CHAPTER 46

The role of mesenchymal stem/stromal cells in the management of critical limb ischemia

P.K. Gupta*, Chullikana Anoop, Balasubramanian Sudha, R Mathiazhagan, Raj Swathi Sundar, and Majumdar Anish Sen

Stempeutics Research Pvt Ltd, Akshay Tech Park, Whitefield, Bangalore, India

Chapter menu

46.1 Introduction

Peripheral arterial occlusive disease, also commonly known as peripheral artery disease (PAD), is a manifestation of atherosclerosis caused by obstruction of peripheral arteries (Figure 46.1). Critical limb ischemia (CLI) is the severe subset and end stage of PAD and is characterized by severe pain at rest, nonhealing ischemic skin lesions, and, finally, gangrene of the extremity due to inadequate blood supply to the limb [1]. If no therapeutic intervention is made during this stage of the disease the inevitable outcome is limb amputation [2]. The blockage of arteries is primarily due to atherosclerosis.

Another cause of PAD, especially in Mediterranean and Asian countries, is thromboangitis obliterans, also known as Buerger's disease. This is a recurring progressive inflammation and thrombosis of small and medium arteries and veins of the hands and feet. It is thought to be an autoimmune disease that is strongly associated with use of tobacco products, primarily from smoking, but also from smokeless tobacco [3]. Buerger's disease was first reported by Felix von Winiwarter in 1879 in Austria, but it was not until 1908 that the disease was given its first accurate pathological description by Leo Buerger of Mount Sinai Hospital in New York City.

Other causes of PAD include other autoimmune disorders like systemic lupus erythematosus and acute conditions such as arterial embolism. The prevalence of PAD has been estimated to be around 27 million in Europe and North America [4]. It occurs in approximately 3–12% of the general population and increases with age [5–7]. Buerger's disease accounts for a variable proportion of patients with peripheral vascular disease throughout the world: 0.75% in North America, 0.5–5.6% in western Europe, 16–66% in Korea and Japan, 45–63% in India, and 80% in Israel among Jews of Ashkenazi descent [3,8]. Various risk factors causally related to PAD include diabetes mellitus, hyperlipidemia, hypertension, and smoking. Coexisting coronary artery disease and cerebrovascular disease are highly prevalent in patients with PAD, particularly in the elderly population. CLI, which is the most severe form of the disease, is seen in 1% of the general population [9].

Diagnostic studies include measurement of hemodynamic changes by ankle–brachial pressure index (ABPI) (Figure 46.2), transcutaneous partial oxygen pressure (TcPO2), toe brachial

* Corresponding author: Director, Medical Services, Stempeutics Research Pvt Ltd, Akshay Tech Park, No. 72 & 73, 2nd Floor, EPIP Zone, Phase I-Area, Whitefield, Bangalore – 560066, India. Email: pawan.gupta@stempeutics.com.

The Biology and Therapeutic Application of Mesenchymal Cells, First Edition. Edited by Kerry Atkinson.

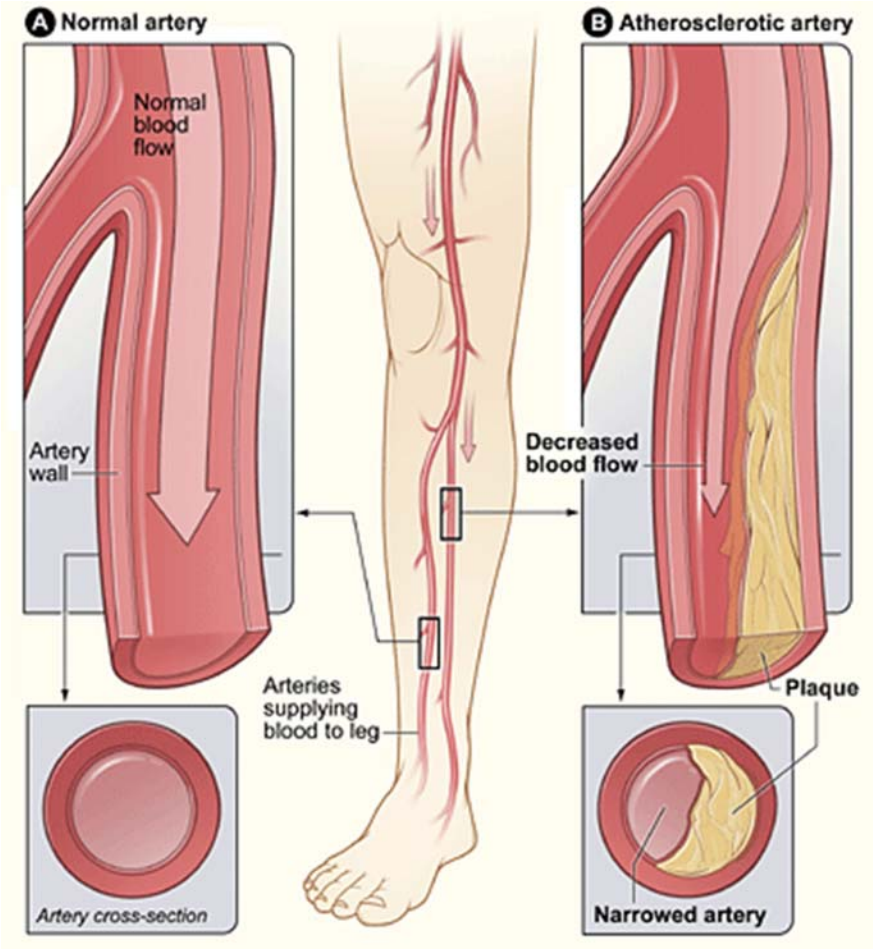

Figure 46.1 The illustration shows how PAD can affect arteries in the legs. (A) A normal artery with normal blood flow. The inset image shows a cross-section of the normal artery. (B) An artery with plaque build-up that is partially blocking blood flow. The inset image shows a cross-section of the narrowed artery. Source: this image, reproduced here from Wikimedia Commons, is in the public domain. The author is the National Heart, Lung and Blood Institute, The National Institutes of Health, USA. Its attribution is as follows: http://www.nhlbi.nih.gov/health/health-topics/topics/pad/.

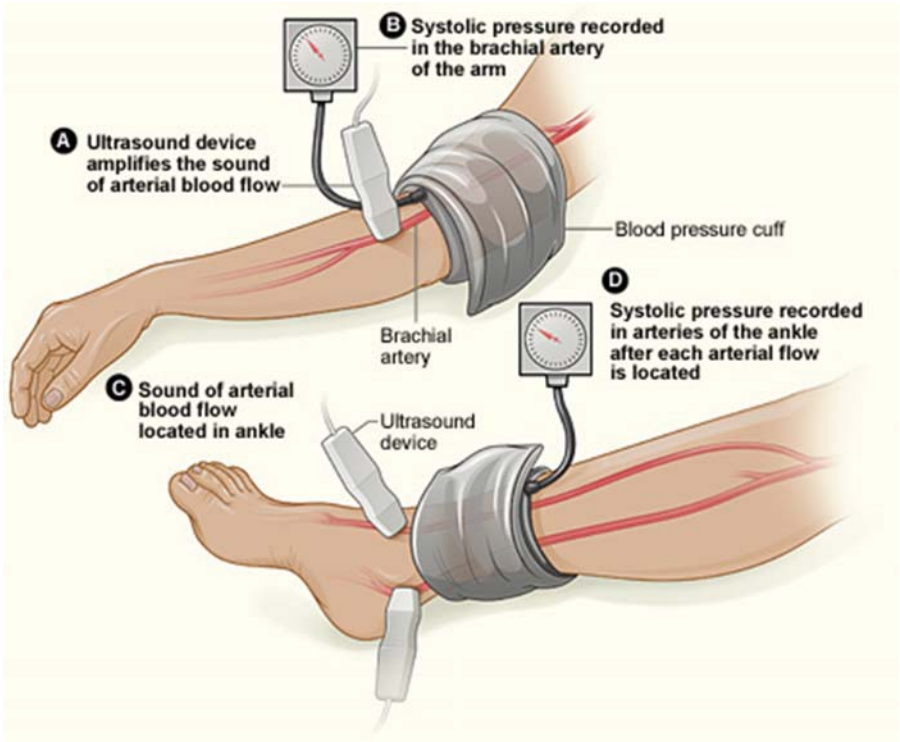

Figure 46.2 The illustration shows the ankle–brachial index test. The test compares blood pressure in the ankle with blood pressure in the arm. As the blood pressure cuff deflates, the blood pressure in the arteries is recorded. *Source:* this image, reproduced here from Wikimedia Commons, is in the public domain. The author is the National Heart, Lung and Blood Institute, The National Institutes of Health, USA.

pressure index, exercise testing to elicit symptoms, segmental pressure monitoring, and Doppler examination of the vascular system.

Magnetic resonance angiogram and computed tomography angiography can also aid in the diagnosis [10]. The management of CLI is based on risk factor management and surgical or endovascular revascularization aiming to improve blood flow to the affected extremity [11]. Most patients with CLI are managed with analgesics and medications that inhibit platelet aggregation, such as cilostazol, in addition to lipid-lowering drugs. Cilostazol is a phosphodiesterase inhibitor with a therapeutic focus on cyclic adenosine monophosphate. It inhibits platelet aggregation and is a direct arterial vasodilator. Its main effects are dilation of the arteries supplying blood to the legs and decreasing platelet coagulation.

If revascularization has failed or is not possible, amputation is often necessary. Approximately 10% of CLI patients will undergo primary amputation, with healing rates varying between 30 and 90% and re-amputation rate at between 4 and 30%. Overall, approximately 40–50% of CLI patients will lose their leg within 6–12 months and approximately 15% will also require contralateral amputation within 2 years [12]. In CLI patients cardiovascular mortality increases substantially with approximately 20% of patients dying during the first 6 to 12 months after the onset of CLI, and 2, 5 and 10 year mortality rates of approximately 35%, 70% and 100% respectively [12]. It has therefore become urgent to develop new modalities of therapy that will induce revascularization, including neoangiogenesis and remodeling of the vascular system, to prevent the complications of CLI, thus improving limb salvage and increasing the overall survival of these patients.

46.2 Mesenchymal stem/stromal cells and angiogenesis

Several types of cells, particularly bone-marrow-derived mesenchymal stromal cells (BM-MSCs), have been shown to differentiate in the presence of appropriate growth stimuli along specific pathways including endothelial cells and perivascular cells. Mesenchymal stromal cells (MSCs) were isolated first from the bone marrow [13] and subsequently from a variety of other tissues, including adipose tissue, placenta, umbilical cord and cord blood, dental pulp, and amnion. MSCs are usually isolated by their plastic adherence property and can be expanded in large-scale culture for clinical use. Although no specific marker has been identified to isolate the MSC population, the International Society of Cell Therapy has defined these cells as being positive for CD73, CD105, CD90, and major histocompatibility complex class I antigens (human leukocyte antigen (HLA)-A, -B, and -C) and being negative for the hematopoietic markers CD45, CD34, CD14, CD19, and CD11b as well as major histocompatibility complex class II antigens (such as HLA-DR) [14]. The lack of a specific marker to identify MSCs has made it difficult to categorically determine the similarities or differences between the biological properties of these cells isolated from different tissues. Interestingly, BM-MSCs have been shown to possess several unique biological properties that are potentially beneficial for their use in both autologous and allogeneic cell therapy. Their intrinsic self-renewing ability and differentiation potential into chondrocytes, adipocytes, and osteocytes have been well documented [15,16].

Furthermore, MSCs are able to migrate and home to injured tissues, where they can act either by differentiating into cells of the musculoskeletal system if the injury is a musculoskeletal one and/or by secreting trophic factors that mediate paracrine signaling [16]. MSCs possess anti-inflammatory and immunomodulatory properties by a number of mechanisms, including inhibition of secretion of tumor necrosis factor-α (TNF-α) and interferon-γ and by increasing secretion of interleukin (IL)-10, indolamine 2,3 deoxygenase, prostaglandin E_2, and nitric oxide [17]. This unique immunomodulatory property makes these cells suitable for allogeneic, as well as autologous, use since they are nonimmunogenic as well as actively suppressive of T cells, B cells, natural killer cells, and macrophages and thus avoid rejection by the recipient's immune system [18].

It is now accepted that the myriad of growth factors and cytokines secreted by the MSCs *in vitro* and *in vivo* are critical for their therapeutic activity (Figure 46.3).

The major immunomodulatory cytokines secreted by MSCs include prostaglandin E_2, transforming growth factor-β1 (TGF-β1), hepatocyte growth factor (HGF), stromal derived factor-1, nitric oxide, indolamine 2,3-deoxygenase, IL-4, IL-6, IL-10, IL-1 receptor antagonist, and soluble tumor necrosis factor-α receptor.

With regard to angiogenesis, MSCs secrete vascular endothelial growth factor (VEGF), insulin-like growth factor-1 (IGF-1), epidermal growth factor, and angiopoietin (Ang)-1. These factors are released to recruit endothelial lineage cells and initiate vascularization [21]. MSCs also express mitogenic proteins such as TGF-α, TGF-β, HGF, epidermal growth factor, basic fibroblast growth factor, and IGF-1, which increase fibroblast, epithelial, and endothelial cell division [22]. These important factors are

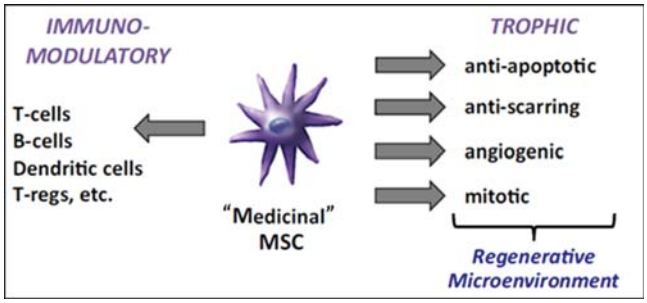

Figure 46.3 The bioactive molecules secreted by medicinal MSCs are immunomodulatory and affect a variety of immune cell lineages [17]. Other secreted molecules establish a regenerative microenvironment by establishing a powerful trophic field [19]. *Source:* adapted from Caplan 2011 [20], pp. 11–15. Reproduced with permission of Elsevier.

known to play a key role in angiogenesis and act by a paracrine pathway [23,24]. Both VEGF and the angiopoietins (Ang-1 and Ang-2) play a crucial role in the process of neoangiogenesis. While Ang-1 and Ang-2 participate in the stabilization of the newly formed vessels, VEGF exerts its proangiogenic function by binding to one of its receptors, specifically VEGF receptor 2 (VEGFR2) also known as kinase insert domain receptor), which is expressed exclusively by endothelial cells and their precursors. This binding triggers a cascade of events leading to the formation of new blood vessels, including recruitment of support cells such as pericytes for small capillaries and smooth muscle cells for larger vessels [25]. MSCs have also been shown to possess anti-inflammatory, antiapoptotic, antifibrotic, mitogenic, and wound healing properties [26]. The anti-inflammatory properties of MSCs may be primarily responsible for the marked benefit of these cells in Buerger's disease, which is an inflammatory disease with a likely autoimmune basis affecting small and medium-sized arteries [27].

46.3 Potency assays for cells to be used in critical limb ischemia

The criteria for establishing the safety and quality of cellular therapies, both during production and at the time of lot release, are the use of potency assays to measure biologic activity appropriate to their intended clinical use. Potency testing, as stipulated by regulatory guidelines, should be a quantitative measure that reflects directly on the fundamental mechanistic pathway underlying the therapeutic response to verify the manufacturing process and is a measure of biological activity. A potency assay also provides the basis for comparability assessment after process changes, such as scale-up, site transfer, and new starting materials (e.g., a new MSC donor). Unlike defined component therapies with known molecular mechanisms, the therapeutic benefit of cellular therapies is often driven by its influence on a combination of mechanistic pathways and is often the result of a complex mixture of factors [28]. Cell viability is an important component of the potency of cell-based therapy. However, additional parameters of biological activity should also be tested [29] (and see Chapters 43 and 44).

Release specifications, which are acceptance criteria to be met by a product lot in order to allow administration to a patient, need to be defined in terms of identity, purity, and potency. Critical biological activity or potency for cell products can be defined by repeated testing of new cell lots in preclinical models. However, this is often not practical because the time taken for its execution is long and thus complicates lot release and clinical inventory requirements. Therefore, development of rapid, robust, consistent surrogate assays is required to ensure maintenance of biologic activity of cell therapies before their release for clinical use. Core requirements for potency testing as defined by the US Food and Drug Administration are presented in Table 46.1 [30].

Table 46.1 US Food and Drug Administration core requirements for potency testing [30].

Potency testing of cell therapy product must:

- indicate product-specific biologic activity
- measure identity and activity of active component
- provide test results for product release
- provide data to establish stability specifications
- meet labeling requirements
- comply with biologics regulations and good manufacturing practice

Potency testing methods must:

- have predefined acceptance or rejection criteria
- include appropriate reference materials, standards, controls
- be amenable to validation
- have established and documented accuracy, sensitivity, specificity, and reproducibility
- provide quantitative data

Lehman *et al.* demonstrated that by using an *in vitro* endothelial tube formation assay a surrogate angiogenic potency assay could be developed that reflects the proangiogenic activity of multipotent adult progenitor cells (MAPCs) [28]. Serum-free conditioned media collected from MAPC culture induced endothelial tube formation. Three proteins, chemokine (C–X–C motif) ligand 5 (CXCL5), IL-8, and VEGF, were required and were linked to angiogenic potency. Depletion of any of these factors from the media prevented tube formation, while adding back increasing amounts of these cytokines into the depleted serum-free conditioned media established the lower limits of each of the cytokines required to induce angiogenesis. Thus, these markers were adopted as a surrogate potency assay for MAPC angiogenic activity.

46.3.1 Ixmyelocel-T

Ledford *et al.* demonstrated that ixmyelocel-T, which is an autologous patient-specific, expanded, multicellular therapy cultured from bone marrow mononuclear cells (BM-MNCs), contains a unique expanded population of M2-like macrophages that may limit tissue injury and promote repair [31]. It also contains large numbers of MSCs. M2 macrophages promote tissue repair and regeneration through various mechanisms, including immunomodulation and scavenging of tissue debris. It was proposed that delivering high numbers of these cells to ischemic tissues might limit tissue injury and promote repair. The authors demonstrated that macrophages in ixmyelocel-T therapy expressed the surface markers of M2 macrophages, CD206, and CD163. CD206 (also known as mannose receptor) is a 175 kDa type I membrane protein. It is a pattern recognition receptor belonging to the C-type lectin superfamily. CD163 is a scavenger receptor for the hemoglobin–haptoglobin complex.

After stimulation with lipopolysaccharide, they showed minimal secretion of the proinflammatory cytokines IL-12

and TNF-α compared with conventional M1 and M2 macrophages. Ixmyelocel-T macrophages efficiently ingested apoptotic BM-MNCs. They concluded that ixmyelocel-T therapy contained a unique population of M2-like macrophages that are characterized by expression of M2 markers, decreased secretion of proinflammatory cytokines after inflammatory stimuli, and efficient removal of apoptotic cells and that they may have a potential role in tissue repair and regeneration.

46.3.2 Stempeucel®

Stempeutics Research in Bangalore has developed a cell product called stempeucel®, which is an adult human bone-marrow-derived, cultured, pooled allogeneic MSC population. The cells show greater than 90% positivity for MSC-associated markers CD73, CD90, and CD105 and do not express CD34, CD45, or HLA-DR or co-stimulatory molecules (Figure 46.4) [32,33].

The probable mechanism of action of stempeucel in CLI is through secretion of growth factors, chemokines, and cytokines that are known for their pro-angiogenic, anti-inflammatory, and immunomodulatory functions [34]. BM-MSCs are known to secrete angiogenic cytokines such as VEGF, IL-6, IL-8, and Ang-1, and they have been shown to be potently angiogenic [35]. VEGF has a unique feature compared with the other growth factors in the regulation of new blood vessel formation in that it has a high degree of specificity for endothelial cells. VEGF treatment of cultured endothelial cells promotes cell survival, proliferation, migration, calcium influx, and branching morphogenesis [36]. In order to understand the exact mechanisms involved, stempeucel was initially screened for the expression of angiogenic genes such as VEGF A and its receptor VEGFR2, IL-6, Ang-1, HGF, keratinocyte growth factor (KGF), IGF-1, and TGF-β by reverse transcriptase polymerase chain reaction (Figure 46.5). The results clearly demonstrated that stempeucel expressed most of the angiogenic genes and that VEGF could potentially be a surrogate potency marker for stempeucel [31–33].

A possible potency assay for an MSC-based product for use in CLI could be its ability to initiate the process of neoangiogenesis. The potency assay could indicate the mechanism of action of the cellular product and could be used for quality control (stability) throughout the manufacturing process. Different studies [28,31] have demonstrated that various surrogate angiogenic markers can be established to demonstrate the potency of the product in CLI. These include VEGF, CXCL5, IL-8, endothelial tube formation, markers of M2 macrophages (CD206, CD163), IL-6, Ang-1, HGF, KGF, IGF-1, and TGF-β.

Selection of an angiogenic marker or markers is important in order to demonstrate the ability of the product to effect the desired therapeutic result. Developing at least two different methods increases the chances that one of them will be suitable for the product and will satisfy the requirements of the regulatory authorities [37].

46.4 Preclinical studies

46.4.1 Preclinical safety studies

Many studies have demonstrated that MSCs are nontoxic and nontumorigenic when tested in various preclinical animal models. MSCs from different tissue sources have been studied for potential side effects according to the regulatory criteria of respective countries (see Chapter 44). Studies involving both allogeneic and xenogeneic MSCs have shown that these cells are nontoxic. Similar results have been found in numerous clinical trials. Ra *et al.* studied the safety of human adipose-tissue-derived MSCs (ADMSCs) in humans and animals [38]. Immunodeficient mice were exposed to different doses of human ADMSCs intravenously and were observed for 13 weeks. There were no side effects and no mortality was observed. Tumorigenicity testing in BALB/c-nu (nude) immunodeficient mice showed no evidence of tumor formation even at the highest cell dose used.

Isakova *et al.* injected BM-MSCs into the central nervous system of young adult rhesus macaque monkeys to evaluate their safety and feasibility as vectors for direct intervention in neurologic disorders. The study demonstrated that the procedure of cell implantation was well tolerated and there was no observed toxicity attributable to the injected MSCs. Importantly, unlike the study by Ra *et al.*, in which xenogeneic cells were used, the study by Isakova *et al.* used syngeneic MSCs isolated from the iliac crest of a male rhesus macaque [39].

There have been concerns over the possibility of aggregation of these cells producing emboli in the lungs [40]. However, Eggenhofer *et al.* demonstrated the MSCs delivered by intravenous infusion have a short life and do not migrate to other organs [41], although other studies of intravenously injected MSCs have shown that after initial lodgment in the lungs they can subsequently migrate to sites of inflammation beyond the pulmonary capillary bed (see Chapters 22 and 23).

We have also conducted preclinical safety studies using BM-MSCs. These studies included acute toxicity studies (14 days single dose and 14 days repeat dose) by intravenous and intramuscular administration of stempeucel, as well as longer toxicity studies (90 days single dose) in two animal species (rats and rabbits) by two routes (intravenous and intramuscular) of administration. The toxicity studies were conducted using human-equivalent doses in respective animals to a maximum dose of 20×10^6 BM-MSCs/kg body weight. In addition, tumorigenicity and prenatal developmental toxicity studies have also been performed in immunocompromised mice and Sprague Dawley rats respectively. Results have shown that these cells are safe, nontumorigenic, nonteratogenic, and did not induce genotoxicity (Mathi *et al.* unpublished results).

Studies using a wide range of doses (2×10^6 cells/kg body weight to 250×10^6 cells/kg body weight) of MSCs have been used in preclinical studies and have not shown signs of toxicity in rodents and large animal studies, including subhuman primates.

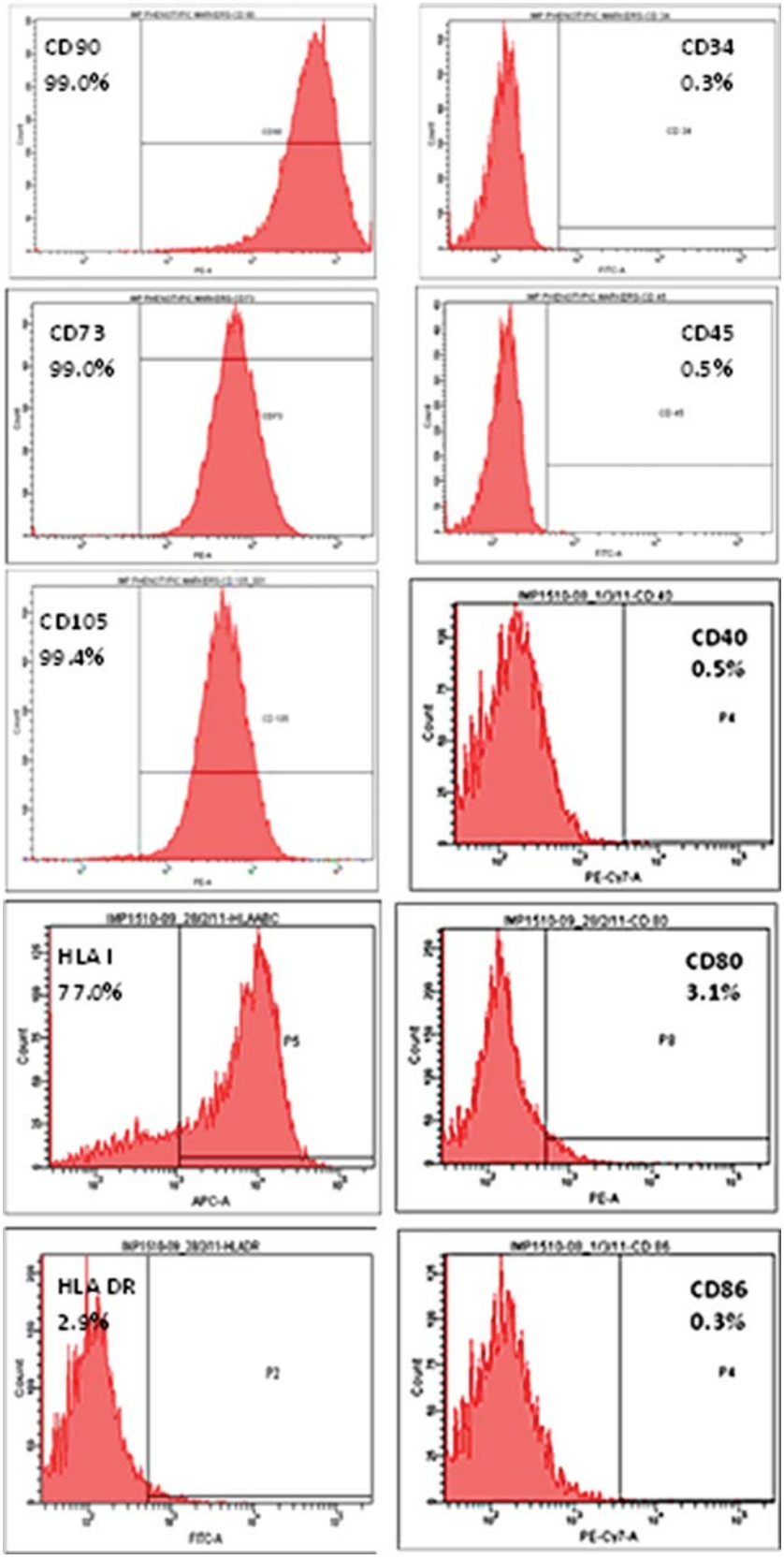

Figure 46.4 Immunophenotype analysis of stempeucel. Stempeucel is positive for expression of the characteristic mesenchymal stromal cell markers CD90, CD73, and CD105 and negative for hematopoietic markers CD34 and CD45, as well as HLA-DR and the immune co-stimulatory markers CD80 and CD86.

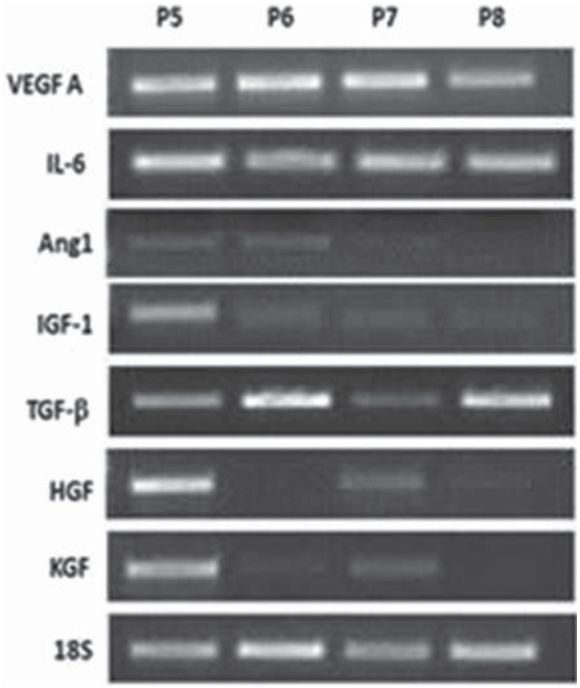

Figure 46.5 Reverse transcriptase polymerase chain reaction showing that stempeucel expresses most of the angiogenic genes, including VEGF A, IL-6, Ang-1, IGF-1, TGF-β, HGF, and KGF from passage 3 to passage 7.

46.4.2 Preclinical efficacy studies

It is also important to demonstrate efficacy of any cell therapy product in an animal model of disease before administering the same cells to humans with the same disease. Unlike conventional small molecules, stem cell products face a unique challenge in this area. The disparity lies in determining the suitability of using animal stem cells in animals or human stem cells in immunocompromised/immunocompetent animals. A common regulatory requirement is to have animal data for the same test product that is intended to be tested in humans. This infers a regulatory preference for testing a human cell product in an animal disease model that closely resembles the human disease condition for which the product is intended. However, to validate the concept of efficacy, scientists have more commonly tested animal cells in animals.

In the context of this chapter, numerous animal models have shown that transplantation of MSCs or mononuclear cells (MNCs) from different sources augmented arteriogenesis in the ischemic limb of the animal [42,43]. Kim *et al.* compared the angiogenic potency of human mesenchymal stem/stromal cells derived from adipose tissues (ADSC) and bone marrow in a nude mouse model of hind limb ischemia [42]. Two weeks after transplantation the laser Doppler perfusion index was significantly higher in the ADSC group and BM-MSC groups compared with animals in the control group. The authors demonstrated that human MSCs can be a potential source for therapeutic angiogenesis.

Iwase *et al.* studied whether MSCs or MNCs would be a better option for treatment of CLI in a rat model of hind limb ischemia [43]. Three weeks after injection the laser Doppler perfusion index was noted to be significantly higher in the MNC group than in the control group. Capillary density was found to be higher in the MSC group than in the MNC-treated group. The numbers of endothelial cells and vascular smooth cells derived from the injected cells were also higher in the MSC group than in the MNC group. The study concluded that MSCs provided greater improvement in hind limb ischemia than MNCs.

We have performed preclinical efficacy studies in an animal model of hind limb ischemia in BALB/c nude mice to determine the efficacy of stempeucel. We used an extended femoral ligation method as the model and injected stempeucel intramuscularly at two different doses around the ligation. In the control group, all animals developed foot necrosis, whereas in the stempeucel group the highest dose of 5×10^6 MSC-treated animals showed 43% protection from foot necrosis. The results clearly demonstrated that animals treated with the highest dose of stempeucel had a better outcome than animals treated with the lower dose.

Based on this preliminary study a second efficacy study was performed with stempeucel at a dose of 5×10^6 cells. The results clearly showed a significant improvement in limb function, limb salvage, and histology scores, including muscle fiber area in animals treated with stempeucel, compared with the animals treated with the vehicle control (unpublished results). Histological analysis of limb muscles at day 28 after stempeucel injection revealed significant increase in the total muscle fiber area and significantly reduced muscle degeneration, inflammation, and muscle necrosis (Figure 46.6).

These findings suggest that allogeneic human BM-MSCs have the therapeutic potential to reduce inflammation and muscle degeneration and to promote neoangiogenesis for the healing of ischemic ulcers in CLI patients.

46.5 Clinical trials in critical limb ischemia

46.5.1 Safety of mesenchymal stromal cells in clinical trials

Lalu *et al.* conducted a systematic review of the literature and a meta-analysis of clinical trials to evaluate the safety of MSCs given intravenously or intra-arterially to humans [44]. A total of 1012 participants with various clinical conditions were included. Eight studies were randomized controlled clinical trials and enrolled 321 of the total 1012 patients studied. Both adult populations and mixed adult and pediatric populations were included. Studies using differentiated MSCs or additional cell types were excluded. There was a significant association between MSCs and transient fever at or shortly after MSC administration, which was not associated with long-term sequelae. Importantly, the pooled analysis recorded no serious adverse events due to administration of the MSCs and specifically found no association between MSCs and tumor formation. Other potential risks, such

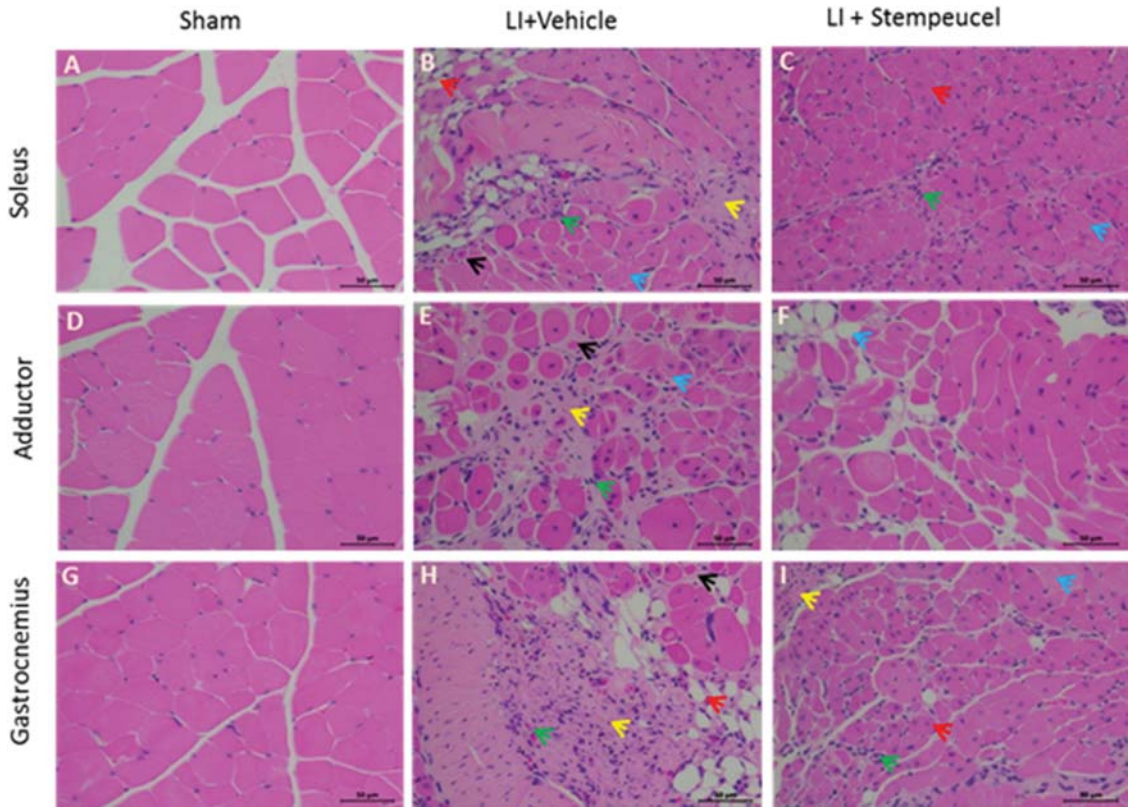

Figure 46.6 Histology (hematoxylin and eosin stain) of sham-treated, arterial ligation+vehicle and arterial ligation+stempeucel animals after 28 days. (A, D, G) Cross-sections of skeletal muscles (A; soleus; D: adductor; G: gastrocnemius) of sham-treated animals showing the normal histology of muscle fibers with a peripheral nucleus arranged in muscle bundles with occasional blood vessels seen in between. (B, E, H) Cross-sections of the skeletal muscles (B; soleus; E: adductor; H: gastrocnemius) of animals after arterial ligation treated with vehicle showing severe vacuolar degeneration (red arrow), infiltration of inflammatory cells (green arrow), necrosis of muscle fibers (yellow arrow), degeneration of muscle fibers (blue arrow), and atrophy of muscle fibers (black arrow). (C, F, I) Cross-sections of the skeletal muscles (C; soleus; F: adductor; I: gastrocnemius) after arterial ligation in animals treated with stempeucel showing very mild degeneration of muscle fibers (blue arrow), infiltration of inflammatory cells (green arrow), and necrosis of muscle fibers (yellow arrow). Many regenerating muscle fibers (red arrow) with a centrally placed nucleus are observed. (See color plate section.)

as acute infusional toxicity, organ system complications, infection, or death, were also not associated with MSC administration. Hence, one can conclude that systemic administration of MSCs is safe.

46.5.2 Efficacy of mesenchymal stromal cells in clinical trials of critical limb ischemia

Several clinical trials have been conducted using AD- or BM-MSCs in CLI, both in atherosclerotic PAD and in Buerger's disease. Administration has been fairly standardized, with cells being administered in most studies by intramuscular injection into the gastrocnemius muscle. A few studies have also tried intra-arterial injections. There has been a preference for administering the MSCs locally around the ischemic ulcers for better healing.

On searching clinicaltrials.gov using the keywords "stem cells" and "CLI" we found 46 completed or ongoing clinical trials. Fifteen studies were using mesenchymal stem/stromal cells as the investigational agent, while other studies were using autologous MNCs from the bone marrow. TACT (Therapeutic Angiogenesis using Cell Transplantation) was the first large report on the use of BM-MNCs in the treatment of CLI [45]. In this pilot study they investigated the efficacy and safety of autologous implantation of BM-MNCs in patients with ischemic limbs because of PAD. They concluded that administration of autologous BM-MNCs was safe and effective for obtaining therapeutic angiogenesis, presumably because of the ability of marrow-derived cells to supply endothelial progenitor cells and to secrete various angiogenic factors or cytokines. They further suggested that the success of BM-MNCs transplantation was due to a higher number of $CD34^+$ hematopoietic stem cells and immature precursor cells in the bone marrow compared with peripheral blood. This was the first study using cells for the treatment of limb ischemia, and it was followed by a number of studies across the globe. The studies published in clinicaltrials.gov using mesenchymal stem cells are shown in Table 46.2.

Idei *et al.* assessed long-term clinical outcomes after BM-MNC administration in 51 patients with CLI, including 25 patients with PAD and 26 patients with Buerger's disease. Forty-six CLI

Table 46.2 Clinical trials using MSCs in CLI registered with clinicaltrials.gov.

	Type of cells	Condition	Trial phase	Enrolment	Primary outcome measures	Sponsor or collaborators	NCT number
1	MNCs and MSCs	CLI	Phase II	50	Change in angiogenesis Change in blood supply	National University of Malaysia Cytopeutics PTL	NCT01456819
2	MSCs	CLI	Phase I Phase II	20	Adverse events and symptomatic relief	Stempeutics Research Pvt Ltd	NCT00883870
3	BM-MSCs	CLI/Buerger's disease	Phase II	126	Relief of rest pain Healing of ulcerations or reduction of ulcer area in the target limb	Stempeutics Research Pvt Ltd	NCT01484574
4	ADMSCs	CLI/diabetes mellitus	Phase I Phase II	33	Angiographic assessment of neovasculogenesis (angiogenesis plus arteriogenesis). Major adverse events (death, target limb amputation)	Iniciativa Andaluza en Terapias Avanzadas - Fundación Pública Andaluza Progreso y Salud/Iniciativa Andaluza en Terapias Avanzadas	NCT01257776
5	MSCs and MNC	Autologous transplantation. Diabetic foot	Phase I	40	Magnetic resonance angiography	Third Military Medical University	NCT00955669
6	UCMSCs	Diabetic foot/ CLI	Phase I Phase II	50	Angiographic evaluation of angiogenesis at ischemic limb. Pain	Qingdao University	NCT01216865
7	ADMSCs	CLI	Phase I Phase II	20	Major adverse events	K-Stemcell Co Ltd/Pusan National University Hospital	NCT01663376
8	BM-MSCs	CLI/PAD	Phase I Phase II	30	Collection of adverse events Safety laboratory values ECG findings Analysis of inflammation markers	Apceth GmbH & Co. KG	NCT01351610
9	ADMSCs	CLI	Phase I/ phase II	33	Number of adverse events and serious adverse events	Iniciativa Andaluza en Terapias Avanzadas – Fundación Pública Andaluza Progreso y Salud/Iniciativa Andaluza en Terapias Avanzadas	NCT01745744
10	ADMSCs	CLI	Phase I Phase II	60	Safety	Kasiak Research Pvt Ltd	NCT02145897
11	BM-MSCs	Diabetic foot	Phase II	30	Survival. Major amputation. Primary wound healing. Ipsilateral relapse	Ruhr University of Bochum	NCT01065337
12	ERCs	Peripheral vascular diseases	Phase I Phase II	15	Safety	Medistem Inc.	NCT01558908
13	ADMSCs	Non-revascularizable critical ischemia of the lower limbs	Phase I Phase II	10	Safety	Instituto de Investigación Hospital Universitario La Paz/ Hospital Universitario La Paz	NCT01824069
14	BM-MSCs	Type I diabetes mellitus with ulcer/type II diabetes mellitus with ulcer	Phase I Phase II	10	Adverse events	Sheba Medical Center	NCT01686139

(*continued*)

Table 46.2 (*Continued*)

	Type of cells	Condition	Trial phase	Enrolment	Primary outcome measures	Sponsor or collaborators	NCT number
15	BM-MSCs	Critical ischemia	Phase I Phase II	48	Serious adverse events	Iniciativa Andaluza en Terapias Avanzadas – Fundación Pública Andaluza Progreso y Salud/Iniciativa Andaluza en Terapias Avanzadas	NCT02287974
16	UCMSCs	Diabetes mellitus/PAD	Phase I Phase II	30	Angiographic evaluation of angiogenesis	Institute of Hematology & Blood Diseases Hospital	NCT02287831
17	Combination cell therapy with MSCs and EPCs	CLI/severe leg ischemia/PAD/ peripheral vascular disease	Phase II	35	Enhancement of vessel formation accessed by nuclear perfusion scan	TCA Cellular Therapy	NCT00721006
18	Combination cell therapy	CLI/peripheral vascular disease	Phase I	10	Safety	TCA Cellular Therapy	NCT00518401
19	Expanded autologous adipose-derived adult stroma/stem cells	Peripheral vascular diseases/ cardiovascular diseases	Phase I Phase II	13	Adverse events and tolerability	University Hospital, Toulouse/Unity Research 5241 UPS/CNRS/French Blood Establishment (Midi-Pyrénées)/Clinical Investigation Centre	NCT01211028
20	Peripheral blood-derived autologous angiogenic cell precursors	CLI	Phase II	22	Safety Rest pain Pain-free walking distance Ulcer size Gangrene dimension and intensity Improvement of tissue perfusion	Salus Ltd/TheraVitae Ltd	NCT01584986

MNCs: mononuclear cells; MSCs: mesenchymal stromal cells; BM-MSCs: bone marrow-derived mesenchymal stromal cells; ADMSCs: adipose-tissue-derived mesenchymal stromal cells; EPCs: endothelial progenitor cells; UMSCs: umbilical-cord-derived mesenchymal stromal cells.

patients who had no BM-MNC injection served as control subjects. The median follow-up period was 4.8 years. The 4-year amputation-free rates after BM-MNC implantation were 48% in PAD patients and 95% in Buerger's disease, while they were 0% in control PAD patients and 6% in control Buerger's disease. The 4-year overall survival rates after BM-MNC implantation were 76% in PAD patients and 100% in Buerger's disease, while they were 67% in control PAD patients and 100% in control Buerger's disease. Multivariable Cox proportional hazards analysis revealed that BM-MNC implantation correlated with prevention of major amputation and that hemodialysis and diabetes mellitus correlated with major amputation. In the patients with Buerger's disease, ABPI and TcPO2 were significantly increased after 1 month and remained high during the 3-year follow-up period. However, in patients with PAD, ABPI and TcPO2 significantly increased after 1 month but then gradually decreased during the 3-year follow-up period and returned to baseline levels. The authors concluded that BM-MNC transplantation is safe and effective in patients with CLI, especially in patients with Buerger's disease [27].

We have earlier evaluated and reported the results of a study of 13 patients in a phase I trial to investigate the safety and efficacy of intra-arterial injection of BM-MSCs in CLI patients. There was significant pain relief in these patients at 6 months after cell administration compared with their baseline level. Visual analog scale scores decreased from 2.29 ± 0.29 to 0.5 ± 0.34 ($P < 0.05$), ABPI increased significantly from 0.56 ± 0.02 to 0.67 ± 0.021 ($P < 0.01$), and TcPO2 also increased significantly in the foot from 13.57 ± 3.63 to 38 ± 3.47 ($P < 0.05$). Similar improvements were seen in the leg as well as in the thigh region. There was 86% limb salvage and six of seven ulcers showed complete or partial healing. The authors concluded that intra-arterial MSCs could be safely administered to patients with CLI and that significant therapeutic benefits were observed in these patients [46]. (A visual analog scale is a psychometric response scale that can be used in questionnaires. It is a measurement instrument for subjective characteristics or attitudes that cannot be directly measured. When responding to a visual analog scale item, respondents specify their level of agreement to a statement by indicating a position along a continuous line between two endpoints.)

The ulcer healing properties of BM-MSCs was demonstrated by Dash *et al.* [47]. This study was undertaken to assess the efficacy and feasibility of autologous BM-MSCs in the treatment of chronic nonhealing ulcers in patients with diabetic foot ulcers or Buerger's disease of the lower extremities. A total of 24 patients with nonhealing ulcers of the lower limb were enrolled and randomized into cell and control groups. In the cell group the patients received autologous cultured BM-MSCs together with standard wound dressing. The control group received only the standard wound dressing regimen. Patients were followed up for at least a 12-week period. Wound size, pain-free walking distance, and biochemical parameters were measured before therapy and at 2-week intervals following the intervention. The cell group had a significant improvement in pain-free walking distance and a reduction in ulcer size compared with those in the control group. There was no significant alteration in biochemical parameters observed during the follow-up period, indicating normal liver and renal function following the intervention. The study indicated that autologous implantation of BM-derived MSCs in nonhealing ulcers accelerated the healing process and significantly improved clinical parameters.

Lu *et al.* conducted a study to compare autologous bone-marrow-derived mesenchymal stem/stromal cells with the BM-MNCs for the treatment of diabetic CLI and foot ulceration [48]. The ulcer healing rate in the BM-MSC group was significantly higher than that in the BM-MNC group at 6 weeks after injection and reached 100% 4 weeks earlier than in the BM-MNC group. After 24 weeks of follow-up the improvements in limb perfusion induced by BM-MSC administration were more significant than those receiving BM-MNCs in terms of painless walking time, ABPI, TcPO2, and magnetic resonance angiography analysis. There was no significant difference between the groups in terms of pain relief and amputation and there were no serious adverse events related to either type of cell injections. The authors concluded that BM-MSC administration may be better tolerated and more effective than BM-MNCs for increasing lower limb perfusion and promoting foot ulcer healing in diabetic patients with CLI.

Teraa *et al.* conducted a meta-analysis of all randomized controlled trials (RCTs) that studied bone-marrow-derived cell therapy compared with standard care with or without placebo in CLI patients. Major amputation and amputation-free survival were considered as the primary endpoints. A total of 12 RCTs cumulatively including 510 CLI patients were analyzed. The meta-analysis showed significant beneficial effects of bone-marrow-derived cell therapy on both subjective and surrogate objective endpoints, including pain score, pain-free walking distance, ABPI, and TcPO2 measurements. Overall, the RCTs showed reduced amputation rates in the therapeutic arms of the trials with a relative risk (RR) of major amputation of 0.58 (95% confidence interval (CI), 0.40–0.84;$P = 0.004$). However, when only the placebo-controlled RCTs were considered, the beneficial effect on major amputation rates was considerably reduced and no longer significantly different (RR = 0.78; 95% CI, 0.40–1.51; $P = 0.46$). Amputation-free survival did not significantly differ between the bone-marrow-cell-treated group and the control group. This meta-analysis underlined the promising potential of bone-marrow-derived cell therapy in CLI patients, but importantly indicated the need for placebo-controlled clinical trials once safety and feasibility issues were satisfactorily resolved in phase I trials [49].

46.5.3 Clinical trials in India

Few clinical trials using allogeneic or autologous MSCs or MNCs in CLI have been conducted in India. The first initiative was undertaken by the Department of Biotechnology, Government of India, which conducted a multicenter trial using autologous BM-MNCs in patients with CLI, the results of which are yet to be published.

Motukuru *et al.* enrolled 36 patients with nonreconstructible Buerger's disease and injected autologous BM-MNCs into the calf muscles of the affected limbs in a clinical trial conducted at Jain Institute of Vascular Sciences, Bhagwan Mahaveer Jain Hospital, Karnataka, India [48]. They reported no procedure-related complications. Three patients (12%) underwent major amputations within 6 months. The others had improvement in ulcer healing, an increase in the mean ABPI, and an improvement in mean TcPO2 levels, resulting in limb salvage in all at 6 months. They concluded that BM-MNCs transplantation into ischemic limbs was a relatively safe procedure with no demonstrable side-effects at 6 months follow-up.

46.5.4 Stempeutics research experience in critical limb ischemia

The off-the-shelf allogeneic BM-MSC product developed by Stempeutics has undergone two clinical trials in CLI. The phase I/II trial included all patients with CLI (both atherosclerotic CLI and Buerger's disease), while in the phase II trial we exclusively included patients with CLI due to Buerger's disease as per Shionoya's diagnostic criteria [51]. These criteria are a smoking history, onset before the age of 50 years, infrapopliteal arterial occlusions, either upper limb involvement or phlebitis migrans, and absence of atherosclerotic risk factors other than smoking. All five criteria need to be present in order to make a diagnosis of Buerger's disease. Phlebitis is inflammation of a vein. When it occurs repeatedly in different locations it is known as thrombophlebitis migrans or migrating thrombophlebitis and can be an early indicator of gastric or pancreatic cancer.

46.5.5 Phase I/II study in patients with critical limb ischemia

The phase I/II results of stempeucel in CLI patients have been published [52]. Briefly, 10 patients each were randomized to either the cell arm or the placebo arm. A BM-MSC dose of 2 million MSCs/kg body weight administered intramuscularly was shown to be safe in patients with CLI. Improvement was observed in the rest pain scores in both arms, and no measurable differences were observed between the two arms. A significant

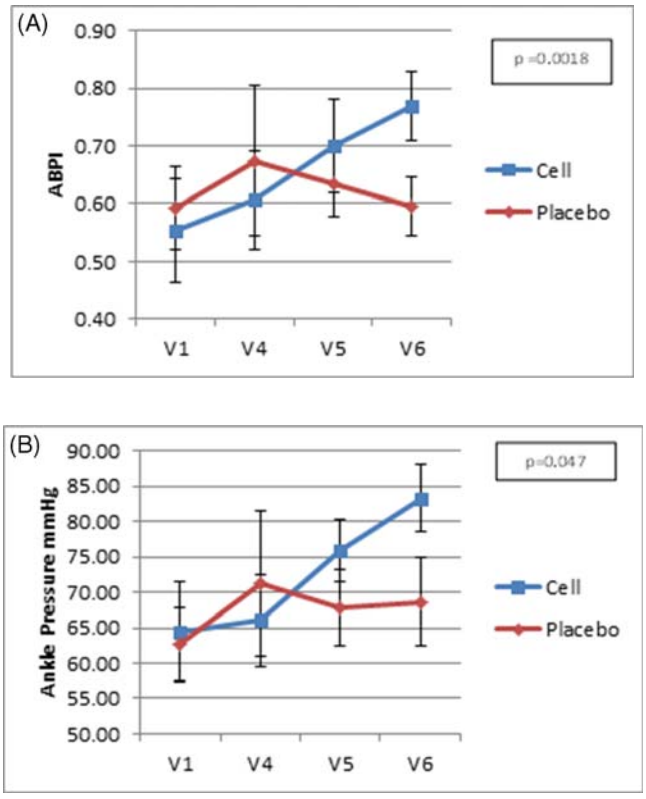

Figure 46.7 Phase I/II study efficacy parameters. Results of the ankle-brachial index test (A) and ankle pressure (B) are shown as mean plus/minus standard deviation. VI: screening; V4: 1 month; V5: 3 months; V6: 6 months of follow-up.

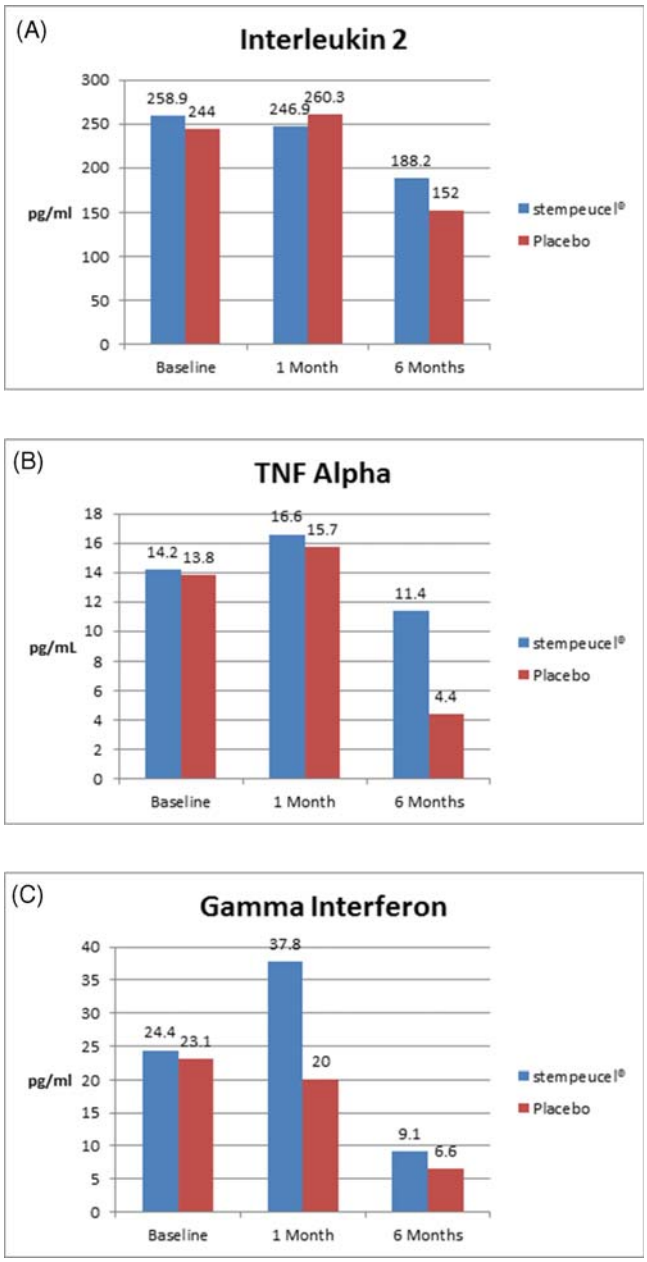

Figure 46.8 Immunological response markers in stempeucel-treated arm and placebo arm at baseline, 1 month, and 6 months follow-up for IL-2 (A), TNF-α (B), and interferon-γ (C).

increase in ABPI and ankle pressure was seen in the MSC arm compared with the placebo group (Figure 46.7).

It is noteworthy that although a small group of 10 patients was recruited in each arm of the trial, we found a significant increase in the blood flow of patients in the lower limbs that received stempeucel. The incidence of adverse events in the BM-MSC arm was 13 versus 45 in the placebo arm, while the incidence of serious adverse events was similar in both the arms: five in the MSC group and four in the placebo group. The serious adverse events were related to disease progression and not related to the administration of cells or placebo.

In addition to routine safety laboratory parameters, such as measurement of parameters of renal and hepatic function, levels of the proinflammatory cytokines IL-2, TNF-α, and interferon-γ were documented. $CD4^+$, $CD8^+$, and $CD25^+$ lymphocyte numbers before and after stempeucel or placebo injection were measured by flow cytometry at baseline, 1 month, 3 months, and 6 months. Results showed that both immunological response (Figure 46.8) and CD markers (Figure 46.9) were comparable in both the placebo and cell arms.

No significant difference in blood lymphocyte profile or in serum cytokine level was observed between stempeucel and placebo-administered patients, suggesting that the administered allogeneic cells did not elicit a T cell proliferative response *in vivo*. With respect to the levels of the proinflammatory cytokines, we observed a possible difference between the MSC recipients and the placebo control patients at baseline, 1 month, and 6 months after treatment, but they were either comparable to the baseline values or were within the normal range of these cytokines. Collectively, these data indicate that allogeneic BM-MSC

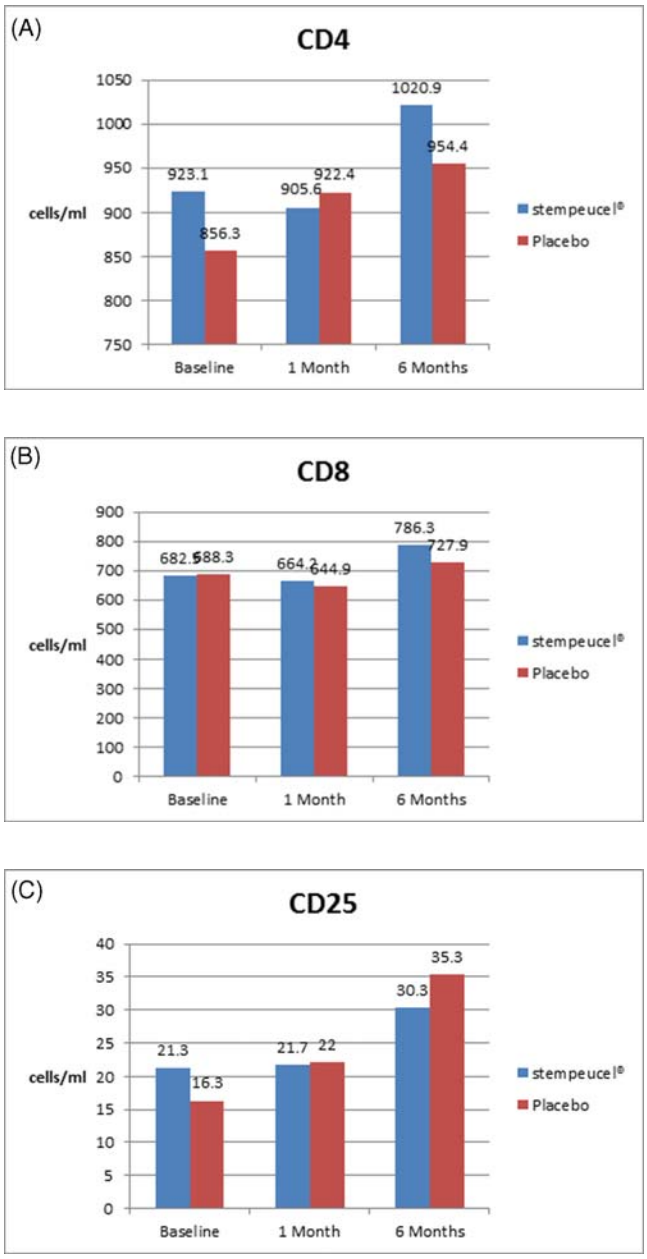

Figure 46.9 CD markers in stempeucel arm and placebo arms at baseline, 1 month, and 6 months follow-up for $CD4^+$ cells (A), $CD8^+$ cells (B) and $CD25^+$ cells (C).

administration in CLI patients did not adversely alter the immunological profile.

It was concluded that BM-MSCs were safe when injected intramuscularly at a dose of 2 million cells/kg body weight. The safety profile exhibited by BM-MSCs, coupled with the observed improvements in some of the important clinical parameters in Buerger's disease, warranted a separate study with a larger number of patients. An open-label nonrandomized dose-finding phase II study was next conducted in patients with Buerger's disease.

46.5.6 Phase II study in patients with Buerger's disease

The second trial initiated in patients with CLI due to Buerger's disease was an open-label, nonrandomized, dose-finding phase II study evaluating different dose levels of stempeucel in patients with CLI due to Buerger's disease. Thirty-six patients were accrued to one arm of the study and received 1 million MSCs/kg, and 36 were accrued to the second arm of the study and received 2 million MSCs/kg. Eighteen patients were accrued to the control arm. Patients in the cell groups received the respective dose of stempeucel into the calf muscle of the affected limb and 2 mL of stempeucel around ischemic ulcers. Patients belonging to the control arm received standard of care only. Six months follow-up of the patients has been completed, and these patients will be followed up for 2 years.

The safety profiles of both the 1 million MSCs/kg and 2 million MSCs/kg groups were comparable to that of control group. All the adverse events were manifestations of the underlying disease. None of the serious adverse events reported were related to stempeucel as per the investigators' feedback and were due to progression of the disease. There was significant improvement in both the primary endpoints of the trial: relief of rest pain (Figure 46.10) and healing of ulceration.

Furthermore, stempeucel recipients showed a significant increase in blood flow in the lower limbs, as evidenced by a significant increase in ankle systolic pressure (Figure 46.11) and total walking distance in the 2 million MSCs/kg dose group.

Hence, it was concluded that BM-MSCs at a dose of 2 million MSCs/kg body weight has a role in alleviating the symptoms of CLI in Buerger's disease.

46.6 Conclusions

CLI represents the most severe manifestation of PAD, diminishes quality of life and global function, and is often associated with very high short-term mortality. Prompt clinical diagnosis, vasodilator agents, and surgical revascularization represent the current standard of care. However, in some patients this care is either not possible or ineffective in providing clinical benefit. Effective new biological and angiogenic therapies would fill a real clinical need. Current literature favors the administration of autologous BM-MNCs or allogeneic BM-MSCs; the latter are relatively safe, easy to expand *ex vivo*, and appear to be efficacious. Issues that remain unresolved include the optimal cell type, optimum cell dosage, and optimal route of administration. Cells that have immunomodulatory and proangiogenic properties appear likely to be the cell of choice for the treatment of CLI due to PAD.

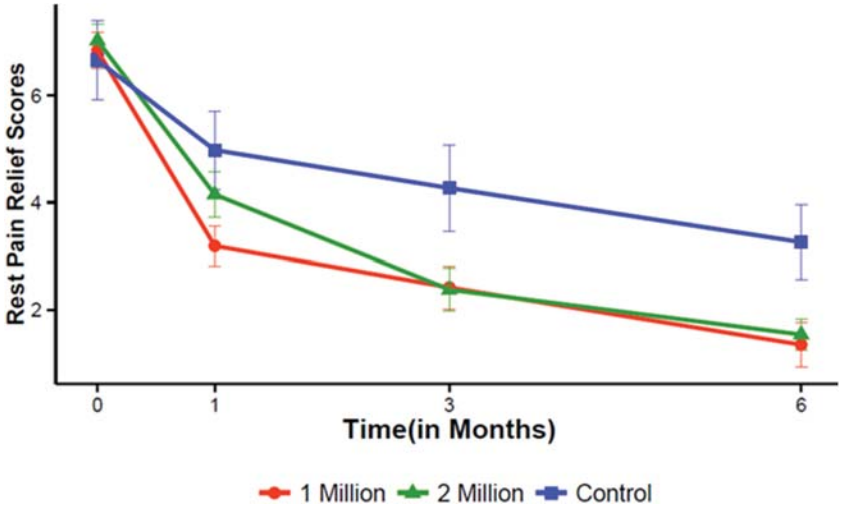

Figure 46.10 Phase II study results of rest pain scores, shown as mean plus/minus standard error, at time of screening and at 1 month, 3 months, and 6 months follow-up.

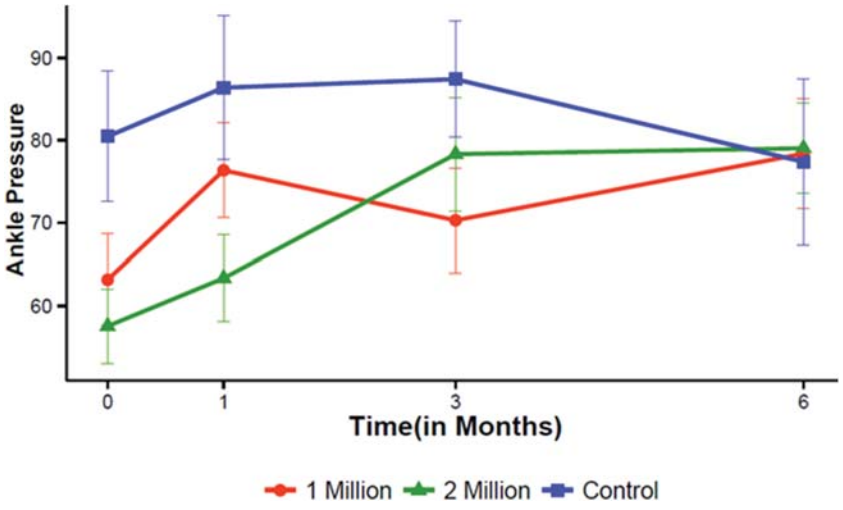

Figure 46.11 Phase II study results of ankle pressure index, shown as mean plus/minus standard error, at time of screening, and at 1 month, 3 months, and 6 months follow-up.

References

1 A. T. Hirsch, Z. J. Haskal, N. R. Hertzer, *et al.* ACC/AHA 2005 Practice guidelines for the management of patients with peripheral arterial disease (lower extremity, renal, mesenteric, and abdominal aortic):a collaborative report from the American Association for Vascular Surgery/Society for Vascular Surgery, Society for Cardiovascular Angiography and Interventions, Society for Vascular Medicine and Biology, Society of Interventional Radiology, and the ACC/AHA Task Force on Practice Guidelines (Writing Committee to Develop Guidelines for the Management of Patients With Peripheral Arterial Disease): endorsed by the American Association of Cardiovascular and Pulmonary Rehabilitation; National Heart, Lung, and Blood Institute; Society for Vascular Nursing; TransAtlantic Inter-Society Consensus; and Vascular Disease Foundation. *Circulation* 2006;**113**(11): e463–e654.

2 G. Piazza and M. A. Creager. Thromboangiitis obliterans. *Circulation* 2010;**121**(16):1858–1861.

3 J. W. Olin. Thromboangiitis obliterans (Buerger's disease). *N. Engl. J. Med.* 2000;**343**(12):864–869.

4 J. J. Belch, E. J. Topol, G. Agnelli, *et al.* Critical issues in peripheral arterial disease detection and management: a call to action. *Arch. Intern. Med.* 2003;**163**(8):884–892.

5 M. H. Criqui. Peripheral arterial disease – epidemiological aspects. *Vasc. Med. Lond. Engl.* 2001;**6**(3 Suppl):3–7.

6 W. R. Hiatt, A. T. Hirsch, J. G. Regensteiner, and E. P. Brass. Clinical trials for claudication assessment of exercise performance, functional status, and clinical end points. *Circulation* 1995;**92**(3):614–621.

7 W. S. Aronow. Management of peripheral arterial disease of the lower extremities in elderly patients. *J. Gerontol. A. Biol. Sci. Med. Sci.* 2004;**59**(2):M172–M177.

8 J. L. Mills and J. M. Porter. Buerger's disease (thromboangiitis obliterans). *Ann. Vasc. Surg.* 1991;**5**(6):570–572.

9 B. Sigvant, K. Wiberg-Hedman, D. Bergqvist, *et al.* A population-based study of peripheral arterial disease prevalence with special focus on critical limb ischemia and sex differences. *J. Vasc. Surg.* 2007;**45**(6):1185–1191.

10 R. W. Sprengers, D. J. Lips, F. L. Moll, and M. C. Verhaar. Progenitor cell therapy in patients with critical limb ischemia without surgical options. *Ann. Surg.* 2008;**247**(3):411–420.
11 H. Lawall, P. Bramlage, and B. Amann. Treatment of peripheral arterial disease using stem and progenitor cell therapy. *J. Vasc. Surg.* 2011;**53**(2):445–453.
12 The European Agency for the Evaluation of Medicinal Products, Evaluation of Medicines for Human Use. Note for guidance on clinical investigation of medicinal products for the treatment of peripheral arterial occlusive disease. CPMP/EWP/714/98 rev 1, April 25, 2002.
13 A. J. Friedenstein, J. F. Gorskaja, and N. N. Kulagina. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. *Exp. Hematol.* 1976;**4**(5):267–274.
14 M. Dominici, K. Le Blanc, I. Mueller, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy* 2006;**8**(4):315–317.
15 D. J. Prockop. Marrow stromal cells as stem cells for nonhematopoietic tissues. *Science* 1997;**276**(5309):71–74.
16 M. F. Pittenger, A. M. Mackay, S. C. Beck, *et al.* Multilineage potential of adult human mesenchymal stem cells. *Science* 1999;**284**(5411):143–147.
17 S. Aggarwal and M. F. Pittenger. Human mesenchymal stem cells modulate allogeneic immune cell responses. *Blood* 2005;**105**(4):1815–1822.
18 J. Stagg. Immune regulation by mesenchymal stem cells: two sides to the coin. *Tissue Antigens* 2007;**69**(1):1–9.
19 A. I. Caplan. What's in a name? *Tissue Eng. Part A* 2010;**16**(8):2415–2417.
20 A. I. Caplan and D. Correa. The MSC: an injury drugstore. *Cell Stem Cell* 2011;**9**(1):11–15.
21 T. J. Burdon, A. Paul, N. Noiseux, *et al.* Bone marrow stem cell derived paracrine factors for regenerative medicine: current perspectives and therapeutic potential. *Bone Marrow Res.* 2011;**2011**:e207326.
22 M. B. Murphy, K. Moncivais, and A. I. Caplan. Mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp. Mol. Med.* 2013;**45**:e54.
23 L. Chen, E. E. Tredget, P. Y. G. Wu, and Y. Wu. Paracrine factors of mesenchymal stem cells recruit macrophages and endothelial lineage cells and enhance wound healing. *PLoS ONE* 2008;**3**(4):e1886.
24 A. M. Hocking and N. S. Gibran. Mesenchymal stem cells: paracrine signaling and differentiation during cutaneous wound repair. *Exp. Cell Res.* 2010;**316**(14):2213–2219.
25 C. Botti, C. Maione, A. Coppola, *et al.* Autologous bone marrow cell therapy for peripheral arterial disease. *Stem Cells Cloning* 2012;**5**:5–14.
26 A. I. Caplan. Adult mesenchymal stem cells for tissue engineering versus regenerative medicine. *J. Cell. Physiol.* 2007;**213**(2):341–347.
27 N. Idei, J. Soga, T. Hata, *et al.* Autologous bone-marrow mononuclear cell implantation reduces long-term major amputation risk in patients with critical limb ischemia: a comparison of atherosclerotic peripheral arterial disease and Buerger disease. *Circ. Cardiovasc. Interv.* 2011;**4**(1):15–25.
28 N. Lehman, R. Cutrone, A. Raber, *et al.* Development of a surrogate angiogenic potency assay for clinical-grade stem cell production. *Cytotherapy* 2012;**14**(8):994–1004.
29 European Medicines Agency, Evaluation of Medicines for Human Use. Guideline on potency testing of cell based immunotherapy medicinal products for the treatment of cancer. EMEA/CHMP/BWP/271475/2006, October 10 2007.
30 C. A. Bravery, J. Carmen, T. Fong, *et al.* Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. *Cytotherapy* 2013;**15**(1):9–19.
31 K. J. Ledford, F. Zeigler, and R. L. Bartel. Ixmyelocel-T, an expanded multicellular therapy, contains a unique population of M2-like macrophages. *Stem Cell Res. Ther.* 2013;**4**(6):134.
32 R. Pal, M. Hanwate, M. Jan, and S. Totey. Phenotypic and functional comparison of optimum culture conditions for upscaling of bone marrow-derived mesenchymal stem cells. *J. Tissue Eng. Regen. Med.* 2009;**3**(3):163–174.
33 M. K. Mamidi, K. G. Nathan, G. Singh, *et al.* Comparative cellular and molecular analyses of pooled bone marrow multipotent mesenchymal stromal cells during continuous passaging and after successive cryopreservation. *J. Cell. Biochem.* 2012;**113**(10):3153–3164.
34 S. H. Ranganath, O. Levy, M. S. Inamdar, and J. M. Karp. Harnessing the mesenchymal stem cell secretome for the treatment of cardiovascular disease. *Cell Stem Cell* 2012;**10**(3):244–258.
35 T. Kinnaird, E. Stabile, M. S. Burnett, *et al.* Marrow-derived stromal cells express genes encoding a broad spectrum of arteriogenic cytokines and promote *in vitro* and *in vivo* arteriogenesis through paracrine mechanisms. *Circ. Res.* 2004;**94**(5):678–685.
36 N. Ferrara and T. Davis-Smyth. The biology of vascular endothelial growth factor. *Endocr. Rev.* 1997;**18**(1):4–25.
37 T. Pritchett and L. Little. "Hard cell": potency testing for cellular therapy products. *Bioprocess. Int.* 2012;**10**:36–38.
38 J. C. Ra, I. S. Shin, S. H. Kim, *et al.* Safety of intravenous infusion of human adipose tissue-derived mesenchymal stem cells in animals and humans. *Stem Cells Dev.* 2011;**20**(8):1297–1308.
39 I. A. Isakova, K. Baker, J. Dufour, *et al.* Preclinical evaluation of adult stem cell engraftment and toxicity in the CNS of rhesus macaques. *Mol. Ther.* 2006;**13**(6):1173–1184.
40 D. J. Prockop and S. D. Olson. Clinical trials with adult stem/progenitor cells for tissue repair: let's not overlook some essential precautions. *Blood* 2007;**109**(8):3147–3151.
41 E. Eggenhofer, V. Benseler, A. Kroemer, *et al.* Mesenchymal stem cells are short-lived and do not migrate beyond the lungs after intravenous infusion. *Alloimmunity Transplant.* 2012;**3**:297.
42 Y. Kim, H. Kim, H. Cho, *et al.* Direct comparison of human mesenchymal stem cells derived from adipose tissues and bone marrow in mediating neovascularization in response to vascular ischemia. *Cell. Physiol. Biochem.* 2007;**20**(6):867–876.
43 T. Iwase, N. Nagaya, T. Fujii, *et al.* Comparison of angiogenic potency between mesenchymal stem cells and mononuclear cells in a rat model of hindlimb ischemia. *Cardiovasc. Res.* 2005;**66**(3):543–551.
44 M. M. Lalu, L. McIntyre, C. Pugliese, *et al.* Safety of cell therapy with mesenchymal stromal cells (SafeCell): a systematic review and meta-analysis of clinical trials. *PLoS ONE* 2012;**7**(10):e47559.
45 E. Tateishi-Yuyama, H. Matsubara, T. Murohara, *et al.* Therapeutic angiogenesis for patients with limb ischaemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. *Lancet* 2002;**360**(9331):427–435.
46 A. K. Das, B. J. J. Bin Abdullah, S. S. Dhillon, *et al.* Intra-arterial allogeneic mesenchymal stem cells for critical limb ischemia are safe

and efficacious: report of a phase I study. *World J. Surg.* 2013;**37**(4):915–922.

47 N. R. Dash, S. N. Dash, P. Routray, *et al.* Targeting nonhealing ulcers of lower extremity in human through autologous bone marrow-derived mesenchymal stem cells. *Rejuvenation Res*. 2009;**12**(5):359–366.

48 D. Lu, B. Chen, Z. Liang, *et al.* Comparison of bone marrow mesenchymal stem cells with bone marrow-derived mononuclear cells for treatment of diabetic critical limb ischemia and foot ulcer: a double-blind, randomized, controlled trial. *Diabetes Res. Clin. Pract.* 2011;**92**(1):26–36.

49 M. Teraa, R. W. Sprengers, Y. van der Graaf, *et al.* Autologous bone marrow-derived cell therapy in patients with critical limb ischemia: a meta-analysis of randomized controlled clinical trials. *Ann. Surg.* 2013;**258**(6):922–929.

50 V. Motukuru, K. R. Suresh, V. Vivekanand, *et al.* Therapeutic angiogenesis in Buerger's disease (thromboangiitis obliterans) patients with critical limb ischemia by autologous transplantation of bone marrow mononuclear cells. *J. Vasc. Surg.* 2008;**48**(6 Suppl):53S–60S, discussion 60S.

51 S. Shionoya. Diagnostic criteria of Buerger's disease. *Int. J. Cardiol.* 1998;**66**(Suppl 1):S243–S245, discussion S247.

52 P. K. Gupta, A. Chullikana, R. Parakh, *et al.* A double blind randomized placebo controlled phase I/II study assessing the safety and efficacy of allogeneic bone marrow derived mesenchymal stem cell in critical limb ischemia. *J. Transl. Med.* 2013;**11**:143.