, according to the Rutherford criteria, 39 of the 45 patients treated had improved and of those 30 showed an increase in BPI of more than 0.1. Since this ground-breaking study, there have been many that have differed in the study design, cell numbers and populations, as well as in the numbers of patients involved. Several recent major studies are discussed here.

The A Prospective, Randomized, Placebo-Controlled Trial of Intra-Arterial Progenitor Cell Transplantation of Bone Marrow Mononuclear Cells in Patients with Peripheral Arterial Occlusive Disease (PROVASA) study conducted in Germany adapted an innovative design employing a double-blind placebo-controlled pilot trial using BM MNC for induction of neovascularization in patients with peripheral arterial occlusive disease (101). Initially 40 patients were randomized with 1:1 i.a. administration of BM MNC or placebo. After a 3-month follow-up period, patients in the placebo arm crossed over to the active treatment arm and, at the same time, patients in the initial treatment arm received a second dose of cells. Thus it was possible to compare the 3-month follow-up of the cross-over group with the potential effects of the double-dose group. This is also one of the few studies to use an i.a. route for cell delivery. The primary endpoint of the study was change in BPI, but this showed no significant difference at follow-up. However, there was a significant improvement in the secondary endpoints, including ulcer healing and rest pain reduction; other secondary endpoints, such as death, limb salvage and amputation-free survival rates, were not significantly affected. The study also showed that patients who already had gangrene or tissue loss did not respond to therapy (102), possibly because they had an advanced, irreversible form of the disease. The study demonstrated that the major predictors of healing were the total number of cells delivered, repeated dose and greater functionality of cells measured as by in vitro assays.

Another major study published simultaneously in the same journal by a Japanese group (103) examined the long-term clinical outcomes of 41 patients with CLI, including 25 patients with PAD and 26 with Buerger’s disease (BD). An additional 46 CLI patients with similar baseline demographic patterns served as controls. This study was non-randomized and did not have a placebo arm. The results showed remarkable improvement in the BM MNC-treated group during a median follow-up period of 4.8 years. The 4-year amputation-free rates in the treated group were 0% for PAD and 6% for BD, compared with 48% and 95% in the untreated groups. The overall survival rates were 76% for PAD and 100% for BD groups of treated patients, while 67% and 100%, respectively, of the untreated patients survived. One significant observation made was that, while there was an improvement in the BPI and TcPO₂ levels in both groups of treated patients, the improvement persisted only in the BD group, while the PAD group gradually returned to baseline levels during a 3-year follow-up. In a perceptive editorial comment, Gupta & Losordo (102) pointed out that the amputation levels in the control group were much higher than one would expect from the literature. They also noted that the control group did not stop smoking and suggested that this might have had major effects on the outcome. However, this study confirmed that improved parameters such as BPI or TcPO₂ are observed only with long-term follow-up in BD patients, whereas PAD patients typically revert over long follow-up periods. One of the major strengths of this study was an in vitro experiment, where the group clearly demonstrated that autologous cells maintained phenotypic characteristics in BD patients, while cells had impaired functional characteristics in the atherosclerotic group. This suggests that the better functioning stem cells in these patients may be responsible for the improved results. We believe that this could also offer an argument for using an allogeneic rather than autologous mode of cell therapy.
Another study reported by a group from The Duke University Medical Centre and the Indiana University School of Medicine in the USA examined the role of autologous BM MNC in CLI (104). This was a phase I non-randomized study that addressed the limitations of the previously discussed studies and recruited 29 patients, of whom most (21) had rest pain and the others had ulcers with or without rest pain. They were treated with i.m. injected BM MNC and observed for amputation-free survival at 1 year along with the primary endpoint, as well as First toe pressure (FTP), Toe-brachial index (TBI), Ankle-brachial index (ABI) and TcPO$_2$. The amputation-free survival was 86.3%; there was a significant increase in the other parameters, and three out of nine ulcers healed completely. The authors are planning a large-scale phase III trial to test the hypotheses that have emerged from this trial. They have also shown that contrast arteriography or MRI angiography are not useful, because they failed to yield any interpretable data. Two serious adverse events were noted, one of which was related to an angiography procedure. The second patient had angina and ST segment depression, and it was determined that this was related to the large volume of BM aspiration and the consequent drop in hemoglobin level. Two deaths were also recorded; one was a suicide because the patient was depressed by the lack of improvement in his condition, and the second was the result of complications following an above-knee amputation. The authors also noted that complications as a result of large-volume BM aspiration have been reported in several studies other than their own, making this an important constraint for autologous therapy.

To our knowledge, the first study to compare MSC with BM MNC for the treatment of diabetic ischemic limbs and foot ulcers was published last year (105). This Chinese study randomized 41 patients, or more properly 82 lower limbs, to receive MSC, MNC and normal saline (NS). Thus 41 limbs were injected with NS, 20 with MSC and 21 with MNC. Two patients each in the MSC and MNC groups were withdrawn from the study because of worsening symptoms. The cells were injected at 20 sites in the gastrocnemius muscle, each injection being 0.5–1 mL. After 24 weeks of follow-up, the ulcer healing was significantly better in the MSC group. In addition, the parameters of limb perfusion (pain-free walking time, ABI, TcPO$_2$ and MRI analyses) were significantly improved in the BM-MSC group compared with its counterparts. However, amputation rates were equal in the two groups. This is probably also the first study to use a randomized design to compare different cell types for this indication. Despite some weaknesses in the design of the study, it is certainly an important contribution to the field.

Unanswered questions

Although sufficient numbers of cells for infusion can be obtained in vitro, and autologous and allogeneic models of transplantation using different kinds of adult stem cells have been tried in multiple ways, limited data are available and even the mode of action still remains elusive (106). Some of the key questions that need to be addressed are discussed below. Nevertheless, we strongly believe that aggressive multimodal therapy with combinations of cells delivered at the affected site, as well as intravascular, timed repeated injections, may result in reducing surgical intervention.

Best choice of cells

The optimal source of cells for regenerative therapy is a major question. A wide variety of cells have been used successfully for the treatment of CLI. These include BM MNC, PB MNC, CD34$^+$ cells, endothelial cells, SMC and MSC. However, there has been no comparison between the different methods and the best therapy is still a matter of debate. It was originally proposed that the cells that were relevant to angiogenesis were the CD34$^+$ progenitor cells. Studies have also shown that MSC as well as cells of myeloid/monocyte lineage can be used for this purpose (107). While some data are available from studies that have compared different types of cells for cardiac ischemia, these studies show no difference between cell types. In any case, the extent of their relevance to skeletal muscle ischemia in the lower limb is a contentious issue.

Autologous or allogeneic cells

The vast majority of published studies have been on autologous cells. A few studies, including Pluristem’s (www.ClinicalTrials.gov (accessed 27 September 2011); Table I), are exploring the use of allogeneic cells. The Pluristem study used placental-derived MSC. Garcia-Olmo et al. (108) showed the use of allogeneic MSC derived from adipose tissue to treat Crohn’s fistula. In subsequent studies, researchers have shown that combined (autologous/allogeneic) therapy is effective against the treatment of ischemic ulcers (109). The Osiris study on acute myocardial infarction has shown the safety of these cells for indications related to angiogenesis (110). It is now well accepted that MSC can be used from allogeneic sources, and the therapeutic use of allogeneic cells has several advantages. There is no need for BM aspiration, which can be a constraint as well as a cause of severe adverse events, as documented by several studies (104,110). It is also possible that the use of MSC from healthy donors will erase the difference in
Table I. Details of registered clinical trials for CLI.

<table>
<thead>
<tr>
<th>Clinical trial identification</th>
<th>Status</th>
<th>Disease indications</th>
<th>Investigational drug/study phase</th>
<th>Patients enrolled</th>
<th>Route of injection</th>
<th>Sponsor</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00956332</td>
<td>Recruiting</td>
<td>PAD; peripheral vascular disease; chronic CLI</td>
<td>Cell suspension of endothelial and smooth muscle cells/phase I and phase II</td>
<td>18</td>
<td>Intra-arterial</td>
<td>MultiGene Vascular Systems Ltd, Israel</td>
</tr>
<tr>
<td>NCT00721006</td>
<td>Completed</td>
<td>CLI; severe leg ischemia; PAD; peripheral vascular disease</td>
<td>Mixture of stem cells/phase II</td>
<td>35</td>
<td>Calf muscle of the diseased leg</td>
<td>TCA Cellular Therapy, United States</td>
</tr>
<tr>
<td>NCT00533104</td>
<td>Completed</td>
<td>Peripheral vascular disease</td>
<td>BM MNC and PB MNC/phase I and phase II</td>
<td>40</td>
<td>Intra-muscular</td>
<td>CHU de Reims, France</td>
</tr>
<tr>
<td>NCT01079403</td>
<td>Recruiting</td>
<td>Diabetes; limb ischemia; PAD; peripheral vascular disease</td>
<td>Autologous AD MSC/phase I and phase II</td>
<td>36</td>
<td>Intra-arterial</td>
<td>FundacionProgreso y Salud, Spain</td>
</tr>
<tr>
<td>NCT00533104</td>
<td>Completed</td>
<td>Peripheral vascular disease; diabetic foot; limb ischemia; leg ulcers</td>
<td>ALDH-br BM cells versus MNC/phase I and phase II</td>
<td>20</td>
<td>Intra-arterial</td>
<td>CHU de Reims, France</td>
</tr>
<tr>
<td>NCT00922389</td>
<td>Recruiting</td>
<td>Diabetic foot; CLI; leg ulcers</td>
<td>G-CSF and PB MNC/phase I and phase II</td>
<td>36</td>
<td>Subcutaneous</td>
<td>Beike Biotech India Pvt Ltd</td>
</tr>
<tr>
<td>NCT00518401</td>
<td>Completed</td>
<td>CLI; peripheral vascular disease</td>
<td>Infusion of MSC (Apceth)/phase I and phase II</td>
<td>30</td>
<td>Percutaneous transluminal angioplasty</td>
<td>Apceth GmbH and Co. KG, Germany</td>
</tr>
<tr>
<td>NCT01351610</td>
<td>Recruiting</td>
<td>CLI; advanced peripheral occlusive disease</td>
<td>Autologous AD MSC/phase I and phase II</td>
<td>36</td>
<td>Intra-arterial</td>
<td>FundacionProgreso y Salud, Spain</td>
</tr>
<tr>
<td>NCT01257776</td>
<td>Recruiting</td>
<td>CLI; diabetes</td>
<td>Autologous BM MNC/phase I and phase II</td>
<td>20</td>
<td>Calf muscle</td>
<td>Aldagen, United States</td>
</tr>
<tr>
<td>NCT00987363</td>
<td>Recruiting</td>
<td>Arterial occlusive disease; diabetic foot; gangrene; ischemia; peripheral vascular disease</td>
<td>Autologous BM MNC/phase I and phase II</td>
<td>60</td>
<td>Intra-arterial</td>
<td>FundacionProgreso y Salud, Spain</td>
</tr>
<tr>
<td>NCT00221143</td>
<td>Completed</td>
<td>Leg pain; ulcer; peripheral vascular disease</td>
<td>Autologous PB EPC (CD34⁺)/phase I and phase II</td>
<td>15</td>
<td>Not found</td>
<td>Translational Research Informatics Center, Japan</td>
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<tr>
<td>NCT01065337</td>
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<td>Diabetic foot</td>
<td>TRC (BM MNC + BM MSC)/phase I and phase II</td>
<td>30</td>
<td>Intra-arterial</td>
<td>Ruhr University of Bochum, Germany</td>
</tr>
<tr>
<td>NCT00468000</td>
<td>Active, not recruiting</td>
<td>PAD; peripheral vascular disease; CLI</td>
<td>Autologous BM cells + electrolyte solution/phase I and phase II</td>
<td>150</td>
<td>Intra-arterial</td>
<td>Aastrom Biosciences, United States</td>
</tr>
<tr>
<td>NCT00919958</td>
<td>Active, not recruiting</td>
<td>PAD; peripheral vascular disease; CLI</td>
<td>Allogeneic PLX-PAD cells (placental MSC)/phase I</td>
<td>15</td>
<td>Intra-arterial</td>
<td>Pluristem Ltd, Germany</td>
</tr>
<tr>
<td>NCT00951210</td>
<td>Active, not recruiting</td>
<td>PAD; peripheral vascular disease; CLI</td>
<td>Allogeneic PLX-PAD cells (placental MSC)/phase I</td>
<td>12</td>
<td>Intra-arterial</td>
<td>Pluristem Ltd, United States</td>
</tr>
<tr>
<td>NCT00523731</td>
<td>Completed</td>
<td>PAD; CLI</td>
<td>Autologous ACP or Vescell TM/phase I and phase II</td>
<td>6</td>
<td>Intra-arterial</td>
<td>TheraVita Ltd, Israel</td>
</tr>
<tr>
<td>NCT01232673</td>
<td>Active, not recruiting</td>
<td>CLI</td>
<td>UC MSC/phase I and phase II</td>
<td>50</td>
<td>Intra-arterial</td>
<td>Qingdao, University China</td>
</tr>
<tr>
<td>NCT01243533</td>
<td>Not yet recruiting</td>
<td>CLI due to PAOD</td>
<td>BMAC/phase II</td>
<td>40</td>
<td>Calf vessels of the ischemic limb</td>
<td>University Hospital, Ostrava Czech Republic</td>
</tr>
<tr>
<td>NCT01019681</td>
<td>Recruiting</td>
<td>CLI</td>
<td>UCBSC/phase I</td>
<td>25</td>
<td>Intra-arterial</td>
<td>Northwestern University, United States</td>
</tr>
<tr>
<td>NCT00956699</td>
<td>Recruiting</td>
<td>Diabetic foot</td>
<td>Autologous BM MSC and BM MNC/phase I</td>
<td>40</td>
<td>Intra-arterial</td>
<td>Third Military Medical University, China</td>
</tr>
<tr>
<td>NCT01019681</td>
<td>Recruiting</td>
<td>Arterial occlusive disease</td>
<td>BMAC/phase I and phase II</td>
<td>60</td>
<td>Ischemic limbs</td>
<td>Harvest Technologies, India</td>
</tr>
<tr>
<td>NCT01019681</td>
<td>Recruiting</td>
<td>CLI; arterial occlusive disease; vascular disease</td>
<td>Autologous CD133⁺ cells/phase I</td>
<td>24</td>
<td>Intra-arterial</td>
<td>University of Wisconsin, Madison, USA</td>
</tr>
</tbody>
</table>

(Continued)
the results obtained in atherosclerotic patients compared with BD, which was speculated to be because of defects in the BM itself. Having said that, it must be admitted that there is still no published report on the use of these cells, whereas autologous cells have had their safety proven for almost a decade.

**Mode of delivery: i.m. or i.a. or combined?**

The rationale for using an i.m. injection has been the creation of a depot of cells with paracrine activity in the ischemic area (99). However, studies done, albeit on cardiac tissues, have shown a very short survival time for these cells. The local depot has been shown to augment vascular tissue by a variety of mechanisms, which include cell differentiation, cell-to-cell contact and other paracrine mechanisms (111). The actual method of delivery has mostly been by multiple injections along a symmetrical grid based on the BM itself. Having said that, it must be admitted that there is still no published report on the use of these cells, whereas autologous cells have had their safety proven for almost a decade.

The passage of the cells in the circulatory system appears to preserve the nutrients and oxygen status and improve the chances of survival, although the uptake from the circulatory system has been shown to be suboptimal (113). A murine study comparing two methods of administration was unable to show much difference (97). However, clinical studies that address this question are few. One study that focused on i.m. versus combined i.m./i.a. administration of BM MNC to address this question looked at 27 patients, of whom 12 were injected with a combination of i.a. and i.m. injections and the rest just i.a. injections (116). This study showed improvement in both groups and was unable to show any difference between the two.

Another clinical study has evaluated the safety and efficacy of i.a. administration of autologous BM MNC in diabetic patients with severe below-the-knee arterial ischemia (117). All the patients presented a notable improvement after 12 months of follow-up, although the clinical manifestation differed among patients. Unfortunately, local injection of cells in the target limb had no beneficial effects. This study concluded that i.a. perfusion of BM MNC is safe, generates a significant increase in the vascular network in ischemic areas, and promotes remarkable clinical improvement in diabetic patients with CLI.

**Dosage: single or multiple?**

The dosages of cells used have ranged from $0.1 \times 10^9$ to $50 \times 10^3$, and positive results have been observed even with the lowest doses (99). Most studies have tended to follow the lead of the TACT study, using around $1.6 \times 10^9$ MNC. There has been one reported study investigating the correlation between the number of MNC injected and outcome (118). The eight patients were divided into two groups, which received either $6.0 \times 10^4$ to $1.58 \times 10^7$/kg or $1.0 \times 0.60 \times 10^6$/kg. There was a strong correlation between the number of cells and BPI that was not so pronounced for the TcPO2 levels. It has been pointed out that the large differences in the numbers of injected cells across various studies may be explained by the different methods used for counting the cells (99,107). Further, it has been suggested that using an automated cell counter may be the cause of higher counts because of the possibility of incorrectly including granulocytes when counting monocytes (99). The PROVASA study has

<table>
<thead>
<tr>
<th>NCT01049919</th>
<th>Recruiting</th>
<th>CLI; PAD</th>
<th>Autologous BMAC/phase I</th>
<th>152</th>
<th>Intra-muscular</th>
<th>Biomet Biologics, LLC, United States</th>
</tr>
</thead>
<tbody>
<tr>
<td>NCT00434616</td>
<td>Active, not recruiting</td>
<td>PVD; diabetic foot; peripheral arterial occlusive disease; leg ulcer; gangrene</td>
<td>Autologous BM cell concentrate; transplantation/phase II and phase III</td>
<td>90</td>
<td>Not found</td>
<td>Franziskus-Krankenhaus, Germany</td>
</tr>
<tr>
<td>NCT00498069</td>
<td>Active, not recruiting</td>
<td>Ischemia</td>
<td>BMAC/phase I and phase II</td>
<td>48</td>
<td>Limb ischemic tissues</td>
<td>Harvest Technologies, United States</td>
</tr>
<tr>
<td>NCT0088370</td>
<td>Active, not recruiting</td>
<td>CLI</td>
<td>BM MSC+/plasmalyte A/phase I and phase II</td>
<td>20</td>
<td>Intra-muscular</td>
<td>Stempeutics Research Pvt Ltd, Bangalore, India</td>
</tr>
<tr>
<td>NCT00616980</td>
<td>Completed</td>
<td>PAD; peripheral vascular disease; CLI</td>
<td>Autologous stem cells (CD34+)/phase I and phase II</td>
<td>28</td>
<td>Intra-muscular</td>
<td>Losordo Douglas, Maryland, USA</td>
</tr>
</tbody>
</table>

Apeth, applied cell therapy; ALDH-br, Aldehyde Dehydrogenase-Bright; TRC, Tissue repair cells; ACP, Angiogenic Cell Precursors; Vescell, trade name; BMAC, Bone-Marrow Aspiration Concentrate; UCBSC, Umbilical Cord Blood Stem Cell; UC, umbilical cord; PAOD, Peripheral Arterial Occlusive Disease; PVD, Peripheral Vascular Disease.
clearly shown that the use of multiple doses (2) can improve outcomes (101). This finding is very important and provides a rationale for using multiple doses in future trials.

Are results better with thromboangitis obliterans compared with atherosclerosis, and if so why?

Several studies have shown that the results of therapy for thromboangitis obliterans (TAO) are better than those for PAD (119). This could be because of the less active BM of the older PAD patients, who frequently also suffer from several other systemic diseases. The only study that has compared treatment results for these two conditions has been discussed above. We believe that using allogeneic MSC may be able to obliterate these differences, at least partially. Obviously this hypothesis must be established in a large clinical trial.

What are the possible adverse events?

Most trials with cell therapy for CLI have shown remarkably good safety data. The only exception is the TAO study, discussed above (119). An editorial comment by Hirsch (1) hypothesized regarding the causes of sudden death reported by Myomi-to’s group (106), for which they suggested two possible causes. First, migration of cells to sites distant from the targeted area may cause unanticipated effects. Second, cell differentiation may not be a fully directed process, and this might cause an atherogenous response as demonstrated in pre-clinical models. The unexpected development of an Arterio venous (AV) fistula reported in this study may be the result of a robust angiogenic response to the therapy. The stem cells may cause proliferation of both arteries and veins, leading to the fistula. However, Hirsch group did not discount the possibility that this unique adverse event may have been a pre-existing condition diagnosed only through the angiographic surveillance conducted by the clinical trial.

Conclusions and future directions

It is clear that stem cell technology is a rapidly advancing field that promises to have a substantial impact on the future treatment of CLI. The novel angiogenic cell-based drug approach aims to prevent the death of cells by inhibiting insult-activated pathologic steps and/or induction of biochemical pathways that induce survival. Exogenous cell transplantation elicits endogenous repair via angiogenesis, offering the complementary advantage of generating cells in unlimited numbers as well as control over fate, cell number, timing and site of infusion. To summarize, while the laboratory results and pre-clinical and clinical trials reviewed here offer encouraging data supporting the potential benefits of cell transplantation in CLI, well-organized studies are required to ensure the safety and efficacy of progenitor cell therapy prior to widespread clinical trials. In-depth research into strategies for optimal cell dosing, cell delivery method, techniques for in vivo cell tracking and outcome of long-term follow-up needs to be undertaken to ensure the safety of potential clinical trials while affording them the best possible chances of success. We propose that several of these key questions still need to be answered by higher quality randomized clinical trials. Above all we call for caution, to ensure the highest standards of safety and scientific precision as this exciting field moves rapidly forward.

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Cell therapy for CLI


