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Concise Review: Cell Therapy for Critical Limb Ischemia: An Integrated Review of Preclinical and Clinical Studies

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ABSTRACT

Critical limb ischemia (CLI), the most severe form of peripheral artery disease, is characterized by pain at rest and non-healing ulcers in the lower extremities. For patients with CLI, where the extent of atherosclerotic artery occlusion is too severe for surgical bypass or percutaneous interventions, limb amputation remains the only treatment option. Thus, cell-based therapy to restore perfusion and promote wound healing in patients with CLI is under intense investigation. Despite promising preclinical studies in animal models, transplantation of bone marrow (BM)-derived cell populations in patients with CLI has shown limited benefit preventing limb amputation. Early trials injected heterogenous mononuclear cells containing a low frequency of cells with pro-vascular regenerative functions. Most trials transferred autologous cells damaged by chronic disease that demonstrated poor survival in the ischemic environment and impaired function conferred by atherosclerotic or diabetic co-morbidities. Finally, recent preclinical studies suggest optimized blood vessel formation may require paracrine and/or structural contributions from multiple progenitor cell lineages, angiocrine-secretory myeloid cells derived from hematopoietic progenitor cells, tubule-forming endothelial cells generated by circulating or vessel-resident endothelial precursors, and vessel-stabilizing perivascular cells derived from mesenchymal stem cells. Understanding how stem cells co-ordinate the myriad of cells and signals required for stable revascularization remains the key to translating the potential of stem cells into curative therapies for CLI. Thus, combination delivery of multiple cell types within supportive bioengineered matricies may represent a new direction to improve cell therapy strategies for CLI. STEM CELLS *2017; 00:000–000*

SIGNIFICANCE STATEMENT

It remains a challenging era for the clinical development of improved cell therapy strategies for critical limb ischemia (CLI). For the first time, we have the capacity to generate the cells to model complete vessel formation from exogenous allocgeneic and or autologous sources using combinatorial delivery of vessel-forming endothelial precursor cells, with pro-angiogenic hematopoietic progenitor cell, and vessel-stabilizing mesenchymal stem cell, within implantable decellularized matricies in vivo. Unfortunately, the morbidity and mortality from CLI remains unacceptably high and the need for well-controlled translational studies in this area cannot be overemphasized. Thus, careful preclinical evaluation of emerging concepts and technologies are critical for the expedited development of cell therapy trials for CLI.

INTRODUCTION

The human body possesses tremendous capacity to heal itself. Central to all regenerative processes are somatic stem cells; rare cells found within every organ that can replace damaged cells or deliver signals that coordinate tissue repair. However, after acute events like heart attack or stroke, the severity of injury can overwhelm the regenerative response; or during chronic diseases such as atherosclerosis or diabetes, relentless damage can exhaust the stem cell pool, leading to diminished regenerative capacity and progressive organ dysfunction.

Critical Limb Ischemia: An Emerging Epidemic

Peripheral artery disease (PAD) is characterized by ischemia in the lower extremities due to narrowing of arteries with atherosclerotic plaque accumulation [1]. Presently, PAD affects 8–12 million individuals in North America and >200 million worldwide. Due to increasing rates of obesity in an aging population, PAD incidence is predicted to double by 2050 [2]. Critical limb ischemia (CLI) is the most severe form of PAD and represents the manifestation of pervasive atherosclerosis attributable to age, smoking, hypercholesterolemia, and diabetes [3]. The Inter-Society Consensus for the Management of PAD [4] estimated that 25% of patients diagnosed with CLI will die within 1 year and an additional 30% will receive limb amputation. Patients typically present with a spectrum of symptoms including pain at rest, non-healing ulcers and tissue necrosis with gangrene [5]. Treatment options for patients are usually limited to surgical arterial reconstruction, endovascular therapy, or limb amputation. Surgical revascularization is not recommended in patients with severe co-morbidity, sepsis/limb gangrene, or in non-ambulatory individuals [6]. Consequently, the use of experimental, cell-based therapies has emerged as a last resort to prevent amputation in patients with no treatment options [7].

In this concise review, we detail the current understanding of stem cells that mediate angiogenic, vasculogenic, and arteriogenic processes within ischemic tissues. We summarize the present status of randomized cell therapy trials (RCT) in CLI patients with rest pain. We further define the emerging concept of "stem cell exhaustion" and provide evidence that autologous cell dysfunction may limit treatment efficacy in patients with atherosclerotic and diabetic co-morbidities. Finally, we discuss areas where recent preclinical advancements, such as the delivery of multiple allogeneic progenitor cell lineages within within bioengineered constructs, can be used to improve the success of future cell therapy outcomes in patients with CLI.

Stem and Progenitor Cells That Govern Vascular Regeneration

During tissue development, growth and repair blood vessel remodeling is governed by distinct but co-operative angiogenic, vasculogenic, and arteriogenic processes [3, 8]. *Angiogenesis*, first described by Folkman in the 1970s [9, 10], is defined as expansion of the vasculature via formation of new capillary networks from pre-existing vessels (Fig. 1A). Angiogenesis includes two broad processes, sprouting and intussusceptive angiogenesis. Sprouting angiogenesis is triggered in tissues with regional ischemia via the activity of hypoxia inducible factor-1 alpha

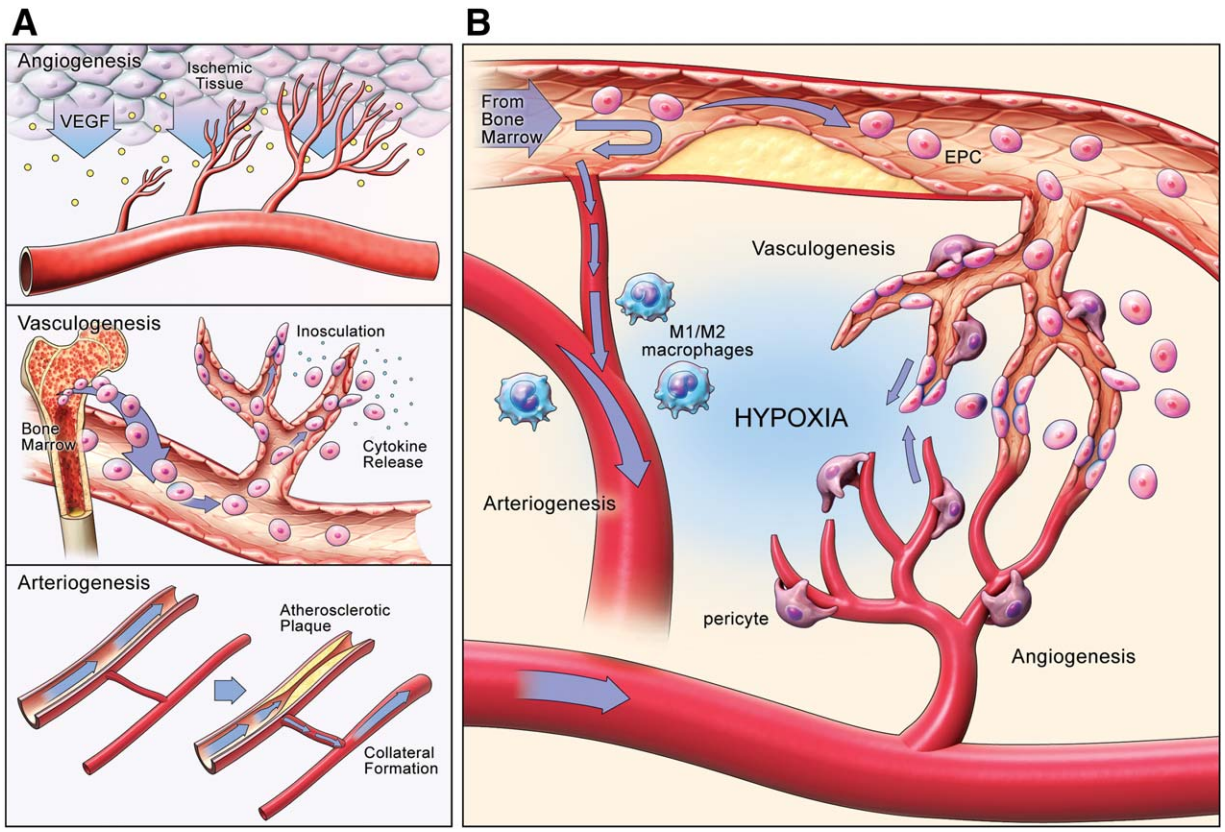

Figure 1. Multicellular mechanisms of blood vessel regeneration. **(A):** *Angiogenesis*, the sprouting or intussusception of new vessels from within pre-existing vessels; *vasculogenesis*, the *de novo* synthesis of new vessels from circulating precursor cells; and *arteriogenesis*, the positive remodeling and use of pre-existing collateral channels, all represent critical processes requiring the co-ordination of circulating and tissue–resident progenitor cells during vessel regeneration. **(B):** Schematic representation of the roles of mesodermal progenitor cell lineages during a co-ordinated, provascular response in vivo. Myeloid hematopoietic progenitor cells secrete angiocrine signals that stimulate *angiogenesis*. Circulating and vessel-derived endothelial precursor cell inosculate into vessel walls during *vasculogenesis*. MSCs differentiate into wrapping pericytes that stabilize vessels and secrete chemokines that recruit accessory cells (M2 macrophages) implicated in *arteriogenesis*. Abbreviations: EPC, endothelial precursor cell; VEGF, vascular endothelial growth factor.

(HIF-1α). Under low oxygen, HIF-1α is stabilized and translocated to the nucleus to orchestrate a cascade of gene expression events including the production of vascular endothelial growth factor (VEGF) and other potent pro-angiogenic cytokines. Collectively, these signals coordinate a well-characterized series of events including sprouting, ECM remodeling, endothelial cell migration, proliferation, and luminogenesis to generate new capillaries (for review see ref. [11]). In contrast, intussusceptive angiogenesis is a dynamic intravascular process initiated by transluminal pillar formation followed by arborization and vascular splitting [12]. Although the molecular control of intussusceptive angiogenesis is less well understood, this process can dramatically modify the microcirculation by pruning or duplicating vessels creating an organ specific angioarchitecture. Highly studied in the context of tissue ischemia and tumor biology [13], angiogenesis is best envisioned as a dynamic process of microvessel advancement and regression to increase capillary density.

Post-natal *vasculogenesis* is described as de novo synthesis of new blood vessels formed by the activities of endothelial precursor cells (EPC) (Fig. 1A). Vasculogenesis was thought to be restricted to the blood islands during embryonic development until Asahara et al. first identified circulating EPC, which homed to areas of ischemia and integrated into vessels [14]. However, the identity and regenerative potential of EPC remained controversial for many years [14–17], due to overlapping phenotype with circulating angiogenic cells (CAC) of hematopoietic origin [18]. Yoder et al. first delineated pro-angiogenic cells of hematopoietic or endothelial lineages using umbilical cord blood [19]. Non-adherent CAC formed early outgrowth (4–7 days) colonies under endothelial culture conditions and co-expressed both endothelial cell (EC) surface markers and the pan-leukocyte marker CD45. CAC have subsequently been described as myeloid hematopoietic progenitor cells (HPCs), or cells of the monocyte lineage that secrete paracrine factors to support angiogenesis without integrating into vessels [20–22]. In contrast, adherent endothelial colony forming cells (ECFC) formed late outgrowth (14–17 days) colonies with cobblestone appearance that did not express CD45 and were clonally distinct from CAC. Circulating ECFCs are extremely rare in peripheral blood, are highly proliferative in culture, and are able to inosculate into vessels after transplantation [19]. Thus, ECFCs represent the true building blocks of blood vessels, and vasculogenesis is mediated by collaboration between circulating CAC and vessel-resident ECFC to maintain vascular health [23–25].

Arteriogenesis describes positive remodeling of pre-existing collateral channels into functional arteries forming a "natural bypass" in the limb (Fig. 1A). Normally, there is little flow in these high resistance collaterals; however, when a major artery becomes occluded, flow to collaterals is increased and arteriogenic remodeling is triggered by recruited monocytes and macrophages that mediate matrix restructuring [26], and stabilized by supportive smooth muscle cells or pericytes that wrap larger diameter vessels. Isolated from perivascular sights in multiple human tissues, Crisan et al. [63] first established that CD146-expressing pericytes fulfilled the criteria defining mesenchymal stem cells (MSCs) [27]. MSCs differentiate into bone, cartilage, and adipose tissues and are described as potent biofactories that home to sites of ischemia [28–31], and secrete a broad spectrum of pro-angiogenic and immunomodulatory factors to support angiogenic [32] and arteriogenic processes [33]. Thus, MSCs represent a third progenitor cell lineage that stabilize vessels and provide secreted cues to support vessel maturation (Fig. 1B).

Cell Therapy Trials for CLI: What Can We Learn from the Historical Perspective?

To date over 50 phase I/II clinical trials have investigated a variety of cell therapies for patients with PAD. Here, we summarize the results of RCT performed on CLI patients with pain at rest (Rutherford score 4–6) due to atherosclerosis obliterans (ASO) rather than thromboangiitis obliterans (TAO) or Buerger's Disease. To focus on studies with reliable statistical power that diligently followed patient outcomes, RCT were only selected if they transplanted >10 patients, and if patient follow-up was 3 months or longer. Rigorous blinding was also required in study design because placebo-effect factors predominantly in cell therapy trials [34]. For a systematic review, and meta-analyses of RCTs and non-randomized trials please refer to Rigato et al., 2017 [35]. The majority of trials used unselected MNC harvested from autologous bone marrow (BM) or from peripheral blood (PB) after G-CSF-stimulated mobilization. In addition, a variety of more homogenous cell types have been studied, including BM-derived cells $CD34^+$ cells, tissue repair monocytes ($CD14^+/CD45^+$), progenitor cells with high aldehyde dehydrogenase (ALDH)-activity, or culture-expanded MSC from autologous or allogeneic BM. Throughout these studies, therapeutic details such as patient selection, cell dosage, and delivery modalities have continued to evolve (Table 1).

Intra-Muscular Transplantation of BM- or PB-Derived MNC

The landmark Therapeutic Angiogenesis by Cell Transplantation (TACT) trial was the first study to demonstrate that intramuscular (IM) transplantation of autologous BM MNC into patients with bilateral CLI could improve ABI and tissue oxygen saturation ($TcPo_2$) compared with autologous PB MNC administered to the contralateral leg [36]. Although the TACT trial did not report amputation rates, a critical parameter of treatment success, follow-up studies by Matoba et al., have shown improved resting pain, pain-free walking time, and ulcer healing at 3-years post-transplantation [37]. Subsequent studies from independent groups collectively established safety and bioactivity with BM MNC showing consistently improved ABI scores, pain-free walking, wound healing, and Rutherford scores when compared with placebo [38–40]. However, overall clinical benefit was considered modest, because with exception of Prochazka et al., the primary endpoint of limb amputation was not significantly improved [38]. Huang et al., and Ozturk et al., reported autologous MNC from G-CSF-mobilized PB also improved ABI and rest pain compared with placebo [44]. Cell harvesting was less invasive and cell numbers accrued were increased compared with BM aspirate, but again amputation rates were similar to placebo [45, 46] (Table 1).

Intra-Arterial Transplantation of BM-Derived MNC

Preclinical cell tracking studies consistently demonstrated only transient engraftment after IM injection, with poor MNC survival and retention in ischemic tissue and little integration into host vasculature [21, 53–55]. Thus, it was postulated that after intra-arterial (IA) delivery, cells would better distribute into zones with sufficient oxygen to prolong beneficial pro-angiogenic function. The PROVASA trial was the first randomized, placebo-controlled trial investigating IA-injection of BM MNC [41], and showed dose-dependent ulcer healing and improved rest pain compared with placebo. Unfortunately, limb salvage did not differ between groups. Recently, the JUVENTAS trial, performed in Utrecht, the Netherlands, and powered with 160 patients, also concluded that IA infusion of autologous MNC did not reduce amputation rates [42]. van Tongeren et al. combined IA and IM-injection to achieve a nonsignificant trend toward lowered amputation rates compared with IM-injection alone, suggesting that development of more specific micro-injection methods, perhaps into geniculate collaterals, was warranted.

IM Transplantation of Marker-Selected Cell Types

Multiple preclinical studies have suggested that transfer of more homogeneous, marker purified cells would increase the proportion of "active" cells that mediate beneficial effects leading to improved perfusion in mice after femoral artery ligation [17, 56–58]. $CD34^+$ cell selection seemed to be a promising approach in the ACT34-CLI (Autologous Cell therapy using CD34 cells for CLI) trial performed by Losordo et al., showing that IM transfer of G-CSF-mobilized CD34+ cells at high dose trended toward lower amputation incidence ($p = .054$) compared with placebo in a small cohort of patients [47]. A similar approach used selection based on high ALDH activity, a protective oxidizing enzyme and highly expressed in progenitor cells of multiple mesodermal lineages [53, 59–62]. Notably, $ALDH^{hi}$ cells highly coexpressed CD34, and accelerated recovery of perfusion in NOD/SCID mice with femoral artery ligation [53]. In a phase I RCT performed by Perin et al, IM-transplantation of autologous BM $ALDH^{br}$ cells improved ABI and Rutherford scores at 6 months, but did not improve limb salvage rates compared with BM MNC. Thus, finding a purified cell population superior to mixed MNC has proven challenging in clinical trials. The RESTORE-CLI trial transplanted autologous "tissue repair cells" (TRC), consisting of a mix of expanded $CD90^+$ MSC and $CD14^+$ cells. IM-administration showed a trend toward increase amputation free survival at 1 year [49]. Finally, the VesCell trial transplanted PB-derived, culture-expanded angiogenic cell precursors showing improved amputation rates at 3 months but no difference at 2 years.

IM Transplantation of BM-Derived MSC

Culture-expanded MSCs have also been pursued as a potential candidate for cell therapy due to beneficial pro-angiogenic and immunosuppressive secretory functions [51, 52, 63]. BM MSC injected into the ischemic hind limb of rats lead to greater perfusion and capillary density increase than BM MNC injection [64]. Comprehensive proteomic analyses have revealed that human MSC-derived exosomes contain a broad spectrum of pro-angiogenic cytokines and micro-RNAs [65], and we recently identified a subset of MSC with enhanced pro-angiogenic secretory function [66]. Compared with BM-derived MNC transplantation in 41 CLI patients with diabetes [51], autologous transfer of MSC significantly improved ABI, pain free walking time, and accelerated ulcer healing. Notably, MSC administration led to significantly increased collateral vessel scores compared with MNC-injection, indicating a link between MSC and the induction of arteriogenesis [51]. In a phase I study of 20 CLI patients, IM-injection of allogeneic BM MSC increased ABI at 6 months compared with placebo [52]. Thus, human MSC represent a promising population for the development of improved, second-generation cell therapies for CLI.

What Has Gone Wrong and What Can We Do Better?

After nearly 2 decades of clinical trials, the field still awaits a pivotal phase III cell therapy trial that improves limb salvage in patients with CLI. Invasive revascularization procedures remain the gold standard option for the treatment for CLI and cell-therapy is reserved only to those patients who are ineligible for surgical procedures. Nonetheless, lessons have been learned and gradual improvements in efficacy have been achieved. In 2013, a meta-analysis of 10 randomized, placebo-controlled trials (499 CLI patients) performed by Peeters Weem et al., showed that cell therapy demonstrated significant improvements in ABI, resting pain, and pain-free walking time, but provided no benefit for amputation rates, amputation free survival compared with placebo controls [67]. Updated in 2017, a similar meta-analysis by Rigatto et al., included 19 RCT (837 CLI patients) concluded cell therapy modestly reduced the risk of amputation by 37%, improved amputation free survival by 18%, and improved wound healing by 59% [35]. Finally, in an uncontrolled study, Madaric et al. [68], analyzed the outcomes of 55 patients transplanted with BM MNC and concluded that responding patients with limb salvage and wound healing (33 of 55) at 1 year were transplanted with significantly higher MNC ($p = .032$) and CD34+ cell dose ($p = .001$) compared with nonresponders that required limb amputation (22 of 55). Therefore, in contrast to cell therapy trials for heart disease [69], the benefits of cell therapy for CLI are clearly evident, and considering up to 50% of CLI patients may not be candidates for revascularization therapies, cell therapy should be considered safe option for improving ABI, rest pain, and ulcer healing. However, it is quite remarkable that efficacy of limb salvage in individual trials has not improved over the past 10 years, highlighting the need for well-designed RCTs with patient numbers sufficient to support statistical analyses. Nonetheless, we have identified the following six "domains" where preclinical advancements could improve future cell therapy trials.

Development of Transplantation Models Relevant to CLI Patients with Co-morbidities

Since Asahara et al. first discovered circulating EPC in 1997 [14], the regeneration of blood vessels using stem cells has

undergone intense preclinical investigation [70]. The surgical hindlimb ischemia model performed by femoral artery ligation in immunodeficient mice has been used for proof-of-concept to establish the efficacy of transplanted human cell populations [71]. Cells were usually administered by intravenous or multiple IM injections proximal to the ligation site, blood flow was monitored noninvasively by laser Doppler perfusion imaging, and vessel formation was quantified in muscle using a battery of immunohistochemical stains. High-impact, preclinical studies suggesting infused BM MNC or selected $CD34^+$ progenitor cells could home to ischemic tissues and improve perfusion [20, 21, 72–77], generated great excitement and perhaps prematurely spawned clinical trials. Unfortunately, acute surgical resection has little resemblance to chronic occlusion during atherosclerosis. Thus, development of improved transplantation models that integrate chronic inflammation, hyperlipidemia, and hyperglycemia comorbities are needed to permit better evaluation of cellular therapies before clinical testing.

Stem Cell Exhaustion Impacts Autologous Cell Regenerative Function in CLI Patients

In end-stage CLI patients, angiogenic, vasculogenic, and arteriogenic mechanisms (Fig. 1) may be severely compromised or in some cases absent. The concept termed "stem cell exhaustion" is generally defined as the acceleration of cellular aging and senescence in adult stem cells and is an emerging concept in preclinical studies associated with ischemic disease of multiple etiologies [78–84]. Although senescence is a normal process during chronological aging due to telomere shortening, cellular aging can be accelerated by accumulation of oxidative damage, reducing self-renewal, and inducing premature differentiation [85]. Chronic atherosclerosis and diabetes in patients with CLI may impact the vascular regenerative niche where oxidative stress, chronic inflammation, glucotoxicity, and lipotoxicity culminate in progenitor cell dysfunction including aberrant proliferation, differentiation, migration, mobilization, or signaling.

In 2001, Vasa et al. were the first to report that the number and migratory function of EPC was severely reduced in patients with coronary artery disease [84]. In landmark articles published in the *New England Journal of Medicine*, Hill et al. reported a strong correlation between the number of circulating EPC and Framingham risk factor score [86], and Werner et al., observed increased levels of circulating $CD34^+KDR^+$ EPC were associated with reduced risk of death from cardiovascular causes, and reduced risk of first major cardiovascular events [83]. Shortly, thereafter, patients with type 1 diabetes [87], type 2 diabetes [79, 82], and metabolic syndrome [80] were all reported to have EPC depletion and impaired function. Atherosclerosis is associated with systemic inflammation and chronic arterial injury that may overwhelm the ability of EPC to maintain homeostasis [88]. Similarly, MSC from patients with atherosclerosis adopt a proinflammatory secretome via increased secretion of IL-6, IL-8, and MCP-1 reversing the normally immunosuppressive nature of MSC [89]. Unfortunately, for CLI patients with diabetic and atherosclerotic co-morbidities, chronic metabolic assault may culminate in a "perfect storm" causing progenitor cell dysfunction that impacts downstream progeny (Fig. 2). As a result, trials transplanting autologous cells to treat CLI may have transferred cells with compromised function. Although HLA-matching will be required, the use of allogeneic cells from healthy BM or UCB may provide alternate sources of progenitor cells less burdened by chronic comorbidities.

Selection of Patient Populations Suitable for Cell Therapy

It is well defined that CLI has several etiologies and ASO patients comprise the majority of the CLI patients [90]. BM cells from CLI patients with atherosclerosis demonstrate impaired function and reduced EPC numbers compared with CLI patients with Buerger's Disease [84, 91]. Thus, autologous cell therapy may be more favorable in CLI patients with Buerger's disease that were excluded from many of the trials in Table 1. Also worth mentioning is the degree of ischemia in patients considered for cellular therapy. Walter et al. demonstrated that patients with Rutherford stage 6 did not respond to cellular therapy compared with patients with Rutherford stage 4–5 [41]. Autologous approaches may be more useful at earlier stages of disease pathology such as in intermittent claudication patients [92], where the delivery of cells may have a greater effect due to the preservation of regenerative potential by both transplanted and recipient-derived cells.

There also exists great heterogeneity between PAD patients in the ability to mediate meaningful vascular repair. This is most evident when assessing arteriogenic bypass mechanisms. Patients with similar severity of arterial occlusion can demonstrate dramatically different impairments in perfusion. For example, a patient with near full occlusion of the superficial femoral artery may appear asymptomatic due to robust generation of geniculate collaterals that connect the deep femoral artery with the popliteal artery, thus providing a natural bypass around the occlusion. While microvascular remodeling by angiogenesis may be sufficient to improve perfusion in the small tissue volume of a mouse hindlimb, the much larger volume of the human leg may require larger conduits to provide adequate flow. Therefore, development of methods to therapeutically target arteriogenic processes is warranted and more attention should be paid to understanding the mechanisms governing this understudied process in preclinical studies.

Purification, Expansion, and Cotransplantation of Cells with Complementary Functions

After $>$15 years of investigation, it remains unclear which cell type and source yields superior benefit on a per cell basis. Cells have traditionally been obtained from either BM or G-CSF mobilized PB (Table 1). In any cell therapy application, it is essential to identify "active" cells that mediate beneficial effects [19, 93, 94]. Early trials for CLI used unpurified, heterogeneous cell preparations with low representation of "active" cells with documented pro-angiogenic functions [36, 38, 95, 96]. MNC contain an extremely low frequency ($<$1%) of "active" cells. The frequency of pro-angiogenic HPC in human BM is $\approx$ 1 in 10,000 MNC, whereas the frequency of nonhematopoietic MSC or EPC is estimated at 1 in 10^6 to 10^7 MNC or fewer. MNC can be efficiently purified by CD34 [47] or CD133 [53] expression, and ALDH-activity also select for progenitor cells with enhanced vasculogenic functions [60]. Preclinical studies aimed at understanding how these cell

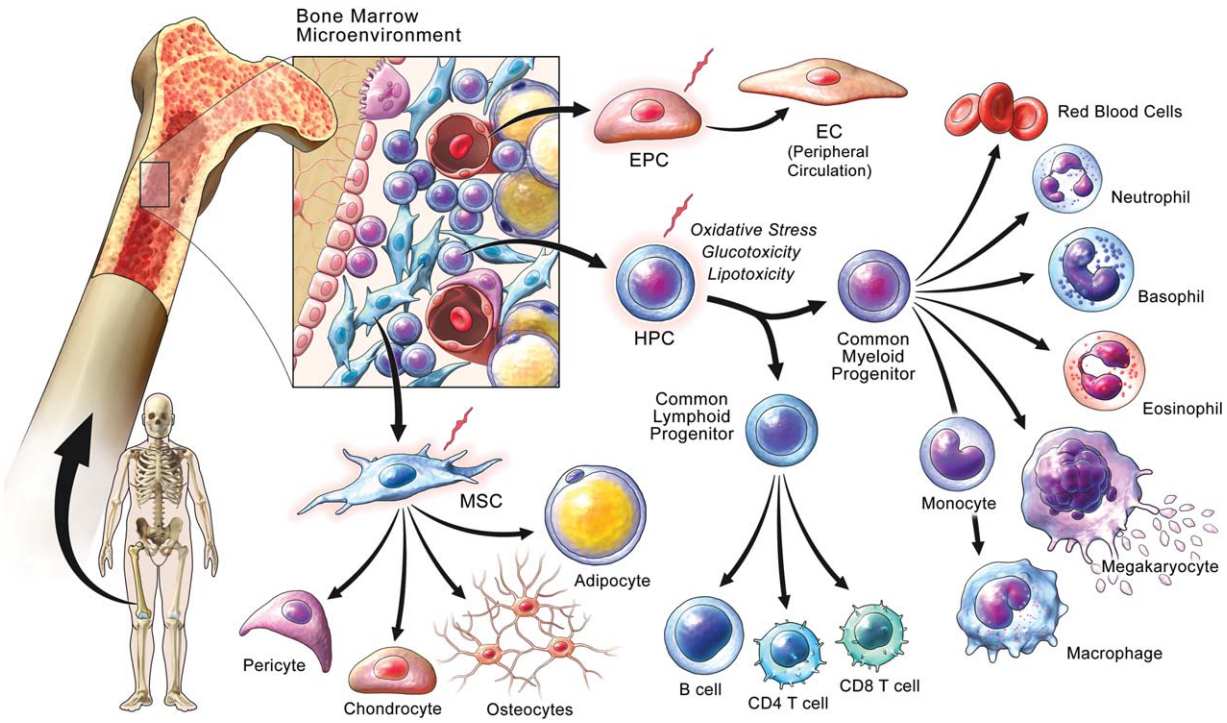

Figure 2. Pro-vascular progenitor cell "exhaustion" in patients with critical limb ischemia. Human bone marrow is a rich reservoir of progenitor cells that co-ordinate blood vessel repair. Myeloid hematopoietic progenitor cells secrete angiocrine signals that stimulate *angiogenesis*. Circulating and vessel-resident endothelial precursor cell act as the building blocks of blood vessels and inosculate into the vessel wall during *vasculogenesis*. Multipotent stromal cells (aka mesenchymal stem cells) generate vessel wrapping pericytes and smooth muscle cells that stabilize newly formed vessels and secrete trophic factors that recruit accessory cells (M2 macrophages) implicated in activation of arteriogenic remodeling and collateral vessel perfusion. Unfortunately, in critical limb ischemia patients with diabetic with atherosclerotic co-morbidities, chronic exposure to oxidative stress, systemic inflammation, lipotoxicity, and glucotoxicity result in regenerative cell depletion and dysfunction within the progenitors cells that formulate a vascular regenerative niche. Abbreviations: EC, endothelial cell; EPC, endothelial precursor cell; HPC, hematopoietic progenitor cell; MSC, mesenchymal stem cell.

types can be combined to formulate a niche for perfused neovessel formation (Fig. 1), remains the key to translating the potential of stem cells into a curative therapy for CLI.

Pro-angiogenic progenitor cells are rare in human BM and UCB and large cell numbers will be required for human therapy. Over the past 10 years, the field has developed clinically applicable (serum-free and xeno-free) expansion media for HPC, EPC, and MSC using defined conditions. Bbioengineering approaches such as "batch-fed" automated systems and large-scale bioreactors have emerged for the safe, robust, and cost-efficient expansion of lineage-restricted progenitor cells [97, 98]. However, extended culture is known to negatively impact regenerative function. Using the established principle that ALDH-expression decreases as cells mature [59, 99], we have used high ALDH-activity to reselect subsets from expanded lineages with enhanced pro-vascular functions [66]. A similar strategy can be employed using cell surface marker expression for CD34, CD133, and CD146, representing HPC, EPC, and MSC, respectively. Concurrently, improved genomic and proteomic screening technologies may be used to identify critical pathways required to promote transplanted cell cross-talk and improve pro-vascular function. Finally, ex vivo expansion also provides a "window of opportunity" to modulate stem cell function using pharmacological agents [100]. Indeed, CLI-mediated dysfunction in MSC can be reverted by the culturing process [101], and augmenting the potency of cells during expansion represents one potential strategy to rejuvenate cells before transplantation [99].

Extracellular Vesicles Contain Potent Pro-Angiogenic Signaling Content

During the past decade, extracellular vesicles (EVs), including exosomes and microvesicles, have emerged as important mediators of cell-cell communication in vascular development, growth, and maturation [102]. EVs contain a number of bioactive molecules including, peptides, proteins, lipids, and nucleic acids (micro-RNAs and mRNA) that act as regulators of EC function to promote or inhibit angiogenesis in a paracrine and endocrine fashion. Although EVs are produced by many cell types, EVs from multiple progenitor cell lineages are released into the peripheral circulation and target distant sites with potent pro-angiogenic stimuli. Mathiyalagan et al. have recently shown that human CD34+ cell-derived exosomes injected into the ischemic hindlimb of mice improved limb perfusion via EC uptake of miRNA-126-3p that suppressed expression of the sprouty-family gene, SPRED1, and simultaneously upregulated VEGF, ANGF1, ANG2, and MMP9 expression [103]. ECFC-derived EVs incorporate into ECs via interaction with α4 and β1 integrins, and stimulate angiogenesis via delivery of mRNA associated with eNOS production and PI3K/AKT pathway [104]. Similarly, MSC-derived exosomes

Table 1. Summary of randomized, controlled trials using cellular therapy for CLI

Reference trial name	Patient population	Cell type and dose	Delivery	No. of subjects	Follow up	Outcomes
Unselected MNC from autologous bone marrow						
Tateishi-Yuyama et al. [36] *TACT Trial*	Rutherford Score 4–6, ABI <0.6	10^9 BM MNC vs. PB MNC (BM $\approx$ 1% $CD34^+$)	40 IM injections	22 patients with bilateral CLI, Group B	6 months	• Improved ABI, TcPO2, rest pain, pain-free walking with BM MNC vs. PB MNC • *Amputation not reported*
Barc et al. 2006 [115]	CLI with rest pain, ABI <0.5	BM MNC, unspecified dose	10 IM injections	29 patients, 14 BM MNC 15 std. care	6 months	• No diff. in ABI or pain • 7/15 amputation in control • 3/14 amputation in MNC
Matoba et al. [37] *TACT Trial*	Rutherford Score 4–6	10^9 BM MNC vs. PB MNC	40 IM injections	74 patients	Up to 3 yrs	• Improved rest pain, ulcer healing with BM MNC • *Amputation not reported*
Procházka et al. [38]	Rutherford Score 4–6, foot ulcers	BM MNC, unspecified dose	40 IM injections (1mL)	96 patients 42 BM MNC 54 std. care	4 months	• Reduced amputation rate ($p<.05$) with BM MNC vs. control
Benoit et al. [39] *BMAC Trial*	Rutherford Score 4–5	10^9 BM MNC vs. blood as placebo	40 IM injections	48 patients 34 BM MNC 14 placebo	6 months	• Nonsignificant trend for reduced amputation rate vs. placebo
Li et al. [40]	ASO with rest pain, ABI <0.5	$5 \times 10^8 - 1.2 \times 10^9$ BM MNC	50–120 IM injections	58 patients 29 BM MNC 29 placebo	6 months	• Improved rest pain, ulcer healing, ABI with BM MNC • *No diff. in amputation rate*
Walter et al. [41] *PROVASA*	Rutherford Score 4–6	10^8 BM MNC single or double dose	IA injection	40 patients 21 BM MNC 19 placebo	6 months	• Improved ulcer healing, rest pain vs. placebo • *No diff. in amputation rate*
Teraa et al. [42] *JUVENTAS*	CLI with rest pain and ulcers	2×10^8 BM MNC or placebo	IA (3X every 3 weeks)	160 patients 81 BM MNC 79 placebo	6 months	• No difference in all cause mortality rate • *No diff. in amputation rate*
Van Tong-eren et al. [43]	Rutherford Score 4–6, no option	10^9 BM MNC	IA + IM or IM only	27 patients 15 IM 12 IA + IM	6 months	• Improved ABI, pain score compared with baseline • *No diff. in amputation rate*
Unselected MNC from autologous G-CSF mobilized peripheral blood						
Huang et al. [44]	Rutherford Score 4–5	10^9 G-CSF MPB MNC vs. placebo	40 IM injections x 2	28 patients 14 PB MNC 14 placebo	3 months	• Improved rest pain, ulcer healing, blood perfusion • *No diff. in amputation rate*
Huang et al. [45]	Rutherford Score 4–6	10^9 G-CSF MPB MNC vs. 10^8 BM MNC	40 IM injections x 2	150 patients 76 PB MNC 74 BM MNC	3 months	• Improved rest pain and ABI with MPB vs. BM • *No diff. in amputation rate*
Ozturk et al. [46]	Diabetic Patients with CLI	10^9 G-CSF MPB MNC	Multiple IM injections	40 patients 20 PB MNC 20 std. care	3 months	• Improved rest pain and ABI with MPB vs. BM • *No diff. in amputation rate*
Marker-selected or expanded cells from autologous bone marrow or peripheral blood						
Losordo et al. [47] *ACT34-CLI*	Rutherford Score 4–5	10^5 or 10^6 G-CSF MPB 34^+ cells per kilogram	8 IM injections	28 patients 7 LD, 9 HD 12 placebo	1 year	• Nonsignificant trend for reduced amputation rate with high dose vs. placebo
Perin et al. [48] *CLI-001*	Rutherford Score 4–5	BM $ALDH^{br}$ cells vs. MNC	10 IM injections	21 patients 11 ALDHbr 10 BM MNC	6 months	• Improved Rutherford Score and ABI at 12 weeks • *No diff. in amputation rate*
Powell et al. [49] *RESTORE*	Rutherford Score 4–6	Ixmyelocell-T (cultured BM MSC and HPC)	20 IM injections	72 patients 42 Ixmyelo-T 24 placebo	1 year	• Improved rates of mortality, gangrene • *No diff. in amputation rate*
Szabo et al. [50] *VesCell*	ABI <0.45 or $TcPO_2 < 40$ mmHg	Expanded PB angiogenic cell precursors	30 IM injections	20 patients 10 ACP	3 months and 2 years	• Improved pain and amputation rate at 3 month • No diff. in amputation rate at 2 years

Table 1. *Continued*

Reference trial name	Patient population	Cell type and dose	Delivery	No. of subjects	Follow up	Outcomes
Expanded bone marrow-derived mesenchymal stem cells (MSC)				10 std. care		
Chen et al. 2009 [116]	Rutherford Score 5–6, Ulcers	BM MSC	30 IM injections	45 patients 22 BM MSC 23 Std. Care	3 months	• Improved ulcer healing, ABI and $TcPO_2$ • *No diff. in amputation rate*
Lu et al. [51]	Rutherford Score 5–6, Ulcers	BM MSC vs. BM MNC	30 IM injections	41 patients 21 BM MSC 20 BM MNC	6 months	• Improved ulcer healing, ABI and TcO_2 in BM MSC • *No diff. in amputation rate*
Gupta et al. [52]	Rutherford 4–6	2×10^6cells per kilogram allogeneic BM MSC	40–60 IM injections	20 patients 10 BM MSC 10 placebo	6 months	• Improved rest pain, ABI • Reduced adverse events • *No diff. in amputation rate*

Abbreviations: ABI, ankle-brachial index; ACP, ● ● ●; ALDH, aldehyde dehydrogenase; ASO, atherosclerosis obliterans; BM, bone marrow; BMAC, bone marrow angiogenic cells; CLI, critical limb ischemia; G-CSF, granulocyte colony stimulating factor; HPC, hematopoietic progenitor cell; IA, intra-arterial; IM, intra-muscular; MNC, mononuclear cells; MPB, mobilized peripheral blood; MSC, mesenchymal stem cells; PB, peripheral blood.

modify EC function via the transfer of platelet-derived growth factor, fibroblast growth factor, EGF, VEGF, Wnt-pathway, and nuclear factor kappa-light-chain-enhancer of activated B cells proteins [65], and via the inhibitory actions of miRNA-31 suppressing HIF-1α [105]. Thus, the therapeutic application of MV-injection is drawing increasing interest for the treatment of CLI.

Refinement of Cell Delivery Technologies to Improve Cell Survival and Function

A critical caveat with any cell therapy trial is the lack of persistence of injected cells in the damaged or diseased tissue. Preclinical survival data in immunodeficient animals suggest most human cell lineages only engraft transiently ($<$7 days) [53, 60], truncating the duration of the regenerative stimulus. As shown in Table 1, most of the trials have used direct IM or IA injection, and both methods are considered sub-optimal since cells might not reach their target site due to compromised vasculature [106]. In addition to studying the site of injected cells, the dosage and frequency of cell injection cells needs further investigation. To date, there are few studies that have addressed cell dosage in humans, and this work will be necessary to optimize the delivery of marker-selected or expanded stem cell populations.

To address the cell survival after transplantation, recent progress has been made using 3D, biodegradable, implantable scaffolds designed to provide anchorage for cells and to support paracrine delivery of pro-regenerative factors into the ischemic region. Decellularized bioscaffolds have generated great interest due to their potential to enhance regeneration [107–109], by creating off-the-shelf scaffolds enriched in structural ECM components [107], which can support cell attachment, infiltration, and constructive tissue remodeling in vitro and in vivo [110–112]. Importantly, in vivo studies have demonstrated that human decellularized adipose tissue scaffolds provided a constructive microenvironment for angiogenesis with no evidence of a negative host response following implantation into immune competent Wistar rats [113]. In fact, these constructs promote the recruitment of endogenous, proregenerative M2 macrophages in scaffolds preseeded with MSC [114]. These emerging delivery modalities may some day represent a novel paradigm shift in the effectiveness of future cell therapies for CLI.

Conclusion

It remains a challenging era for the clinical development of cell therapies for CLI. For the first time, we have the capacity to generate all the cell types required to model complete vessel formation from exogenous allogeneic or autologous sources using combinatorial delivery of vessel-forming EPC, with pro-angiogenic HPC, and vessel-stabilizing MSC, within implantable decellularized matricies. Unfortunately, morbidity and mortality from CLI remains unacceptably high, underscoring the need for translational studies to carefully evaluate emerging concepts and technologies and expedite the development of cell therapies for CLI.

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Author Contributions

M.Q: manuscript conception and design; collection, analysis, and interpretation of data; manuscript writing and editing; D.C.T., S.V., and M.A.-O.: collection, analysis, and interpretation of data; manuscript writing and editing; D.A.H.: manuscript conception and design; collection, analysis and interpretation of data; manuscript writing and editing; final approval of manuscript.

Disclosure of Potential Conflicts of Interest

D.A.H. holds the Sheldon H. Weinstein Chair in Diabetes Research at Western University, Canada. All other authors indicated no potential conflicts of interest.

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