Autologous Peripheral Blood Mononuclear Cell Implantation for Patients With Peripheral Arterial Disease Improves Limb Ischemia

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Background Implantation of bone marrow mononuclear cells, including endothelial progenitor cells, into ischemic limbs has been shown to improve collateral vessel formation. In the present study the safety and feasibility of autologous peripheral blood mononuclear cells (PBMCs) implantation after granulocyte-colony stimulating factor (G-CSF)-induced mobilization was investigated in patients with severe peripheral arterial disease.

Methods and Results Six cases were enrolled: 5 of thromboangitis obliterans and 1 of arteriosclerosis obliterans. Following administration of G-CSF (10μg·kg⁻¹·day⁻¹), PBMCs were harvested and injected intramuscularly (5 legs and 1 arm) for 2 days for the patients with ischemia of the legs. No serious adverse events related to G-CSF administration, harvest or implantation were observed during this study period. Improvement in the ankle–brachial pressure index (ΔABI: >0.1) was seen in 4 patients at 4 weeks and ischemic ulcers improved in 3 of 3 patients. The mean maximum walking distance significantly increased from 203 m to 559 m (p=0.031) at 4 weeks and was sustained for 24 weeks. Significant improvement was seen in physiological functioning sub-scale of Short Form-36.

Conclusion Implantation of PBMCs collected after G-CSF administration could be an alternative to therapeutic angioplasty in patients with severe peripheral arterial disease. (Circ J 2005; 69: 1260–1265)

Key Words: Granulocyte-colony stimulating factor; Peripheral arterial disease; Peripheral blood mononuclear cells; Therapeutic angiogenesis

In hematological and cancer diseases, peripheral blood has largely replaced bone marrow as the source of stem cells in autologous transplantation, because of faster hematologic recovery. The safety of granulocyte-colony stimulating factor (G-CSF) administration and apheresis has already been established, although frequent side-effects of G-CSF administration are myalgias, arthralgias, headache, and fever; however, long-term serious complications have not been reported. Pretreatment with G-CSF can mobilize not only hematopoietic stem cells, but also endothelial progenitor cells from bone marrow to peripheral blood. Therefore, we conducted an open-labeled clinical trial of implantation of autologous peripheral blood mononuclear cells (PBMCs) after mobilization with G-CSF in patients with severe PAD.

Methods

Patients Six cases of PAD were enrolled, all male patients: 5 cases of TAO, and 1 of ASO. All the patients had a history of intermittent claudication, rest pain, non-healing ischemic ulcers, or all three, and were not candidates for surgical revascularisation. None of the patients had responded to conventional medical therapy for at least 8 weeks. Exclusion criteria were: diabetes mellitus with proliferative retinopathy; malignant disease; recent onset (within 3 month) of myocardial infarction or brain infarction; uncontrolled myocardial ischemia; persistent severe heart failure (ejection fraction <30%); hematological disease; current serious infectious disease; aged older than 80 years; and diseases with life expectancy of less than 1 year. We
obtained written informed consent from all patients and the Medical Ethics Committee of University of the Ryukyus School of Medicine approved the protocol. Our study started in February 2003, at the University of the Ryukyus. By September 2004, 17 patients had been screened for this therapy, but 11 patients were excluded because of indication and exclusion criteria.

**Procedure**

After administration of 10μg·kg⁻¹·day⁻¹ G-CSF (filgrastim, Kirin Brewery Co, Tokyo, Japan) by subcutaneous injection for 4–5 days, PBMCs were harvested using an AS104 cell separator (Fresenius Medical Care, Tokyo, Japan). During the preparation of the PBMCs, the sedimentation process reduced the volume of PBMCs to approximately 30–50 ml. Within a few hours after PBMCs harvest, patients were injected with 0.75–1.0 ml of mononuclear cells in each of 30–50 sites in the ischemic limb muscles, under epidural anesthesia. Injections were spaced 2–3 cm apart, using a 25-gauge needle. PBMCs harvest and injection were done for 2 days for lower limb ischemia. On the second day, approximately 50 ml of PBMCs were injected in between the first injection sites. We assessed ischemic status by measuring the ankle–brachial pressure index (ABI), transcutaneous oxygen pressure (TcPO₂), and exercise treadmill test (2.4 km/h, 12% incline, pain-free walking distance, maximal walking distance). The health-related quality of life of the patients was assessed using the Short Form (SF)-36 questionnaire."
esis for the other patients. Although the number of harvested peripheral blood mononuclear cells (PBMNCs) was slightly low in all patients on second day of harvest (2.35 (0.99)×10^10 vs 1.36 (0.76)×10^10 p=0.061), a comparable number of CD34+ cells (1.12 (0.36)×10^8 vs 1.07 (0.47)×10^8, p=0.697) were collected on the second PBMNCs harvest day.

The remainder of the collected peripheral blood cells was returned to the patient by intravenous continuous infusion. No adverse events occurred during PBMNCs harvest. Patients were injected with approximately 30 or 100 ml of

![Graph](image1)

**Fig 2.** Number of (a) mononuclear cells and (b) CD34+ cells collected during apheresis. Although the number of harvested peripheral blood mononuclear cells (PBMNCs) was slightly low in all patients on second day of harvest (2.35 (0.99)×10^10 vs 1.36 (0.76)×10^10 p=0.061), a comparable number of CD34+ cells (1.12 (0.36)×10^8 vs 1.07 (0.47)×10^8, p=0.697) were collected on the second PBMNCs harvest day.

![Graph](image2)

**Fig 3.** Changes in (a) ankle-brachial pressure index (ABI) and (b) transcutaneous oxygen pressure (TcPO2) after the treatment.

![Graph](image3)

**Fig 4.** Change in walking capacity after the treatment. (a) Pain-free walking distance and (b) maximal walking distance were assessed by treadmill exercise test at 2.4 km/h and 12% incline. *p<0.05 vs baseline.
PBMNCs in the arm or leg, respectively. Local edema and muscle pain, except flare reaction and local fever, at the injection sites were observed in all patients from the day following implantation, but abated within 1 week.

At 4 weeks’ follow-up, there was an increase in ABI (ΔABI >0.1) in 4 of 5 patients who received PBMNCs implantation in the leg, however, it nearly returned to the baseline level at 24 weeks. The TcPO2 level increased in 2 patients and was unchanged in 2 patients (Fig 3).

The pain-free walking distance improved from 72.1±33.5 m to 189.3±108.9 m (p=0.053) at 4 weeks. After PBMNCs implantation, leg pain did not affect continuous walking and as a result, the maximum walking distance significantly increased from 203±95.4 m to 559±318 m (p=0.019) at 4 weeks (Fig 4) and continued to improve up to 24 weeks (858±305.9 m, p=0.019).

Ischemic ulcers of the thumb (patient no. 1) and big toe (patient nos. 5, 6) showed improvement either at 4 weeks (patient nos. 1, 6) or 8 weeks (patient no. 5) after implantation (Fig 5).

Pain relief occurred within 1 week after implantation and daily use of analgesics was reduced (patient nos. 1, 5, 6). The bodily pain subscale rating on the SF-36 improved from 47±29.5 to 64±29.1 at 4 weeks in all except 1 patient (no. 4) and the physical functioning subscale significantly improved from 51±17.7 to 70±10.5 (p=0.032) at 4 weeks and to 81±4.8 (p=0.032) at 24 weeks (data not shown).

Discussion

We here report the results of 6 cases of PAD for which we used autologous PBMNCs implantation, in order to demonstrate the safety and feasibility of this procedure. There was improvement in the ABI, ulcer healing, TcPO2, maximum walking distance, and health-related quality of life, without any serious adverse reactions, similar to previous reports of the use of bone marrow cells.5,6 The mobilizing effect of G-CSF was sustained for several days, and we could collect comparable numbers of CD34+ cells on the second day of PBMNCs harvest (Fig 2). In this study, PBMNCs were harvested and implanted into ischemic leg muscles over 2 successive days to enhance neovascularization. The average total number of harvested mononuclear cells and CD34+ cells for each patient were 3.98×10¹⁰ and 2.01×10⁸ cells, respectively, which is equivalent to or more than those harvested from bone marrow in previous studies.5,10 Although the minimum number of mononuclear cells or CD34+ cells for improvement of tissue ischemia has not been established, repeatability is one of the advantages of PBMNCs implantation over bone marrow mononuclear cells implantation. In a recent report the number of implanted CD34+ cells positively correlated with the efficacy of bone-marrow-derived cells implantation in PAD patients,11 which supports our method.

It is unclear whether the composition of mononuclear cells from peripheral blood harvested after G-CSF administration is similar to that from bone marrow. Tateishi-Yuyama et al have showed the advantages of bone marrow cells over peripheral blood cells without G-CSF administration for improvement of ischemic status in patients with PAD5 but a recent study performed in patients with myocardial infarction demonstrated intracoronary infusion of PBMNCs mobilized with G-CSF, essentially following the identical procedure used in the present study, resulted in a similar improvement of left ventricular systolic function as with bone marrow cell implantation.10

Unfortunately, there is not yet an established method for evaluating neovascularization after cell implantation. The most important objectives of any intervention in patients with PAD are symptomatic relief, limb salvage, and functional improvement. In the present study subjective symptoms such as ischemic pain were greatly reduced within 1 week, and analgesic use was reduced. In 3 patients there...
was improvement of the ulcers and limbs were salvaged after PBMCs implantation. The SF-36 is responsive to therapeutic interventions and improvements in PAD symptoms, particularly the physical functioning subscale;13,14 and in the present study that showed significant improvement after PBMCs implantation and was maintained during 24 weeks of follow-up.

Although the increased ABI nearly returned to baseline level, the improvement in maximum walking distance was maintained during the 24 weeks' follow-up. We do not know the exact reason for this discrepancy. In patient no. 5, the ABI level did not decrease at the baseline and there was no difference after PBMCs implantation, although his big toe ulcer was obviously improved at 8 weeks after treatment, which suggests that the ABI level does not accurately reflect improvement in peripheral circulation. Inseri et al reported that in diabetic neuropathy ischemic change resulting from a decrease of vasa nervorum can be restored by administration of vascular endothelial growth factor (VEGF)5,16 and Iba et al showed that implantation of PBMCs, which have a high concentration of VEGF and various angiogenic factors, into ischemic limbs enhanced collateral vessel formation.17 It could be hypothesized that sustained improvement of microvascular ischemia around the peripheral nerves in ischemic tissue after PBMCs implantation may restore peripheral nerve function and produce the improvement in the subjective signs and symptoms.

G-CSF administration may promote leukocytosis, hypercoagulability, or plaque vulnerability, and induce acute vascular events;18–20 however, none occurred during the follow-up period in this study. Risk factors for coronary artery disease were entirely controlled in all the present patients. We assessed the carotid and coronary artery by ultrasonography, and 201Tl-dipyridamole scintigraphy or coronary angiography in all patients before G-CSF administration.

There is a hypothetical concern that implanted cells may differentiate into mesenchymal cells, such as osteoblasts, fibroblasts, smooth muscle cells and myogenic cells, instead of endothelial cells in the injected tissue, suggesting that selective implantation of endothelial lineage cells might be suitable for therapeutic angiogenesis. In fact, Inaba et al demonstrated that implantation of selected CD34+ cells is also effective as angiogenic therapy for PAD patients.10 However, recent studies showed the potential of bone marrow cells to promote secretion of angiogenic cytokines, such as VEGF and basic fibroblast growth factor (bFGF), and suggest that it is these secreted cytokines rather than cell incorporation that promote functional recovery.5,17,21,22 Not only CD34+ cells but also the CD34– fraction in bone marrow mononuclear cells secrete angiogenic cytokines, and enhance angiogenesis. It has been reported that PBMCs also secrete several angiogenic cytokines such as VEGF, bFGF, platelet-derived growth factor-AB, and transforming growth factor-β. Therefore, we implanted the entire mononuclear cell population obtained after G-CSF administration.

The present results demonstrate that implantation of autologous PBMCs mobilized with G-CSF is a safe and feasible strategy for therapeutic angiogenesis in patient with PAD. The major limitation of this study is the lack of a control group, such as PBMCs implantation alone or a G-CSF alone group. It is difficult to obtain equal number of PBMCs and CD34+ cells without G-CSF administration and G-CSF per se may increase the peripheral blood flow by mobilization of endothelial progenitor cells from bone marrow to peripheral artery. However, Kang et al reported that G-CSF-mobilized peripheral blood cells improved cardiac function in patients with acute myocardial infarction, though G-CSF alone had no effect on cardiac function.21 Interestingly, the improvement in the finger ulcer in patient no. 1 was sustained at least for 14 month after PBMCs implantation, although an ulcer of the big toe developed 12 months later (= patient no. 5), suggesting that the effect of G-CSF per se is not as effective as that of PBMCs implantation. Further follow-up examinations of the patients are needed to define whether the beneficial effects will be sustained long-term.

The advantage of PBMCs over bone marrow mononuclear cells is its repeatability, which thereby enhances the formation of collateral vessels, and it is a procedure that does not require general anesthesia. Recently, potent angiogenic effects of PBMCs without mobilization with G-CSF were reported;23 so randomized trials are required to compare the clinical outcome of PBMCs with or without G-CSF administration versus the use of bone marrow mononuclear cells.

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References


