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Cell-Based Therapies for Peripheral Arterial Disease

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Abstract

Chronic critical limb ischemia (CLI) is defined as the end-stage of lower limb ischemia due to atherosclerotic peripheral arterial disease (PAD) or vasculitis including thromboangitis obliterance (Buerger's disease). CLI patients are at very high risk of amputation and experience poor physical function, leading to severe morbidity and mortality despite the development of surgical bypass technique or endovascular approach. Therefore, exploring novel strategies for blood flow recovery of ischemic limbs is urgently needed for patients with CLI. Although researchers initially focused on gene therapy using proangiogenic growth factors, recent discovery of somatic stem/progenitor cells including bone marrow (BM)-derived endothelial progenitor cells (EPCs) and mesenchymal stem cells (MSCs) has drastically developed the field of therapeutic angiogenesis for CLI. In 2002, the first clinical trial of intramuscular injection of BM-derived mononuclear cells (BM-MNCs) demonstrated safety, feasibility and efficacy for CLI patients. Since then, at least 50 clinical trials of BM- and peripheral blood (PB)-derived MNC therapy, 4 trials of CD34+ cell (an EPC-enriched fraction) therapy and 8 trials of MSC therapy have been performed for CLI. Overall, the results of these early phase clinical trials regarding stem/progenitor cell therapies may be safe, feasible and effective. However, only few late-phase clinical trials have been conducted. Currently, at least 3 phase III trials including 2 trials using BM-MNCs and 1 trial using granulocyte-colony stimulating factor (G-CSF)-mobilized PB-MNCs are ongoing. This review provides an overview of the preclinical and clinical reports to demonstrate the usefulness and the current limitations of the cell-based therapies.

Keywords: Angiogenesis; Cell therapy; Critical limb ischemia; Neovascularization; Peripheral artery disease

Introduction

Peripheral arterial disease (PAD) is commonly referred to ischemia of extremities secondary to atherosclerotic occlusion. More than 25 million patients suffer from PAD in the developed countries [1-5]. An additional cause of PAD is vasculitis including thromboangitis obliterance (TAO) (Buerger's disease), which can also lead to severe limb ischemia. Chronic critical limb ischemia (CLI) is defined as the end-stage of lower limb ischemia. The clinical manifestations consist of rest pain and/or skin ulceration or gangrene. The annual incidence of CLI is estimated to be 500-1,000 cases per million people and an estimated 250,000 major amputation are performed annually in the United States and Europe, resulting in a significant socioeconomic burden and severe reduction in quality of life [5]. Prognosis of the CLI patients is quite poor. The 1-year mortality and major amputation rate are reported to be 25% and 30%, respectively [5]. Currently, revascularization of the ischemic limb with surgical bypass techniques or endovascular approaches is believed to be the best option for limb salvage. However, 25-40% of patients with CLI are not candidates for either of these options due to a lack of autologous vein graft, extensive lesions in the tibial and peroneal arteries or medical co-morbidity [5-7]. Therefore, new strategies for blood flow recovery are urgently required for such no-option patients with CLI. The challenge to improve blood flow to CLI has provoked extensive research programs and numerous innovative approaches in the fields of molecular biology and pharmacology.

In early studies of vascular regeneration, angiogenic recombinant proteins including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), hepatocyte growth factor (HGF) and cell-mobilizing cytokines such as granulocyte colony stimulating factor (G-CSF) and granulocyte-macrophage colony-stimulating factor (GM-CSF) have been tested to promote neovascular formation. Although preclinical and early-phase clinical results were promising in short-time [8-11], protein therapy did not achieve a long-term clinical effect [12]. Subsequently, gene therapy was proposed as a therapeutic option for cardiovascular diseases. Gene therapies using naked/plasmid-encoding angiogenic factors were developed to improve duration of transgene expression over direct recombinant protein injection.

Isner et al. demonstrated the effect of intramuscular gene therapy with naked plasmid DNA encoding VEGF for ischemia reduction in animal models of hindlimb ischemia [13] and confirmed the effect of VEGF165 plasmid DNA in patients with limb ischemia for the first time in 1996 [14,15]. Adenovirus-mediated VEGF121 gene therapy has also been reported to be effective in improving endothelial function and lower-extremity flow reserve in patients with PAD [16]. Thus, VEGF gene therapy appears to be promising, however, its efficacy and safety remains to be controversial because 2 phase II randomized clinical trials failed to meet the primary endpoint of significant amputation reduction [17] or improvement of peak walking time at 12 weeks, while an adverse event, peripheral edema relating to AdVEGF121 was observed [18]. Recently, Muona et al. reported a 10-year safety follow-up in patients receiving local VEGF gene transfer to ischemic lower limbs [19]. This study demonstrated that there were no differences in the causes of death or in the incidence of cancer, diabetes or diabetic retinopathy between the patients receiving VEGF-mediated gene therapy and the control patients. However, there were no differences in the number of amputations between the 2 groups.

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HGF is another angiogenic factor, which regulates proliferation and migration of vascular endothelial cells through tyrosine phosphorylation of its specific receptor, c-Met. In preclinical studies, gene transfer using naked plasmid DNA encoding HGF induced therapeutic angiogenesis in animal models [20-24]. The first pilot (phase I/IIa) study of intramuscular injection of naked human HGF plasmid in patients with atherosclerotic PAD or TAO provided early evidence of safety and feasibility. Powel et al. performed another phase I/II double-blind placebo-controlled study with HGF plasmid for CLI (HGF-STAT trial) [25]. Transcutaneous oxygen pressure ($TcPO_2$) significantly increased at 6 months in the high-dose group (4.0 mg at days 0, 14 and 28) compared with the placebo, low-dose (0.4 mg at days 0, 14 and 28), and middle-dose (4 mg at days 0 and 28) groups. However, there were no differences in ankle-brachial pressure index (ABPI), toe-brachial pressure index (TBPI), pain relief, wound healing and major amputation between the groups. In a randomized, double-blind, placebo-controlled clinical trial of HGF plasmid in patients with CLI, the primary endpoints including rest pain and ulcer size significantly decreased in the HGF treated group compared with the placebo group [26].

FGF is also an angiogenic factor, which has been studied in cardiovascular diseases. FGF is composed of 23 members, FGF-1 through FGF-23. In particular, FGF-1 (acidic or aFGF), FGF-2 (basic or bFGF) and FGF-4 are highly potent endothelial mitogens [27]. Among them, FGF-1 and FGF-2 have been extensively examined. Comerota et al. first demonstrated the safety and efficacy of increasing single and repeated doses of intramuscular naked plasmid DNA encoding FGF type 1 (NV1FGF) administered to patients with unreconstructable end-stage PAD in an open-label phase I trial [28]. In this study, a significant reduction in pain and ulcer size was observed after FGF-1 gene administration associated with an increased $TcPO_2$ and ABPI as compared with baseline pretreatment values. Furthermore, the phase II TALISMAN trial demonstrated that administration of NV1FGF significantly reduced the risk of all amputations and major amputations at 12 months as compared with placebo, although improvement of ulcer healing was similar between the NV1FGF-treated group and the control group [29]. However, the phase III TAMARIS trial including 525 patients with CLI demonstrated no beneficial effects on either the primary endpoint of time to major amputation or death at 1 year or secondary endpoints including major amputation and death from any cause [30]. Clinical usefulness of FGF gene therapy has been controversial so far.

While outcomes of angiogenic molecular therapy trials remain controversial, accumulating knowledge of the potential of stem/progenitor cells as therapeutic agents in both animal studies and clinical trials has shifted the interest in regenerative medicine from molecular to cell-based approaches.

In this review, we provide an overview of the basic characteristics and clinical trials of cell-based therapies for PAD and discuss regarding the current problems and the future perspectives.

Cell-Based Therapies for PAD

Transplantation of stem or progenitor cells is an attractive approach for therapeutic neovascularization. Stem/progenitor cells possess the capability of self-renewal and differentiation into organ-specific cell types as well as paracrine effects via the release of pro-angiogenic growth factors. Some of the stem/progenitor cells including bone marrow (BM)-mononuclear cells (MNCs), granulocyte colony stimulation factor (G-CSF)-mobilized peripheral blood (PB)-MNCs, unmobilized PB-MNCs, endothelial progenitor cells (EPCs), mesenchymal stem cells (MSCs) are under investigation.

EPCs and crude MNCs

In 1997, EPCs were first identified in adult human PB as CD34 antigen-positive (CD34+) MNCs [31]. They are phenotypically characterized by expression of antigens associated with haematopoietic stem cells (HSCs) including CD133, CD34, c-kit, VEGFR-2, CD144 (vascular endothelial (VE)-cadherin) and Sca-1. The discovery of circulating EPCs changed the traditional paradigm that 'vasculogenesis' occurs exclusively in the developing embryo. EPC levels in the PB are low under normal conditions; however EPCs residing in the BM are mobilized into PB in response to physiological and pathological stimuli, such as myocardial and peripheral ischemia [32,33]. Mobilized EPCs recruit to the foci of neovascularization where they form structural components of the growing vasculature [34]. Accumulated recent insights into the mechanism of EPC-mediated neovascularization reveal that EPCs secrete paracrine factors including VEGF-A, VEGF-B, stromal cell-derived factor-1 (SDF-1) and insulin-like growth factor-1 (IGF-1) [35]. The paracrine effect of EPCs inhibits cell death, enhances cell proliferation, activates resident stem/progenitor cells in the ischemic tissue, and recruits additional stem/progenitor cells to the ischemic site [36-40]. On the other hand, Gehling et al. reported that a cell population positive for AC133 (CD133), a more immature HSC marker, consists of progenitor and stem cells with not only hematopoietic potential but also the capacity of endothelial differentiation [41]. Accumulated studies revealed that PB-, BM- and umbilical cord blood-derived CD34+ or CD133+ cells are enriched for endothelial lineage, can express endothelial markers and form endothelial structure *in vitro* and *in vivo* [42-45].

The discovery of EPCs guided to the development of stem/progenitor cell-based strategies for ischemic cardiovascular diseases. Since then, BM- or PB-MNCs including EPCs as well as the EPC-enriched fraction purified from the crude MNCs have been preclinically applied for ischemic cardiovascular diseases including PAD. The promising results from these experimental studies in rodents promoted the initiation of clinical pilot trials.

BM-MNCs: As shown in Table 1, a number of clinical trials of BM-MNC therapy have ranged from small, pilot to randomized, placebo-controlled trials. BM-derived MNCs are usually separated by density gradient centrifugation or plasmapheresis.

In 2002, the first set of clinical trials of intramuscular implantation of BM-MNCs in patients with PAD was reported [46]. BM-MNC therapy resulted in improvement of rest pain, ABPI, $TcPO_2$, and pain-free walking distance at 24 week follow up compared with baseline. Improvement in rest pain, ulcer size, and pain-free walking distance maintained at 2 years after BM-MNC therapy [47]. A multicenter, randomized, double-blind, placebo-controlled trial is the PROVASA (Intraarterial Progenitor Cell Transplantation of Bone Marrow Mononuclear Cells for Induction of Neovascularization in Patients with Peripheral Arterial Occlusive Disease) trial [48]. A total of 40 patients with CLI were enrolled, received either intraarterial administration of BM-MNCs or placebo, and at the end of 3 months, the placebo group were crossed over to active treatment (an initial administration of BM-MNCs) and the active group received the second administration of BM-MNCs. This study demonstrated the dose-dependent improvement in ulcer healing and significant reduction in rest pain, despite no difference in limb salvage rate, amputation-free survival and the primary end point which was an increase in ABPI

Trial name/Author	Year	Study design	Number of patients			Route of administration	Follow-up duration	Outcomes
			Total	Treated	Control			
TACT Tateishi-Yuyama et al. [46]	2002	Patient series RCT	45	45 25 BM-MNCs 20 PB-MNCs	0	IM	24 weeks	ABPI ↑, $TcPO_2$↑, Pain-free walking time ↑
Higashi et al. [93]	2004	Patient series	7	7	0	IM	4 weeks	$TcPO_2$↑, Pain-free walking time ↑, Blood flow response to acetylcholine↑
Miyamoto et al. [94]	2006	Patient series	8	8	0	IM	2 years	Rest pain scale↓, Ulcer healing↑ (at 4 weeks) Sudden death (n=1), Skin ulcer (n=1), Rest pain (n=1), Arteriovenous shunt (n=1) (Long-term)
Motukuru et al. [95]	2008	Patient series	36	36	0	IM	6 months	ABPI↑, $TcPO_2$↑, Ulcer healing↑
De Vriese et al. [96]	2008	Patient series	16	16	0	IM	12 weeks	ABPI→, $TcPO_2$↑, Rest pain scale↓, Muscle capillary density↑
Cobellis et al. [97]	2008	Patient series	10	10	0	IA	12 months	ABPI↑, Pain-free walking distance↑, Rest pain scale↓, Blood flow↑, Capillary density↑
Chochola et al. [98]	2008	Patient series	24	24	0	IM	1 year	Fontaine grade↓, Ulcer healing↑, Collateral vessel↑, QOL↑
Franz et al. [99]	2009	Patient series	9	9	0	IM+IA	3 months	ABPI →, Rest pain scale →, Limb salvage →
BONMOT-1 Amman et al. [100]	2009	Patient series	51	51	0	IM	6 months	Rutherford's category↓, ABPI↑, $TcPO_2$↑, Ulcer healing↑, Total walking distance↑
Prochazka et al. [101]	2010	RCT non-blinded	96	42	54	IA	120 days	Limb salvage↑,
PROVASA Walter et al. [48]	2011	RCT double-blind crossover	40	19	21	IA	6 months	Ulcer healing↑, Rest pain scale↓, ABPI →, Limb salvage →
Ruiz-Salmeron et al. [102]	2011	Patient series	20	20	0	IA	12 months	ABPI↑, Wound healing↑, Angiographic blood flow ↑ (at 3 months)
Murphy et al. [103]	2011	Patient series	29	29	0	IM	1 year	FTP↑, TBPI↑, Rest pain scale↓, QOL↑ (at 12 weeks)
Idei et al. [104]	2011	Cohort	97	51	46	IM	3 years	Amputation-free survival↑ ABPI↑, $TcPO_2$↑in patients with TAO. ABPI→, $TcPO_2$→ in patients with Atherosclerotic PAD.
Benoit et al. [105]	2011	RCT double-blind	48	34	14	IM	6 months	Amputation rate↓
Klepanec et al. [106]	2012	Patient series	41	41	0	IM (n=21), IA (n=20)	6 months	Rutherford's category↓, $TcPO_2$↑, Rest pain scale↓, QOL↑

ABPI indicates ankle brachial pressure index; BM, bone marrow; CLI, critical limb ischemia; FTP, first toe pressure; IA, intra-arterial; IM, intramuscular; MNC, mononuclear cell; PAD, peripheral arterial disease; PB, peripheral blood; QOL, quality of life; RCT, randomized controlled trial; TAO, thromboangitis obliterance; $TcPO_2$, transcutaneous oxygen pressure.

Table 1: Clinical trials of autologous BM-MNC administration for CLI.

between the groups. Furthermore, patients with Rutherford's category 6 (extensive gangrene and impending amputation) CLI at baseline did not respond to administration of BM-MNCs. All these patients underwent major amputation within 3 months after treatment, presumably owing to too far progression, which is the characteristic of advanced staged CLI. Administration of BM-MNCs may not be suitable for Rutherford's category 6 CLI. The PROVASA investigators speculated that the choice of change in ABPI as the primary endpoint was not appropriate for this study because the change of ABPI did not correlate with improvements in the clinically most relevant secondary endpoints including ulcer healing and rest pain reduction. The discordance between clinical endpoints and functional endpoints like ABPI, TBPI or TcPO2 was similarly reported in a gene therapy trial for therapeutic angiogenesis in CLI [25] and remains a cardinal issue in design and execution of clinical trials in CLI. To establish the appropriate endpoints for cell therapy trials, various characteristics of cell therapy, as distinct from conventional revascularization including bypass surgery and endovascular intervention, should be considered.

Currently, 2 phase III clinical trials are ongoing (ClinicalTrials.gov NCT01245335; NCT01818310). In these clinical trials, a hard endpoint including amputation-free survival is selected as a primary endpoint.

G-CSF-mobilized PB-MNCs: G-CSF-mobilized PB-MNCs have been also investigated in CLI as shown in Table 2. PB-MNCs are usually mobilized using several doses of subcutaneous G-CSF and harvested by plasmapheresis. Both intramuscular and intraarterial injections of unfractionated mobilized PB-MNCs improved ABPI and maximum walking distance in small clinical trials [49-52]. A phase III clinical trial is ongoing (ClinicalTrials.gov NCT 01833585), and the final results may provide a definitive evidence of clinical usefulness of this cell-based therapy in CLI.

Huang et al. compared the therapeutic effect of intramuscular administration of BM-MNCs with G-CSF-mobilized PB-MNCs in patients with CLI [53]. One hundred fifty patients with CLI were randomized to receive intramuscular injection of BM-MNCs or G-CSF-mobilized MNCs. At 12 weeks after therapy, improvement of ABPI, skin temperature and rest pain was significantly greater in PB-MNC group than BM-MNC group despite no difference in pain-free walking distance, ulcer healing, and amputation rates between the 2 groups (Table 3). Further studies comparing the therapeutic potential between the different types of cell therapies would be intriguing.

Non-mobilized PB-MNCs: Tateno et al. reported the results of a pilot clinical trial of intramuscular injection of non-mobilized PB-MNCs in patients with CLI [54]. In this study, 29 patients with CLI caused by atherosclerotic PAD or TAO received intramuscular injection of PB-MNCs twice within 1-month period. Rest pain was significantly improved until 2 months after treatment, and maximal walking distance significantly increased until 6 months. These improvements were preserved for 12 months (Table 2). Furthermore, a long-term retrospective study demonstrated that intramuscular injection of non-mobilized PB-MNCs for CLI might be safe and potentially effective. However, improvement of ischemic symptoms was less in atherosclerotic PAD patients on hemodialysis compared with non-hemodialysis PAD or TAO patients. Major adverse events including death, major amputation and cardiovascular events frequently occurred in PAD patients on hemodialysis [55]. Horie et al. also demonstrated that PAD patients on hemodialysis had a worse prognosis than non-hemodialysis PAD patients after intramuscular administration of G-CSF-mobilized PB-MNCs [56]. Choi et al. demonstrated that the number and functional activity of EPCs decreased in patients with end-stage renal disease [57]. To overcome the therapeutic limitation in PAD patients on hemodialysis, novel strategies such as transplantation of other type of stem/progenitor cells or cell culture for quantitative expansion and qualitative improvement may be required.

Fractionated EPCs (CD34+/ CD133+/ aldehyde dehydrogenase bright ($ALDH^{br}$) cells): BM-derived EPCs comprise a small fraction (0.1-2%) of total MNCs. The advantage of the administration of fractionated EPCs is a higher concentration of EPCs compared with that of crude MNCs resulting in greater therapeutic potency. Onodera et al. reported that treatment with small number of harvested CD34+ cells was a negative independent predictor of amputation and death following either BM- or PB-MNC implantation in patients with CLI [58]. This finding suggests an important role of EPCs for therapeutic neovascularization and may provide a reasonable rationale for transplantation of CD34+ cells purified from crude MNCs in patients with CLI.

In a phase I/IIa clinical trial, our group evaluated the safety and feasibility of G-CSF-mobilized CD34+ cells in no-option patients with atherosclerotic PAD or TAO representing CLI [59]. CD34+ cells were isolated from the G-CSF-mobilized apheresis product using a magnetic cell sorting system, and then intramuscularly transplanted in a dose-

Trial name/Author	Year	Study design	Number of patients			Product	Route of administration	Follow-up duration	Outcomes
			Total	Treated	Control				
Huang et al. [107]	2004	Patient series	5	5	0	G-CSF-mobilized PB-MNCs	IM	3 months	ABPI ↑ Laser Doppler blood perfusion ↑
Ishida et al. [49]	2005	Patient series	6	6	0	G-CSF-mobilized PB-MNCs	IM	24 weeks	ABPI → $TcPO_2$ →, Maximal walking distance↑
Huang et al. [51]	2005	RCT, non-blinded	28	14	14	G-CSF-mobilized PB-MNCs	IM	3 months	ABPI ↑, Laser Doppler blood perfusion↑, Ulcer healing↑, Angiographic score↑
Lenk et al. [50]	2005	Patient series	7	7	0	G-CSF-mobilized PB-MNCs	IA	12 weeks	ABPI ↑, $TcPO_2$↑, Pain-free walking distance ↑, Flow-dependent vasodilation ↑, Flow reserve in response to adenosine↑, Endothelium-dependent vasodilation↑
Lara-Hernandez et al. [52]	2010	Patient series	28	28	0	G-CSF-mobilized PB-MNCs	IM	14 months	ABPI ↑, Rest pain scaled ↓, Limb salvage↑
Tateno et al. [54]	2006	Patient series	29	29	0	non-mobilized PB-MNCs	IM	12 months	Rest pain scale↑, Maximal walking distance↑

G-CSF indicates granulocyte-colony stimulating factor.

Table 2: Clinical trials of autologous G-CSF-mobilized PB-MNC therapy and non-mobilized PB-MNC administration for CLI.

Trial name /Author	Year	Study design	Number of patients			Product	Route of administration	Follow-up duration	Outcomes
			Total	Treated	Control				
Huang et al. [53]	2007	RCT	150	150	0	BM-MNCs (n=74) G-CSF-mobilized PB-MNCs (n=76)	IM	12 weeks	ABPI ↑, Skin temperature ↑, Rest pain scale ↓ in the G-CSF-mobilized PB-MNC treated group. No significant difference in $TcPO_2$, pain-free walking distance, Ulcer healing and amputation rate.

Table 3: Clinical trials of autologous BM-MNCs versus G-CSF-mobilized PB-MNCs for CLI.

escalating manner into 17 patients (10^5/kg, n=6; $5x10^5$/kg, n=8; or 10^6/kg, n=3). CD34+ cell therapy significantly improved Rutherford's category, pain scale, and skin ulcer size and blood perfusion at 12 weeks after treatment; although no significant dose-response relationship was observed. Furthermore, the safety and efficacy of CD34+ cell therapy was sustained for up to 4 years after cell therapy [60]. Furthermore, our phase II trial almost reproduced the clinical outcomes in the previous phase I/IIa trial, indicating the safety, feasibility and potential effectiveness of CD34+ cell transplantation for CLI patients [61]. Recently, in the ACT34-CLI (Autologous CD34+ Cell Therapy for Critical Limb Ischemia Investigator) study, a double-blind, randomized, placebo-controlled, phase I/IIa pilot clinical trial, 28 patients with CLI were randomized to receive intramuscular injection of $1x10^5$ (low-dose, n=7) or $1x10^6$ (high-dose, n=9) cells/kg of mobilized CD34+ cells or an equal volume of diluent [62]. A favorable trend towards improvement of amputation-free survival rate was observed in the cell-treated groups, especially in the high-dose group, compared with control group at 6 and 12 months after treatment (Table 4).

In a phase I trial, Burt et al. evaluated the safety and feasibility of intramuscular implantation of autologous G-CSF-mobilized CD133+ cells, another EPC-enriched fraction, in 9 patients with CLI [63]. In this uncontrolled study, leg amputation was observed in 2 out of 9 patients at 12 months after treatment. The 7 amputation-free patients showed significant improvement in QOL score at 3 and 6 months, but not 12 months. There was a favorable trend towards improvement in pain-free walking distance and exercise capacity at 12 months (Table 4).

Storms et al. developed a method of isolation and purification of stem/progenitor cells based on the specific cell function. They isolated a very primitive population, Lin-CD34+CD38lo/- of HSCs from human cord blood, using a fluorescent substrate for the cytosolic aldehyde dehydrogenase (ALDH), which is expressed at high level in HSCs [64]. These cells, referred to ALDH-bright ($ALDH^{br}$) cells, were also isolated from human BM and PB. Gentry et al. demonstrated that $ALDH^{br}$ cells derived from human BM showed more hematopoietic colony forming activity and generated more endothelial colonies compared with $ALDH^{br}$ cells-depleted BM-MNCs [65]. A preclinical study demonstrated that intravenous administration of human BM-derived $ALDH^{br}$ cells was more effective for promoting angiogenesis and restoring blood flow than BM-MNCs or $ALDH^{dim}$ cells in immunodeficient mice with hindlimb ischemia [66].

In a phase I/II randomized, controlled trial of $ALDH^{br}$ cell therapy for patients with CLI, 21 patients were randomized to receive intramuscular injection of BM-derived $ALDH^{br}$ cells (n=11) or BM-MNCs (n=10) [67]. No therapy-related serious adverse events were observed. At 12 weeks after treatment, Rutherford's category and ABPI significantly improved compared with baseline in the $ALDH^{br}$ treated group but not in the BM-MNC treated group. However, there was no significant change in ischemic ulcer grade and $TcPO_2$ in either group (Table 4).

Larger randomized clinical trials are warranted to clarify the efficacy of EPCs fractionated by various methods for the treatment of PAD patients.

Mesenchymal stem cells (MSCs)

Mesenchymal stem cells (MSCs) are a subset of stem cells which exist in the BM stroma and can differentiate into the mesenchymal lineages, including osteoblasts, chondrocytes, myoblasts, and adipocytes [68]. MSCs can be distinguished from BM hematopoietic cells by their ability to adhere to the culture dish [69]. No specific markers of MNCs exist. However, it is generally recognized that MSCs express CD105, CD73, CD44, CD90, CD71, and Stro-1 and cell adhesion molecules VCAM-1 and ICAM-1, but not hematopoietic markers CD45, CD34, CD14 or CD11 [70]. MSCs have been demonstrated to be an attractive source of cell therapy for the treatment of ischemic diseases [71-73].

Umbilical cord MSCs, BM-MSCs and Ixmyelocel-T: The first clinical trial of intramuscular administration of allogeneic human umbilical cord-derived MSCs has shown that allogeneic umbilical cord-derived MSC therapy improved ulcer healing time and rest pain, and increased capillary formation in 4 patients with TAO [74]. Dash et al. reported that intramuscular injection of autologous BM-derived MSCs in patients with non-healing ulcers of lower limb accelerated ulcer healing and improved pain-free walking distance [75]. Recent clinical trials have used combination cell product, BM-MSCs and BM-MNCs. Lasala et al. reported a phase II clinical trials of a combination cell therapy in patients with limb ischemia [76]. In this study, 26 patients received intramuscular injection of a combination of up to $30x10^6$ BM-MSCs and $30x10^8$ BM-MNCs into the more ischemic leg and a placebo product into the less ischemic contralateral leg. Walking time and ABPI significantly improved. Technetium-99m-tetrofosmin scintigraphy demonstrated that blood flow of the treated limbs significantly increased compared with the contralateral control legs. On the other hand, Lu et al. compared the therapeutic effect of BM-

Trial name /Author	Year	Study design	Number of patients			Product	Route of administration	Follow-up duration	Outcomes
			Total	Treated	Control				
Kawamoto et al. [59] Kinoshita et al. [60]	2009	Patient series	17	17	0	G-CSF-mobilized CD34+ cells	IM	52 weeks	Efficacy score (TBPI, Rest pain scale, Total walking distance) ↑
ACT34-CLI Losordo et al. [62]	2012	RCT double-blind	28	16	12	G-CSF-mobilized CD34+ cells	IM	12 months	Amputation rate ↓ (p=0.058) compared with control
Fujita et al. [61]	2014	Patient series	11	11	0	G-CSF-mobilized CD34+ cells	IM	52 weeks	Rutherford's category ↑, Rest pain scales ↓, Skin perfusion pressure ↑, TBPI ↑, $TcPO_2$ ↑, Pain-free walking distance ↑, Total walking distance ↑
Burt et al. [63]	2010	Patient series	9	9	0	G-CSF-mobilized CD133+ cells	IM	12 months	QOL ↑ (at 6 months)
Perin et al. [67]	2011	RCT	21	21	0	$ALDH^{br}$ (n=11) BM-MNCs (n=10)	IM	12 weeks	Rutherford's category ↓, ABPI ↑ in the $ALDH^{br}$ treated group.

$ALDH^{br}$ indicates aldehyde dehydrogenase bright; EPCs, endothelial progenitor cells; TBPI, toe brachial pressure index.

Table 4: Clinical trials of fractionated EPCs (CD34+/ CD133+/ $ALDH^{br}$ cells) therapy for CLI.

MSCs with BM-MNCs in 20 diabetic patients with bilateral CLI [77]. They demonstrated that the ulcer healing rate was significantly higher in BM-MSC group than BM-MNC group at 6 weeks after treatment and achieved complete ulcer healing 4 weeks earlier in BM-MSC group than BM-MNC group. At 24 weeks, pain-free walking time, ABPI and $TcPO_2$ significantly improved in BM-MSC group compared with BM-MNC group. However, there was no significant difference between the groups in terms of pain relief and amputation (Table 5).

Ixmyeloid-T is generated in a closed and automated culture system that expands the number of CD90+ mesenchymal and CD14+ monocytic stem/progenitor cells obtained from a small amount of a patient's own BM [78]. In the phase II, prospective, randomized, double-blind, RESTORE-CLI (Use of Tissue Repair Cells in Patients with Peripheral Arterial Disease to Treat Critical Limb Ischemia) trial [79], a total of 46 patients with CLI were randomized to receive intramuscular injection of Ixmyelocel-T or placebo. By the time of interim analysis, 33 patients completed the 12-month follow-up and 13 patients completed at least 6 months of follow-up. Ixmyelocel-T treatment resulted in a significantly prolonged time to the first occurrence of treatment failure defined as major amputation, all-cause mortality, doubling of the total wound surface area from baseline, or de novo gangrene. There was a trend towards improvement of amputation-free survival after Ixmyeloid-T treatment; however the difference was not statistically significant (Table 5). Following the promising results, a pivotal phase III clinical trial, REVIVE (ClinicalTrials.gov NCT 01483898) has started, although this study ended recently due to slow recruitment of the CLI patients.

Further clinical trials will be required to identify the clinical usefulness of MSCs in patients with PAD.

Adipose-derived stem/progenitor cells (ADSCs): Adipose tissue has been also represented as a cell source for therapeutic angiogenesis in ischemic diseases. Adipose tissue is mainly composed of two classes of cells. One is mature adipocyte, which forms the major part of adipose tissue volume. The other is stromal cell termed stromal vascular fraction (SVF) [80]. Several studies have revealed that SVF contains multipotent mesenchymal stem/progenitor cells, which have the capability to differentiate into various lineages including adipocytes, fibroblasts, osteoblasts, chondrocytes, pericytes and myocytes [81,82]. Recently, mesenchymal stem cells in adipose tissue are named adipose-derived stem/progenitor cells (ADSCs or ASCs) or adipose-derived regenerative cells (ADRCs) which have the capability of regenerating injured tissue [80]. The advantage of ADSCs is that they can be isolated from a small amount of human subcutaneous adipose tissue through minimally invasive procedures including liposuction and excision of subcutaneous adipose tissue and expanded *ex vivo* [82]. Several studies demonstrated that the transplantation of ADSCs increased angiogenesis in rodent models of hindlimb ischemia [83,84]. However, the mechanism underlying neovascularization induced by ADSCs has not been fully understood. Traktuev et al. reported that ADSCs could not differentiate into EPCs or endothelial cells, but that ADSCs could differentiate into pericytes and play a role in vascular stabilization [85]. Recent studies have shown that ADSCs can secrete multiple pro-angiogenic growth factors and cytokines including VEGF, HGF and SDF-1 [83,84,86,87]. In particular, SDF-1 is thought to play a pivotal role in ADSC-mediated angiogenesis via acceleration of EPCs recruitment into ischemic foci [84].

The first phase I clinical trial, ACellDREAM (Adipose CELL Derived Regenerative Endothelial Angiogenic Medicine) study was reported in 2014 [88], in which 7 patients with no-option CLI received intramuscular injection of autologous ADSCs. ADSC injection resulted

Trial name /Author	Year	Study design	Number of patients			Product	Route of administration	Follow-up duration	Outcomes
			Total	Treated	Control				
Kim et al. [74]	2006	Patient series	4	4	0	HLA matched UCB-derived MSCs	SC or IM	4 months	Rest pain ↑swithin 14 days, Wound healing ↑uwithin 120 days, Angiographic collateral vessels ↑
Dash et al. [75]	2009	RCT	24	12	12	BM-MSCs	IM	12 weeks	Pain-free walking distance ↑, Ulcer size ↓
Lu et al. [77]	2011	RCT double-blind	41 (82 limbs)	41 limbs	41limbs	BM-MSCs (20 limbs) BM-MNCs (21 limbs)	IM	24 weeks	**BM-MSCs vs Control** ABPI ↑, $TcPO_2$ ↑, Rest pain scale ↓, Pain-free walking time ↑, Angiographic score ↑, Ulcer healing ↑ **BM-MSC vs BM-MNCs** ABPI ↑, $TcPO_2$ ↑, Rest pain scale ↓, Pain-free walking time ↑, Angiographic score ↑, Ulcer healing ↑, Rest pain scale →, Amputation rate →
Lasala et al. [108]	2010	Patient series	10	10	0	BM-MSCs +BM-MNCs	IM	10 months	Total walking time ↑, ABPI ↑, QOL ↑
Lasala et al. [76]	2012	Patient series	26	26 limbs	26 limbs	BM-MSCs +BM-MNCs	IM	4 months	ABPI ↑, Total walking time ↑, QOL ↑, Scintigraphic limb perfusion ↑
RESTORE-CLI Powell et al. [79]	2011	RCT	46	32	14	Ixmyelocel-T	IM	6 months 12 months	Amputation-free suivival ↑ Ulcer healing ↑
ACellDREAM Bura et al. [88]	2014	Patient series	7	7	0	ADSCs	IM	6 months	$TcPO_2$ ↑ Ulcer healing ↑

ADSC indicates adipose-derived stem cell; HLA, human leukocyte antigen; MSC, mesenchymal stem cell; SC, subcutaneous; UCB, umbilical cord blood.

Table 5: Clinical trials of MSCs for CLI.

in improvement of $TcPO_2$ and wound healing (Table 5). Several phase I/II clinical trials of ADSCs for CLI are ongoing.

Meta-analysis of BM-derived cell trials in patients with CLI

Recently, Teraa et al. performed a meta-analysis of 12 randomized clinical trials (RCTs) of BM-derived cell therapy in patients with CLI [89]. This meta-analysis studied BM-derived cell therapy, including 7 trials using BM-MNCs, 3 trials using BM-MSCs, 2 trials using G-CSF-mobilized MNCs and 1 trial using Ixmyelocel-T, comparing with standard of care groups with or without placebo in a total of 510 patients with CLI. This meta-analysis demonstrated that BM-derived cell therapy resulted in beneficial effects on major amputation rate and subjective and objective surrogate endpoints including pain score, pain-free walking distance, ABPI and $TcPO_2$. However, amputation-free survival did not significantly differ between the treated group and the control group. Subgroup analysis revealed that in the 7 placebo-controlled RCTs including 4 trials using BM-MNCs, 1 trial using BM-MSCs, 1 trial using G-CSF-mobilized MNCs and 1 trial using Ixmyelocel-T, the beneficial effect on major amputation rate was reduced and was not significant.

Conclusions

Summarizing the results of the previous clinical trials, stem/progenitor cell therapies may be safe and feasible. Theoretically, stem/progenitor cell therapies may be superior over protein or gene therapy due to not only direct vasculogenic properties but also paracrine action by secreting multiple growth factors besides a single angiogenic factor.

Early phase clinical trials revealed the efficacy of BM-MNCs, G-CSF-mobilized PB-MNCs and BM-MSCs for CLI. However, a meta-analysis of the early phase RCTs revealed that there was no significant improvement of amputation-free survival in the treatment group compared with the control group, especially in the placebo-controlled trials [89].

Phase I/II trials revealed that intramuscular injection of EPCs (CD34+, CD133+ or $ALDH^{br}$ cells) for CLI might be safe, feasible and effective. Following these favorable outcomes, a multicenter phase III clinical trial for CD34+ cells is in preparation in our institution.

ADSCs have generated great interest as a new tool of cell therapy, which may be a hopeful strategy for CLI.

In either cell type, well-designed larger placebo-controlled RCTs are warranted to prove the safety, feasibility and efficacy of the cell-based therapies. Guidelines or recommendations for adequate clinical trial methodology such as patient selection criteria, endpoints, and study design, etc. was recently proposed for pharmacotherapy [90], endovascular intervention [91] and bypass surgery [92] in patients with CLI. However, there is a lack of consensus on relevant methodology in the pivotal clinical trials of cell therapy in patients with CLI. It would be also urgently needed to establish such consensus for cell therapy in CLI.

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