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ORIGINAL ARTICLE

Autologous peripheral blood CD133+ cell implantation for limb salvage in patients with critical limb ischemia

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We report the safety and feasibility of autologous CD133+ cell implantation into the lower extremity muscles of patients with critical limb ischemia, whose only other option was limb amputation. Nine patients participated in the study: seven patients suffering from arteriosclerosis obliterans, one with thromboangiitis obliterans (Buerger's disease) and one with thromboembolic disorder. Autologous PBSC were collected after the administration of G-CSF (10 mcg/kg/day). CD133+ cells were selected using the CLINIMACS cell separation device and were injected i.m. without earlier cryopreservation using a 22-gauge needle into multiple sites 3 cm apart in the gastrocnemius/soleus muscle, or depending on clinical circumstances, in the foot or quadriceps muscle, or both, of the involved leg. There were no complications from either leukapheresis or injection. Stem cell injection prevented leg amputation in seven of the nine patients. In this small cohort of patients with end-stage critical limb ischemia, quality of life (Short Form-36) physical component score improved significantly at 3 ($P = 0.02$) and 6 ($P = 0.01$) months, but not at 1 year ($P = 0.08$). There was a trend towards the improvement in pain-free treadmill walking time ($P = 0.13$) and exercise capacity ($P = 0.16$) at 1 year. Lower extremity limb salvage was achieved for seven of the nine treated patients.

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Introduction

Initial presentation of peripheral arterial disease (PAD) is intermittent claudication with pain in the calf, thigh or buttock that is elicited by exertion and relieved with a few minutes of rest. Over time, the disease progresses to critical limb ischemia with symptoms of ischemic rest pain, ulceration or gangrene.[1,2] The mainstay of treatment for PAD has been endovascular treatment or surgical revascularization.[3] In patients with critical limb ischemia for which interventional or surgical limb salvage is not possible, 40% will lose their leg within 6 months, whereas 20% will die during this period.[1] Patients with critical limb ischemia, who have exhausted options for operative revascularization procedures, are traditionally treated by limb amputation.[4] Recent studies have suggested that unselected BM and/or CD34+ selected peripheral blood hematopoietic stem cells (HSC) may benefit patients with PAD by contributing to angiogenesis. The mechanism(s) of HSC-induced angiogenesis is thought to be either an indirect paracrine effect from stem cell-mediated angiogenic factors such as vascular endothelial growth factor or direct contribution of HSC-derived endothelial progenitor cells (EPC) to new vessel formation.[5]

As CD133+ is a marker of early EPC phenotype,[6–8] we conducted a study injecting CD133+ stem cells collected from the peripheral blood of nine patients with critical limb ischemia. This is one of the first studies using pre-selected CD133+ cells to induce therapeutic angiogenesis in patients with critical limb ischemia.

Patients and methods

Patients

Nine patients with critical limb ischemia were enrolled: one man with thromboangiitis obliterans (Buerger's disease), one woman with thromboembolic disease and seven patients with arteriosclerosis obliterans (four men and three women). The patient with Buerger's disease underwent the procedure twice. Table 1 illustrates patients'

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Table 1 Patients' characteristics

Patient	*Age*	*Gender*	*Diagnosis*	*Treated leg*	*LE ulcers*	*HTN*	*HL*	*CAD*	*DM*	*Smoking*
1	85	F	ASO	L	No	Yes	Yes	No	No	No
2	26	M	TAO	R	No	No	No	No	No	Yes
3	51	F	TE	L	No	No	No	No	No	Yes
4	82	M	ASO	R	Yes	Yes	Yes	Yes	Yes	Yes
5	60	F	ASO	R	No	Yes	Yes	No	No	Yes
6	78	F	ASO	L	Yes	Yes	No	No	Yes	No
7	74	M	ASO	R	No	Yes	No	No	Yes	Yes
8	81	M	ASO	R	No	Yes	Yes	Yes	No	Yes
9	76	M	ASO	R	No	Yes	Yes	Yes	Yes	Yes

Abbreviations: ASO = arteriosclerosis obliterans; CAD = coronary artery disease; DM = diabetes mellitus; F = female; HL = hyperlipidemia; HTN = hypertension; L = left; LE = lower extremities; M = male; R = right; TAO = thromboangiitis obliterans; TE = thromboembolic disorder.

Table 2 Patients' prior therapy

Patient	*Treated leg*	*Bypass grafts*	*Angioplasties*	*Amputations*	*Other*
1	L	L leg × 2/R leg × 1			
2	R	R leg × 1	R leg × 1	R hallux	
3	L	Mitral valve repair			
4	R	L leg × 2			CABG
5	R	R leg × 2			Aortic dissection
6	L	R leg × 2/l leg × 2		R BKA	
7	R	L leg × 6	R angioplasty		Carotid EA
8	R		LE angioplasties		Carotid EA-CABG
9	R				CABG/Aneurysm repair

Abbreviations: BKA = below the knee amputation; CABG = coronary artery bypass graft; EA = endarterectomy; L = left; LE = lower extremities; R = right.

characteristics. Patients with arteriosclerosis obliterans had a mean age of 77 years (range 60–85 years). Co-existing diseases included type II diabetes mellitus in four patients, five with hyperlipidemia, seven with medically treated hypertension and seven with a history of smoking. At the time of evaluation, two patients had ischemic non-healing ulcerations of the lower extremities. All patients had rest pain, were not candidates for surgical revascularization and faced the prospect of amputation of the affected leg. Earlier therapy received by the patients is summarized in Table 2. Six patients had an earlier lower extremity surgery for bypass grafts, three had earlier lower extremity angioplasties, two had earlier lower extremity amputations and five had earlier carotid, coronary artery or aortic vascular surgery.

Patient eligibility for the study was determined by the vascular surgery service at the Northwestern University Feinberg School of Medicine. Inclusion criteria for the study were all of the following: (1) ischemic peripheral vascular disease with rest pain defined as pain that occurs at night and at rest, which involves the foot and peak walking time <6 min on graded treadmill on two exercise tests separated 2 weeks apart (2) ankle–brachial index (ABI) <0.8 or Doppler waveforms at the posterior tibial artery and dorsalis pedis artery that are monophasic with toe pressure <30 mm Hg and (3) a non-surgical candidate for revascularization for example, an earlier vascular reconstruction, inability to locate a suitable vein for grafting, diffuse multi-segment disease or extensive infra-popliteal disease not amenable to a vascular graft. The study was approved by the Northwestern University IRB and Food and Drug Administration (IND # 11608).

Procedure

As PAD and coronary artery disease have similar risk factors (age, smoking, hypertension, hyperlipidemia and diabetes), before study enrollment, every patient was evaluated by a cardiologist with further cardiac testing as deemed necessary by cardiology before stem cell collection.

Stem cells were mobilized by administering G-CSF at 10 mcg/kg/day for 4–5 days. Therapeutic anticoagulation with twice a day lovenox was administered while on neupogen to avoid the theoretical risk of neupogen-related thrombosis. PBSC were collected by leukapheresis and CD133 were selected using a CLINIMACS cell separation system (Miltenyi Biotech, Bergisch Gladbach, Germany). Once purified, the CD133+ stem cells were reduced to a volume of 3–4 ml in PBS with 5% albumin, and injected without earlier cryopreservation into the patient's affected limb. Injection sites were determined by a vascular surgeon for individual patients based on Magnetic Resonance Imaging, Magnetic Resonance Angiography (MRA) and/or angiograms, and Doppler ultrasound to identify areas where blood flow was reduced or obstructed. Injection sites[7–18] were identified in the vastus medialis, rectus femoris, gastrocnemius and soleus muscles. Patients received vancomycin 1.0 g i.v. and dilaudid 1.0 mg IV before the procedure. The injection sites were disinfected with betadine, draped in sterile fashion and injected using a 22-gauge spinal needle. Each injection delivered a volume of 0.2–0.5 ml and 2.5–5 million cells.

Assessed parameters

The primary end point was limb salvage, defined as alive without limb amputation at 12 months. Secondary end

points were relief of rest pain, new collateral vessel formation, ABI, oxygen consumption (VO2), six-minute walk,[9] Summary Performance Score and Short Form-36 (SF-36). Collateral vessel formation was assessed by MRA (or angiogram), ABI by Doppler ultrasound, oxygen consumption (VO2) per Gardener Protocol Graded Treadmill Test,[10] and Summary Performance Score[11,12] by a composite score of one leg stand, side-by-side stand, semi-tandem stand, tandem stand, chair stand, repeated chair stand and four-meter walk.[11,12] Quality of life was assessed by the SF-36 quality of life questionnaire.[13,14] Follow-up and tests were scheduled at 2 days, 5 days, and then 1, 3, 6 and 12 months after the procedure.

Statistical analysis
Data were analyzed by performing Student's t-tests, except for SF-36 scores, which were analyzed by Wilcoxon signed-rank test.

Results

Pre-treatment evaluation
All patients underwent echocardiogram for cardiac evaluation. One patient had a nuclear medicine stress test and two had adenosine stress tests and subsequent cardiac catheterization before stem cell collection.

Stem cell collection
The apheresis (pre-selected) stem cell yield was a mean of $166.6 \pm 68.4 \times 10^6$ CD133+ cells and $191 \pm 85 \times 10^6$ CD34+ cells. After CD133+ selection, the mean number of cells was 82.5 ± 57.46 CD133+ cells and 88.8 ± 59.7 CD34+ cells. Although the numbers are too small for significance, there seems to be a difference in the yield of CD34 and CD133 stem cells/kg per apheresis between the younger (26- and 51-year-old) patients and the septuagenarian and octogenarian patients (Table 3).

Toxicity
G-CSF administration was without serious adverse events, only mild lumbago and arthralgia that were easily controlled with analgesics and resolved when the G-CSF administration was stopped. There were no thromboembolic complications. No infections occurred as a result of the injection procedure, and the only side effects were mild muscle pain and local edema that resolved by the 2-day follow-up visit.

Results
Although all patients were at risk for limb amputation, seven of the nine patients were able to avoid amputation. Two patients, both with lower extremity ulcers before treatment, subsequently underwent amputation (both below the knee) of the treated leg. Of the patients, who subsequently underwent amputation after treatment, one already had dry gangrene of his hallux and the other had gangrene with severe hyperesthesias and no detectable lower extremity blood flow. For the seven patients that were able to avoid amputation, rest pain resolved within days of injection. All patients have been followed for at least 1 year and only one of the patients had recurrence of rest pain after a 10-month interval, whereas the remaining six patients remained pain-free at rest for the follow-up period of 12 months.

Two patients showed improvement in collateral blood flow by MRAs and/or angiograms. There was no improvement in the lower extremity blood flow on serial ABIs. Other functional parameters, such as maximum VO2 (l per min), six-minute walk distance and Summary Performance Scores also showed no improvement. Some functional parameters, such as treadmill pain-free walking time and treadmill exercise capacity, improved but did not reach statistical significance. Quality of life SF-36 scores improved in physical component score (PCS), but not mental component score (MCS). PCS scores were statistically significant at 3 ($P = 0.02$) and 6 ($P = 0.01$) months but not at 1 year ($P = 0.08$), whereas MCS scores never reached statistical significance. Table 4 summarizes parameters measured before transplant and during the follow-up period.

Discussion

We report the results of autologous CD133+ stem cell implantation in nine cases of critical limb ischemia being

Table 3 Stem cell apheresis (preselected) and CD133+ selected product

Patient	*Age (years)*	*Apheresis (l)*	*No. of CD34/CD133 ($\times 10^6$) cells/kg per l of apheresis*	*PB CD34/ µl*	*CD34+ cells preselected ($\times 10^6$)*	*CD133+ cells preselected ($\times 10^6$)*	*CD34+ cells after selection ($\times 10^6$)*	*CD133+ cells after selection ($\times 10^6$)*
1	85	1 (10)	0.32/0.27	32	200	167	81	76
2A	26	1 (15)	0.16/0.12	41	218	165	156	141
2B	26	1 (15)	0.14/0.11	17	168	125	33	32
3	51	1 (15)	0.23/—	53	212	—	182	170
4	82	2 (10,15)	0.03/—	6	66	—	44	—
5	60	1 (12)	0.16/0.18	11	116	136	61	51
6	78	3 (15,15,15)	0.05/0.03	8	150	97	59	43
7	74	2 (15,15)	0.15/0.12	14	385	310	193	171
8	81	3 (15,15,15)	0.04/—	5	125	—	30	25
9	76	3 (10,10,10)	0.08/—	12	270	—	49	34

Abbreviation: PB = peripheral blood.

Table 4 Evaluation tests before and after stem cell injection for all the nine patients with critical limb ischemia

Test	*Pre-treatment (mean/s.d.)* P-*value*	*At 3 months (mean/s.d.)* P-*value*	*At 6 months (mean/s.d.)* P-*value*	*At 12 months (mean/s.d.)* P-*value*
Improved				
No amputation –natural history is 50% at 6 months		7/9	7/9	7/9
Free of rest pain– natural history is none unless amputated	0/9	7/7	7/7	6/7
SF-36; physical component score	31.1/10	37.2/5.3, $P=0.02$	39/8.1, $P=0.01$	41.5/10, $P=0.08$
Borderline improvement				
Pain-free treadmill walking time (time in seconds)	471/37	552/458, $P=0.71$	738/510, $P=0.28$	858/527, $P=0.13$
Exercise capacity (ml of oxygen/ kg/min)	15.8/5.1	16/5.3, $P=0.99$	18.2/6.53, $P=0.46$	20/5.2, $P=0.16$
No improvement				
ABI—treated leg	0.42/0.30	0.51/0.35, $P=0.54$	0.39/0.24, $P=0.83$	0.54/0.29, $P=0.38$
ABI—untreated leg	0.81/0.23	0.68/0.24, $P=0.30$	0.64/0.17, $P=016$	0.71/0.23, $P=0.39$
Maximum VO_2 (L/min)	1.3/0.47	1.3/0.50, $P=0.85$	1.5/0.58, $P=0.48$	1.6/0.45, $P=0.27$
6-min walk (distance in feet)	1027/45	1053/426, $P=0.92$	1088/485, $P=0.81$	1439/1102, $P=0.75$
Summary performance score (SPS)	6.0/4.3	7.4/4.4, $P=0.51$	8.0/4.7, $P=0.91$	7.5/5.2, $P=0.51$
SF-36; mental component score	57.6/10	52.4/14.8, $P=0.81$	57.6/11.8, $P=0.451$	53.1/10, $P=0.578$

Abbreviations: ABI = ankle–brachial index; Max VO_2 = maximal oxygen consumption/aerobic capacity; SF-36 = Short Form-36.

considered for amputation. As PAD occurs in an elderly patient population with high risk for other vascular diseases including coronary artery disease, pre-treatment evaluation included careful evaluation by a cardiologist for coronary artery disease that could become symptomatic during fluid shifts associated with stem cell mobilization. As neupogen has pro-coagulant side effects, we also used therapeutic anticoagulation with lovenox when patients received G-CSF. With these precautions, there were no cardiovascular side effects from stem cell mobilization. Stem cells were injected fresh, immediately after CD133+ selection, in order to avoid injection of the cryopreservant DMSO into muscle compartments.

This study demonstrates that stem cells may be mobilized into the blood and collected by leukapheresis in septuagenarians and octogenarians, although circulating peripheral blood CD34+ cells and the number of stem cells/kg per apheresis procedure seem to be reduced compared with younger patients. It also shows that elderly patients with severe peripheral vascular disease may undergo leukapheresis safely, provided a careful pre-mobilization evaluation for co-existing coronary artery disease is carried out. As this was a small salvage study designed to prevent leg amputation, no attempt was made to optimize or escalate the injected stem cell dose, rather the total number of leukapheresed and CD133+ selected stem cells were injected. As our data demonstrate, most CD133+ selected cells are also CD34+, as both stem cell markers are characteristic of functional endothelial precursors.[8]

After stem cell injection, rest pain resolved rapidly with marked symptomatic improvements by post-injection evaluation on day 2, and seven of the nine patients were able to avoid limb amputation for the 1 year of follow-up. Of the two patients who did lose their limbs, one already had pre-treatment dry gangrene of the hallux and the other had progressed to lower limb gangrene with no detectable posterior tibial or dorsal pedal pulses by palpation or Doppler ultrasound (ABI of 0) and was suffering painful hyperesthesia from necrotic tissue compartments at the time of attempted stem cell salvage.

The decrease in pain levels correlated with improvement in treadmill walking time for six of the seven patients who were able to take this test (two of the patients were unable to walk on the treadmill at baseline). Improvement also occurred in exercise capacity. These benefits, however, did not reach the threshold of statistical significance. Pain-free walking and increased walking times on the treadmill were retained by five of the patients for a year of follow-up. Only one of the seven patients whose limbs were salvaged has experienced rest pain recurrence after a 10-month pain-free period.

There was no improvement in the measured ABIs, in contrast with the results reported by some investigators.[15] Ishida *et al*.,[16] however, also noticed that the ABI level does not necessarily correlate with improvement in symptoms. The formation of new collateral vessels was suggested (by MRA or angiogram) in two patients. If stem cell infusion leads to the generation of small-caliber collateral vessels, it is probable that angiograms and ABI would not be able to reliably detect benefit, as no established method for evaluating micro-revascularization exists. Angiogams, MRAs and ABI tests were designed to measure large blood vessel flow after surgical intervention to revascularize large arteries. By contrast, stem cell injections are thought to improve microvascular collateral blood flow.

Parameters such as Max VO2 and Summary Performance Scores did not correlate with the clinical improvement in our patients, perhaps because of the advanced age and other co-existing comorbidities. The SF-36 PCS improved significantly in our patients at 3 and 6 months, but benefit was only marginally significant ($P=0.08$) at 1 year. The SF-36 PCS is a measurement of treatment effect for critical limb ischemia, whereas the mental component score is limited in helping assess therapeutic outcomes in severe PAD[17,18] because of the advanced age of many of the participants and the presence of significant other age-related co-morbidities, such as coronary artery disease and diabetes mellitus, that affect the assessed parameters.

Table 5 Studies of stem cell therapy for peripheral vascular disease

Author (ref.)	*Trial type (or name)*	*No. of patients*	*Type*	*Route*	*Stem cell type*[a]	*% amputated*[b]
Nizankowski *et al.*[22]	Unblinded	10	CLI	IM	BM-MNC	30%
Tateishi-Yuyama *et al.*[15]	TACT	47	CLI	IM	BM-MNC & PBSC	NA
Kawamura *et al.*[23]	Unblinded	92	CLI & C	IM	PBSC	C−6% CLI−49%
Durdu *et al.*[24]	Unblinded	28	CLI	IM	BM-MNC	3.6%
Kim *et al.*[25]	Unblinded	27	Buerger's disease	Bone Fen.	BM-MNC	0%
Hernandez *et al.*[26]	Unblinded	12	CLI	IM	BM-MNC	8%
Inaba *et al.*[27]	Unblinded	5	Unknown	IM	CD34+	0%
Bartsch *et al.*[28]	TAM-PAD	13	C	IM & IA	BM-MNC	0%
Kolvenbach *et al.*[29]	Unblinded	15	CLI	IM	CD34+ and CD133+	30%
Koshikawa *et al.*[30]	Unblinded	7	Hand ischemia	IM	BM-MNC	0%
Lenk *et al.*[31]	Unblinded	7	CLI	IA	CPC	14%
Kajiguchi *et al.*[32]	TACT	7	CLI	IM	BM-MNC	43%
Ishida *et al.*[33]	unblinded	6	CLI & C	IM	PBSC	0%

Abbreviations: C = claudication; CLI = critical limb ischemia; CPC = circulating blood-derived progenitor cell; Fen. = fenestration; IA = intra-arterial; MNC = mononuclear cells; NA = not available; TACT = therapeutic angiogenesis using cell transplantation; TAM-PAD = transplant of autologous mononuclear bone marrow cells in peripheral arterial disease.
[a]Unless otherwise stated the bone marrow and peripheral blood stem cells are unmanipulated.
[b]The time interval patient is followed after stem cell injection before amputation is often not reported.

Despite the small number of patients and limitations on assessment of the microvascular blood flow, the primary outcome was successful, in that symptomatic relief of rest pain was achieved in all patients and the limb was salvaged in seven of the nine patients. Earlier studies of therapeutic angiogenesis have used a variety of stem cell sources, including unselected BM, unselected PBSC, MSC and purified CD34+ cells obtained from the marrow or peripheral blood. Stem cells from all these sources have produced various degrees of clinical benefit.[5] This suggests that paracrine, anti-apoptotic growth factor, or other factors produced by stem cells may mediate the response independent of direct differentiation into endothelial cells. For example, BM-derived cells are able to promote the secretion of angiogenic cytokines, such as vascular endothelial growth factor and basic fibroblast growth factor, which promote angiogenesis.[19–21]

Our results show that the procedure is safe with no major complications or adverse events, relieves pain and delays amputation in patients being considered for limb amputation. Future trials should consider CD133+ cell injection earlier in the disease course, that is, intermittent claudication without critical limb ischemia. Other studies using mostly unmanipulated BM or PBSC have suggested that the rate of amputation may be decreased after stem cell implantation (Table 5). Future studies will be required to clarify whether a CD133+ selected stem cell product is superior to other stem cell products for patients with PAD.

Conflict of interest

The authors declare no conflict of interest.

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