Chapter 6
Angiogenesis: Perspectives from Therapeutic Angiogenesis

Monique Bethel, Vishal Arora, and Brian H. Annex

6.1 Introduction

Systemic atherosclerosis remains the number one cause of morbidity and mortality worldwide. Peripheral arterial disease (PAD) is one form of systemic atherosclerosis, and PAD alone is estimated to affect over 200 million people worldwide [1]. PAD is defined as reduced ankle-brachial blood pressure index (ABI). Smoking and diabetes are the major risk factors for PAD, and the prevalence of PAD rises sharply with advancing age [2]. PAD is a systemic disease, and its presence raises the risk of disease in other vascular beds including the coronary arteries and renovascular and cerebrovascular system [2]. Thus, PAD affects both legs and life. In patients with PAD, symptoms may manifest as intermittent claudication, which is defined as exertional pain in the lower extremity, typically in the calf, that is relieved with rest. However, many patients with significant vascular obstruction do not have classic symptoms or even any symptoms at all. In such patients with PAD, the initial clinical manifestation of the disease may be critical limb ischemia (CLI) where patients are at a very high risk for amputation and stroke. Mainstays of medical therapy for PAD include antiplatelet therapy and optimal control of other risk factors for PAD including hypertension, diabetes, and hyperlipidemia [3]. Tobacco use is a stronger risk factor for PAD than for coronary artery disease [4]. Patients that are smokers should be aggressively encouraged to quit. Currently, there are few proven medical therapies that treat symptoms of PAD and improve exercise capacity. Cilostazol is a phosphodiesterase-3 inhibitor that has weak antiplatelet and arterial dilating properties and is one of the few medications shown to improve symptoms and functional capacity in patients with PAD [3]. Unfortunately, the side effect profile of the medication leads to discontinuation in a substantial number of patients, and studies of cilastazol were conducted when

M. Bethel · V. Arora · B. H. Annex (✉)
Division of Cardiology and Department of Medicine, Medical College of Georgia at Augusta University, Augusta, GA, USA
e-mail: BANNEX@augusta.edu

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baseline medical therapies for patients with PAD were limited [3]. A structured exercise program, involving repeated exercise to submaximal claudication, has also been shown to improve exercise capacity [3]. For patients who are symptomatic despite optimal medical therapy, or those that have progression to symptoms at rest, non-healing ulcers, or gangrene, the treatment options are endovascular therapies or bypass surgery [5]. The development of critical limb ischemia is a poor prognostic indicator, both for the affected limb and overall mortality. Dormandy et al. found yearly all-cause mortality rates of 10–20% in this population [6], and Fridh et al. showed 3-year combined incidence of death or amputation in patients with critical limb ischemia was 48.8% [7]. The large public health burden and limited treatment options for PAD have spurred research into alternative therapies, one of which is stem cell therapy.

The hope of stem cell therapy was enormous: what if stem cells could be taken from a patient and put into an ischemic limb to promote revascularization? This would be an attractive option, as there would be no problems with rejection and the cells could potentially integrate and function for long periods. Despite promising findings in numerous small studies, the results of large studies have been largely disappointing. This chapter will review the background of stem cell therapy in PAD, important research studies, and the current status of this therapy as a treatment option for PAD.

6.2 Embryologic Origins

As the human embryo grows, one of the first organ systems to develop is a circulatory system to support necessary biological functions [8]. This occurs via two processes: vasculogenesis, which is the development of new blood vessels de novo, and the other is angiogenesis, which is the formation of new blood vessels from those already in existence [9]. In vasculogenesis, hemangioblasts, which are precursors to hematopoietic stem cells and endothelial cells, form conglomerations of cells called blood islands under the influence of fibroblastic growth factor. The hemangioblasts in the center of these islands differentiate into hematopoietic cells, and the cells on the periphery differentiate into angioblasts [9]. The angioblasts on the periphery form vacuoles that coalesce, undergo liquefaction, and ultimately form the lumen of the blood vessel. Eventually, these peripheral angioblasts terminally differentiate into endothelial cells [9].

Subsequent development of the circulatory system proceeds as angioblasts migrate and then fuse to form new vessels or merge with small capillaries to form branches or a capillary network [8]. The primitive capillary network then forms into larger arteries and veins, a complex process that is due to hemodynamic and local influences [10]. The big picture of embryonic circulatory development is that an initial vascular plexus is formed and remodeled many times over [8]. A more fixed adult pattern emerges, and endothelial cell proliferation, which is active in the fetus and infant, becomes quiescent in the adult [8]. However, the adult still maintains a population of cells with the ability to form new blood vessels, which might be required during wound healing or may be pathologically involved in the development of tumors or malignancies. Importantly, the skeletal muscle has satellite cells which have the capacity to form myocytes and endothelial cells [11].

6.3 Bone Marrow Mesenchymal Cells

As a topic, stem cell therapy is inclusive of a host of distinct cells. For example, the bone marrow contains two main categories of stem cells: hematopoietic stem cells and mesenchymal stem cells (also known as stromal cells, MSCs) [12]. Hematopoietic stem cells give rise to the cellular components of blood, i.e., erythrocytes, lymphocytes, platelets, etc. MSCs are a multipotent cell line that can differentiate into the bone, fat, muscle, and also blood vessels. In adults, these cells can be extracted from the bone marrow or peripheral blood. Whole blood or bone marrow is placed into a solution, and after several minutes of centrifugation at high speeds, red blood cells and platelets fall to the bottom, and a mononuclear cell layer rises to the top [13]. Bone marrow MSCs (BM-MSCs) or peripheral blood MSCs (PB-MSCs) can be found in the monocyte fraction of cells separated by a density gradient. This layer can easily be extracted and put into culture or injected into a patient as a means of therapy. In cell culture, the cells may be driven down a certain differentiation pathway based on exposure to cytokines or growth factors, or potentially modified. Oswald et al. were able to grow endothelial-like cells in culture after exposing BM-MSCs to vascular endothelial growth factor (VEGF) [14]. Beyond the potential of BM-MSCs to differentiate into endothelial cells, there is evidence that these cells also secrete vascular growth factors such as VEGF [15], fibroblast growth factor, and hepatocyte growth factor [16]. These characteristics made BM-MSCs an appealing option for study in the treatment of PAD.

While attractive for study, there are limitations to this approach. First, there is patient-to-patient and preparation-to-preparation variation in the cells and their characteristics. The manner in which the cell is delivered is another variable. The major limitation of this approach is that the fate of the cells after delivery is unknown [17].

6.4 Vascular Endothelial Growth Factor

The direct delivery of cytokines as protein or gene has been studied in PAD. Vascular endothelial growth factor (VEGF), perhaps the most extensively studied angiogenic agent, is a cytokine first described by Senger in 1983 [18]. It was found to markedly increase vascular permeability, promoting ascites formation in rodent species with cancer. Over time, several unique VEGF proteins have been discovered, including VEGF-A through E and placental growth factor. Each of these genes are encoded from different chromosomes, and within each gene, splice variations are also found [19]. The different VEGF proteins preferentially activate receptors VEGFR-1 and VEGFR-2 which promote angiogenesis or VEGFR-3 which promotes lymphangiogenesis [19]. VEGFR-2 is considered the dominant VEGFR in post-natal angiogenesis, and activation of VEGFR-2 increases signaling through the PLCγ-PKC-MAPK pathway to cause endothelial cell proliferation [19]. VEGFR activation has been exploited to promote angiogenesis in animal models of PAD. Most studies of gene therapy for PAD have involved different isoforms of VEGF [20], and many were

small Phase I trials studying safety and Phase II trials looking at efficacy. As will be shown with stem cell therapy, progress in gene therapy has been limited by many small studies that show benefit in some outcomes, but large, randomized, placebo-controlled studies with positive findings are rare. In the RAVE trial, patients were randomized in a double-blind, placebo-controlled study of VEGF gene therapy in patients with severe, life-limiting intermittent claudication in a single limb, and it represented one of the first larger trials of this experimental therapy [21]. Patients were randomized to receive a low-dose intramuscular (IM)) injection of adenoviral VEGF121 (n = 32), a high dose of adenoviral VEGF121 (n = 40), or placebo (n = 33). The primary endpoint of this trial – change in pain-free walking time – was not met. Similarly, secondary outcomes, including change in ABI and claudication onset time, were not different between the three groups at 12 and 26 weeks. Amputations occurred rarely during the period of observation, with one occurring in the placebo group at day 114 and in the low-dose group at day 293. Over the ensuing years, different vector constructs for delivery, different isoforms of VEGF, or different routes of administration would be tested (Table 6.1).

Table 6.1 Comparison of two of the major clinical classifications of PAD

Fontaine Classification		Rutherford Classification			
Grade	Symptoms	Grade	Category	Clinical Symptoms	Objective Criteria
Stage I	Asymptomatic	0	0	No symptoms	Normal treadmill or hyperemia test
Stage II	Mild claudication		1	Mild claudication	Can complete standard treadmill exercise test. Ankle pressure after exercise >50 mmHg but at least 20 mmHg lower than resting value
Stage IIA	Claudication at a distance >200 m	I	2	Moderate claudication	Between categories 1 and 3
Stage IIB	Claudication at a distance <200 m		3	Severe claudication	Cannot complete standard treadmill testing. Ankle pressure <50 mmHg
Stage III	Rest pain	II	4	Rest pain	Resting ankle pressure <40 mmHg, toe pressure <30 mmHg, flat ankle or metatarsal pulse volume recording
Stage IV	Necrosis and/or gangrene	III	5	Minor tissue loss-focal gangrene, non-healing wound	Resting ankle pressure <60 mmHg, toe pressure <40 mmHg, flat ankle or metatarsal pulse volume recording
			6	Major tissue loss extending above the transmetatarsal level; foot not salvageable	Same as category 5

6.5 Fibroblastic Growth Factor

When compared to the VEGF systems, the FGF system is far more complicated with more than 20 different ligands and receptors [22]. Moreover, the FGF systems acts in concert with VEGF and platelet-derived growth factor (PDGF). Specifically, FGF has been shown to activate VEFG pathways, and murine endothelial cells lacking FGF signaling have been shown to become unresponsive to VEGF [23]. FGF also increases expression of the PDGF receptor on vascular smooth muscle cells, which plays a role in physiologic angiogenesis as well as pathophysiologic atherosclerosis [24]. Due to these complex interactions, the precise role of FGF in angiogenesis has yet to be elucidated. Several trials evaluated the safety and efficacy of FGF gene therapy in the treatment of PAD. There was some evidence of benefit, such as in the TRAFFIC trial, a randomized, placebo-controlled trial of recombinant FGF-2 administered intra-arterially (IA) in a single dose or two divided doses [25]. The administered dose was 30 μg/kg, which was the highest dose injected into the coronary arteries before causing hypotension in other studies. At 90 days, there were significant increases in pain-free walking time (PWT) in both treatment groups with no significant increase in PWT in the placebo group. ABI also significantly increased in the treatment groups compared to placebo. However, at 180 days, PWT increased in the placebo group to levels similar to the treatment groups. Other FGF studies (using gene based delivery) have shown a significant decrease in rest pain [26], but inconsistent findings on reduction of amputations [27, 28], and there was no mortality benefit shown [27, 28].

6.6 Hypoxia-Inducible Factor 1-Alpha (HIF-1α)

HIF-1α is a "master" transcription factor that is highly conserved across species [29] and is expressed on numerous cell types. HIF-1α is responsive to states of cell injury and exerts this effect by regulating cell metabolism and survival of cells in conditions of hypoxia [30] by transcriptional regulation of many proteins, including erythropoeitin [31] and other genes involved in glucose metabolism [29]. It has been called a master regulator because its expression leads to upregulation of a host of other cytokines including VEGF, PDGF, angiopoietin, and SDF-1 [32]. It is expressed in BM-MSCs, and in addition to the roles described above, HIF-1α appears to regulate the migration of BM-MSCs to areas of ischemia or tissue damage through expression of SDF-1 [33]. This led to trials of HIF-1α as a therapy in patients with PAD, especially after the lackluster results in prior trials focusing on VEGF gene therapy. Creager et al. studied adenovirus supplemented with the herpes virus transactivator to locally overexpress HIF-1α in ischemic muscle tissue of patients with intermittent claudication [32]. There was no increase in pain-free walking time (PWT), which was the primary outcome in this study.

6.7 Hepatocyte Growth Factor (HGF)

Despite its name, this cytokine was chosen for study in PAD due to its potent angiogenic properties. In the early 1990s, it was shown to induce endothelial cells in culture to form tube-like structures [34], to stimulate endothelial cell proliferation and migration [34], and to release pro-angiogenic factors [35]. This cytokine is expressed by adult BM-MSCs [36] and also has anti-thrombotic and anti-fibrotic properties [37]. There were several clinical trials with HGF delivered intramuscularly via plasmid. All were Phase II trials, and most were small; the largest trial included 79 participants. Depending on the outcome studied, each trial had some positive outcome and often in the primary endpoint, but no outcome was consistent across all the trials. Only one study showed significant improvement in the ABI [38]; others showed improvement in rest pain [38, 39], ulcer size [38, 39], and QoL [39]. For the more concrete outcomes such as amputations, the data were more discouraging, with one study showing no difference between the groups [39, 40]. In other studies, rates of amputations were not reported [38].

6.8 Early Studies of Stem Cell Therapy

In a landmark study in 1997, Asahara et al. isolated human angioblasts from peripheral blood [41]. Using the cell surface marker CD34 to isolate the progenitor cells, the isolates were grown in culture for several weeks. CD34 is a cell surface marker that identifies a progenitor cell that may differentiate into several different cell types, including hematopoietic cells and endothelial cells [42]. With time, the cells were observed to form networks and tube-like structures in culture. The investigators took these findings one step further and injected these human cells into athymic mice with hind-limb ischemia induced by femoral artery ligation. Histological examination of the tissue several weeks later showed the human cells had been incorporated into the capillary walls of the affected limb. The human cells did not appear in the normal limb. In an additional experiment using rabbits [41], CD34+ cells were isolated from the peripheral blood of the animals, grown in culture, labeled, and then given back to the animals after hind-limb ischemia induction. Again, the labeled cells were found in areas of active revascularization.

Further work has shown that in addition to CD34 expression, expression of AC133 [43] and the VEGF-2 receptor more specifically identifies an endothelial cell precursor [43, 44]. As the progenitor cell terminally differentiates, expression of AC133 diminishes, and the cells begin to express adhesion molecules and to produce nitric oxide [45].

Other investigators isolated endothelial progenitor cells from bone marrow in animals [46]. Shi et al. used a bone marrow transplantation model in dogs, where bone marrow cells from a donor animal were injected into a recipient animal [47]. The endothelial progenitors were identified by possessing cell surface markers for

CD34, von Willebrand factor, and low-density lipoprotein. The bone marrow cells were then injected into another dog treated with immunosuppressant therapy to prevent graft-versus-host disease. Further, an impervious Dacron graft was placed in the descending thoracic aorta. As the graft was impervious, there could be no ingrowth of native capillaries from the surrounding tissue. After 12 weeks, the graft was stained for endothelial cells, and it was observed that only donor cells were identified in the graft material, signifying that these endothelial progenitors were able to migrate from the bone marrow to the peripheral circulation and incorporate into sites of vascular tissue.

Preclinical cell therapy studies such as these paved the way for clinical trials in humans with the goal that if an isolation and production process could be replicated, then stem cells derived in this fashion could represent a novel therapeutic approach to the treatment of PAD. As the cells would be derived from the patient (i.e., autologous), there would be no immunological phenomena which would result in rejection and destruction of the cells. Theoretically, the advantage to this strategy over gene therapy would be the potential of the cells to maintain local levels of angiogenic factors and to be incorporated into new vessels. Still, to this day, direct evidence for this effect is lacking.

6.9 Trials of Stem Cell Therapy in Humans

6.9.1 Bone Marrow Mesenchymal Stem Cells (BM-MSCs)

One of the first human studies of stem cell implantation for treatment of PAD was the TACT trial conducted by Tateishi-Yuyama et al. in 2002 [48]. The TACT trial included two groups of patients: Group A had unilateral limb ischemia, and Group B had bilateral limb ischemia. Both groups required an ABI less than 0.6 in the affected limb, rest pain, and/or, a non-healing ulcer and were deemed not amenable to surgical treatment. Group A received an injection of BM-MNCs in the affected limb, and the contralateral limb was injected with normal saline. In the second group with bilateral ischemia, half of the limbs were randomized to receive BM-MNCs, and the other half received an injection of peripheral blood mononuclear cells (PB-MNCs), which had been noted to contain only 1/500th the concentration of endothelial cell precursors [48]. Outcomes were measured at 4 and 24 weeks following the injections. There were three primary clinical outcomes of this trial including change in ABI, transcutaneous oxygen saturation (TcO_2), and resolution of rest pain. All of the primary outcomes in this trial were met with significant increases in ABI and TcO_2 and reduction in rest pain. The secondary outcomes assessed included new collateral vessel formation which was measured with digital subtraction angiography (DSA) and pain-free walking time (PWT). Collateral vessel formation was described on a scale from 0 to 3, with 0 being no collateral vessel formation and 3+ being “rich” collateral vessel formation. On average, new

collateral vessel formation in group A was graded 1 and 1.1 in group B. For those in group B who received PB-MNCs in one limb, there was less robust formation of collateral vessels in that limb compared to the limb injected with BM-MSCs.

Safety was a critical focus of study. There were two deaths in Group A with unilateral ischemia. The cause of death was determined to be myocardial infarction in both patients and was considered unrelated to the treatment. There were no reports of edema or pain at the injection sites for up to 72 hours following the procedure. The safety outcomes of these trials will be discussed later in the chapter.

The TACT trial provided evidence of the safety and efficacy of this strategy for treatment of critical limb ischemia in patients who were not candidates for surgical revascularization and opened the door for a multitude of studies further examining this method. This line of investigation started with several studies that examined intra-arterial (IA) and/or intra-muscular (IM) administration of BM-MSCs.

In another small pilot trial, seven patients with CLI were treated with BM-MSC using the same technique described by Tateisi-Yuyama [49]. The primary outcomes of this trial included change in the ABI, PWT, TCO_2, and leg blood flow (LBF), measured at 4 and 24 weeks after the injection. LBF was measured by plethysmography, a noninvasive technique that measures changes in volume in a segment of the body [50]. There were significant increases in TcO_2, pain-free walking time, and LBF at 4 weeks. ABI increased as well, but this change did not quite meet statistical significance. At 24 weeks, there was no significant difference in the measured variables compared to baseline with the exception of PWT, which was still significantly increased at 24 weeks compared to baseline measures at 24 weeks. Endothelial dependent vasodilatory response to acetylcholine was enhanced in the group that received the bone marrow cells, compared to a control group of patients with leg ischemia that did not. This indicated that BM-MSCs may also improve endothelial function in this patient population.

Cobellis et al. studied 19 individuals with critical limb ischemia as defined by the Fontaine classification system [51]. Fontaine stage III or IV PAD includes the presence of rest pain or ulceration and/or gangrene (Table 6.1) [52]. The control group consisted of nine patients who were clinically similar to the treatment group but did not undergo the experimental treatment for "personal reasons." The treatment group received two infusions of BM cells that were filtered for large particles but were otherwise non-selective. A second infusion was given 45 days later. Outcomes were measured at 6 and 12 months and included perfusion as measured by laser Doppler flowmetry assessed under several conditions as well as capillary density and neoangiogenesis (new capillary formation). Perfusion was significantly increased at 6 months with the exception of perfusion measured with the leg in a lowered position. These changes largely persisted at 12 months. There were no significant changes in capillary density or enlargement, but there were significant increases in neoangiogenesis at 6 months in the tibia, foot, and toe. Only neoangiogenesis at the toe remained significant at 12 months. The majority of patients, 80%, also had clinical improvement with increases in the pain-free walking distance.

Several years later, a study of diabetic patients with severe limb ischemia with BM-MSCs administered once intra-arterially was undertaken [53]. These patients

showed improvement in ABI, wound healing, and symptoms. This study also included an angiographic evaluation at 3 months with novel findings of two patterns of neovascularization: one pattern consisted of increased branching of the existing vessels, and the other pattern showed an increase in the diameter of the existing vessels. Though unrelated to the experimental therapy, mortality remained high in this small cohort, with 4 of the 20 patients dying in 1 year. The overall amputation rate was high, with seven patients having minor amputations, though most occurred before the BM injection.

So far, the studies described to this point have demonstrated efficacy on multiple fronts as well as an acceptable safety profile. However, the sample sizes remained small and the target patient population highly selected. Additionally, outcome measures were inconsistent.

Franz et al. conducted a study of patients with severe PAD in whom the only viable treatment option remaining was amputation [54]. Patients received BM-MSCs intramuscularly and intra-arterially and were followed for 3 months. Though the study was small, the patient sample was interesting in that the sample was high risk, not only in terms of the ischemic limb but also in the presence of comorbidities: eight of the nine patients were smokers, seven were diabetics, four had previously suffered strokes, four had concomitant coronary artery disease, and all had hypertension. The primary outcomes were ABI measurements, major or minor amputations, symptoms (rest pain), wound healing, and amputations.

There were no significant differences in ABI at 3 months compared to baseline. Minor amputations occurred in two patients, and three patients ultimately needed major amputations; however, the authors cite three examples in which the patients required a less extensive amputation after treatment than would have been done without treatment. Of the six patients who did not require major amputation, five did not have rest pain at follow-up. There was complete ulcer healing in three patients. Overall, eight of the nine patients derived some benefit from the experimental therapy. This was one of the first trials of this particular therapy in the United States. These investigators continued recruiting additional patients and published additional data on a total of 20 patients (21 limbs) [55]. In this larger cohort, there were four major and two minor amputations, and of the 18 limbs with a 3-month follow-up, only 1 limb did not demonstrate any of the criteria defining success.

Many of the early trials of stem cell therapy for PAD involved IM injections of stem cells, but there were questions about the best route for delivery, and the potential benefits of IA versus IM administration need to be considered. Bartsch et al. were the first to report results on the administration of BM-MSCs both IM and IA [56]. This study involved patients with moderate PAD, Fontaine class 2b disease [52]. Patients were deemed not to be surgical candidates. A control group ($n = 12$) was comprised of patients with similar clinical characteristics that could not or were unwilling to undergo the stem cell therapy. Following the treatment, patients were assessed at 2 and 13 months. Primary outcomes included walking distance and parameters of perfusion, including venous occlusion plethysmography and capillary venous oxygen saturation via transcutaneous oximetry. Importantly, before the administration of the BM-MSCs, the patients in the treatment group ($n = 13$) had

ischemic pre-conditioning which was achieved by having the patients exercise to claudication, followed by compression of the thigh above systolic pressure. After this, IA injection was given and was followed by a second compression of the thigh. In the final step, BM-MSCs were administered via IM administration. This maneuver was designed with the intention to increase the contact time of the stem cells with the target ischemic tissue. At both 2 and 13 months following the injections, there was a significant increase in total walking distance, while there was no change in the control group. Additionally, the ABI and measures of oxygen saturations and flow significantly increased in the treatment group. These changes were sustained at the 13-month mark. In contrast, the control group showed significant decreases in ABI and flow when assessed at an average of 4 months. There were no other significant changes in the other outcomes measured other than what was expected, but this does give some idea of the natural history of moderate PAD in this patient population.

In the OPTIPEC trial, Smadja et al. quantified the levels of "endothelial precursor cells" circulating in the peripheral blood of patients with CLIPAD who had received BM-MNC as therapy [57]. Additionally, BM-MSCs were grown in culture, and cell marker expression was measured. Importantly, this study also quantified the levels of neo-angiogenesis that had occurred histologically by comparing amputated limbs of individuals who received treatment compared to age- and gender-matched controls with CLI that did not receive therapy and also had amputations. In this study, 11 patients received BM-MNCs injected multiple times in the ischemic gastrocnemius muscle. These patients had significantly fewer circulating early and late endothelial cell precursors compared to controls free of cardiovascular disease and cancer. Most of the patients (8 of 11, 73%) went on to have amputations. Histological studies of the amputated limbs were conducted to quantify the levels of neoangiogenesis that had occurred. These were then compared to amputated limbs of age- and gender-matched individuals who did not receive BM-MSC. In the patients who demonstrated new vessel formation in the anatomic specimen, there were higher levels of colony-forming units endothelial cells (CFU-EC) grown in cultures. CFU-EC are groups of cells in culture that have differentiated down the pathway to the endothelial cell lineage but are not terminally differentiated and typically grow in close association with T-lymphocytes [58]. New vessel formation was defined as vessels observed in unusual locations; vessels identified in this manner were subjected to immunohistochemical staining to confirm the endothelial origin of the cell. This study also showed that patients with PAD had fewer circulating early and late endothelial cell precursors compared to control patients free of cardiovascular disease and cancer.

Hur et al. had shown that "early" EPCs isolated from peripheral blood, i.e., cells with peak growth in culture at approximately 3 weeks followed by death at 4 weeks, secreted larger amounts of angiogenic cytokines [58]. This is compared to late EPC, whose first appearance in culture was at 2–3 weeks, with peak growth at 4–8 weeks, and persisted for up to 12 weeks. These late EPC cells better incorporated into a cell culture of human umbilical vein endothelial cells, produced more nitric oxide, and formed capillary tubes better than early EPC [58].

Van Tongeren et al. also attempted to address the question of optimal method of delivery for BM-MSCs in a small (n = 27), randomized but un-blinded trial [59]. The study subjects had CLI, or persistent claudication (at least 12 months) with maximal walking distance of <100 m. The subjects had no options for surgical or percutaneous revascularization and had a life expectancy of at least 1 year. Subjects were randomized to IM (n = 12) or IA + IM (n = 15) administration of BM-MSCs isolated by the typical protocol. Primary endpoints were pain-free walking distance, complete healing of any ulcers, and avoidance of amputation at 1, 6, and 12 months. Secondary outcomes included changes in ABI and a pain levels. New vessel formation was measured via digital subtraction angiography (DSA) at 6 months following the procedure and compared to baseline anatomy established by DSA 1–2 weeks prior to the procedure. Subjects were followed for a mean of 24 months. Of the original 24 patients, one died from pneumonia prior to the 6-month time point, and the other became extremely ill so as not to be able to participate in the final outcome measures; these two patients were excluded from the final analysis. Therefore, 25 patients were included in the final analysis. Of these, nine had major amputations within 3 months of the BM-MSC infusion and were also excluded from the final analysis.

Overall, in the remaining cohort that did not undergo amputation, there was significant improvement in pain-free walking distance at 6 and 12 months (81 ± 56 m vs 257 ± 126 m vs 282 ± 139, at baseline, 6 months, and 12 months, respectively). Similarly, there were significant increases in the ABI compared to baseline at both 6 months and 12 months. There was no difference in these outcomes based on the method of administration. Most interesting were the results of the DSA, which showed increase in collateral vessel formation in seven patients, no difference compared to baseline in four patients, and deterioration of vessels in four patients. For one patient, DSA values were not able to be compared. Based on the findings of the DSA, "responders" were compared to "non-responders" in terms of the overall number of BMCs received, the number of CD34+ cells, and the number of CFU grown in culture, and there was no significant difference in any of these measures. This led to a quandary to explain the positive clinical benefit with no definite anatomical explanation. The authors proffered an explanation that there may have been undersized collateral vessels unable to be visualized by DSA. The smallest vessel that can be imaged via DSA is approximately 200 microns in diameter [60], a parameter that has not changed significantly over the years [61].

The RESTORE-CLI trial was a novel Phase II, randomized, double-blind, placebo-controlled trial. The novelty of this trial was that in the treatment arm, BM-MSCs were expanded to include a higher concentration of CD90+ cells (mesenchymal stem cells) and CD14+ cells of the monocyte/macrophage lineage [62]. Outcomes in this study included time to first treatment failure, defined as major amputation in the treated limb, all-cause death, and/or new tissue necrosis. This endpoint occurred significantly later in the treatment group compared to the control group. A Cox proportional hazard ratio analysis was included and illustrated that the time-to-event curves separated early and maintained distance throughout the observation period. A post hoc analysis of patients with existing wounds found an even

greater treatment effect in this subset of patients. There was a trend toward longer amputation-free survival in the treatment group, but this did not meet statistical significance. Another highlight of this study was a much smaller volume injected due to the proprietary processing of the BM-MSCs that resulted in higher concentration of the target cells, a process that took approximately 2 weeks. However, this and future studies that thought to use this approach also introduced an important limitation to the study, as several patients did not have enough aspirate to create the final injection product.

In 2010, Iafrati et al. published another randomized, double-blind, placebo-controlled pilot trial of BM-MSCs used for therapy of CLI in patients deemed not to be candidates for surgical revascularization [63]. In this trial, a rapid, point-of-care system was used to process the BM-MSCs and have them ready for reinjection in less than 15 minutes. Control patients received an injection of diluted peripheral blood. Both the treatment and control groups underwent iliac crest puncture, but the treatment group (n = 34) had 240 mL of bone marrow removed, while the control group (n = 14) had only 2 mL removed. A total volume of 40 mL of the BM-MSCs was injected under ultrasound guidance in small aliquots into the affected limb. Patients had follow-up at 1, 4, 8, and 12 weeks after the procedure for amputation, ABI, TCO_2, Rutherford class, pain, walking distance, and quality of life (QoL). The study was not sufficiently powered to determine statistical significance, but there was a trend for lower amputation rates in the treatment group (17.6% vs 28.6%), a finding that did not meet statistical significance. There was also a trend for greater improvement in pain. A composite endpoint that the patient was (1) alive, (2) did not have a major amputation in the treated limb, (3) had an improvement in the Rutherford class, and (4) did not have worsening of pain was also measured. More patients in the treatment group met these criteria for success compared to the placebo group, 17/34 (50%) vs 3/14 (21.4%), though this too did not meet statistical significance. In the QoL assessment, again, there were trends favoring the treatment arm, though none met significance. With the exception of mental health, the treatment group showed greater improvement or less decline in all factors related to QoL. Similar findings were observed with the ABI and TCO_2, with trends in improvement in both in the treatment groups. Beyond the small size of the study that hampered statistical analysis of the findings, this study also had difficulties with collecting some of the follow-up data, particularly walking distance, ABI, and TCO_2 measurements.

This was also one of the few studies to quantify the level of blinding. The patients and investigators were questioned on the treatment day about which group they thought the patients were assigned. The blinding index is the percentage of incorrect guesses added to the percentage of undecided answers; if this is greater than 50%, the study is appropriately blinded [64].

As time progressed, larger trials testing the efficacy of BM-MSCs were conducted. The PROVASA trial was performed in Germany and randomized patients to receive IA BM-MSCs or placebo as a first treatment [65]. This next part of the trial was also double-blinded. However, all patients ultimately received IA BM-MSCs after 3 months in the trial in an open-label fashion. The primary outcome was

improvement in ABI, and this outcome was not met in this trial. The investigators did observe positive outcomes including improved wound healing and reduced rest pain. However, for other outcomes, such as amputation-free survival and limb salvage, there was no difference between the treatment and placebo groups. Median follow-up time was 28 months. Notably, patients with the most advanced CLI, Rutherford 5 or 6 [52], had the worse outcomes. All patients with category 6 went on to have an amputation. Wound healing was a strong positive outcome in this study, as ulcer area significantly declined at 3 months in the group randomized to receive BM-MSC treatment initially ($p = 0.014$). A dose-response effect was shown in this study with regard to ulcer healing, and additional doses of BM-MSCs showed greater decrease in wound area. A similar dose response was noted for pain relief. TCO_2 levels generally increased in the BM-MSC treatment group. The TCO_2 trend in the placebo group was an initial decrease followed by an increase seen after the placebo group crossed over.

The largest randomized stem cell trial to date, JUVENTAS, was published in 2015 [66]. Conducted in the Netherlands, this study included the typical patient population of patients with CLI that was not amenable to revascularization. An additional strength of this study was the randomized, double-blind, placebo-controlled design. Study recruits were randomized to receive multiple IA injections of BM-MSCs via the femoral artery or matching peripheral blood, processed to have the same appearance as the bone marrow aspirate. All subjects underwent bone marrow aspiration. The original sample was divided into three aliquots, with 2/3 cryopreserved for future administration. The cryopreservation consisted of addition of 10% dimethyl sulfoxide followed by freezing in liquid nitrogen. Subjects received additional doses at 3-week intervals.

The primary outcome was major amputation, defined as any amputation occurring above the ankle joint up to 6 months after receiving therapy. Other outcomes included the following: combined major amputation or death, minor amputations, ulcer size, rest pain, pain-free walking distance, ABI, transcutaneous O2 pressure, clinic status, and quality of life. This trial included a large number of outcomes which were measured at 2 months and 6 months. The cell counts injected were the highest for the initial injection and were smaller on subsequent injections. The same was true for the number of CD34+ cell and CFU, suggesting a loss of cells with time and cryopreservation. There was no significant difference in the isolates obtained from the treatment and placebo groups.

There was little positive data in this trial. There was no difference in amputations at either time point or overall. There was no difference in the composite end point of death or major amputation. The study also included composite endpoints fashioned after previously published studies [62, 63], and no significant difference was observed. There was also no difference in any of the secondary endpoints, including ABI, TcO2, QoL, or ulcer area. The investigators also conducted a meta-analysis of the previous trials (including their own) and found a very small benefit to the cell-based therapies that disappeared when only properly blinded and placebo-controlled studies were included.

6.9.2 Peripheral Blood Mononuclear Cells (PB-MNCs)

As another method of stem cell therapy, peripheral blood MNCs (PB-MNCs) were also studied. The obvious advantage of this approach is the ease of material acquisition. Lenk et al. administered an average of 39×10^6 PB-MNCs to seven patients with CLI not amenable to surgical revascularization [67]. The patients were given granulocyte colony-stimulating factor (G-CSF) as a stimulus for production/mobilization of PB-MNCs for 4 days prior to harvesting the cells. Isolation of PB-MNCs from the blood involved a gradient separation system similar to the protocols using BM-MNCs: the cells were grown in culture for 4 days and then administered IA to the patients. A small sample of the cells from culture was tested by flow cytometry to determine the expression of CD34. Outcomes assessed included ABI, TCO_2, PWT, and endothelial function. There were significant improvements in all of these outcomes at 12 weeks after the procedure. Flow cytometry analysis showed that approximately 50% of the cells were positive for CD34, as well as markers of endothelial cells lineage [67].

Larger trials of IM injections of PB-MNCs were conducted by Lara-Hernandez et al. [68]. The patient population ($n = 28$) included severe CLI with no options for surgery. The cells were obtained by apheresis after stimulation with G-CSF for 5 days. The investigators reported "high" levels of EPCs as determined by the expression of CD34 and CD133. There was no control arm. There were significant improvements in ABI and pain. The limb salvage rate was 74.4% after 1 year.

Another promising study was conducted in diabetics with CLI [69]. In this randomized controlled trial, patients received two IM injections 40 days apart of unselected PB-MNCs after granulocyte colony-stimulating factor (G-CSF) stimulation. Control group received IM prostaglandin E1. Compared to the control group, the treatment group showed significant improvements in rest pain, wound healing (Huang et al.), and amputations. PWT was also higher in the treatment group, but this narrowly missed statistical significance.

Losordo et al. studied low and high doses of enriched CD34+ PB-MNCs administered IM in a double-blind, placebo-controlled pilot trial [70]. Amputations occurred more frequently in the control arm (66.7%) compared to the low-dose (42.9%) and in the high-dose (22.2%) group though the difference did not meet statistical significance ($p = 0.137$). Other outcomes studied, including wound healing, PWT, rest pain, and QoL, also did not show differences between the treatment and control groups. The study was small and not powered to detect statistical differences.

Next, trials were conducted comparing PB-MNCs to BM-MNCs. There were mixed results. The TACT trial described above favored BM-MSCs, as did an extension of the TACT trial examining long-term outcomes [71]. One trial favored PB-MNCs [69] but also found improvements in the patients treated with BM-MSCs.

Table 6.2 summarizes the trials discussed. The trials were small Phase I or II trials with few participants. Most of the trials included individuals with advanced PAD; however, two trials included individuals with less severe disease, and both

Table 6.2 A summary of human BM-MSC therapeutic angiogenesis clinical trials

Author/trial	Phase/year	Disease	Treatment	Cell number	Subject (*n*) Treatment/Control	Findings
TACT	Phase II 2002	Rutherford grade III, category 5	Intramuscular Unselected BM-MNCs and PB-MNCs	$2.7–2.8 \times 10^9$	25 unilateral 22 bilateral Diabetes excluded	Improved ABI, TcO2 Improved rest and pain-free walking Evidence of increased collateral vessels
Higashi et al.	Phase I 2004	Rutherford grade III, category 5	Intramuscular BM-MNCs	1.6×10^9 CD34+ 3.8×10^7	7/0 Diabetes, CAD excluded	Improved ABI, pain-free walking, TcPO2 Increased vasodilation dependent leg blood flow by plethysmography
Bartsch et al.	Phase II 2007	Fontaine class 2b	Intra-arterial + intramuscular BM-MNC	83×10^6	13/12	Improved pain-free walking, ABI Increased oxygen saturation and both rest and peak blood flow
OPTIPEC	Phase I 2008	Rutherford grade III, category 5	Intramuscular BM-MNCs	Not reported	11/0	Evidence of endothelial cell proliferation in distal amputated limb
Van Tongeren et al.	Phase II 2008	Fontaine IIB or greater	Intra-arterial + intramuscular vs intramuscular alone BM-MNCs grown in culture	1.23×10^9 total CD34+ 3.07×10^6	12/15	Both groups improved pain-free walking and ABI
Cobellis et al.	Phase II 2008	Fontaine III (21%) Fontaine IV (79%)	Intra-arterial Unselected BM-MSCs	10^9	10/9	Increased perfusion and neoangiogenesis Increased PWT

(continued)

Table 6.2 (continued)

Author/trial	Phase/year	Disease	Treatment	Cell number	Subject (*n*) Treatment/Control	Findings
Franz et al.	Phase I 2009	Rutherford grade II, category 4 $\underline{n} = 1$ Rutherford grade III, category 5, $n = 6$; category 6 = 2	Intramuscular Intra-arterial	Not reported	9/0	8/9 patients had improvement 6/9 patients avoided amputation
Ruiz-Salmeron et al.	Phase I-IIa 2011	Rutherford grade III Category 4 $n = 3$ Category 5 $n = 11$ Category 6 $n = 6$	Intra-arterial Unselected BM-MSCs	266.2×10^6 CD34+ 4.37×10^6	20/0	Improved wound healing, ABI and symptoms
Iafrati et al.	Phase II 2011	Rutherford grade IV or V	Intramuscular BM-MSCs rapidly prepared for injection	3.23×10^9	34/14	Decreased amputations Improved pain, QoL and Rutherford class Improved ABI
PROVASA	Phase II 2011	Rutherford grade II, category 4 or greater Fontaine stage III or greater	Intramuscular BM-MNCs	1st tx: 153×10^6 Second tx: 155×10^6 Third tx: 165×10^6 CD34+ First tx: 3.6×10^6 Second tx: 2.5×10^6 Third tx: 2.4×10^6	19/21	Improved ulcer size, rest pain No difference in ABI, amputation, death

(continued)

Table 6.2 (continued)

Author/trial	Phase/year	Disease	Treatment	Cell number	Subject (*n*) Treatment/Control	Findings
RESTORE-CLI	Phase II 2012	Rutherford grade II, category 4	Intramuscular BM-MSCs	136×10^6 total CD90+ 25×10^6	48/24	Longer time to treatment failure Increased amputation-free survival (32%, NS)
JUVENTAS	Phase II 2015	Rutherford category 3 or greater	Intra-arterial BM-MNCs	1st tx: 199×10^6 Second tx: 144×10^6 Third tx: 144×10^6	81/79	No difference in amputation, death, ABI, ulcer size, quality of life, rest pain, TcPO2

Abbreviations: TCO_2 transcutaneous partial pressure of oxygen, *PWT* peak walking time, *BM-MNC* bone marrow mononuclear cells, *NS* not significant

were positive trials. The mode of delivery for therapy was mostly IM injection, with only two studies exclusively administering cells intra-arterially. There were several studies that compared IM and IA injections, and there was little evidence that one method was superior to the other. An IA injection would call for cannulation of an artery, which requires special equipment and carries risks of bleeding and arterial injury, though there were few reports of these events occurring. In many of the trials, unselected BM-MSCs were administered as treatment, with cell counts on the order of 10^9; however, several of the trials administered substantially fewer cells, on the order of 10^6. The RESTORE-CLI trial used a proprietary process to isolate higher concentrations of MSCs and HSCs and was a positive trial. Some, but not all of the studies, quantified the types of cells being injected. There was also some heterogeneity among the primary outcomes, which makes direct comparison of the trials difficult.

6.10 Safety Outcomes

All medical therapies must be assessed as a balance of benefit vs. risk. In the von Tongeren trial [59], two patients developed heart failure following the BM-MSC extraction and injection. The procedure took place under general anesthesia, and this was implicated as the cause of the complication versus the volume of BM-MSCs received or any other aspect of the bone marrow extraction. In the PROVASA trial, three adverse events were associated with the treatment procedure: thrombus formation in a previously placed stent after inflation of a low-pressure balloon, one hematoma, and one pseudoaneurysm associated with the IA administration of the BM-MSCs [65]. The JUVENTAS trial reported a large number of adverse events at 213, but only one, a femoral hematoma, was directly attributed to the procedure [66]. The RESTORE-CLI trial also reported a high number of adverse events, though there was no significant difference in the number of adverse events in the treatment and control arms; many of the adverse events reported were also sequelae of the disease process, including pain, wound infection, and necrosis. In this trial, an event of wound infection in the hallux of the infected limb was thought to be possibly due to the treatment [62]. Other safety outcomes that were anticipated but not observed included rhabdomyolysis, kidney injury, or proliferative retinopathy [63]. A small drop in hematocrit was noted but did not require any therapy [63].

The studies involving PB-MNCs were also generally safe. Losordo reported 60 serious adverse events, with no differences in incidence of events between the treatment and control arms [70]. While the vast majority was felt not to be related to the procedure, one patient developed hypotension with G-CSF treatment, and another had worsening of rest pain after the injection that required hospitalization. Huang et al. documented one patient with bone pain and malaise during treatment with G-CSF [69]. No deaths that occurred in these trials were attributed to the procedure or the treatments received.

6.11 Perspectives on Stem Cell Therapy

Despite the grave nature of CLI, owing to the lack of positive data, stem cell therapy has not emerged as a proven strategy for the treatment of PAD. In the most recent AHA/ACC [72] and ESC [73] clinical guidelines on the treatment of PAD, there are no recommendations supporting the use of stem cell therapy. There are several potential reasons for the overall lack of success. Several studies have called into question the quality of stem cells from a population of patients with PAD, unfortunately the patients most in need of treatment. Imanishi et al. found that endothelial progenitor cells (EPCs) from patients with hypertension reached senescence and had decreased telomerase activity compared to age-matched control patients without hypertension [74]. Similarly, EPCs isolated from the peripheral blood of healthy smokers were found to have impaired migratory and proliferative response and decreased ability to form precapillary structures in cell culture [75]. Patients with type I diabetes have also been noted to have fewer EPCs with reduced function in culture, even when the cells were grown in culture with normal glucose levels [76]. Hypercholesterolemia has also been associated with lower EPC numbers and dysfunction [77]. Taken together, it is likely that stem cells from patients with PAD are dysfunctional at baseline when compared to similar cells from a non-PAD population.

It has also been shown that the established cell markers used to identify EPCs in bone marrow cells may lead to contamination of the product with cells of the hematopoietic lineage [78]. This raises questions about the actual mechanisms involved in the effects of BM-MSCs in the treatment of PAD: it may be that other mechanisms besides EPC-mediated angiogenesis are involved. Along the same lines, one study in mice with induced hind-limb ischemia found that BM-MSCs did not incorporate into developing blood vessels and that there has been false-positive identification of EPCs from surrounding cells [79]. There is also the question of whether EPCs, also called endothelial colony-forming cells (ECFCs) [80], exist in the general population of BM-MSCs; true EPCs may actually be found in the peripheral blood [81]. The majority of studies highlighted in this chapter used BM-MSCs as compared to PB-MSCs. Evidence is emerging that ECFCs and BM-MSCs may act in concert to support angiogenesis [82](Lin et al); therefore, a strategy using BM-MSCs alone may be inadequate to produce a clinical effect.

Other considerations include the severity of disease in the patient population. These trials included patients with the most severe PAD who were not candidates for surgery. Some have argued that the time to try such therapies may be at an earlier stage of disease.

6.12 Future Directions

Has stem cell therapy for PAD reached a dead end? In a meta-analysis of the trials published in 2019, Gao et al. demonstrated that the cumulative evidence shows a clear benefit for stem cell therapy in terms of improving rest pain and pain-free

walking distance [83]. The evidence for ulcer healing and ABI were less certain, but the preponderance of the studies included in the analysis favored stem cell therapy. Conversely, the data for amputations favored placebo, and this analysis also highlighted the high potential for bias in a large proportion of the studies, particularly related to blinding.

It can, however, be argued that further studies may reveal the true benefit of stem cells. As nearly every study examined patients with the most severe PAD, stem cell therapy considered at an earlier time point in the natural history may prove beneficial. Furthermore, there may be a specific cell population that would provide a benefit.

There may also be benefit from stem cells derived from adipose tissue. Bura et al. conducted a Phase I trial of MSCs obtained from adipose tissue as stem cell therapy in patients with CLI [84]. This small trial of seven patients demonstrated the safety of this technique, as no adverse events were reported. In terms of efficacy, there were overall decreases in wound area and increases in TCO_2 ($p < 0.05$).

Unfortunately, the proportion of studies examining treatment for PAD is low: according to a study published in 2014, 1.7% of all active trials registered in ClinicalTrials.gov from October of 2007 to September 2010 were devoted to examining interventions for PAD [85]. More recently, Biscetti et al., in their review of stem cell therapy in PAD, noted a lack of well-designed Phase III trials [86]. Taken together, this suggests that there are few studies on the horizon.

6.13 Conclusions

PAD remains at epidemic proportions. Current medical therapy is limited, and the jury is still out on the true benefit of novel therapies such as stem cell therapy. Before abandoning the option of stem cell therapy, future studies should focus on well-designed trials that limit bias and explore the optimal population of patients with PAD who may benefit from such therapy.

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