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CELL THERAPIES AS NEW PLAYERS IN BUERGER'S DISEASE

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CONTENTS

SUMMARY

Buerger's disease is an inflammatory, nonatherosclerotic, occlusive vascular disease that mainly affects the small- and medium-sized arteries. Smoking cessation is the only definitive therapy and endovascular or surgical revascularization is generally not indicated or ineffective, because the arterial lesions are mostly peripheral, diffuse and segmental. Thus, exploring new strategies for revascularization of ischemic extremities is an important issue for patients with Buerger's disease. Stem and progenitor cells derived from bone marrow, peripheral blood, umbilical cord blood or adipose tissue have been identified as a new therapeutic option to induce neovascularization. Encouraging results in preclinical studies have rapidly led to several pilot clinical trials. Preliminary clinical outcomes revealed safety, feasibility and potential effectiveness on several important endpoints, such as ankle–brachial pressure index, transcutaneous partial oxygen pressure, rest pain scale, ulcer size and amputation rate. Somatic stem/progenitor cell therapies seem to be a promising tool for the treatment of severe ischemic extremities due to Buerger's disease. Large-scale, randomized, placebo-controlled and double-blind studies will be required to optimize the cell type and dose, the isolation method, the administration route, etc. These future investigations may also provide important clues to understand the mechanisms of the effects of cell therapies.

Key words: Buerger's disease – Cell therapy – Neovascularization – Critical limb ischemia

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CLINICAL FEATURES OF BUERGER'S DISEASE

Buerger's disease, also known as thromboangiitis obliterans, is a nonatherosclerotic peripheral vascular disease in which small- and medium-sized arteries of the extremities are primarily involved. Pathologically, it is characterized by diffuse, inflammatory, hyperplastic and nonsuppurative changes in all layers of the affected vessels, as well as thrombotic occlusion. The two cardinal symptoms of limb ischemia are intermittent claudication and rest pain: the latter symptom occurs in patients with critical limb ischemia (CLI) and may coincide with ischemic ulceration and gangrene. The annual incidence of Buerger's disease is reported to be 12.6 per 100,000 in the U.S. (1). Although Buerger's disease is observed worldwide, it is more prevalent in the Middle East and Far East. Buerger's disease is frequently observed in young and middle-aged males.

Although the cause of Buerger's disease has not been sufficiently elucidated, exposure of some patients with special genotypes, mainly human leukocyte antigen HLA-A9 and HLA-B5, to environmental factors, mainly nicotine, may be the basis of the etiology and pathogenesis of Buerger's disease. Discovery of anti-elastin, anti-collagen I and III antibodies, anti-nicotine and anti-vascular antigen antibodies in the blood of patients allowed to put forward a theory of immunological character of the disease (2).

Treatment of Buerger's disease is not well established. The only proven strategy to prevent disease progression and avoid amputation is the complete discontinuation of cigarette smoking or other use of tobacco in any form. Because the arterial lesions are mostly peripheral, diffuse and segmental, endovascular or surgical revascularization is generally not indicated or ineffective. Pharmacological therapy also does not usually increase blood flow in the ischemic area. Recent progress in vascular and cellular biology has opened up a therapeutic avenue for patients without any current options. Therapeutic angiogenesis by administration of angiogenic growth factors or stem/progenitor cells may constitute a potential alternative treatment strategy for such intractable disease.

CELL THERAPY: PRECLINICAL INVESTIGATIONS

Experimental studies demonstrated that bone marrow-derived endothelial progenitor cells (BM-EPCs), a small fraction of mononu-

clear cells (MNCs), can be mobilized into the circulation, incorporate into sites of active ischemia, proliferate and differentiate into endothelial lineage in order to increase neovascularization (3-5). Postnatal neovascularization is now believed to occur via two possible mechanisms: sprouting of preexisting resident endothelial cells (angiogenesis) and recruitment and direct differentiation of BM-EPCs into mature endothelial cells (vasculogenesis) (Fig. 1). A notion of the pivotal role of adult EPCs in pathophysiological neovascularization encouraged therapeutic application of BM-MNCs or BM-EPCs for ischemic diseases.

BM- or peripheral blood-derived MNCs (PB-MNCs) have been tested for therapeutic neovascularization, as they are easily obtained without specific procedures for EPC isolation. Shintani et al. reported that intramuscular administration of autologous BM-MNCs into rabbit ischemic hindlimb induces a more drastic formation of collateral vessels by angiography, higher capillary density by histology and greater blood perfusion by laser Doppler imaging compared with BM–fibroblast transplant (6). Tateno et al. demonstrated that intramuscular administration of PB-MNCs augments limb perfusion similar to that of BM-MNCs in a mouse model of hindlimb ischemia. PB-MNC injection upregulates the expression of angiogenic growth factors via enhancement of IL-1β expression in ischemic muscle tissue (7). These reports suggest the usefulness of BM- or PB-MNCs for therapeutic neovascularization in limb ischemia.

EPCs and hematopoietic stem cells (HSCs) share many surface markers, such as CD31, CD34 and CD133. Because no simple definition of EPCs exists, various methods for EPC isolation have been reported. Magnetic isolation of $CD34^+$, $CD133^+$ or vascular endothelial growth factor receptor 2 (VEGFR-2, FLK-1)-positive cells was performed to purify EPC-enriched fraction in MNCs without cell culture. Culture of MNCs with angiogenic growth factors was also established to enrich EPC fraction. Therapeutic application of "purified" EPCs was first accomplished by Kalka et al. Intravenous transplant of cultured human EPCs results in drastic improvement of physiological blood flow, histological capillary density and limb salvage ratio in athymic nude mice with hindlimb ischemia (8). Murohara et al. also reported that cultured human cord blood EPCs participate in the formation of capillary networks post-local transplant into

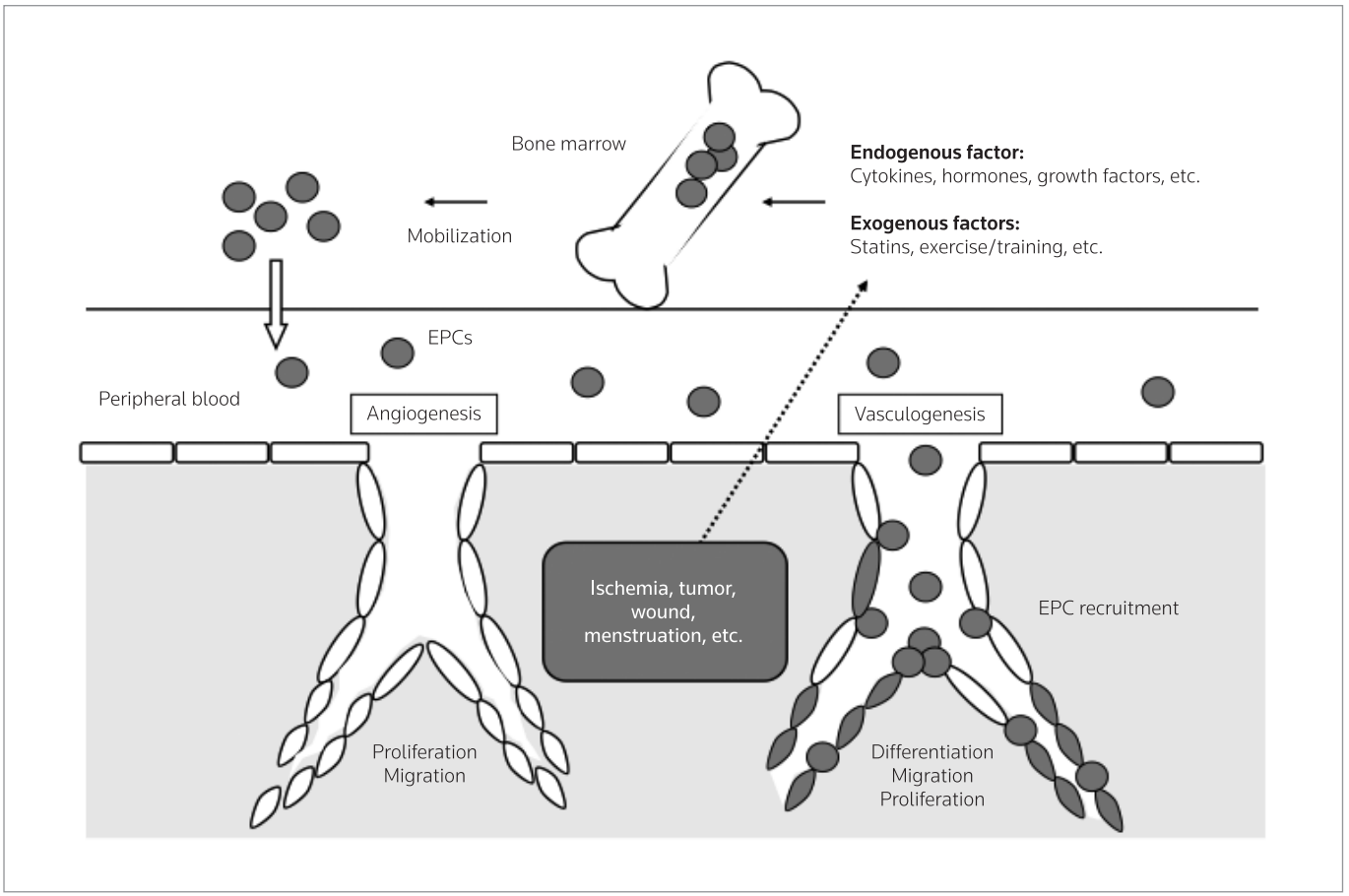

Figure 1. Postnatal neovascularization by angiogenesis and vasculogenesis. Left panel shows the concept of angiogenesis, in which preexisting endothelial cells proliferate and migrate to form new vessels in response to the endogenous or exogenous stimuli. Right panel indicates the concept of vasculogenesis; a variety of factors released from the jeopardized tissue or surrounding area affect bone marrow (BM) remotely and mobilize endothelial progenitor cells (EPCs) from the BM into the circulation. Then, EPCs home to the site and participate in neovascularization by differentiation, proliferation and migration.

immunodeficient rats (9). Lopez-Holgado et al. represented that freshly isolated human $CD133^+$ cells improve blood perfusion and capillary density in a murine model of hindlimb ischemia (10). Finney et al. demonstrated that umbilical cord blood $CD133^+$ cells exhibit robust vasculogenic functionality compared with BM-MNCs in response to hindlimb ischemia (11).

As for the mechanisms underlying the therapeutic effects of EPCs, the importance of paracrine action by cytokine secretion, as well as direct differentiation into endothelial lineage, has been demonstrated (12, 13). Secreted angiogenic cytokines may stimulate mobilization of BM stem/progenitor cells into the circulation, thereby contributing to neovascularization in ischemic tissue. Nonetheless, these favorable results encourage the clinical application of EPC therapy in patients with peripheral arterial disease.

On the other hand, recent investigations indicate the potential limitation of BM-derived cell therapy for ischemic diseases. Heeschen et al. reported that, despite similar content of EPCs, BM-MNCs obtained from patients with chronic ischemic cardiomyopathy have significantly impaired migratory and EPC colony-forming activities in vitro compared to those from healthy subjects. In vivo, intravenously injected BM-MNCs from the ischemic patients have hampered neovascularization capacity in a mouse model of hindlimb ischemia (14). Another previous report demonstrated that EPC colony-forming activity of PB-MNCs inversely correlates with both Framingham risk score and flow-mediated brachial artery reactivity (15). Number and migratory activity of circulating EPCs inversely correlate with coronary risk factors (16). EPCs obtained from patients with type 2 diabetes represent impaired proliferation, adhesion and incorporation into vascular structures (17). These findings suggest that BM-derived MNCs/EPCs obtained from ischemic patients with multiple atherosclerotic risk factors may have an impaired capacity for therapeutic neovascularization. Although Buerger's disease is basically a nonatherosclerotic disorder, the effect of autologous, BM-derived cell therapies might be deteriorated in patients with Buerger's disease who coincidentally have certain risk factors.

Cell therapies using non-EPC populations have also been investigated. BM-, umbilical cord blood (UCB)- or adipose tissue-derived mesenchymal stem cells (MSCs) reportedly can be obtained by culture expansion. Transplant of the MSCs results in attenuation of hindlimb ischemia (18, 19).

Recently, isolation of aldehyde dehydrogenase-bright (ALDH[Br]) populations based on an intracellular marker of "stemness" was established. BM-ALDH(Br) cells include hematopoietic, endothelial, mesenchymal and neural progenitors. A preclinical study demonstrated that the ALDH(Br) cells were highly effective in restoring blood flow to ischemic hindlimb (20).

As described above, accumulating evidence suggests that therapeutic application of various kinds of somatic stem/progenitor cells, including MNCs, EPCs, MSCs and multipotent stem cells, may be useful for attenuation of CLI. Nonetheless, there is little published information directly comparing the safety and potency among different cellular treatment strategies (21). Preclinical research to determine the optimal cell type for therapeutic neovascularization is thus warranted.

CELL THERAPY: CLINICAL TRIALS

Accumulating preclinical evidence indicates that various kinds of cell therapies, such as transplantation of BM-MNCs, PB-MNCs, PB-EPCs, BM-MSCs, adipose tissue-derived MSCs or UCB-derived MSCs, augment collateral vessel formation in ischemic limbs. These favorable results prompted clinical researchers to explore the feasibility of cell therapies in patients with CLI who are not candidates for conventional revascularization.

BM-derived MNCs

The Therapeutic Angiogenesis by Cell Transplantation (TACT) study by Tateishi-Yuyama et al. first demonstrated the safety, feasibility and efficacy of BM-MNCs in patients with CLI. The protocol consisted of an open pilot study (N = 25), in which safety and feasibility of autologous implantation of BM-MNCs were mainly evaluated, and a randomized, controlled confirmatory part comparing the efficacy of BM-MNCs versus PB-MNCs. In the latter part, patients (N = 22) with bilateral limb ischemia were randomly injected with BM-MNCs to one limb and with PB-MNCs to the other leg. At 4 weeks, ankle–brachial pressure index (ABI) significantly improved in legs injected with BM-MNCs compared with those injected with PB-MNCs. Similar trends were observed for transcutaneous partial oxygen pressure ($TcPO_2$), rest pain scale and pain-free walking time. Significant improvement in such parameters in the BM-MNC-injected legs was sustained at 24 weeks. The authors speculated that the mechanism underlying higher efficacy in the BM-MNC group as compared to the PB-MNC group might be the supply of a greater number of EPCs ($CD34^+$ fraction) and the greater amount of multiple angiogenic factors from the $CD34^-$ fraction in BM-MNCs (22).

The TACT follow-up study investigators assessed the long-term safety and clinical outcomes of BM-MNC therapy by investigating the mortality and leg amputation-free interval as primary endpoints (23). The median follow-up time for surviving patients was 25 months, and the 3-year overall survival was 80% in 74 patients with arteriosclerosis obliterans (ASO) and 100% in 41 patients with Buerger's disease. The 3-year amputation-free rate was 60% in patients with ASO and 91% in patients with Buerger's disease. The TACT follow-up study also reported no cases of non-preferred neovascularization and no increase in mortality compared with conventional therapies.

Durdu et al. performed the first clinical study of BM-MNC implantation in 28 patients with CLI due to Buerger's disease. BM-MNCs were implanted into a single limb and saline was infused into the less ischemic contralateral limb in each patient (24). The median follow-up time was 16.6 months, and follow-up was 100% complete. None of the patients underwent major amputations; however, toe amputation could not be avoided in one patient who continued smoking during follow-up. Change in ABI > 0.15 versus baseline was achieved in 8 patients at 3 months and in 14 patients at 6 months. At 6 months, patients experienced significant improvement in rest pain score, peak walking time and quality of life. Complete ulcer healing in the most important lesion was achieved in 15 patients (83%) with ischemic ulcers. Digital subtraction angiography before and 6 months after BM-MNC implantation indicated the formation of new vascular collateral networks across the affected arteries in 22 patients. Although there were several other studies regarding BM-

MNC implantation in patients with Buerger's disease, limitations for interpretation exist due to the small number of subjects, lack of control groups and use of different outcome parameters in each study (25-29). Despite these limitations, the effects of BM-MNC implantation on perfusion parameters (ABI, $TcPO_2$) and clinical courses (wound healing, walking distance) were remarkably consistent and positive throughout these reports. On the other hand, death in a patient receiving BM-MNC implantation was reported by Miyamoto et al. (27). A 30-year-old male patient with Buerger's disease suddenly died 20 months after cell transplant, although a causal relationship with BM-MNC therapy was unclear. Based on the previous results, BM-MNC implantation appears to be a safe and feasible procedure with minimal short- and long-term adverse events.

PB-MNCs

The safety and feasibility of implantation of granulocyte colony-stimulating factor (G-CSF)-mobilized PB-MNCs were investigated in six patients with severe peripheral artery disease, including five patients with Buerger's disease (30). Following administration of G-CSF (10 μg/kg/day) for 4-5 days, PB-MNCs were harvested and intramuscularly injected in ischemic limbs for 2 days. Improvement in ABI (> 0.1) was seen in four patients at 4 weeks and ischemic ulcers healed in three of three patients. The mean maximum walking distance significantly improved from 203 meters to 559 meters at 4 weeks and the improvement lasted for 24 weeks. No major adverse events occurred during the study period and these results demonstrated that G-CSF-mobilized PB-MNC implantation is a safe and feasible strategy for therapeutic angiogenesis in patients with limb ischemia, especially Buerger's disease.

Horie et al. reported the first multi-institutional study regarding the long-term clinical outcomes of G-CSF-mobilized PB-MNC therapy for patients with lower limb ischemia (31). They reviewed data from 162 consecutive patients and the median follow-up time for surviving patients was 26.4 months. The 2-year survival rate was 65% for the 140 patients with ASO and 100% for the 11 patients with Buerger's disease. The 1-year amputation-free rate was 70% for ASO and 79% for Buerger's disease.

A pilot study was performed to investigate whether therapeutic intervention by autologous PB-MNC implantation is feasible and effective for ischemic limbs (7). The study enrolled 29 patients suffering from CLI, including 10 patients with Buerger's disease, who received PB-MNCs into ischemic limbs twice within a 1-month period. Safety and efficacy were assessed at 2 months, 6 months and 1 year after treatment. Rest pain was significantly decreased and was nearly normalized by 1 year after treatment. The maximum walking distance also significantly improved at 6 months, and this improvement was preserved for a year. Furthermore, they assessed the long-term outcome in patients receiving PB-MNCs (32). None of the Buerger's disease patients died during 5-year follow-up, whereas the overall survival proportion of ASO patients was 67.3% at 2 years and only 45.7% at 3 years. Major amputation occurred in 1 of 14 patients with Buerger's disease and 5 of 28 patients with ASO during the study period.

Considering the poor prognosis of CLI, the results of these pilot clinical trials encourage a future randomized and controlled study of PB-MNC therapy for such intractable patients.

G-CSF-mobilized $CD34^+/CD133^+$ cells

EPCs belong to an immature cell population that is capable of differentiating into mature endothelial cells. In adults, EPCs mainly reside in BM and are more proliferative and migrative than terminally differentiated endothelial cells. EPCs can be clinically isolated as $CD34^+$ or $CD133^+$ MNCs from adult BM or PB. These basic findings encouraged clinical application of G-CSF-mobilized $CD34^+$ cell transplant for improvement of the poor prognosis of CLI patients. Recently, our group reported results from a phase I/IIa clinical trial of intramuscular transplant of autologous and G-CSF-mobilized $CD34^+$ cells in no-option patients with CLI. We enrolled 17 cases with CLI, including 12 patients with Buerger's disease, and all patients received subcutaneous administration of 10 μg/kg/day of G-CSF for 5 days. Leukapheresis was performed on day 5, and on the next day, $CD34^+$ cells were magnetically separated from the apheresis products. After separation, cell transplant was immediately performed into ischemic legs under spinal anesthesia. During the 12-week observation after cell transplant, toe brachial pressure index (TBI), $TcPO_2$, pain-free and total walking distances, Wong-Baker FACES pain rating scale and ulcer size were serially improved in all patients, although no significant dose–response relationship was observed (Fig. 2) (33).

Recently, we analyzed the long-term outcome of the CLI patients receiving G-CSF-mobilized $CD34^+$ cell implantation. No deaths and no major amputations occurred during the 2-year follow-up (34). These results indicate the safety and feasibility, as well as favorable trend in efficacy, of G-CSF-mobilized $CD34^+$ cell therapy in patients with CLI, including Buerger's disease.

In addition to CD34, CD133 is a surface marker of early EPC phenotype. A recent clinical study by Burt et al. showed the safety and feasibility of autologous, G-CSF-mobilized $CD133^+$ cell implantation into lower extremity muscles of nine patients with CLI, including a patient with Buerger's disease (35). PB-MNCs were collected by leukapheresis after G-CSF mobilization (10 μg/kg/day for 4-5 days), and $CD133^+$ stem cells were selected using a magnetic separation system. There were no major complications from either leukapheresis or cell injection. The patient with Buerger's disease underwent the procedure twice. After the procedure, rest pain resolved rapidly by day 2, and seven of nine patients, including a case of Buerger's disease, were able to avoid limb amputation during the 1-year follow-up. Although this study was a small, nonrandomized trial, these initial results suggest the potential effectiveness of the purified EPC population in CLI patients.

UCB-derived MSCs

Kim et al. reported the first clinical trial to demonstrate the efficacy of UCB-derived MSCs in four CLI patients due to Buerger's disease (29). The HLA typings were examined to get a proper match between the patients and preserved UCB. The UCBs matched with the patient's HLA types at the intermediate resolution level. UCB-mononuclear fraction was separated by Ficoll-Paque Plus and the MNCs were cultured and expanded for 4 weeks. The UCB-derived MSCs (1×10^6) were implanted into subcutaneous tissue and muscle of ischemic hands or legs. Angiography performed pretransplant, 1 month and 4 months post-transplant revealed the increase in capillary formation following the cell therapy. Vascular resistance in ischemic area also significantly improved at 1 month.

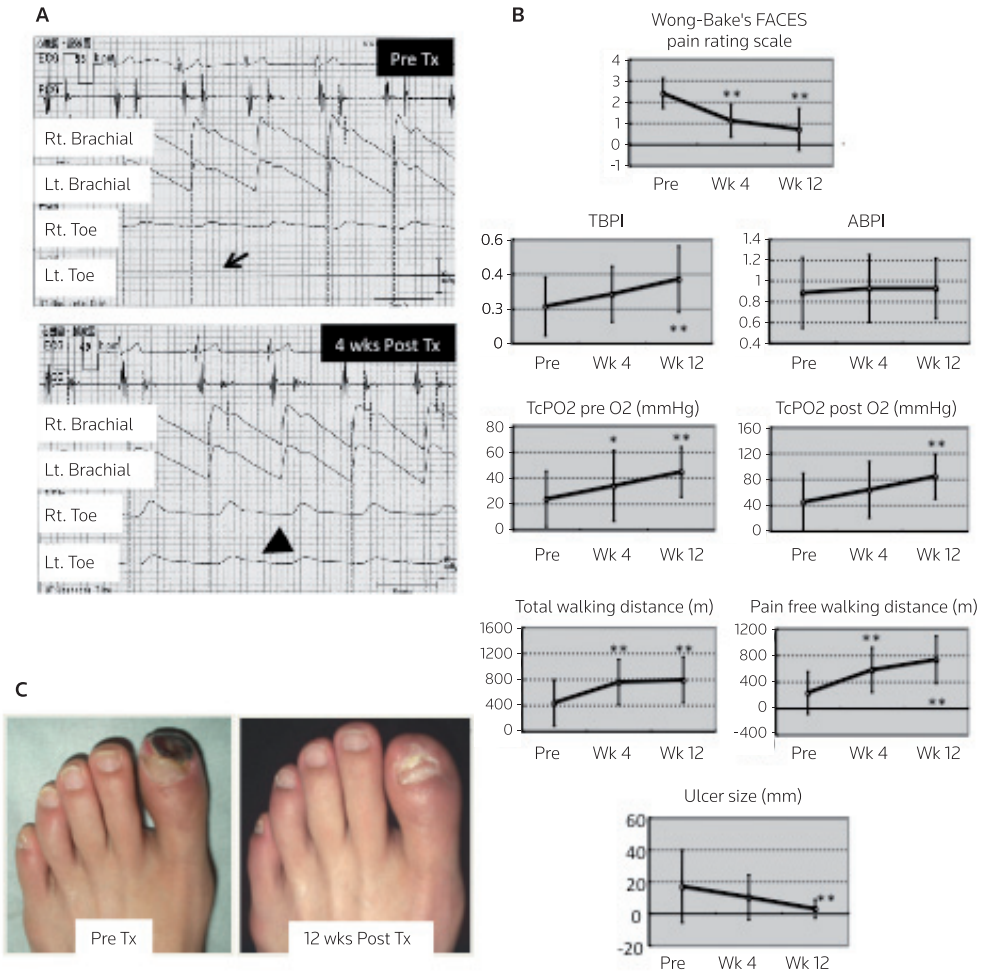

Figure 2. A) Representative recording of the pressure pulse wave in bilateral brachial and toe arteries (a male patient with Buerger's disease aged 21 years) pre- and 4 weeks post-CD34$^+$ cell transplant. A nonpulsatile (flat) wave form (arrow) was observed in the left toe artery pre-cell therapy (Tx); however, recovery of pulsatile pattern (arrowhead) was detected 4 weeks after Tx. **B**) Serial changes in subjective and objective parameters of limb ischemia following CD34$^+$ cell transplant in all patients (N = 17). TBPI, toe brachial pressure index; ABPI, ankle brachial pressure index; TcPO2, transcutaneous partial oxygen pressure. *P < 0.05 vs. baseline; **P < 0.01 vs. baseline. **C**) Representative pictures demonstrating healing of toe ulcer/gangrene following CD34$^+$ cell transplant (a male patient with Buerger's disease aged 36 years).

Interestingly, the UCB-derived MSC implantation relieved ischemic pain of patients more rapidly than forming their new capillaries. Rest pain disappeared between 5 hours and 14 days in all patients and unhealed skin lesions of the 2 patients showed skin regeneration within 120 days. There were no transplant-related major complications, including symptoms and signs of allograft rejection.

BM-derived ALDH(Br) cells

A recent randomized, controlled study showed the safety and efficacy of direct intramuscular injections of autologous BM-ALDH(Br) cells or BM-MNCs into severe ischemic legs of patients with ASO who were not eligible for percutaneous or surgical revascularization (36). ALDH(Br) cells or BM-MNCs were successfully administered to all patients and no therapy-related serious adverse events occurred. Patients treated with ALDH(Br) cells (n = 11) showed significant improvement in Rutherford category from baseline to 12 weeks (4.09 ± 0.30 to 3.46 ± 0.18) and in ABI at 6 weeks (0.22 ± 0.19 to 0.30 ± 0.24) and 12 weeks (0.36 ± 0.18) compared with baseline. Patients in the BM-MNC group (n = 10) showed no significant improvement at 6 or 12 weeks in Rutherford category but showed improvement in ABI from baseline to 12 weeks. No significant

changes from baseline were noted in ischemic ulcer grade or $TcPO_2$ in either group. These results indicate that administration of autologous ALDH(Br) cells appears to be safe and effective in patients with CLI. Further studies to test the clinical usefulness of ALDH(Br) cells in patients with Buerger's disease are warranted.

CONCLUSIONS

Buerger's disease is a vasculitis that mainly affects medium- and small-sized arteries in the extremities, with rare involvement of visceral and cerebral vessels. Patients are predominantly young smokers who present with distal extremity ischemia, ischemic digit ulcers or gangrene. Although smoking is important to the pathogenesis of Buerger's disease, the specific etiological mechanism remains unknown. Previous clinical studies revealed the safety and potential efficacy of cell therapies in patients with Buerger's disease. It is intriguing that patients with Buerger's disease were generally more responsive to the cell therapies compared to patients with ASO in terms of wound healing, rest pain reduction and improvement of exercise tolerance. Patients with Buerger's disease are generally younger and have less coronary risk factors impairing the EPC functions than patients with ASO. This fact may have led to the difference in clinical outcomes post-cell therapies in each patient group. Many previous studies have suggested that angiogenic factors secreted by implanted cells play a critical role in therapeutic neovascularization (5, 37). Tateno et al. demonstrated that the implanted PB-MNCs did not secrete cytokines sufficiently for neovascularization, but instead, stimulated secretion of angiogenic cytokines from ischemic muscle (7). As a mechanism, macrophages present in the implanted cells may contribute to the enhanced production of angiogenic factors by skeletal muscle. Considering the pathophysiology of Buerger's disease, however, antiinflammatory properties of implanted cells might contribute to further improvement of tissue ischemia due to vasculitis. Further investigation would be necessary to elucidate the positive and negative aspects of cell therapy-induced inflammation in Buerger's disease. On the other hand, the large number of $CD34^+$ cells collected was a significantly positive predictive factor for better prognosis following BM-MNC or G-CSF-mobilized PB-MNC implantation in patients with CLI (31, 38). These data suggest the importance of EPC-enriched fraction for therapeutic neovascularization. EPC-enriched fractions, such as $CD34^+$ cells, may have clinical superiority as transplanted cells, because MNCs are a heterogeneous population of cells, including proinflammatory macrophages, hampering the effect of cell therapy.

Exploring new treatment strategies in patients with Buerger's disease who have failed standard treatment options is of utmost importance to rescue ischemic limbs at high risk of amputation and subsequent death. Autologous cell therapies are promising new treatment options for these patients, and previous clinical trials were consistent in clinical benefits, including improvement of ABI and $TcPO_2$, reduction of rest pain and prevention of amputation. However, there is a definite need for randomized, placebo-controlled, double-blind studies to provide absolute evidence of clinical usefulness of these treatment options. Several ongoing clinical trials may shed light upon the issues still remaining to be resolved, such as optimization of cell type, isolation method, cell dose and route of administration. Future investigations regarding the mechanisms underlying the effects of these cell therapies will also be intriguing.

In conclusion, cell therapies appear to be promising tools for the treatment of Buerger's disease. Preliminary evidence indicates their safety, feasibility and potential effectiveness for several important endpoints. Several large-scale studies in patients with CLI are under way to further consolidate the evidence of clinical importance of the stem/progenitor cell therapies.

DISCLOSURES

The authors state no conflicts of interest.

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