A long-term outcome of therapeutic angiogenesis by transplantation of peripheral blood stem cells in critical limb ischemia after interventional revascularization

Alavala Matta Reddy, Byung Kook Kwak, Hyung Jin Shim, Eui-Chan Jang, Ae Ja Park, Eunkyung Park, Chiyoung Ahn

ABSTRACT
A 61-year-old male patient with atherosclerotic critical limb ischemia in the left leg underwent stent insertion into the left superficial femoral artery. Stenting procedures improved Rutherford grade from III-5 to II-4. Granulocyte colony-stimulating factor stimulated the production of white blood cells over four-fold and mononuclear cells (MNCs) 1.5-fold in the whole blood. Transplantation of 7.9×10^9 autologous MNCs into the left femoral artery rapidly decreased the leg pain intensity, with further improvement of Rutherford grades from II-4 to 0-0 without any side effects. In the four-year follow-up, significant improvement was found in terms of ankle brachial index, from nondetectable to 0.67, and peak systolic velocity, from 14.8 to 36.1 cm/s. Limb salvage and decreased resting pain were the notable outcomes of the treatment.

Surgical and interventional revascularization in patients with critical limb ischemia (CLI) could prevent limb amputation, improve quality of life, and prolong survival. The limb salvage rate with direct revascularization is 82% over a four-year period, while indirect revascularization has a salvage rate of 64% (1, 2). However, as the affected arteries are mostly segmented, diffused, and located peripherally, the efficacy of surgical revascularization remains limited (3). Therefore, additional therapy, such as angiogenesis, is absolutely essential to improve the success of limb salvage.

Therapeutic angiogenesis by intramuscular cell transplantation studies using autologous bone marrow mononuclear cells (BM-MNCs) (4, 5) and granulocyte colony stimulating factor (G-CSF)-mobilized peripheral blood mononuclear cells (PB-MNCs) (6) resulted in improvement in ankle brachial pressure index (ABPI), rest pain, transcutaneous oxygen pressure (TcPO$_2$), and pain-free walking distance in peripheral artery disease (PAD) patients with CLI. Comparative analysis between transplantation of BM-MNCs and G-CSF-mobilized PB-MNCs for patients with limb ischemia revealed no significant difference, thus PB-MNCs could be equally effective and more practical for treatment (7).

Until now, there has been no report of a combinatorial approach of surgical or interventional revascularization and consecutive stem cell delivery, especially intra-arterial administration of G-CSF mobilized PB-MNCs. In this case study, we investigated the long-term outcome and efficacy of PB-MNC therapy in an atherosclerotic CLI patient with interventional revascularization for four years.

Case report

In May 2007, we enrolled a 61-year-old male atherosclerotic PAD patient with a history of smoking, ischemic rest pain in the left leg, ischemic gangrene of the left fourth toe, erythematous swelling on the left foot (Fig. 1), and claudication of the right lower extremity. We assessed his left leg condition as Rutherford's grade III-category 5, and right leg as grade I-category 2. He had been treated for diabetes mellitus for 15 years and was taking insulin; he also had hypertension, ischemic heart disease, and a stent inserted in the left common iliac artery. All research protocols were approved by the institutional review board (IRB No. I2007016-74), and the patient provided informed consent for the procedure.

Analysis was performed using computed tomography (CT) assisted angiography on a LightSpeed CT scanner (GE Healthcare, Milwaukee, Wisconsin, USA) and digital subtraction angiography using an Axiom Artis machine (Siemens Inc., Erlangen, Germany). The left superficial femoral artery (SFA) was found diffusely stenotic and completely occluded in the middle, through the collaterals, and the left popliteal artery

From the School of Life and Health Sciences (A.M.R.), Adikavi Nannaya University, Rajahmundry/AP, India; the Departments of Radiology (B.K.K. ✉ kwakbk@cau.ac.kr, H.J.S.), Orthopedic Surgery (E-C.J.), Laboratory Medicine (A.J.P.), and Internal Medicine (E.P.), Chung-Ang University College of Medicine, Seoul, Republic of Korea; the Biologics Research Division (C.A.), Korea Food and Drug Administration, Chungbuk, Republic of Korea.

Figure 1. a–c. Images of the foot before treatment **(a)**, 20 days after transplantation of peripheral blood stem cells following interventional procedures of ballooning and stent insertion **(b)**, and after amputation **(c)**.

(PA), tibioperoneal arteries, and dorsalis pedis were barely visualized (Fig. 2a).

Prestent balloon angioplasty was performed using a 3 mm/10 cm size balloon (Savvy Balloon Catheter, Cordis, Warren, New Jersey, USA) at the left SFA (Fig. 2b). Two stents of 6 mm/10 cm and 7 mm/10 cm (S.M.A.R.T.® Control® Nitinol Stent, Cordis) were implanted at the left SFA. Post-stent balloon angioplasty was performed using a 5 mm/10 cm size balloon (PowerFlex Extreme, Cordis). The left SFA was successfully dilated; however, the PA and tibioperoneal arteries were not treated (Fig. 2c and 2d). The procedure improved the left leg Rutherford grade III-category 5 to II-4 within three days.

G-CSF (Leucostim®, Dong-A Pharmaceuticals, Seoul, Korea) was injected (5 µg/kg) into the patient at 6:00 pm for four days before transplantation, and the blood was analyzed at 6:00 am on the day following transplantation. Stimulation of MNC production and white blood cell (WBC) count by G-CSF was monitored. CD34+/CD133+ cells were counted by flow cytometry on a Cytomics FC500 flow cytometer (Beckman Coulter Inc., Fullerton, California, USA). PB-MNCs were isolated by leukapheresis four days after G-CSF stimulation (four days after the

stent insertion procedure), analyzed (Table), and transplanted on the same day within two hours of isolation.

PB-MNCs were isolated by apheresis using a COBE® Spectra Apheresis System (Gambro, Lakewood, Colorado, USA) with a 12 F 16 cm You-Bend hemodialysis catheter (Arrow International, Reading, Pennsylvania, USA) inserted into the patient's subclavian vein. Apheresis was performed at an 80 mL/min blood flow rate for approximately three hours by processing 15 L of blood. Immediately after isolation, 7.9×10^9 autologous PB-MNCs in a 50 mL volume was infused into the left femoral artery (Fig. 2c).

The ABPI was measured using an ABPI device (VP-2000, Colin Medical Technology, Komaki, Japan) before and after the interventional procedures. Following PB-MNC transplantation, ABPI and Rutherford categories were monitored 3, 7, 14, 30, 60, and 180 days as well as one and four years postprocedure. Doppler ultrasonographic examination of dorsalis pedis artery in left leg was performed using an Acuson Sequoia 512 ultrasound unit (GE Healthcare, Mountain View, California, USA) and a linear array transducer with a 5 MHz frequency and 8L5 probe. Peak systolic velocity (PSV) and end diastolic velocity

(EDV) of blood flow (cm/s) in the dorsalis pedis artery were measured four days prior to and after interventional procedures. Post-stem cell therapy outcome was evaluated by monitoring blood flow 3, 15, and 25 days as well as four years after the procedure.

The combinatorial approach, first with angioplasty and stent insertion followed by PB-MNC transplantation into the left leg alone, was safe and effective in terms of an improvement in Rutherford's clinical categories of chronic limb ischemia from grade III category 5 to 0-0. Furthermore, this approach saved the patient from major amputations without any complications during the long-term follow-up for four years.

Production of WBC in peripheral blood was enhanced four-fold and MNC 1.5-fold by stimulation of bone marrow with G-CSF for four days (Table). G-CSF also stimulated CD133+ and CD34+ cells by 18-fold. Levels of CD133+ cells peaked on the third day and CD34+ cells on the fourth day upon G-CSF stimulation. Other cells, such as red blood cells, hemoglobin, platelets, neutrophils, and nucleated red blood cells, were not dramatically stimulated by G-CSF, except for neutrophils, which increased five-fold (Table).
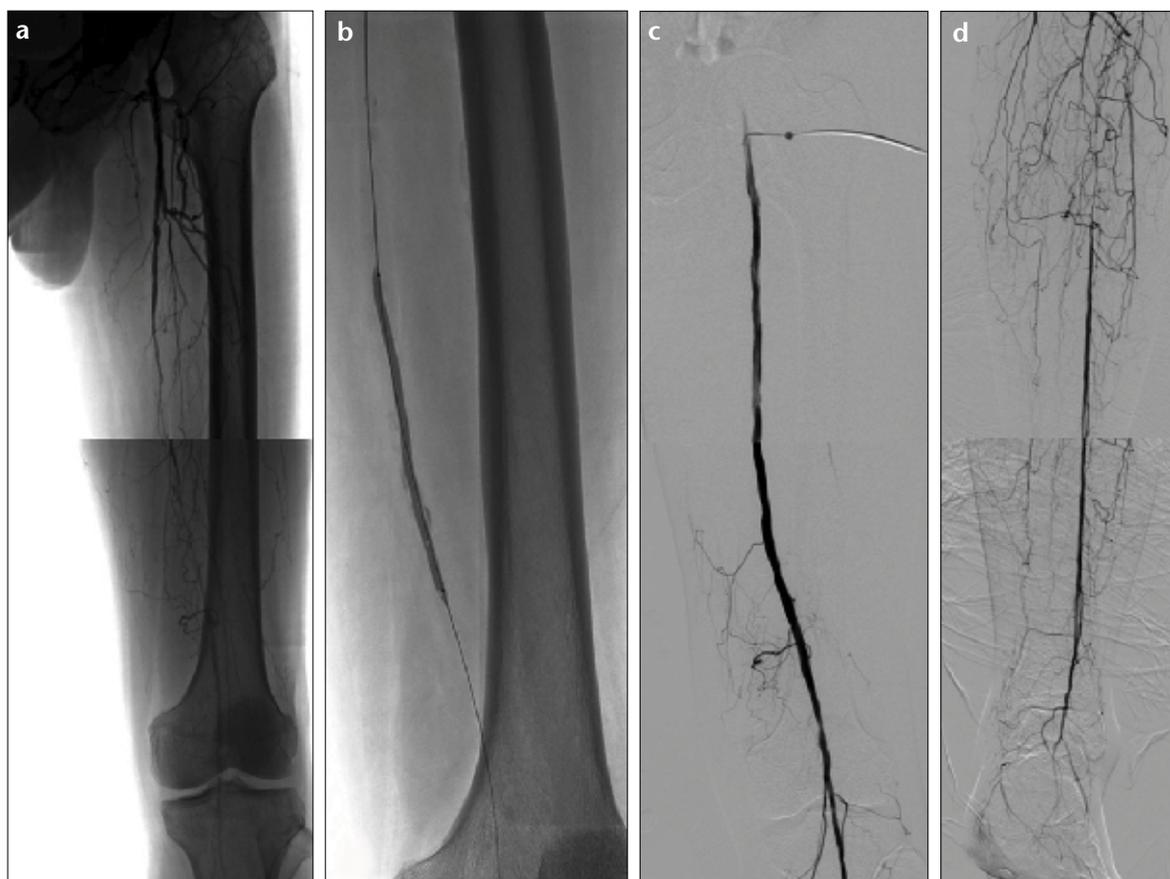
Figure 2. a–d. Angiography images during medical interventional treatment procedures and peripheral blood stem cells transplantation. Before ballooning and stent insertion, left superficial femoral artery (SFA) was diffusely stenotic and completely occluded in the middle **(a)**. Prestent balloon angioplasty was performed using a 3 mm×10 cm balloon at the left SFA **(b)**, and two stents of 6 mm×10 cm and 7 mm×10 cm were implanted; post-stent balloon angioplasty was performed using a 5 mm×10 cm balloon. At stem cell transplantation, after ballooning and stent insertion, left SFA and popliteal artery **(c)** were observed by ipsilateral antegrade infusion of contrast agent, and left tibioperoneal arteries **(d)** were still occluded and barely visualized.

Table. Whole blood and MNC analysis before and during four days of G-CSF injections in the patient with atherosclerotic critical limb ischemia

Cells/blood chemistry	Whole blood analysis					Leukapheresed MNC analysis
	Before G-CSF	G-CSF-1 day	G-CSF-2 days	G-CSF-3 days	G-CSF-4 days	
Total CD133$^+$/μL	2.83	37.00	30.07	56.00	51.60	266.85
CD133$^+$ cells (%)	0.03	0.10	0.08	0.12	0.13	0.17
Total CD34$^+$/μL	1.65	3.04	6.17	12.76	29.62	747.27
CD34$^+$ cells (%)	0.02	0.01	0.02	0.03	0.08	0.48
Total MNC (10^3/μL)	2.29	2.07	3.06	4.52	3.47	158.21
MNC (%) in WBC	24.1	5.4	7.9	10	9	100.8
WBC (10^3/μl)	9.48	38.05	38.56	45.65	38.32	156.99
LYMPH (10^3/μL)	1.7 (17.9%)	1.31 (3.4%)	1.83 (4.7%)	2.26 (5.0%)	2.16 (5.6%)	98.9 (63%)
MONO (10^3/μL)	0.59 (6.2%)	0.76 (2.0%)	1.23 (3.2%)	2.26 (5.0%)	1.31 (3.4%)	59.3 (38%)
NEUTR (10^3/μL)	6.88 (72.6%)	35.7 (94%)	35.2 (91%)	40.7 (89%)	34.5 (90%)	0
EO (10^3/μL)	0.28 (3.0%)	0.22 (0.6%)	0.24 (0.6%)	0.45 (1.0%)	0.34 (0.9%)	0.04 (0%)
BASO (10^3/μL)	0.03 (0.3%)	0.05 (0.1%)	0.03 (0.1%)	0.03 (0.1%)	0.05 (0.1%)	0.78 (0.5%)

BASO, basophils; EO, eosonophils; NEUTR, neutrophils; G-CSF, granulocyte colony stimulating factor; LYMPH, lymphocytes; MNC, mononuclear cells; MONO, monocyt; WBC, white blood cells.

Figure 3. The bleeding at the time of toe amputation seven months after cell therapy.

MNCs collected by leukapheresis were comprised of 63% lymphocytes, 38% monocytes, 0.17% CD133+, and 0.48% CD34+ cells (Table). A total of 7.9×10^9 PB-MNCs in 50 mL normal saline was transplanted by intra-arterial injection into the left SFA (Fig. 2c and 2d).

A remarkable change in visible symptoms was observed, with decreased redness and swelling in the left foot within three days of stem cell transplantation (Fig. 1). In addition, Rutherford classification improved from grade II-4 to grade 0-1 by the time of transplantation, reaching 0-0 in two months and remaining consistent for the four years of the study. At the time of minor amputation of third, fourth, and fifth toes of the left leg (seven months after transplantation), bleeding was observed (Figs. 1 and 3).

Prior to start of the treatment, ABPI was not in the detectable range. Post-peripheral blood stem cells (PBSC) transplantation, it showed a dramatic increase to 0.63 after three days, peaking at 0.68 after two months, and maintaining consistent levels for four years. Doppler ultrasonographic examination of the dorsalis pedis artery in the left leg after interventional treatment by ballooning and stent insertion showed an increase in PSV from 14.8 to 28.5 cm/s and EDV from 11.7 to 19.9 cm/s, respectively. The PBSC transplantation within three days further improved the PSV to 34.4 cm/s and EDV to 22.9 cm/s. After 15 days, PSV and EDV were 28.5 and 19.9 cm/s, respectively, with measurements of 35.3 and 20.8 cm/s after 25 days, and 36.1 and 21 cm/s after four years, respectively.

Discussion

In this report, we presented a combinatorial approach in a CLI patient that is both safe and effective without any side effects, suggesting its potential usefulness as a better option compared to previous single treatment strategies. The major outcomes from this long-term follow-up after treatment were saving the patient from the threat of major amputation, and decreased resting pain, angiogenesis and revascularization.

Several therapeutic studies have reported effective use of autologous stem cells for PAD with a rapid amelioration of symptoms (8), as was observed in the present study. Many investigators, however, still prefer BM-MNCs for treatment (4, 5). We used PBSCs to treat CLI PAD disease, as isolation of PBSCs is easier, convenient, less painful, more practical, and also safe and effective (6, 9). G-CSF increased the number of WBC and CD34 positive cells in the peripheral blood, and thus large volume leukapheresis is commonly used for harvesting the PB-MNC fraction (10). Other medications, such as iloprost (prostacyclin, PGI2), cilostazol (phosphodiesterase-3 inhibitor) and others are widely used in CLI patients to relieve symptoms and signs of ischemia. Because iloprost increases the endothelial progenitor cell number in peripheral blood *in vivo*, it may have a direct effect on therapeutic angiogenesis (11, 12).

As the peripheral arteries are damaged in CLI PAD patients, we aimed to infuse the stem cells by the intra-arterial route in order to directly supply the cells to the affected area, as was performed in other trials in patients with infrapopliteal peripheral artery occlusive disease (9). Intra-arterial administration has many advantages over intramuscular administration, including the use of the native delivery route, the automatic penetration of damaged tissue by the cells, and the arrival of cells to the target tissue through chemotaxis, at least after the second circulation (13, 14).

Bleeding observed at the time of the minor amputation, which occurred months after cell transplantation, confirmed the new and established angiogenesis and vascularization (Suppl. Fig.). The results mainly showed rapid relief of pain without the need for major amputations. Although our results using stem cell therapy in CLI PAD patients are quite promising, it is critical to keep in mind possible unwanted angiogenesis and death due to MNC therapy. Further long-term, randomized controlled clinical trials are required to substantiate the efficacy of this comprehensive approach to treat CLI PAD.

In summary, here we present a case report of a combinatorial approach of interventional revascularization and consecutive stem cell delivery through the intra-arterial administration of G-CSF mobilized PB-MNC in a CLI patient as an effective treatment without any side effects.

Conflict of interest disclosure

The authors declared no conflicts of interest.

References

1. Iida O, Nanto S, Uematsu M, et al. Importance of the angiosome concept for endovascular therapy in patients with critical limb ischemia. Catheter Cardiovasc Interv 2010; 75:830–836.

2. Neville RF, Attinger CE, Bulan EJ, Ducic I, Thomassen M, Sidawy AN. Revascularization of a specific angiosome for limb salvage: Does the target artery matter? Ann Vasc Surg 2009; 23:367–373.

3. JCS Joint Working Group. Guideline for management of vasculitis syndrome (JCS 2008). Japanese Circulation Society. Circ J 2011; 75:474–503.

4. Tateishi-Yuyama E, Matsubara H, Murohara T, et al. Therapeutic Angiogenesis using Cell Transplantation (TACT) Study Investigators. Therapeutic angiogenesis for patients with limb ischemia by autologous transplantation of bone-marrow cells: a pilot study and a randomised controlled trial. Lancet 2002; 360:427–435.

5. Matoba S, Tatsumi T, Murohara T, et al. TACT Follow-up Study Investigators. Long-term clinical outcome after intramuscular implantation of bone marrow mononuclear cells (Therapeutic Angiogenesis by Cell Transplantation [TACT] trial) in patients with chronic limb ischemia. Am Heart J 2008; 156:1010–1018.

6. Huang P, Li S, Han M, Xiao Z, Yang R, Han ZC. Autologous transplantation of granulocyte colony-stimulating factor-mobilized peripheral blood mononuclear cells improves critical limb ischemia in diabetes. Diabetes Care 2005; 28:2155–2160.

7. Huang PP, Yang XF, Li SZ, Wen JC, Zhang Y, Han ZC. Randomised comparison of G-CSF-mobilized peripheral blood mononuclear cells versus bone marrow-mononuclear cells for the treatment of patients with lower limb arteriosclerosis obliterans. Thromb Haemost 2007; 98:1335–1342.

8. Fadini GP, Agostini C, Avogaro A. Autologous stem cell therapy for peripheral arterial disease meta-analysis and systematic review of the literature. Atherosclerosis 2010; 209:10–17.

9. Lenk K, Adams V, Lurz P, et al. Therapeutical potential of blood-derived progenitor cells in patients with peripheral arterial occlusive disease and critical limb ischemia. Eur Heart J 2005; 26:1903–1909.

10. Gertz MA. Current status of stem cell mobilization. Br J Haematol 2010; 150:647–662.

11. Lessiani G, Vazzana N, Cuccurullo C, et al. Inflammation, oxidative stress and platelet activation in aspirin-treated critical limb ischaemia: beneficial effects of iloprost. Thromb Haemost 2011; 105:321–328.

12. Di Stefano R, Barsotti MC, Melillo E, et al. The prostacyclin analogue iloprost increases circulating endothelial progenitor cells in patients with critical limb ischemia. Thromb Haemost 2008; 100:871–877.

13. Staudacher DL, Sela Y, Itskovitz-Eldor J, Flugelman MY. Intra-arterial injection of human embryonic stem cells in athymic rat hind limb ischemia model leads to arteriogenesis. Cardiovasc Revasc Med 2011; 12:228–234.

14. Mäkelä J, Anttila V, Ylitalo K, et al. Acute homing of bone marrow-derived mononuclear cells in intramyocardial vs. intracoronary transplantation. Scand Cardiovasc J 2009; 43:366–373.