

Prevention of Limb Amputation in Patients with Limbs Ulcers by Autologous Peripheral Blood Mononuclear Cell Implantation

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Abstract: There are many cases of amputation of ischemic limbs of dialysis patients due to diabetes, despite the availability of medicine therapy and vascular by-pass operations. As there is extensive ruin of the vascular bed due to diabetes, vascular regeneration therapy by stem cell implantation is effective. Thirty patients with ischemic limbs due to diabetes (not including type-I) and on dialysis for chronic renal failure (19 cases), diabetes (5 cases), dialysis patients without diabetes (4 cases), and arteriosclerosis obliterans (ASO, 2 cases) were treated by autologous peripheral blood stem cell (PBSC) implantation where imminent amputation was under consideration. Granulocyte Colony Stimulate Factor (G-CSF: 5 µg/kg/day) was administered subcutane-

ously for 4 days before PBSC collection, that was carried out using a centrifuge (Spectra and/or CS3000) via the vein. The collected PBSC, containing 4.2×10^7 of CD 34 positive cells, was divided into units of 0.5–1.0 mL and implanted, without any purification, to the ischemic area of the limbs in about 65 points. In 21 cases, normalization of limb temperature was observed by thermograph, and symptoms also improved. The result of this first attempt of PBSC implantation is that we were able to save 22 ischemic limbs. This is the first large report of the application of regenerative medicine to peripheral ischemic limbs. **Key Words:** Angiogenesis, Apheresis, Cell transplantation, Ischemic ulcer, Peripheral blood mononuclear cell, Regeneration.

Amputation of limbs in patients with diabetic foot ulcers and arteriosclerosis obliterans is very serious. Especially, as many cases of ischemic limbs due to diabetes in dialysis patients are being amputated. Vasodilators such as PGE1 are the first choice of treatment, and vascular by-pass operation is frequently needed, but these treatments are not always effective in preventing amputation. Recently, regeneration therapy has shown promise of revascularization and/or neovascularization in ischemic heart disease and ischemic limbs. Effective bone marrow cell and peripheral blood stem cell implantation, and gene therapy have been reported, but gene therapy using vascular endothelial growth factor (VEGF) is

not yet successful enough. There are various reasons for this. First, infection is a problem when gene therapy is used in the clinical field. Second, repeated treatment is necessary. Third, the clinical effects are not clear. These points notwithstanding however, the clinical usefulness of cell therapy is becoming established (1,2).

In the collection of stem cells, two ways have been proven. The first is bone marrow aspiration from both iliac crests under general anesthesia. The second is peripheral blood stem cell (PBSC) separation from peripheral blood using a centrifuge, following which the stem cells are purified. Aspiration of bone marrow cells has some problems associated with general anesthesia, and with anemia due to the collection of about 800 mL of blood. Also, the process of purification is complex. As a result, the viability of separated cells declines somewhat.

Therefore, we used a centrifuge separator to collect the autologous peripheral blood stem cells. The collected suspension was implanted into the ischemic

Received July 2004; revised November 2004.

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area of the limbs as soon as possible. We did not purify the suspension in any way.

After approval by our local ethics committee, all patients provided written informed consent and were willing to comply with planned follow-ups.

In Japan, there are very large numbers of maintenance dialysis patients (about 240 000 patients) and the number of ischemic limbs is increasing also. As patients, they are bothered by rest pain, cyanosis, ulcer, and necrosis of limbs. Therefore, we attempted revascularization of ischemic limbs using implantation of peripheral blood stem cell. This therapy is very easy and has a low responsibility for patients.

PATIENTS

Patients with ischemic limbs (Table 1) who were suffering from rest pain, cyanosis, or ulcer of the limbs, and patients with subjective and objective symptoms that did not improve despite medication (PGE1 and/or anticoagulants) were included in this study. Patients with necrotic limbs, active infection under poor control, and with malignant neoplasm

were excluded, as were patients with myocardial infarction, angina pectoris, and cerebro-vascular disorder since the administration of G-CSF may adversely affect those complications.

Thirty patients were treated (15 male: 15 female, mean age 65.8 years \pm 16.6 SD, range 45–86 years). Twenty-four patients were diabetic (type-II) and the mean duration of their disease was 14.9 years \pm 12.6 (range 0.83–46 years). Twenty-three patients suffered from chronic renal failure (CRF). They have been treated by maintenance dialysis with a mean duration of 6.9 \pm 8.0 (range 0.42–30.33). Only two patients did not have diabetes or CRF.

ASSESSMENT

Before cell implantation, we assessed patients with thermography, plethysmography, 3D computed tomography scan (3D-CT), angiography and through subjective symptoms. Limbs were assessed using Fontaine's classification (3). One patient was at Level I, 8 were at Level III and 21 were at Level IV (Fontaine classification I: coldness and/or numbness of

TABLE 1. *Thirty patients with ischemic limbs*

Case	Age	Sex	Volume of PBSC (mL)	CD34 ($\times 10^7$)	Implant-R	Implant-L	Observation (mo)
1	68	F	57	4	15	64	R (21)
2	68	M	57	1.3	37	38	R (18)
3	73	M	57	3.7	62	–	A (3)
4	68	F	57	5	94	9	A (1.5)
5	56	M	64	9.9	–	133	R (11)
6	74	M	57	12	–	112	A (1)
7	60	M	68	2.3	–	131	R (9)
8	45	F	50	3.6	64	40	R (8)
9	66	M	57	4.8	93	20	R (6.5)
10	70	M	54	2.7	45	69	R (8)
11	54	F	93	8.4	62	130	R (7.5)
12	77	F	57	4.5	35	79	R (8)
13	58	M	57	3.4	17	70	A (4)
14	61	M	57	0.94	40	71	R (8)
15	85	F	57	2.2	71	46	R (7)
16	55	F	57	0.8	33	85	R (7.5)
17	65	F	57	7.1	75	20	R (5)
18	61	M	40	2.3	–	82	R (7.5)
19	75	M	57	1.5	61	60	R (5)
20	48	F	44	2.3	90	–	R (7)
21	45	F	57	2.1	–	110	A (6.5)
22	64	M	60	8.3	108	30	R (2.5)
23	86	F	60	15.9	30	90	R (6)
24	60	F	57	2.7	47	69	A (2)
25	70	M	54	2.5	34	82	A (1)
26	81	F	43	0.68	–	88	R (5.5)
27	69	M	92	4.1	140	40	R (3)
28	70	F	40	0.57	46	40	A (0.5)
29	76	F	52	1.53	60	49	R (4)
30	66	M	70	5.43	132	–	R (1)
					61.1 \pm 33.1	68.8 \pm 34.6	

–, not applicable; A, amputation; CD34, CD34 positive cell count; Implant-L, injection point number of the left arm and foot; Implant-R, injection point number of the right arm and foot; mo, months; PBSC, peripheral blood stem cell; R, rescue.

TABLE 2. Peripheral blood stem cell collection using CS-3000 and SPECTRA

	TBV (L)	VCS (mL)	CD34 ($\times 10^7$)	RBC ($10^6/\mu\text{L}$)	MNC ($\times 10^{10}$)
CS-3000 (15 patients)	9.7 \pm 0.9	57.0 \pm 0.6	3.6 \pm 2.9	2.5 \pm 0.9	2.1 \pm 0.8
Spectra (15 patients)	7.8 \pm 1.1	59.1 \pm 16.6	4.8 \pm 4.2	0.7 \pm 0.3	1.7 \pm 0.6

CD34, CD34 positive cell count; MNC, Mononuclear cell count; RBC, Red blood cell count; TBV, Treated blood volume; VSC, Volume of cell suspension.

the limb, II: intermittent claudication, III: rest pain, IV: ulceration and/or necrosis).

STEM CELLS COLLECTION

The stem cells of the bone marrow were mobilized to the peripheral blood by administration of G-CSF. Each patient was administered 5 $\mu\text{g}/\text{kg}/\text{day}$ of G-CSF subcutaneously for 4 days. A complete blood count (CBC) was done every day. As the white blood cell number reached 20 000–40 000 mm^3 , the peripheral stem cells were collected. We separated the stem cells from the peripheral blood using a Cobe Spectra cell separator (Gambro BCT, Lakewood, CO, USA) and CS3000 (Baxter Healthcare, Deerfield, IL, USA). Using CS3000 (15 patients), the mean processing volume was 9.7 L \pm 0.9 (mean \pm SD). The mean suspension volume was 56.8 mL \pm 0.6. The other patients ($N=15$) were treated using the Spectra. The mean processing volume of their peripheral blood was 7.8 L \pm 1.1. The mean volume of cell suspension was 59.1 mL \pm 16.6. The mean number of CD-34 (+) cells of the former was 3.6 \pm 2.9 $\times 10^7$ and the latter was 4.8 \pm 4.2 $\times 10^7$. Therefore, the Spectra collected PBSC more effectively than CS3000. The suspension of Spectra included fewer red blood cells (Table 2).

STEM CELLS IMPLANTATION

The collected stem cell suspension was divided into units of 0.5–1.0 mL with an injector and was implanted within 1 h into the muscle of the ischemic limbs without any modification. The divided suspension was injected into 50–70 points of the limbs under spinal anesthesia. We did not observe any side-effects, such as redness, pain, inflammation, or allergic reaction on the day after implantation of the PBSC (Fig. 1).

RESULTS

The mean observation time of the rescued cases was 8.3 \pm 4.0 months and in amputated cases it was 2.4 \pm 2.0 (Table 1).

One month later, the subjective symptoms such as coldness and rest pain were investigated. Twenty-one patients felt improvement, and 2 patients felt no change. Plethysmography was done in 29 patients. In their preoperative state, normal pulse waves were observed in 2 patients and not observed in 27 patients. After implantation, sharp pulse waves were observed in 9 patients (Fig. 2). In 10 patients, there was no change, and wave shapes were unclear in 4 patients. On examination of 3D-CT in 19 patients, we did not find arterial shadow in any cases before treatment. One month after the implantation, arterial shadow was found in 10 patients (Fig. 3). We could not find arterial shadow in seven patients. The condition of two patients was unknown. Thermograph was also performed. Prior to cell implantation, 29 limbs of 30 patients showed a blue and/or green color on the thermograph, i.e. those limbs were ischemic. One patient's limb showed normal color. After the

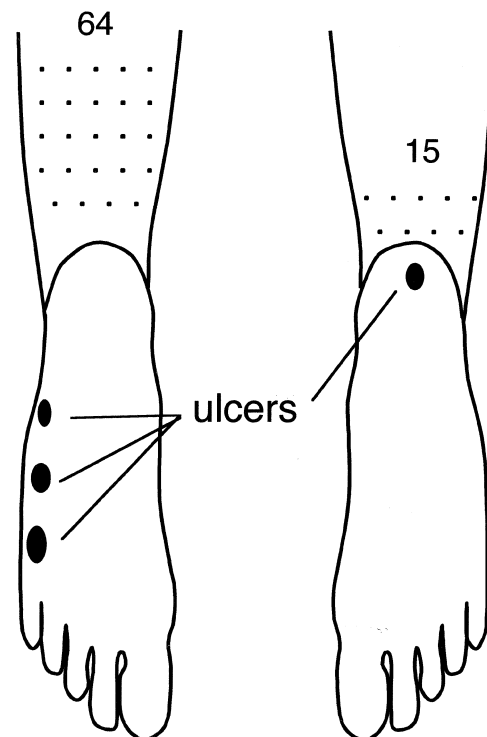


FIG. 1. Case 1: Cell implantation.

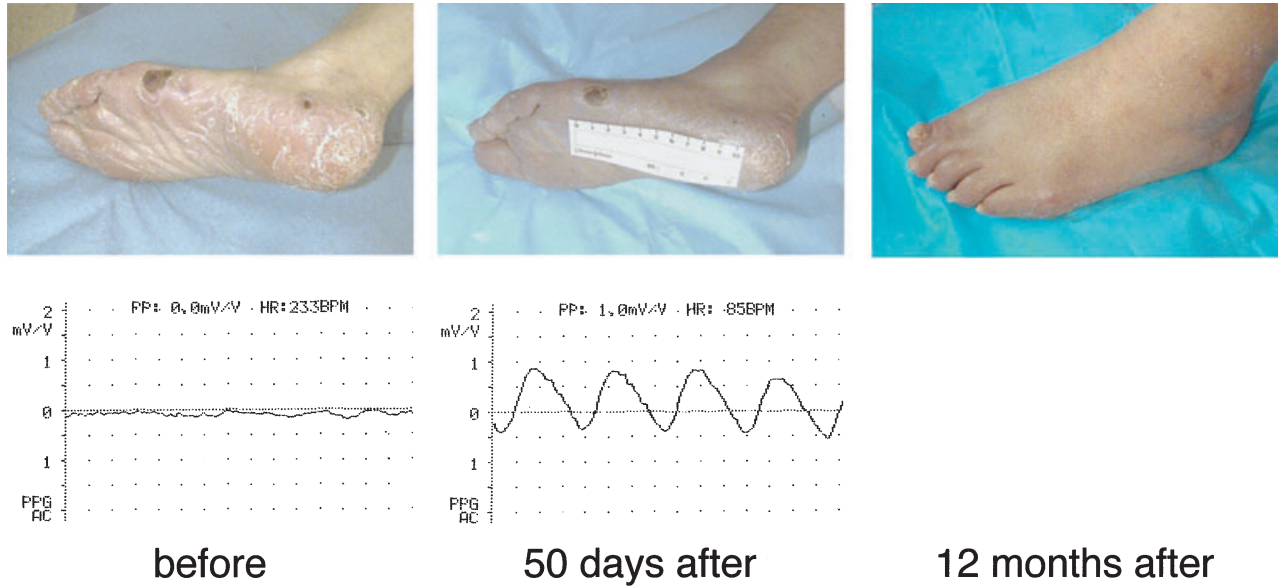


FIG. 2. Case 1: Clinical course.

implantation, a red color was observed in 21 patients (Fig. 4). In two patient's limbs there was no change. One patient's color was becoming dark blue. In six patients, thermograph was not examined, as their limbs were amputated.

In one patient in Fontaine class I, her limb (case #23) was rescued. In Fontaine III (8 patients), all

patient's limbs were rescued. In Fontaine IV (21 patients), 8 patients' (case #3, 4, 6, 13, 21, 24, 25 and 28) limbs were regrettably amputated as their ulcers were complicated by infection, necrosis, and severe pain. The mean observation time (amputated cases) was 2.4 months. This indicates the importance of timing: It seems that categories up to and including Fon-



FIG. 3. Case 4: 3D-CT.

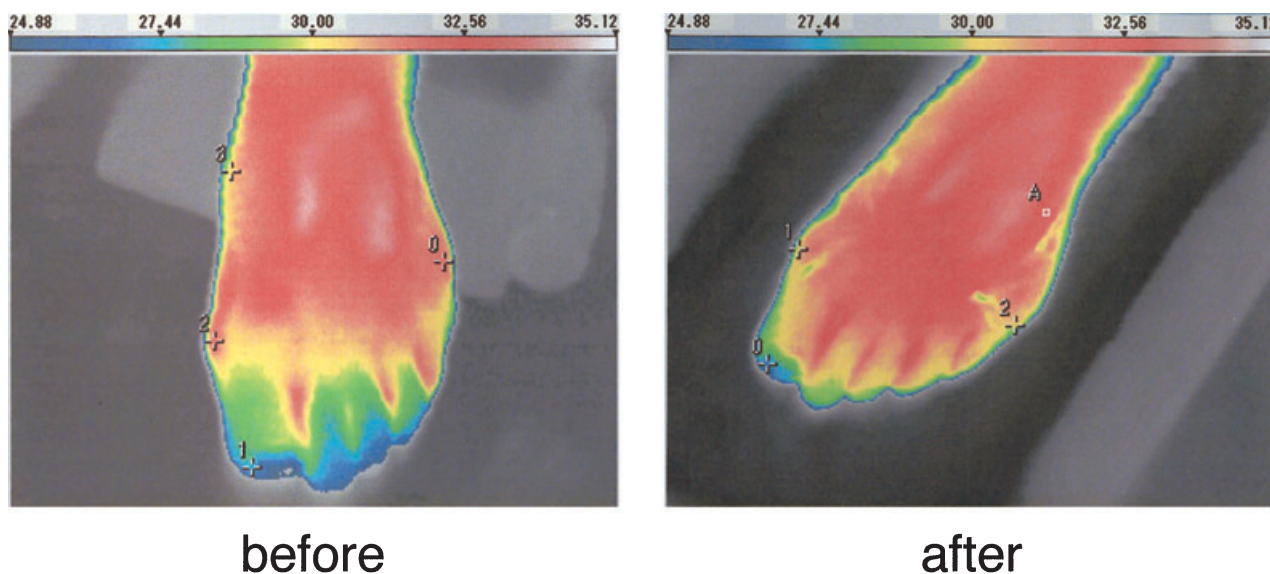


FIG. 4. Case 1: Thermograph.

taine III are the most appropriate indications for SBPC implantation. Fontaine IV may be too late.

DISCUSSION AND CONCLUSION

We have shown PBSC implantation in 30 patients with ischemic limbs. Recently, the autologous stem cells of bone marrow have been implanted to induce angiogenesis and to improve heart function after myocardial infarction (4–6). Usually, 600–1000 mL of bone marrow blood is collected from the iliac crest of individuals under general anesthesia. The aspirated marrow blood is adjusted to the ischemic myocardium under a catheter guide. In the case of ischemic limbs, the same treatment was tried in Japan. Although this method is also certainly very useful, there are some drawbacks. On the collection of peripheral mononuclear cell from the iliac crest, the patients are under general anesthesia and they lose about 800 mL of peripheral blood. Though rare, some dangers accompany general anesthesia. Also, the collection of bone marrow cells brings mild anemia, and in the process of purification of the stem cells, a few are lost or damaged. Collection and treatment of the PBSC using the Spectra, however, is easy, fast, cheap and risk free. The cell suspension from Spectra especially, is clear and red cell involvement is few.

Generally, diabetic ischemia of the limbs is diagnosed as arteriosclerosis obliterans, but, its real pathogenesis is diabetic microvascular angiopathy (microvascular disease). Therefore, vasodilators such

as PGE1 are sometimes effective but in most cases vascular surgery is not effective. As a result, many limbs were amputated. Cell regenerative therapy is more effective than surgical treatment, and probably, implanted stem cells might induce angiogenesis into the ischemic muscles of the limbs. After cell implantation, we observed the contrast medium into the artery by an angiogram. This shows that the blood flow is increased by angiogenesis.

As a result, 73% of patient's limbs, without infection and necrosis, were rescued from amputation. But for the implantation of the SBPC, many patients would have lost their ischemic limbs.

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