Treatment of peripheral arterial disease using stem and progenitor cell therapy

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Peripheral arterial disease (PAD) is a highly prevalent atherosclerotic syndrome associated with significant morbidity and mortality. PAD is most commonly caused by atherosclerosis obliterans (ASO) and thromboangiitis obliterans (TAO), and can lead to claudication and critical limb ischemia (CLI), often resulting in a need for major amputation and subsequent death. Standard treatment for such severe cases of PAD is surgical or endovascular revascularization. However, up to 30% of patients are not candidates for such interventions, due to high operative risk or unfavorable vascular involvement. Therefore, new strategies are needed to offer these patients a viable therapeutic option. Bone-marrow derived stem and progenitor cells have been identified as a potential new therapeutic option to induce angiogenesis. These findings prompted clinical researchers to explore the feasibility of cell therapies in patients with peripheral and coronary artery disease in several small trials. Clinical benefits were reported from these trials including improvement of ankle-brachial index (ABI), transcutaneous partial pressure of oxygen (TcO_2), reduction of pain, and decreased need for amputation. Nonetheless, large randomized, placebo-controlled, double-blind studies are necessary and currently ongoing to provide stronger safety and efficacy data on cell therapy. Current literature is supportive of intramuscular bone marrow cell administration as a relatively safe, feasible, and possibly effective therapy for patients with PAD who are not subjects for conventional revascularization. (J Vasc Surg 2011;53:445-53.)

Clinical Relevance: **This article describes the background and first results of stem and progenitor cell therapy in patients with critical limb ischemia not suitable for revascularization. The principle as far as it is understood and the methods are described. Compelling evidence suggests that progenitor cell therapy might become a useful adjunct to the treatment options at present. Due to poor prognosis and the increasing number of patients, there is a need for new therapeutic methods. The article gives an overview of first encouraging results provided by early-phase clinical trials. Challenges in this new therapeutic option still include open questions such as cell phenotype, processing, dosing, route of optimal delivery, and frequency of application. Validation by more rigorous controlled trials involving homogenous patient populations are required to confirm the first hopeful results.**

Peripheral artery disease (PAD) is a highly prevalent atherosclerotic syndrome that affects approximately 8 to 12 million individuals in the United States and is associated with significant morbidity and mortality.[1] An additional cause of PAD is Buerger disease, also called thromboangiitis obliterans (TAO), which is a nonatherosclerotic, segmental inflammatory disease most frequently affecting the small and medium-sized arteries and veins in the upper and lower extremities and is strongly associated with heavy tobacco use.[2] Risk factors for atherosclerotic PAD are mainly, but not exclusively, smoking and diabetes, and are, therefore, identical with those for atherosclerosis in the cerebrovascular and coronary circulation. Comorbid PAD substantially increases the mortality risk conferred by coronary artery disease and/or cardiovascular disease alone.[3] Critical limb ischemia (CLI) is the endstage of lower extremity PAD in which severe obstruction of blood flow results in ischemic rest pain, ulcers, and a significant risk for limb loss. CLI is not a specific disease per se, rather, it represents a syndrome that may develop from many fundamentally distinct pathophysiological processes. For all stages of the disease, minimization of risk factors is mandatory. The mainstay of therapy for severe, limb-threatening ischemia is either surgical or endovascular revascularization aiming to improve blood flow to the affected extremity. Approximately 20% to 30% of patients with CLI are not considered candidates for vascular or endovascular procedures, however, with amputation often being the only option. This corresponds to about 100,000 major leg amputations in the European Union, and to 120,000 in the United States.[4] Leg amputation due to atherosclerotic PAD gives rise to an acute mortality rate of around 30% and a 5-year prognosis with survival rates of less than 30%.[5,6] Albeit life expectancy is not as severely limited for patients suffering from Buerger disease or TAO, major amputations necessary for these individuals often result in severe handicaps in the usually younger patients.

To alleviate symptoms of PAD, research has been focusing on the use of bone marrow (BM)-derived stem and progenitor cells, which were identified as a potential new

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Competition of interest: none.

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The editors and reviewers of this article have no relevant financial relationships to disclose per the JVS policy that requires reviewers to decline review of any manuscript for which they may have a competition of interest.

0741-5214/$36.00

doi:10.1016/j.jvs.2010.08.060

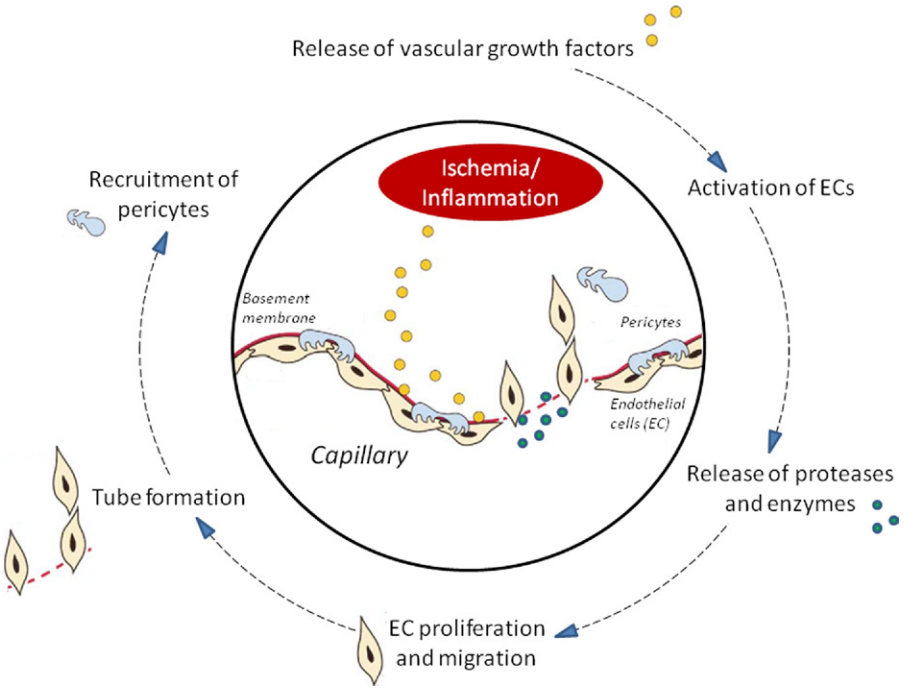

Fig 1. Schematic diagram of putative events in the angiogenic process. *EC,* Endothelial cell. (Figure reproduced with permission from Lawall et al.[4])

therapeutic option to induce therapeutic angiogenesis. The goal was to improve the vascularization of the ischemic leg so that perfusion increases sufficiently for wound healing to occur, and to resolve pain at rest, this ultimately allowed limb salvage for these patients.

Angiogenesis and arteriogenesis. In patients with obstructive artery disease, two different forms of compensatory vessel growth occur, angiogenesis and arteriogenesis. Angiogenesis is the formation of a capillary network, through the activation and proliferation of endothelial cells in ischemic tissue. Therefore, it is also often called capillary growth. It occurs as a sprouting of small endothelial tubes from pre-existing capillary beds in response to local hypoxia. It is mediated by hypoxia-induced release of cytokines (vascular endothelial growth factor [VEGF] and related growth factors). No influx of non-tissue resident cells is needed.[7] The resulting capillaries are small, with a diameter of about 10 to 20 μm, and cannot sufficiently compensate/substitute for a large occluded transport artery due to Hagen-Poiseuille law (Fig 1).

Arteriogenesis, also called collateral growth, is the transformation of pre-existent collateral arterioles into functional collateral arteries, meaning an increase in the diameter of existing arterial vessels capable of compensating for the loss of function of occluded arteries.[8] The original diameter of a small, initially non-perfused arteriole may increase up to 20 times during the process of arteriogenesis.[9,10] It is initiated when shear stresses increase in the pre-existent collateral pathways upon narrowing of a main artery. The increased shear stress leads to an upregulation of cell adhesion molecules for circulating monocytes, which subsequently accumulate around the proliferating arteries and provide the required cytokines and growth factors.[11] Experimental evidence in animal models of limb ischemia indicates that endogenous arteriogenesis can almost fully restore a normal vascular conductance induced by large vessel occlusion. This correlates well with the clinical observation that many patients with PAD and, for example, femoral artery occlusion are free of ischemic symptoms because their collateral network delivers enough blood to meet the perfusion need of the lower limb.[12] The following overview will describe the physiology of arteriogenesis.

Physiology of collateral artery growth (arteriogenesis). Mechanically, arteriogenesis is linked to elevated pressure, which increases radial wall stress, and elevated flow thus increasing endothelial surface stress. The vessel increases in diameter until the stress is normalized. The activation of the collateral endothelium caused by increased fluid shear stress is reflected by the upregulation of adhesion molecules and by the release of cytokines that attract circulating monocytes. These adhere and invade the collateral vessel wall. Increase of T cell numbers and granulocytes has also been reported in addition to this monocyte/macrophage accumulation around growing collaterals, emphasizing the importance of circulating cells to this type of vascular growth.[13] The invading monocytes are BM-derived cells with monocytic and/or macrophagic surface markers. Matrix proteases are hereby activated in the pericollateral space and destruct the tissue surrounding the growing vessel, producing a space into which the collateral

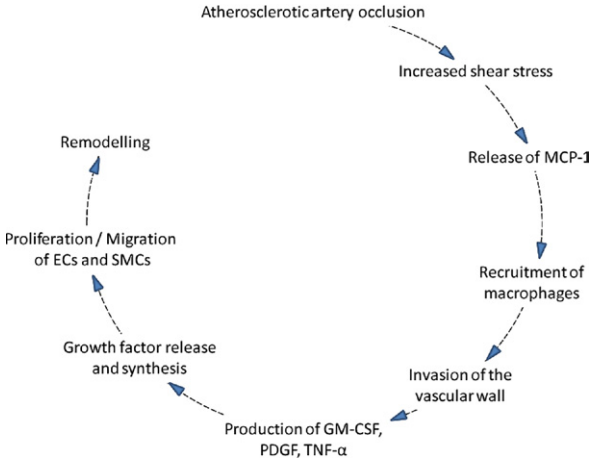

Fig 2. Schematic diagram of putative events in the arteriogenic process. *EC,* Endothelial cell; *GM-CSF,* granulocyte-macrophage colony-stimulating factor; *MCP-1,* monocyte chemoattractant protein-1; *PDGF,* platelet derived growth factor; *SMC,* smooth muscle cell; *TNF-α,* tumor necrosis factor-α. (Figure reproduced with permission from Lawall et al.[4])

arterial wall can expand (Fig 2). These physiologically preformed arterio-arterial collateral connections increase in size and diameter in a temporally and spatially well-defined cascade of events until a working three-layered collateral artery restores blood flow 4 to 6 weeks after initial occlusion of the large artery.[14] The question remains, however, whether these effects can be attributed to the incorporation of stem cells into the wall of the new vessel, or to the cytokines released by chemo-attracted BM cells inducing proliferation of resident endothelial cells. Findings by Kinnaird et al[15] suggest that cultured human BM-derived stromal cells promote arteriogenesis through paracrine mechanisms. This notion is supported by Heil et al,[13] who suggest that in the adult organism, bone marrow cells (BMCs) do not promote vascular growth by incorporating into vessel walls but rather act as "cytokine factories," promoting vascular growth by paracrine effects. Findings by Jin et al[16] also support this concept by which ischemia induces plasma elevation of stem and progenitor cell-active cytokines, including sKitL (Soluble Kit-ligand) and thrombopoietin, and, to a lesser extent, progenitor-active cytokines, such as granulocyte-macrophage colony-stimulating factor (GM-CSF) and erythropoietin. Thrombopoietin and sKitL induced the release of stromal-derived factor-1 (SDF-1) from platelets, thereby increasing systemic plasma levels of SDF-1 (stromal-derived factor-1 also known as CXCL12). This results in a substantial mobilization of CXCR4+VEGFR1+ cells, accelerating revascularization of the ischemic limbs. Jin et al[16] term this unique class of CXCR4+VEGFR1+ cells "hemangiocytes," which represent a heterogeneous population of VEGF-responsive non-endothelial, proangiogenic hematopoietic progenitors consisting of immature and differentiated myelomonocytic cells. Hemangiocytes also express progenitor markers and induce neovascularization by releasing angiogenic factors and by physically supporting the assembly of endothelial cells.

Nonetheless, this regenerative repair mechanism of arteriogenesis by recruitment of monocytic BM cells fails to work adequately in a large number of patients, resulting in advanced peripheral ischemia and, ultimately, in limb loss. The so-called "circulating endothelial progenitor cells" (EPCs), originally identified by Asahara et al[17] in a mouse model, were also recently found to originate from the monocyte-macrophage lineage, thus being likely to be identical to the BM-derived monocytic cells active in the perivascular collateral artery space.[18]

Interestingly, the risk factors observed for advanced ischemia due to insufficient collateralization (diabetes, smoking, hyperlipidemia, and advanced age) are the same for a lower number of circulating, monocytic EPCs.[19-23] This observation strengthens the pivotal position of BM-derived monocytes in PAD repair, enabling imitation and boosting of physiological repair processes to ultimately induce arteriogenesis.

Animal models of cell therapy in limb ischemia. Putative endothelial cell (EC) progenitors which subsequently differentiated to ECs in vitro were first isolated by Asahara et al[17] from human peripheral blood, using separation methods based on cell surface antigen expression. These heterologous, homologous, and autologous EC progenitors were then found to incorporate into sites of active angiogenesis in animal models of ischemia. These findings suggested that EC progenitors could augment collateral vessel growth to ischemic tissues (therapeutic angiogenesis) and deliver anti-angiogenic or pro-angiogenic agents, respectively, to sites of pathologic or utilitarian angiogenesis. Since then, stem cell therapy has been used in a large number of rat, rabbit, and mouse models to improve limb vascularity. These experiments demonstrated that the number of circulating EPCs increase in response to ischemia, and those cells were incorporated into capillaries and interstitial arteries.[24,25] The caveat of these studies was that hind limb ischemia was induced in models of acute, but not of chronic ischemia by unilateral ligation or coagulation of the common femoral artery.[15,26-29] The need for specific animal models with true degenerative arteriosclerotic disease therefore remains, as the latter is the most frequent etiology of human PAD. Notwithstanding, encouraging results of preclinical studies have rapidly led to several small clinical trials in which BM-derived mononuclear cells were also administered to patients with limb ischemia caused by atherosclerotic PAD. In the following, we will attempt to give an overview of clinical trials for the use of autologous cell therapy for patients with PAD.

Autologous stem cell therapy trials. The preclinical studies establishing that BM-derived mononuclear cells (BM-MNC), including EPCs, into ischemic limbs increase collateral vessel formation prompted clinical researchers to explore the feasibility of cell therapies in patients with PAD. The first large report on the use of BM-MNC in limb ischemia was the Therapeutic Angiogenesis by Cell Trans-

plantation (TACT) study by Tateishi-Yuyama et al.[30] The protocol consisted of an open pilot study in which efficacy and safety of autologous implantation of BM-MNC was established, and a randomized controlled confirmatory part, comparing the efficacy of BM-MNC vs peripheral blood (PB)-MNC treatment. In the latter part, patients (n = 22) with bilateral leg ischemia were randomly injected with BM-MNC in one leg (active treatment), or with PB-MNC into the other as a control. At 4 weeks, ankle-brachial index (ABI) was significantly improved in legs injected with BM-MNC compared with those injected with PB-MNC (difference 0.09 [95% confidence interval {CI}, 0.06–0.11]; $P < .0001$). Similar improvements were seen for transcutaneous oxygen pressure (TcO_2; 13 [95% CI, 9–17]; $P < .0001$), rest pain (−0.85 [95% CI, −1.6 to −0.12]; $P = .025$), and pain-free walking time (1.2 [95% CI, 0.7–1.7]; $P = .0001$). Legs injected with PB-MNC cells showed much smaller increases of ABI and TcO_2. The improvements in the BM-MNC-injected legs were sustained at 24 weeks.[30] The authors concluded that the higher efficacy of implantation of BM-MNCs as compared to PB-MNCs was due to the supply of endothelial progenitor cells (included in the CD34+ fraction), and multiple angiogenic factors (released from the CD34− fraction).

The publication of TACT and the first studies on cardiac stem cell therapy[31] raised general interest in stem cell treatment for vasculogenesis, and lead to the use of stem cell/BM-MNC therapy for peripheral ischemia in a number of different countries. An overview of these studies has been recently published by Lawall et al.[4] Despite the limitations presented by not only the variable methods of cell isolation, but also by the variable degrees of ischemia and often small number of study subjects, the outcome of the listed BM-derived cell therapy on perfusion parameters (ABI, TcO_2) and clinical course (wound healing, walking distance) was remarkably consistent and positive throughout the different reports. Pooled results show that autologous cell therapy induces ABI increases between 0.1 and 0.2 points, and $TcPO_2$ increases of 10 to 20 mm Hg O_2. Depending on baseline values, walking distance was shown to improve to a mean of 100 to 200 meters. In addition, no serious side effects were reported.

A recent meta-analysis by Fadini et al[32] searching for effective autologous cell therapy studies for the treatment of PAD yielded 108 studies, 42 of which were clinical trials and 37 of which were potentially appropriate to be meta-analyzed. From these 37 trials in which autologous cell therapy was effective in improving surrogate indexes of ischemia, subjective symptoms, and hard end points (ulcer healing and amputation), 24 had usable data for ABI measurements, 13 had usable data for TcO_2, 20 had usable data for pain, and 11 had usable data for walking distance.

Considering all trials of cell therapy, ABI improved from 0.46 ± 0.04 before therapy to 0.63 ± 0.04 after therapy ($P = .011$), whereas ABI improved by 0.115 ± 0.060 ($P = .054$) when considering only controlled trials. In trials with granulocyte-colony stimulating factor (G-CSF) monotherapy, ABI was not significantly increased, considering all studies (from 0.41 ± 0.09 to 0.59 ± 0.11; $P = .30$) and controlled studies only (difference 0.049 ± 0.22; $P = .83$). TcO_2 increased from 22.8 ± 2.8 to 35.8 ± 2.9 ($P = .0002$) considering all trials of cell therapy, and increased by 12.8 ± 7.0 ($P = .069$) considering only controlled trials. Data on TcO_2 in trials with G-CSF monotherapy were too limited to be analyzed. Walking capacity increased significantly considering all trials (from 75.7 ± 19.4 to 402.3 ± 70.9 meters; $P < .0001$), but was comprehensively reported in only one G-CSF therapy trial with no significant difference vs placebo. Pain (on a 0-10 scale) was also found to be significantly reduced in cell therapy trials, regardless of whether all trials (6.35 ± 0.43 to 2.11 ± 0.37; $P < .0001$) or controlled trials only (−2.39 ± 1.01; $P = .019$) were considered. In G-CSF trials, pain was not significantly reduced. Ulcer healing significantly improved in the active treatment group vs the control group (odds ratio [OR], 3.54, 95% CI, 1.09–11.51; $P = .032$) in controlled cell therapy trials. This was not the case for the one reported trial of G-CSF therapy. Amputation as an outcome was explored in only two controlled trials of cell therapy part of the Fadini et al[32] meta-analysis, indicating a significant benefit in terms of limb salvage as compared to control treatment (OR for amputation 0.09; 95% CI, 0.02–0.44; $P = .0005$). No assessment of incidences of amputation was done in controlled G-CSF trials. The overall conclusion of the Fadini et al[32] meta-analysis is that cell therapy is able to significantly improve ABI, TcO_2, rest pain, pain-free walking distance, ulcer healing, and limb salvage. In contrast, G-CSF monotherapy was not associated with significant improvement of these end points, albeit final conclusions should be deferred because the number of G-CSF testing trials was limited (Fadini et al[32] and references therein).

Importance of cell type and origin. The concept of therapeutic angiogenesis was driven by the theoretical concept that BM-derived EPCs could incorporate into damaged vessel endothelium and promote collateral vessel formation. However, albeit being a promising tool for cell therapy, a clear and physiologically relevant definition of EPCs still remains elusive. Co-recruitment of angiocompetent hematopoietic cells delivering specific angiogenic factors facilitate incorporation of EPCs into newly sprouting blood vessels. It seems that cell therapy for treatment of PAD using either whole BM-MNCs or G-CSF-mobilized whole PB-MNCs is more successful than use of subfractionated cell preparations (eg, CD 133+ or highly purified CD 34+ cells) from peripheral blood after G-CSF mobilization.[33,34] Several studies with growth factor liberated PB-MNC were performed with results very similar to those of BM-MNC trials, which were lately reviewed by Lawall et al.[4] The question of whether G-CSF mobilized PB-MNCs or BM-MNCc achieved better primary outcomes with respect to safety and efficacy of treatment, improved ABI and rest pain were directly addressed in a study by Huang et al[35] for patients with lower limb TAO. Significant improvement of the above outcomes was observed in both groups of the study (group A receiving G-CSF mobilized PB-MNCs,

group B receiving BM-MNCs) after transplantation. Comparative analysis revealed that at 12 weeks after cell implantation, improvement of ABI (difference 0.06 ± 0.01; $P <$ .0001), skin temperature (difference 0.55 ± 0.25; $P =$ .028), and rest pain (difference −0.57 ± −0.15; $P <$ .0001) was significantly better for patients in group A (G-CSF mobilized PB-MNCs) than for those in group B (BM-MNCs). However, there was no significant difference between the two groups for pain-free walking distance, TcO_2, ulcers, and rate of lower limb amputation.[35]

The meta-analysis by Fadini et al,[32] which included trials for the treatment of PAD, CLI, TAO, atherosclerosis obliterans (ASO), and peripheral vascular disease, reports mobilized PB-MNC therapy to be consistently associated with slight but not significantly better improvements in ABI, TcO_2 and pain-free walking distance for all trials than BM-MNC therapy. Pain-scale reduction was significantly better with mobilized PB-MNCs than with BM-MNCs ($P = .006$). However, BM-cell therapy significantly improved a hard end point such as ulcer healing (OR 7.23; $P = .038$), whereas mobilized PB-MNCs did not (OR 2.24; $P = .13$). There were no significant differences in the clinical characteristic between patients treated with mobilized PB-MNCs or BM cells. As stated above, Fadini et al[32] found that G-CSF monotherapy led to nonsignificant improvement in ABI, pain-free walking distance, rest pain, and ulcer healing, this observation being in compliance with the results of a double-blinded randomized, placebo-controlled study showing no superiority of G-CSF over placebo.[36]

With respect to different surface phenotypes of BM-MNCs and PB-MNCs, Romagnani et al[37] were able to demonstrate that PB-MNC-derived EPCs seem to be CD14+ by using the conventional cytofluorometric technique; however, virtually all cells were also found to express low levels of surface CD34 when assessed by the highly sensitive antibody conjugated magnetofluorescent liposomes (AC-MFL) or fluorescence amplification by sequential employment of reagents (FASER). These $CD14+CD34^{low}$ cells represented a variable proportion at individual levels of CD14+ cells, and constituted the dominant population among circulating KDR+ cells, indicating that the major source of EPCs obtainable from PB is a subset of double-positive $CD14+CD34^{low}$ cells showing phenotypic and functional features of multipotent stem cells.[37] In addition, Peichev et al[38] found that AC133, an early hematopoietic stem cell marker, is expressed on a large subset of circulating endothelial precursors but not on the mature endothelium. The percentage of CD34+ cells expressing AC133+ and VEFGR2+ cells is only 2% of circulating CD3+ cells. This figure is much less than the percentage of CED34+ VEGFR-2+ cells previously reported by other groups.

It should also be remembered that autologous BM or autologous peripheral-blood cells were used in all of the above-mentioned studies. Therapies with allogenous cells from another donor or pooled from several donors as in a placental cell concentrate are solely in animal-trials or phase I trials with no publications on their effect in humans so far.

Intramuscular vs intra-arterial administration. Intramuscular and intra-arterial injection or a combination of both has yielded promising results in the treatment of human PAD. The underlying principle of intramuscular injection is the creation of a cell depot with paracrine activity in the ischemic area. However, the mechanisms by which transplanted cells improve the patients' clinical status are thus far unclear. Experimental animal studies indicate that BM-derived cells contribute to vascular and muscle regeneration by physically integrating into the tissue and/or by secreting growth factors.[13,39]

Intramuscular injection was usually performed into the gastrocnemius muscle along a symmetric grid with a fixed number of injections (between 20 and 60) in most human trials (Lawall et al[4]). In the recent pilot, BM outcomes trial 1 (BONMOT-1)[40] and in the follow-up placebo-controlled double blind study (BONMOT-CLI),[41] injections were placed along the occluded native arteries, because the density of preformed collaterals is highest in parallel orientation to the axial arteries, and this is the preferred location for collateral growth. In BONMOT 1 and 2, the number of injections was increased corresponding to the length of the arterial occlusion, from 40 injections for infrapopliteal disease only, to 60 injections if femoral, popliteal, and infrapopliteal disease was present. However, no direct comparisons between different intramuscular injection sites and numbers exist. In the meta-analysis by Fadini et al,[32] the most common route of cell administration was intramuscular (33 trials). Only four trials used intra-arterial route of administration, one trial combined intra-arterial plus intramuscular routes, and one trial compared intramuscular vs intramuscular plus intra-arterial cell administration. ABI and TcO_2 were found to be significantly improved only after intramuscular, and not after intra-arterial cell therapy. However, both significantly and comparably improved pain and pain-free walking distance. Intramuscular cell therapy significantly improved ulcer healing (OR 2.62; $P = .029$), whereas this could not be assessed in the details in trials of intra-arterial cell therapy.[32]

Isolation and dosage of stem cells for therapeutic vasculogenesis. It is necessary to isolate and concentrate monocytic precursor cells. Improvements of current cell therapy aim at establishing effective and straightforward application methods that can be performed easily and in a timely fashion. This sounds self-evident; however, the sometimes complex requirements for cell isolation procedures have been obstacles for the wider application of cell therapy in PAD.

Most studies used between 100 to 800 mL of BM blood as a mean extraction volume. The mononuclear cell fraction was enriched by different separation techniques: (1) by Ficoll density gradient system centrifugation and variations thereof[42-45]; (2) by use of blood centrifugation and plasmapheresis systems (ie, COBE Spectra, Gambro, Sweden; CS 3000-Plus, Baxter Healthcare, Deerfield, Ill)[30]; or (3) by use of point-of-care, bedside centrifugation systems (SmartPReP, Harvest Technologies, Plymouth,

Mass).[40,46] A total BM-MNC number of between 1.5 and 10×10^9 cells was obtained using these techniques. This corresponds to the cell count numbers described in the meta-analysis by Fadini et al,[32] in which the mean number of mononuclear cells implanted or infused was $3.56 \pm 2.81 \times 10^9$, and the mean CD34+ cell count was $5.0 \pm 1.48 \times 10^7$, indicating that about 1.4% of transplanted cells were CD34+.

Both Ficoll and blood separator techniques require a blood handling facility which is good clinical practice-certified, usually either a specialized transfusion service or a dedicated hematology unit, all of which are very labor intensive. An additional sterile cell biology laboratory is necessary if cells need to be expanded by further cultivation steps.[47,48] European Union-wide regulations for both Ficoll and separator techniques are tight, and special permission by the respective authorities is necessary. Attempting to circumvent or overcome these obstacles, a single-step, bedside, closed isolation system was developed recently. This system is independent of specialized hospital subservices without legal hurdles, and shortens total therapy time from 8 to 10 hours to 1 hour. It also seems to be considerably cheaper than separator or Ficoll-based techniques,[40,46] albeit having similar arteriogenic potency. With the advent of simpler techniques, cell therapy may thus gain impetus also in non-university hospitals that currently treat the majority of patients with PAD.

Based on our ample experience with both the Ficoll density gradient system and the bedside isolation method, we strongly believe that simpler techniques will enhance practicability of cell therapy. BM as compared to peripheral blood seems to be the cell source of choice, a finding which also holds true for cardiac applications, because withdrawal of BM (usually between 100-250 mL) is fast (<10 minutes), does not require general anesthesia but sedation only, and yields reproducible cell numbers. In contrast, PB-MNC collection requires expensive G-CSF injections over 5 consecutive days and plasmapheresis for several hours, these procedures being both time-consuming and costly.

The Transplantation of Progenitor Cells and Regeneration Enhancement in Acute Myocardial Infarction (TOPCARE-AMI)[49] and the Reinfusion of Enriched Progenitor Cells and Infarct Remodelling in AMI (REPAIR-AMI)[50] trials showed that Ficoll isolated BM-MNC was able to improve cardiac function. This is in contrast to the findings of the Autologous Mononuclear Bone Marrow Cells in Acute Anterior Wall Myocardial Infarction (ASTAMI) trial, which did not show an improvement of cardiac function after intra-myocardial injection of BM-MNC isolated using the Lymphoprep method.[51] The conclusion drawn by Seeger et al[52] was that different diluents (saline vs heparin plasma), and different buffer solutions and incubation media caused the reduced (about one-third) BM-MNC number and reduced function of the Lymphoprep isolated cells.

With respect to dosage, most trials aimed at using MNC numbers comparable to those in the TACT study (1.6×10^9).[30] There is no trial making direct comparisons of the degree of positive effects between different cell doses, and the only study trying to establish a correlation between clinical response and cell number had only 8 participants.[53] Myocardial Stem Cell Administration after Acute Myocardial Infarction (MYSTAR) is the only published clinical trial with a positive correlation between the rate of improvement of cardiac perfusion and the number of injected stem cells in clinical cardiac stem cell therapy. It demonstrated that the only predictor for a reduction in infarct size was the number of intramyocardially injected cells.[54]

Tolerability and safety considerations. Long-term safety has been questioned by a trial of intramuscular BM cell therapy in which 1 of the 8 participating patients died suddenly at 30 months after the procedure, 2 patients had ulcer worsening, and 1 patient had incompetent angiogenesis.[55] This prompted other researchers to report the long-term status of their patients. In the meta-analysis by Fadini et al,[32] a total of 21 deaths (20 in the cell therapy group plus 1 in the G-CSF group) between 2 months and 3 years after therapy of 761 patients treated are reported. No controlled trial included reported mortality rates in the experimental vs the control groups. Safety data were described in 32 of 41 studies. BM aspiration was well tolerated, the most frequent adverse reaction being local pain, responsive to nonsteroidal anti-inflammatory drugs. Another less common adverse event was mild anemia. G-CSF stimulation was generally well tolerated, with prevalently minor side effects, including flu-like symptoms, myalgia, fever, and bone pain. Fadini et al,[32] therefore, conclude that given the existing data, no worrying safety concerns exist, but that most studies included in the meta-analysis were not properly designed to assess safety in comparison with control treatment, and that systematic reporting of adverse events was rare.

The TACT late study was designed to assess the long-term safety and clinical outcomes of cell therapy by investigating the mortality and leg amputation-free interval as primary end points.[56] The median follow-up time for surviving patients was 25 months, and the 3-year overall survival was 80% of patients with atherosclerotic PAD (11 of 74 patients died). This number increased to 100% in the 41 patients with TAO. The 3-year amputation-free rate was 60% in patients with PAD and 91% in patients with TAO. The TACT late study also reported no cases of unwanted neovascularization, no increase of the expected mortality, and no unwanted neovascularization. BONMOT-1[40] included 51 patients with impending major amputation due to severe critical limb ischemia for BMC transplantation into the ischemic leg with a 3.2-year follow-up. Limb salvage was 59% at 6 months and 53% at last follow-up.[40] From a clinical perspective, the most important finding was that patients with limb salvage improved from a mean Rutherford category of 4.9 at baseline to 3.3 at 6 months. Three severe periprocedural adverse events occurred (two cases of anemia after 500 mL BM aspiration, one case of large bowel puncture) and resolved without sequelae, but no unexpected long-term adverse events occurred. Based on the data presented above, BM cell transplantation seems

Table. Ongoing randomized cell therapy trials in PAD and CLI

NCT-ID	*Acronym (or other)*	*Sponsor/location*	*Condition*	*Intervention*	*Patient no.*	*Outcome measure*	*Phase*	*Completion date*
NCT-00377897	(OPTIPEC)	Univ. of Paris 5; AP-HP	CLI	BM-MNC	20	ABI, $TcPO_2$	I	12/09
NCT00392509	CLI-001	Aldagen	CLI, PAD, PVD	ALDH-br BM cells vs MN-BMC	20	ABI, $TcPO_2$, QoL, LPR	I/II	12/08
NCT00956332	(MGVS-MGA 002)	MultiGene Vascular Systems Ltd	PAD, CLI	MultiGeneAngio	18	AE, improvem. of CLI symptoms	I/II	01/26
NCT01065337	(HDZ-SBE-2004)	Ruhr Univ. Bochum	Diabetic foot	TRC, BMC	30	ABI, $TcPO_2$, amputation status, CPWH	II	02/09
NCT00468000	RESTORE-CLI	Aastrom Biosciences	PAD	Autologous BMC, electrolyte solution (-C)	150	Safety of TRCs in CLI patients, amputation status, ABI, QoL, $TcPO_2$	II	03/11
NCT00523731	(TV-003, ACPs-CLI)	TheraVitae Ltd	PAD, CLI	ACPs or Vescell	6	Attenuation of CLI, reduction of amp. rate, ulcer size	I	03/07
NCT00951210	(1202-2)	Pluristem Ltd	PAD, PVD, CLI	PLX-PAD	12	AE, amp. rate, inc. of death	I	12/10
NCT00919958	(PLX-PAD 1202-1)	Pluristem Ltd	PAD, PVD, CLI	PLX-PAD IM injection	15	AE, SLV, TG	I	05/12
NCT00913900	SCRIPT-CLI	Univ. of Wisconsin, Madison	CLI, AOD, VD	Autologous CD133+ cells	24	Death or amp., vascular hemodynamics and function	I	09/12
NCT00595257	(TriCell/CT/IND-001)	Harvest Technologies	AOD	SmartPReP2 BMAC system	60	Avoid amp., measurem. of HR	I/II	04/10
NCT00616980	ACT34-CLI	Baxter Healthcare	PAD, PVD, CLI	ASC (CD34+)	75	Ulcer healing, funct. improvem., limb salvage	I/II	10/09
NCT00434616	BONMOT-2	Franziskus Hospital Berlin	CLI	A-BMC-c vs placebo	?	Reduction of amp., induce wound healing, ABI, QoL, $TcPO_2$	II/III	03/10

A-BMC-c, Autologous bone marrow cell concentrate; *ABI,* ankle-brachial index; *ACP,* angiogenic cell precursors; *AE,* adverse events; *amp.,* amputation; *AOD,* arterial occlusive disease; *ASC,* autologous stem cells; *BMCs,* bone marrow stem cells; *BM-MNCs,* bone marrow mononuclear cells; *-C,* without cells; *CLI,* critical limb ischemia, *CPWH,* complete primary wound healing; *CTH,* cell therapy; *func.,* function; *HR,* hemodynamic response; *improvem,* improvement; *i.m.,* intramuscular; *inc.,* incidence; *LPR,* level of pain at rest; *PAD,* peripheral arterial disease; *PVD,* peripheral vascular disease; *QoL,* quality of life (as determined by questionnaire form); *SLV,* safety laboratory values; *$TcPO_2$,* transcutaneous partial oxygen tension; *TG,* tumorigenesis; *TRC,* tissue repair cells; *VD,* vascular disease.

to be a safe procedure with minimal short-term and long-term side effects.

Future directions and ongoing randomized controlled trials. A number of ongoing trials involving stem cell therapy in the treatment of PAD and limb ischemia can be found performing a database search of clinical trials at NIH ClinicalTrials.gov. Recent search results (August 2010) yielded the 12 trials listed in the Table which also lists further sub-specifications, such as condition to be treated and intervention method, numbers enrolled, sponsor, and date of completion. Results of these trials will likely provide stronger efficacy data of cell therapy. Albeit trial status in the search database can be indicated as completed (full recruitment), patient treatment and control might still be ongoing.

CONCLUSIONS

CLI represents the most severe manifestation of PAD that profoundly diminishes quality of life and global function and that is often associated with very high short-term mortality. Prompt recognition, vascular specialty referral, and revascularization are the current standards of care. Nevertheless, this care strategy is not always feasible, nor is it always effective. Evaluation of new pharmacological and

angiogenic therapies would fill a real clinical need. Current literature supports that intramuscular BM cell administration is a relatively safe, feasible, and possibly effective therapy for patients with PAD not susceptible to conventional revascularization. This novel therapeutic option could also soon be more widely available due to developments in the private industry sector, which is in the process of conducting promising second-generation trials. However, there is a need for larger, placebo-controlled, randomized multicenter trials to confirm safety and efficacy of this type of therapy, such as the ongoing BONMOT-CLI[41] (or BONMOT-2).

As an increasing number of clinical trial evidence supports routine use of stem cell therapy, more practical aspects of cell therapy will gain importance. Related to this, a single-step, bedside, closed isolation system without the need for specialized hospital subservices and without legal hurdles is especially useful, particularly because it substantially shortens total procedure time to 1 hour, and is considerably less expensive than Ficoll-based techniques. This may also enable non-university hospitals to use stem cell therapy for treatment of their patients with PAD.

Ongoing trials may also shed light upon the open issue still remaining to be resolved, such as selection of optimal cell type, isolation method, cell number, and the role of colony stimulating factors, route of administration, and paracrine stimulation mechanisms.

In conclusion, autologous stem cell therapy seems to be a promising tool for the treatment of ischemic peripheral disease. Preliminary evidence confirms its safety, feasibility, and effectiveness for several important end points, and a number of large end point studies are ongoing to further corroborate this evidence.

AUTHOR CONTRIBUTIONS

Conception and design: HL, PB, BA
Analysis and interpretation: Not applicable
Data collection: HL, BA
Writing the article: HL, PB, BA
Critical revision of the article: HL, PB, BA
Final approval of the article: HL, PB, BA
Statistical analysis: Not applicable
Obtained funding: Not applicable
Overall responsibility: HL, BA

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Submitted Apr 14, 2010; accepted Aug 21, 2010.