Early and Long-term Effects of the Autologous Peripheral Stem Cell Implantation for Critical Limb Ischemia

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Objective: Therapeutic angiogenesis by peripheral blood mononuclear cells (PB-MNCs) implantation has been shown to be a safe and effective treating for critical limb ischemia (CLI). We herein report our investigation of the long-term efficacy of implantation of granulocyte-colony stimulating factor (G-CSF)-induced PB-MNCs to treat patients with CLI for which surgical bypass and/or percutaneous transluminal angioplasty are not possible.

Methods and Methods: Eleven cases were enrolled in this study. Following an injection of G-CSF (250 ug/day) for 3 days, PB-MNCs ($1.1 \pm 0.5 \times 10^{10}$ including $1.5 \pm 0.2 \times 10^7$ CD34-positive cells) were harvested by apheresis and then injected into 13 ischemic limbs.

Results: Resting pain either diminished or improved in 10 cases (91%) at 4 weeks, and ulcer formation was cured in 6 out of 10 limbs (60%) after treatment. The time required to enhance the arteries at the level of foot-joint by angiography which was performed in the abdominal aorta was shortened by 1 month (10 ± 4 seconds) and 6 months (12 ± 1) compared with the pretreatment time (15 ± 5). Three patients died after treatment, and the actuarial survival rate at 3 years was 73%. Freedom from major amputation at 3 years was 92%.

Conclusion: The local injection of G-CSF-induced PB-MNCs showed striking early and long-term effects.

Keywords: peripheral blood mononuclear cells, arteriosclerosis obliterans, thromboangiitis obliterans, critical limb ischemia, angiogenesis

INTRODUCTION

Critical limb ischemia (CLI) due to arteriosclerosis obliterans (ASO) or thromboangiitis obliterans (TAO) is a disease that significantly decreases a patients' quality of life because of leg pain, ulceration, and gangrene.

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Although several therapies, including medical and surgical procedures, may reduce patients' symptoms and improve the condition of their legs, a lot of patients are not candidates for surgery or percutaneous transluminal angioplasty, and 25% of CLI patients requires a major amputation of a leg within 1 year after the first diagnosis of CLI.¹⁾ Recently, a new therapy using bone marrow mononuclear cells (BM-MNCs) has been developed to treat CLI, and the implantation of granulocyte-stimulating factor (G-CSF)-derived peripheral blood mononuclear cells (PB-MNCs) into the ischemic limb have also been shown to be effective.²⁻⁴⁾ However, a few reports⁵⁾ of critical limb ischemia have, so far, examined the long-term (>3 years) effect of PB-MNCs therapy for CLI. We herein report the acute and long-term effects of PB-MNCs therapy against CLI.

Table 1 Profiles of the patients underwent PB-MNCs therapy

Case	Gender	Age	Rest pain	Ulcer	DM	HD	Diagnosis
1 (L)	Male	75	+	–	+	+	ASO
2 (L)	Female	72	+	+	+	–	ASO
3 (R)	Male	69	+	+	+	+	DM
3 (L)	Male	69	+	+	+	+	DM
4 (L)	Male	70	+	+	+	–	ASO
5 (L)	Male	79	+	+	–	–	ASO
6 (R)	Female	83	+	+	+	+	ASO
7 (L)	Male	74	+	–	–	–	ASO
8 (R)	Female	82	+	+	–	–	ASO
9 (R)	Female	81	+	+	+	–	TAO
9 (L)	Female	81	+	+	+	–	TAO
10 (L)	Male	69	+	–	–	–	ASO
11 (R)	Female	83	+	+	–	–	ASO

L: left lower limb; R: right lower limb; HD: hemodialysis; DM: diabetes mellitus; ASO: arteriosclerosis obliterans; TAO: thromboangiitis obliterans

MATERIALS AND METHODS

Patients with CLI suffering from rest pain and/or ulcer formation on the limbs for at least three months, which did not improve despite possible bypass graft operation and/or medical treatment, were included in this study. All patients underwent angiography before this treatment, and 2 radiologists and 3 vascular surgeons evaluated whether PTA and/or bypass grafting was possible. When it was determined that it was impossible to perform PTA and/or bypass grafting, we then considered PB-MNCs therapy. Patients with severe ischemic heart disease, cerebrovascular disease, proliferative diabetic retinopathy, malignancy within one year, or who were unable to give fully informed consent, were excluded. After approval by the medical ethics committee of Shimonoseki City Hospital, 11 patients (13 legs) underwent the PB-MNCs therapy during from December 2006 to May 2008. All patients underwent angiography before and after PB-MNCs therapy. Two radiologists carried out the angiographic examination of the lower body from the brachial artery and injected contrast medium into the abdominal aorta near the bifurcation. We measured the time from the injection of the contrast medium to the starting point of the enhancement of the artery at the level of the foot-joint. Characteristics of the patients are shown in **Table 1**. All patients were suffering from rest pain, and ulcer formation with and without gangrene were

recognized in 10 legs of 8 patients. Six patients were suffering from diabetes mellitus (DM) and 3 patients underwent hemodialysis for chronic renal failure. Nine patients were suffering from arteriosclerosis obliterans (ASO), and the rest was suffering from DM angiopathy (case 3) or thromboangiitis obliterans (TAO; case 9). Previous treatments for patients are listed in the **Table 2**. All patients underwent medical treatment at least 3 months before PB-MNCs therapy. Two patients had already undergone major amputation in the contra-lateral limb. Patients with diabetic angiopathy and TAO and one patient with ASO had only been treated medically before PB-MNCs therapy. The rest of the ASO patients had undergone PTA and/or bypass surgery before PB-MNCs therapy.

PROTOCOL FOR PB-MNCs THERAPY

Patients were injected with 250 µg of G-CSF subcutaneously for 3 days, and the next day PB-MNCs were harvested by cell apheresis using a cell separator (COBE Spectra™, GAMBRO BCT, Lakewood, USA), in a final suspension volume of approximately 50 ml. We harvested $1.1 \pm 0.5 \times 10^{10}$ total mononuclear cells, of which $1.4 \pm 0.5 \times 10^7$ were CD 34 positive cells. Within a few minutes after harvesting PB-MNCs, the collected stem cell suspension was divided into 0.5 ml with a syringe and injected with a 26 gauge needle into 40–60 points of the

Table 2 Previous treatments of the patients underwent PB-MNCs therapy

Case	Previous treatment
1	PTA to Lt. EIA. Graft replacement of Lt. EIA and Rt. FA.
2	PTA and Bilateral FPAK bypass (twice). Amputation of the contra-lateral limb.
3	Medication
4	Lt. FPBK bypass. Amputation of the contra-lateral limb.
5	PTA to Rt. CIA and EIA
6	PTA to bilateral CIA and CFA
7	FF bypass
8	Resection of the gangrene of the Lt. 2nd finger.
9	Medication
10	Lt. FPBK bypass. PTA to Lt. ATA.
11	Medication

PTA: percutaneous transluminal angioplasty; EIA: external iliac artery; FA: femoral artery; FPAK: femoro-popliteal above knee; FPBK: femoro-popliteal below knee; CIA: common iliac artery; CFA: common femoral artery; FF: femoro-femoral; ATA: anterior-tibial artery

Table 3 Results of ulcer after PB-MNCs therapy

Case	Gender	Age	Ulcer	No. Size (mm)	Improvement	Recurrence
2 (L)	Female	72	+	2 (22 × 20, 12 × 8)	+	–
3 (R)	Male	69	+	2 (18 × 10, 10 × 8)	+	–
3 (L)	Male	69	+	1 (8 × 8)	+	–
4 (L)	Male	70	+	1 (5 × 5)	+	–
5 (L)	Male	79	+	1 (5 × 3)	–	–
6 (R)	Female	83	+	1 (8 × 5)	+	+
8 (R)	Female	82	+	1 (5 × 5)	–	–
9 (R)	Female	81	+	1 (8 × 5)	+	–
9 (L)	Female	81	+	1 (10 × 5)	+	–
11 (R)	Female	83	+	1 (5 × 5)	+	+

L: left lower limb; R: right lower limb; No.: number

ischemic limb under venous anesthesia using midazolam (0.5 mg/kg). All patients were discharged from our hospital on the fifth day.

RESULTS

There were no significant side effects of this procedure during the five days of hospitalization. Ten (91%) of 11 patients reported either diminished or deceased rest pain at 2 weeks after PB-MNCs therapy. **Table 3** shows the number, size and fate of the ulcers after treatment. Ulcer formation was completely cured or improved in 8 (80%)

out of 10 legs at 4 weeks after PB-MNCs therapy, while no improvement of ulcer formation was observed in the other 2 cases during the follow-up period. Recurrences of ulcers were observed in 2 patients (**Table 3**). Thus, 60% of ulcers were cured without recurrence after PB-MNCs therapy. **Figure 1** shows an improvement of an ischemic ulcer in one patient (case 2 in **Table 1**). One patient died of pneumonia at 3 months after PB-MNCs therapy. This patient underwent a major amputation, 1 month after PB-MNCs therapy.

The ankle-brachial index (ABI) was improved from a baseline of 0.77 ± 0.21 to 0.91 ± 0.27 and 0.99 ± 0.1 at 1

Fig. 1 Ulcer formation in case 2 (A) was completely healed after PB-MNCs therapy (B).

Fig. 2 The time required to enhance the arteries by angiography at the level of the foot-joint was significantly shortened at 1 month (10 ± 4 seconds, $p < 0.05$) after PB-MNCs therapy compared with the time of pretreatment (15 ± 5).

and 2 months after PB-MNCs therapy, respectively, and the ABI was still higher one (0.84 ± 0.28) and two years (0.90 ± 0.13) later. The time required to enhance the arteries at the level of the foot-joint by angiography was significantly shortened at 1 month (10 ± 4 seconds, $p < 0.05$) and 6 months (12 ± 1) after PB-MNCs therapy compared with the time of before treatment (15 ± 5 , **Fig. 2**).

The actuarial survival rate and freedom from major amputation of the lower limb at 36 months after PB-MNCs therapy were 73% and 92%, respectively (**Fig. 3**). The causes of death were a hepatic coma and sepsis in 2 patients (cases 1 and 6) and pneumonia (case 7) in 1 patient. Because no severe infection was observed in the lower limb of patients in a septic state, we did not carry out amputation of the lower limb. Both patients finally died of hepatic coma and septic shock due to necrosis of the small intestine.

DISCUSSION

In this report, we demonstrated the superior early and long-term results of PB-MNCs therapy for critical limb ischemia. Thanks to PB-MNCs therapy, rest pain was diminished or decreased in 91% of the patients and ischemic ulcer formation was cured without recurrence in 60% of the legs. The actuarial survival rate and freedom from major amputation of the lower limb at 36 months after PB-MNCs therapy were 73% and 92%, respectively.

According to the ACC/AHA guidelines,¹⁾ the prognosis of critical limb ischemia is relatively poor. Twenty-five percents of the patients require major amputation of the diseased limb, and only 50% of the patients will be alive with two limbs at one year after diagnosis of critical limb ischemia. It is, therefore, important to develop new treatment for CLI, especially those who are not indicated for surgery. Recently, angiogenetic therapeutic methods using bone marrow mononuclear cells (BM-MNCs) for CLI have been shown to have beneficial effects.^{6,7)} However, one-third of the treated patients did not respond

Fig. 3 Actuarial survival rate (A) and freedom from major amputation of the lower limb (B) at 36 months after PB-MNCs therapy were 73% and 92%.

well, and it is sometimes difficult to use BM-MNCs in the high-risk patients because general anesthesia is required to obtain a significant number of BM-MNCs. Therefore, less-invasive PB-MNCs therapy has been developed to treat CLI.

Hoshino et al.²⁾ described the efficacy of PB-MNCs therapy for CLI patients with diabetes mellitus on hemodialysis. Skin perfusion pressure, skin temperature, and ABI were maintained at a higher level than those at baseline during the 6 months after PB-MNCs therapy. Huang et al.³⁾ compared the efficacy between BM-MNCs therapy and PB-MNCs therapy to treat CLI. According to their study, comparative analysis revealed that at 12 weeks after cell implantation therapy, improvement of ABI, skin temperature, and rest pain was significantly better in PB-MNCs patients than BM-MNCs patients. Although the exact explanation was not clear, the authors speculated that the role of G-CSF used in PB-MNCs therapy might have exerted some favorable effects to

promote superior results, because G-CSF induced the production of several growth factors. Although other reports⁸⁻¹⁰⁾ have also described the beneficial effects of PB-MNCs therapy, only a few reports have so far examined the long term effects of PB-MNCs therapy. Horie et al.⁵⁾ reported a 2 year survival rate of 65% and a 1 year amputation free rate of 70% in ASO patients after PB-MNCs therapy.

We followed 11 patients for over 3 years and observed 3 deaths and one major amputation after PB-MNCs therapy. Two patients among the 3 dead patients underwent hemodialysis for end-stage renal failure. Although one patient died of sepsis at 4 months after treatment, and the other died of a hepatic coma at 12 months after the treatment. One patient underwent major amputation, 2 months after the PB-MNCs therapy, and later died of severe pneumonia. This was the only patient who required major amputation after PB-MNCs therapy, and the remaining 10 patients have not undergone any major amputation, indicating a superior amputation free ratio of 92% at three years after PB-MNCs therapy. We also showed that the time required to enhance the arteries by angiography at the level of the foot-joint was significantly shortened at 1 month after PB-MNCs therapy. Although it is difficult to clarify the exact mechanism responsible for these superior long term effects, once improved micro-circulation induced by PB-MNCs therapy might continue to enhance the favorable effects for local circulation of the ischemic limbs.

Our study has, therefore, demonstrated that G-CSF-induced PB-MNCs therapy showed striking early and long term results, however, because of the relatively small number of patients enrolled this study, more patients and a longer follow-up duration will be required to confirm our data.

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