Circulation Reports
Circ Rep 2020; 2: 630–634
doi:10.1253/circrep.CR-20-0086

PROTOCOL PAPER

Therapeutic Angiogenesis Using Bone Marrow-Derived Mononuclear Cell Implantation for Patients With Critical Limb-Threatening Ischemia Caused by Thromboangiitis Obliterans

— Study Protocol for a Multicenter Prospective Interventional Trial —

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Background: Patients with thromboangiitis obliterans (TAO) can develop critical limb-threatening ischemia (CLTI) and require limb amputation. Smoking cessation and exercise therapy are recommended as standard treatments, and revascularization by bypass surgery or endovascular therapy (EVT) is required for patients with CLTI. However, there are many cases in which revascularization is difficult because of vascular characteristics, and the patency rate after revascularization remains unsatisfactory. Therapeutic angiogenesis using bone marrow-derived mononuclear cell (BM-MNC) implantation is used clinically, with many trials demonstrating long-term efficacy and safety of the technique in patients with CLTI, especially that caused by TAO. To expand the use of BM-MNCs implantation in clinical practice, further evidence is required in patients with CLTI caused by TAO.

Methods and Results: This trial is a multicenter, prospective, non-randomized interventional trial of an Advanced Medicine B treatment approach. We aim to enroll 25 patients aged 20–80 years with Fontaine classification Stage III or IV, who will undergo BM-MNC implantation. The primary endpoint is the improvement in skin perfusion pressure of the target limb 180 days after BM-MNC implantation, whereas secondary endpoints are improvements in rest pain or ulcer size. We will also investigate rates of major or minor amputation, survival, and adverse events during follow-up.

Conclusions: BM-MNC implantation is expected to be an efficacious and feasible treatment for patients with CLTI caused by TAO.

Key Words: Bone marrow-derived mononuclear cells; Critical limb-threatening ischemia; Therapeutic angiogenesis; Thromboangiitis obliterans

Thromboangiitis obliterans (TAO; Buerger's disease) is more common among men in their 30 s and 40 s, and has a strong causal association with smoking. However, its pathogenesis remains unknown, and it is categorized as an incurable disease.[1] It has been reported that approximately 70% of patients with TAO develop critical limb-threatening ischemia (CLTI) of Fontaine classification Stage III or higher during disease progression, and that approximately 10% of patients undergo major amputation surgery, with approximately 20% of patients requiring minor amputations of the toes or fingers.[2–4] In addition, it has been reported that around 40% of patients with TAO experience work restrictions because of pain at rest, ulcers, and gangrene, and that more than 50% of patients experience some sort of lifestyle restrictions, with major effects on activities of daily living (ADL) and quality of life (QOL).[5]

Smoking cessation and drug and exercise therapies are generally recommended as the standard of care for patients with TAO, but revascularization using bypass surgery and endovascular therapy (EVT) are required for patients with CLTI.[6] However, the patency rate in the chronic phase

Received August 5, 2020; accepted August 5, 2020; J-STAGE Advance Publication released online September 15, 2020 Time for primary review: 1 day

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ISSN-2434-0790

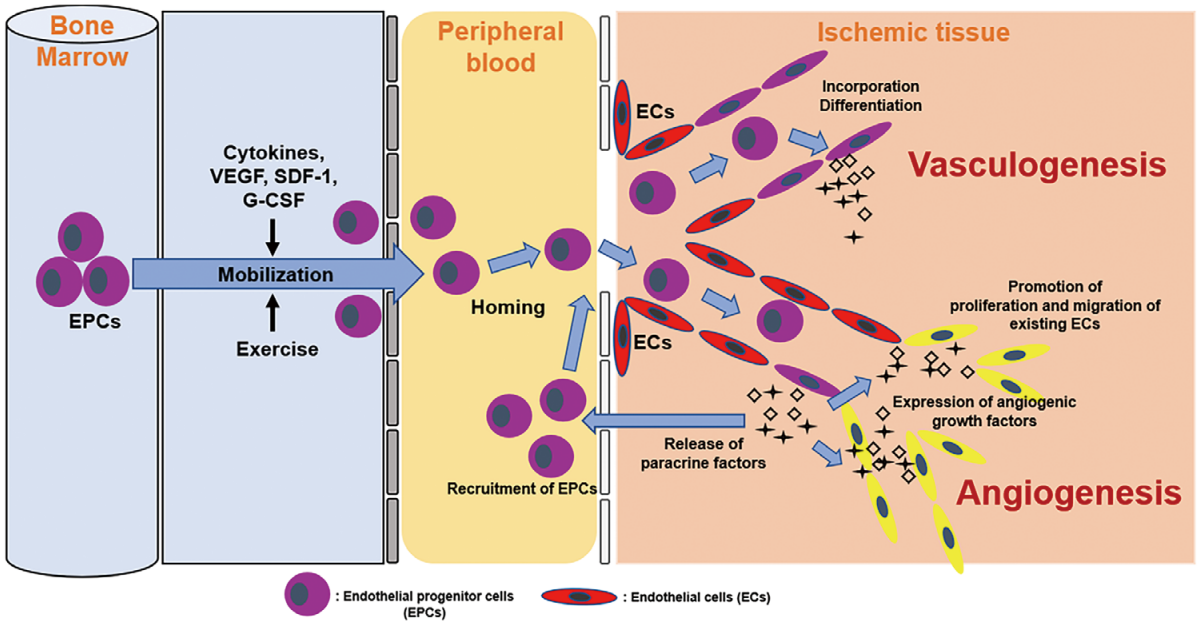

Figure 1. Kinetics of endothelial progenitor cells (EPCs) for post-natal neovascularization. During tissue ischemia, EPCs are mobilized from the bone marrow by cytokines and growth factors (e.g., vascular endothelial growth factor [VEGF], stromal-derived factor 1 [SDF-1], and granulocyte colony-stimulating factor [G-CSF]), leading to increases in the number of EPCs in peripheral blood. In addition, external factors, such as exercise, contribute to an increase in the number of EPCs. The mobilized EPCs are recruited to ischemic tissue, and the accumulation of EPCs directly contributes to vasculogenesis by providing components of the blood vessel wall. Furthermore, EPCs synthesize and secrete various cytokines, thereby promoting the proliferation and migration of existing endothelial cells (ECs). Basic experiments have demonstrated that bone marrow-derived EPCs are involved in neovascularization in the ischemic penumbra by angiogenesis and vasculogenesis.[9,10]

following bypass surgery is low, and the procedure is difficult to perform in many cases due to narrowing of the peripheral blood vessels.[7,8] Furthermore, there is insufficient evidence regarding the benefits of EVT because there is often early recoil or re-occlusion due to vascular properties. Therefore, among TAO patients, there are many with CLTI who are refractory to conventional standard treatments, including revascularization (i.e., patients with "no-option CLTI").

Endothelial progenitor cells (EPCs) derived from bone marrow were reported to promote angiogenesis (**Figure 1**).[9–11] Later, therapeutic angiogenesis to improve peripheral blood flow was clinically introduced for patients with no-option CLTI. Many clinical trials, such as the Japan Trial for Therapeutic Angiogenesis using Cell Transplantation, have reported on the safety and efficacy of therapeutic angiogenesis using autologous bone marrow-derived mononuclear cell (BM-MNC) implantation in patients with no-option CLTI, particularly that caused by TAO.[12–17]

In the future, BM-MNCs will need to be widely commercialized to improve the limb salvage rate of patients with CLTI caused by TAO. Thus, we designed a clinical trial to further evaluate the efficacy of BM-MNC implantation.

Methods

Trial Design

This is a multicenter prospective interventional trial. Kyoto Prefectural University of Medicine is the institution supervising the research, and another 5 institutions have agreed and registered to participate in this clinical trial. The trial was approved by the Ministry of Health, Labour, and Welfare of Japan as Advanced Medicine B after being approved by the Certified Committee for Regenerative Medicine in the Kyoto Prefectural University of Medicine in 2017 (ID: NA8150008). Furthermore, the trial was also registered with the Japan Registry of Clinical Trials (jRCT) in 2019 with the revision of the Specified Clinical Research Act (ID: jRCTb050190082).

The trial will enroll 25 patients with CLTI caused by TAO (**Figure 2**). In accordance with the Declaration of Helsinki, written informed consent will be obtained by trial investigators or subinvestigators from all participants prior to enrollment.

Participants

Inclusion Criteria To be eligible for inclusion in the trial, patients must have severe ischemic limbs and have been previously or newly diagnosed with TAO according to Shionoya's Diagnostic Criteria as follows: (1) onset at <50 years of age; (2) smoking history; (3) lower leg arterial occlusion; (4) the presence or a history of upper limb arterial occlusion or migratory phlebitis; and (5) no risk factors for obstructive arteriosclerosis other than smoking.[1] Participants meeting all these criteria are diagnosed with TAO; those meeting only 4 criteria are diagnosed with subtype TAO.

In addition, to be eligible for inclusion in the trial, participants must meet the following selection criteria: (1) male or female aged between 20 and 80 years; (2) TAO

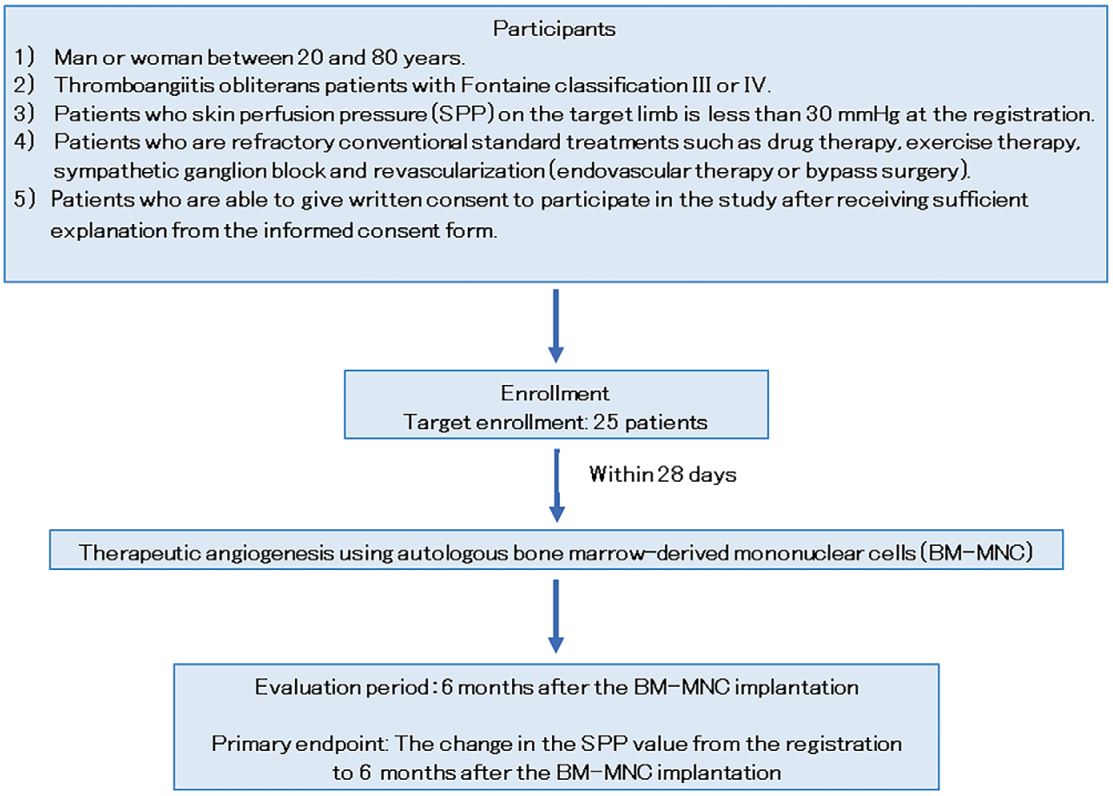

Figure 2. Study flow chart.

patient with Fontaine classification Stage III or IV; (3) skin perfusion pressure (SPP) of the target limb <30 mmHg at the time of registration; (4) refractory to conventional standard treatments, such as drug therapy, exercise therapy, sympathetic ganglion block, and revascularization (EVT or bypass surgery); and (5) able to provide written informed consent to participate in the study after receiving sufficient information about the trial (**Figure 2**).

Exclusion Criteria The exclusion criteria are as follows: refusal to provide informed consent or being considered unsuitable for the trial because of emotional considerations, even if the disease and procedure are suitable; CLTI in both lower limbs and in need of immediate treatment; life expectancy <1 year because of another comorbid condition; diagnosis of malignant tumor; ischemic heart disease that has not been treated with revascularization; untreated severe diabetic retinopathy; serious infection; serious hepatic impairment or renal impairment (excluding chronic maintenance dialysis); serious hematological disorders, such as leukopenia or thrombocytopenia; severe anemia necessitating blood transfusion; pregnancy, potential pregnancy, or breastfeeding; participation in clinical trials of a drug or medical device at the same time as this trial or within 30 days before enrollment in this trial; and other severe acute or chronic medical or psychiatric conditions or abnormal clinical test results for which participation in the trial may result in increased risk or may affect the interpretation of the trial results.

Rationale Underlying Sample Size Calculation

In a previous study, Tateishi-Yuyama et al[11] reported mean±SD variations in transcutaneous oxygen pressure ($TcpO_2$), which can be considered equivalent to SPP, of 18±11 and 16.6±9.9 mmHg (in the two groups injected BM-MNCs), whereas Matoba et al[14] reported a mean $TcpO_2$ of approximately 15 mmHg (SD not listed) in the group with Buerger's disease. In this trial, we will substitute a value of 20 mmHg for baseline SPP value if it is below the limit of detection and 0 mmHg for SPP at 6 months (i.e., SPP after treatment) if it is again below the limit of detection to analyze the primary outcome conservatively. Using an expected difference in SPP values of 10 mmHg, and a standard deviation of 11 mmHg, the sample size was calculated to be 19 using PASS 13 (NCSS Statistical Software) with a significance level of 0.05 and power of 0.95 based on the Wilcoxon signed-rank test. In consideration of the potential exclusion of some patients from analysis, the sample size in this study was set to 25 patients.

Protocol Procedures

Figure 3 shows the screening and examination schedule from enrollment and throughout the follow-up period to the final survey.

Enrollment Patients meeting the selection criteria are enrolled in the study, and all parameters making up the primary and secondary endpoints are measured at the time of registration.

BM-MNC Implantation (Day 0) BM-MNCs will be implanted in trial patients within 28 days of registration.

	STUDY PERIOD							
	Enrollment	Treatment	Follow-up					Close out
TIME POINT		Day 0	Day 1	Day 7	Day 30	Day 90	Day 180	Survey date
Enrollment:								
Eligibility screening	×							
Informed consent	×							
Patient characteristics	×							
Vital signs	×	×	×	×	×	×	×	
Blood examination	×		×	×			×	
Urinalysis	×							
ECG	×							
Chest X-ray	×							
ABI	×					×	×	
Numerical rating scale	×				×	×	×	
SPP/$TcpO_2$	×			×/		×	×	
Ulcer diameter	×					×	×	
Fontaine classification	×				×	×	×	
6 minutes walking distance	×						×	
Lower limb contrast CT	×						×	
BM-MNC implantation		×						
Adverse events		×	×	×	×	×	×	×

Figure 3. Study schedule. The trial plans to evaluate items marked with "×" during the follow-up period. ABI, ankle-brachial pressure index; BM-MNCs, bone marrow-derived mononuclear cells; CT, computed tomography; ECG, electrocardiogram; SPP, skin perfusion pressure; $TcpO_2$, transcutaneous oxygen pressure.

With patients under general anesthesia, approximately 600 mL bone marrow fluid is collected from both iliac bones. The bone marrow fluid collected is then separated by a blood component separator and concentrated into approximately 40–80 mL, containing over 0.5×10^9 BM-MNCs. The BM-MNCs are injected evenly into the skeletal muscle of the target leg below the knee.

Follow-up Period All specified items are measured on Days 1, 7, 30, 90, and 180 after BM-MNC implantation. The protocol therapy is completed 180 days after BM-MNC implantation. During the follow-up period, vasodilators, antiplatelet agents, and analgesics can be reduced or discontinued, but they cannot be changed or the doses increased. In addition, concomitant drug use as part of other clinical trials is prohibited, and pain relief therapy using nerve blocks (e.g., sympathetic ganglion block and epidural block) cannot be performed.

Outcome Survey The outcome survey of all enrolled patients will be performed within 1 year of enrolment of the last patient. The outcome survey will investigate overall survival, limb amputation, and adverse events for all patients enrolled in the trial.

Outcome Measures

Primary Endpoint The primary endpoint is the change in SPP from the time of registration to 180 days after BM-MNC implantation.

Secondary Endpoints The secondary endpoints are: (1) walking distance; (2) changes in pain since the time of registration based on a numerical rating scale; (3) changes in blood vessel volume below the popliteal artery of the affected limb, as determined by contrast-enhanced computed tomography (CT); (4) changes in the ankle-brachial index and $TcpO_2$ from the time of registration; (5) SPP ≥30 mmHg 180 days after BM-MNCs implantation; (6) at least a 30% reduction in the ischemic ulcer area; (7) Fontaine classification; and (8) the time until major amputation of the affected limb. Improvements in blood vessel volume based on contrast-enhanced CT and ulcer size will be evaluated by a third-party specialist as a central evaluation. To evaluate safety, the presence or absence and the severity of adverse events, in addition to overall life expectancy, will be assessed.

Statistical Analysis

The significance of differences in the primary endpoint will

be evaluated using the Wilcoxon signed-rank test. Subgroup analyses according to baseline SPP (<20 vs. ≥20 mmHg) will be conducted. Secondary endpoints will be analyzed using the Wilcoxon signed-rank test for continuous outcome measures, whereas the Kaplan-Meier estimator will be used to estimate the time to major amputation of the affected limb. The significance one-tailed level will be set at 0.05, and the confidence level will be set at 0.95.

Discussion

According to previous clinical trials, the outcomes of BM-MNC implantation are comparable to those of current revascularization strategies in patients with CLTI caused by TAO.[12–16] That is, we believe that BM-MNC implantation may be an option for revascularization in patients with CLTI caused by TAO, even in those who are not refractory to current revascularization therapies. However, few studies have evaluated changes over time in SPP and $TcpO_2$ values, which are indicators of peripheral perfusion, and the direct effects of BM-MNC implantation on wound healing have not yet been fully established. If this clinical trial can determine the efficacy of BM-MNC implantation in improving peripheral perfusion under conditions of limb ischemia, it may be possible to expand the use of BM-MNC implantation clinically. Further, we expect that BM-MNC implantation will lead to improvements in the limb salvage rate, ADL, and QOL in patients with TAO.

BM-MNC implantation is expected to be an efficacious and feasible treatment option for patients with TAO. If the efficacy of BM-MNC implantation for peripheral perfusion can be established, we aim to put BM-MNC implantation into widespread use to further improve the limb salvage rate for patients with TAO.

Acknowledgments

The authors greatly appreciate the help and support of all members of the committee for this project and members of the collaborating research institutes listed in the **Appendix**.

Sources of Funding

This trial has received no external funding.

Disclosures

The authors have no competing interests to declare.

IRB Information

This trial was approved by the Certified Committee for Regenerative Medicine in the Kyoto Prefectural University of Medicine in 2017 (ID: NA8150008) and is registered with the Japan Registry of Clinical Trials (jRCT; ID: jRCTb050190082).

Data Availability

The researchers concerned with this trial take full consideration of participants' personal information and privacy protection in accordance with relevant regulations and laws. In addition, the information obtained in this trial will not be provided to third parties with the exception of request by a public organization.

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Appendix

Research institutes and researchers collaborating with the trial are as follows:

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