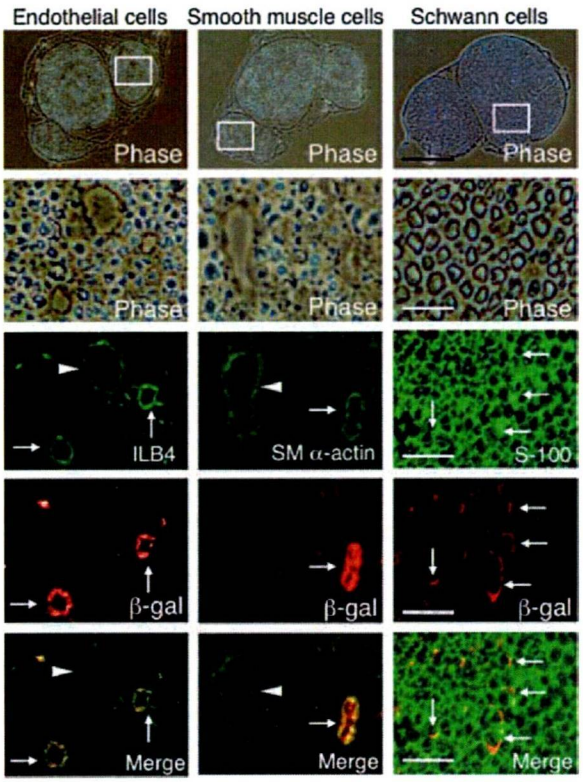

Figure 5. Differentiation of NSCs into endothelial cells, vascular SM cells and Schwann cells in crushed nerves. NSCs from Rosa 26 mice were administered to mice with sciatic nerve crush injury. After 2 weeks, the sciatic nerve was dissected and frozen sections were made. Endothelial cells and vascular SM cells were identified by isolectin B4 (ILB4) (left) and SM α-actin (middle), respectively. Vasculature and cells derived from transplanted NSCs were demonstrated by immunostaining for β-gal. Arrows in the left and middle columns indicate double-positive vasculature. Arrowheads in the left and middle columns denote ILB4 or SM α-actin positive but β-gal–negative portions, inferred to derive from original host cells. Schwann cells (right column) were identified by S-100 antibody. Arrows in the right column indicate cells that are double-positive for S-100 and β-gal. Boxed areas in the top photomicrographs are magnified in the lower photomicrographs of each column. Scale bars: 200 μm (black); 50 μm (white).

sion (10^5 per mouse) significantly improved motor nerve conduction velocity and action potential in injured sciatic nerve 21 and 28 days after surgery compared to the control (PBS) (motor nerve conduction velocity, 21 days: 31.1±5.2 versus 16.5±1.7 m/sec, $P<0.05$, 28 days: 41.9±1.3 versus 23.2±3.4 m/sec, $P<0.01$; and action potential, 21 days: 4.6±0.5 versus 2.6±0.4 mV, $P<0.05$, 28 days: 5.2±0.4 versus 3.2±0.2 mV, $P<0.05$) (Online Figure III). These observations suggest that NSCs can contribute to vasculogenesis and myelination, which jointly promote peripheral nerve regeneration, resulting in favorable therapeutic outcome in injured nerve.

NSC Differentiation Into Vascular and Neural Lineage in Ischemic Brain

In the next experiments, physiological NSC differentiation profiles in the central nervous system were evaluated using a forebrain ischemic model. A sublethal forebrain ischemia was induced by bilateral common carotid artery occlusion for 18 minutes. Clonally isolated NSCs from Rosa 26 mice were injected into the cerebral ischemic mice in the next day and euthanized 2 weeks later. Immunostaining of frozen sections from forebrain revealed the recruitment of β-gal–expressing CD31-positive endothelial cells and SM α-actin–positive SM cells in the vascular structure. Colocalization of β-gal–expressing astrocytes, which were identified by the expression of glial fibrillary acid protein, was also detected around the β-gal–expressing vascular structures. There were, however, no β-gal–expressing neurons, identified by the MAP-2 immunostaining (Figure 6a).

NSC Differentiation Into Endothelial Lineage in Ischemic Myocardium

Finally, to determine whether NSCs can differentiate into vascular cells in response to a nonneural pathological insult, NSCs were isolated from Rosa 26 mice and expanded ex vivo by forming neurospheres. Dissociated NSCs were injected via a tail vein into C57B6/J mice with myocardial infarction induced by coronary artery ligation. Immunohistochemical analysis using an antibody directed against β-gal revealed that β-gal–positive cells (red fluorescent) expressed endothelial-like morphology and immunopositivity for FITC-conjugated BS lectin (green fluorescent), which was infused systemically just before euthanasia of the animals, (Figure 6b) suggesting that NSCs recruited to neovascular foci with the endothelial cell characteristics. The finding was more evident in ischemic myocardium 2 weeks (Figure 6b, lower) than 1 week (Figure 6b, upper) after surgery. Expression of β-gal in intact myocardium was observed only in rare cells; none was detected in vehicle-treated mice (data not shown). However, some of the injected NSCs surprisingly recruited to nonischemic intact capillaries in liver that was isolated from the mice underwent myocardial infarction surgery (Figure 6c). These findings thus demonstrate that NSCs can differentiate into vascular cells in the setting of nonneural tissue ischemia or even in nonischemic tissue.

Discussion

In the present study, we have shown the following series of evidences: (1) triple characteristics of neural, endothelial, and SM progenitors in clonally isolated NSC-derived neurosphere; (2) differentiation capacity of the expanded neurosphere/NSCs into neural, endothelial, and SM lineages; (3) neural- or vascular growth factor–dependent NSC differentiation into functional vascular lineage cells involving specific interactive cell–cell signaling; and (4) in vivo NSC contribution to both neurogenesis and vasculogenesis in not only neuronal tissue but also nonneural tissue in adults.

Our results established that NSCs retain the ability in adult mammals to differentiate into endothelial and SM lineage cells along with neural lineage cell differentiation. Vascular cell–like phenotypes with the expressions of Notch signaling molecule in neurospheres were induced under certain culture conditions such as stimulation of VEGF signaling or blocking NGF signaling. Particularly, the blocking of NGF signaling in neurospheres resulted in the induction of more endothelial

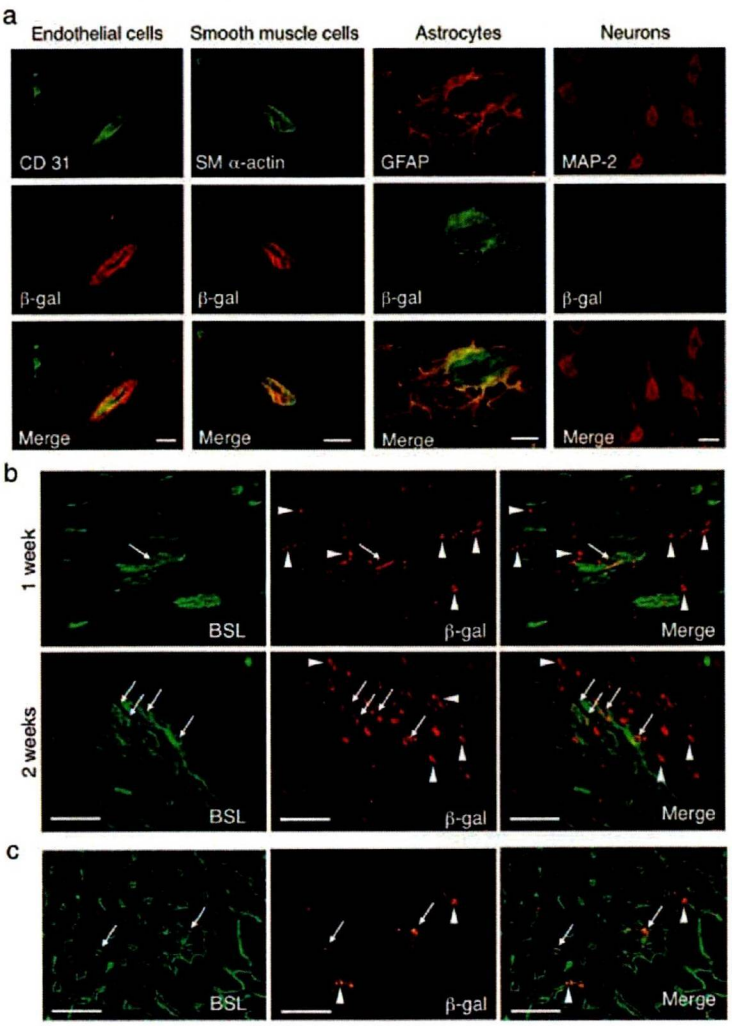

Figure 6. Recruited NSC differentiation in ischemic brain, ischemic myocardium, and liver. Clonally isolated NSCs from Rosa 26 mice were dissociated and administered (1×10^5 cells/mouse) to mice with sublethal forebrain ischemia and myocardial infarction. a, Differentiation of NSCs into endothelial cells, vascular SM cells, and astrocytes in ischemic brain 2 weeks after surgery. Endothelial cells, vascular SM cells, astrocytes, and neurons were identified by CD31, SM α-actin, glial fibrillary acid protein (GFAP), and MAP-2, respectively. Vasculature and cells derived from transplanted NSCs were demonstrated by immunostaining for β-gal. Yellow in the merged images indicates endothelial cells, vascular SM cells, and astrocytes differentiated from the administered NSCs. No neurons from injected NSCs were detected. Scale bars: 20 μm. b, Differentiation of NSCs into endothelial cells indicated with perfused FITC-conjugated BS lectin (BSL) (green) in ischemic myocardium 1 and 2 weeks after surgery and nonischemic liver (c) harvested 2 weeks after surgery/NSC injection. Vasculature and cells derived from transplanted NSCs were visualized by immunostaining for β-gal (red). Arrows indicate double-positive portion of the vasculature. Arrowheads denote BS lectin–positive but β-gal–negative portions. Scale bars: 50 μm.

specific gene expressions involving Delta-like 4 ligand than the stimulation of VEGF signaling, suggesting that because neurospheres themselves produce both VEGF and NGF (Figure 1), and these growth factors are thought to play a role in neurospheres as autocrine factors for the growth/differentiation, suppression of NGF-involved neuronal signaling pathways would be more critical for their differentiation into vascular lineage cells rather than more than stimulation of VEGF signaling. These findings may give rise to a novel mechanistic insight that the direction of NSC differentiation toward vascular or neuronal lineage is determined depending on the balance of exposure to angiogenic and neurogenic factors. Indeed, transplanted NSCs differentiated into vascular cells not only in neural tissues but also nonneural tissues in vivo. However, the differentiation of recruited NSCs into neuronal cells could not be detected immunohistologically (data not shown) in ischemic myocardium and nonischemic liver (Figure 6b). These findings suggest that the microenvironments, which are not always specific for the nervous system, are important in vascular differentiation from NSCs. Also, because undifferentiated neurospheres exhibit distinct expression patterns of Notch ligands and Eph B4 as well as both vascular and neuronal markers, cell–cell interaction through Notch signaling or Eph/ephrin signaling might also play a crucial role in vascular differentiation from NSCs.

Consistent with the observation in a previous study that adult neurogenesis occurs within an angiogenic niche where active vascular recruitment occurs,[26] our findings indicate that foci of neurogenesis and vasculogenesis within a given cluster may derive from common stem or progenitor cells. Transplanted NSCs practically contributed to vasculogenesis as well as gliogenesis both in damaged peripheral and central nervous system in vivo. Unexpectedly, no neurons derived from exogenously transplanted NSCs were observed in infarct brain in our hands, whereas transplanted NSC-derived vascular cells and astrocytes were observed. Although the differentiation potential of adult NSCs into neurons is quite limited in vitro (<1%),[24] NSCs in adult brain stem cell niches in subventricular zone and hippocampus evidently provide neurons during homeostasis and regeneration.[33,34] The discrepancy between our observation and previous reports may be attributable to the reason why. (1) NSCs were transplanted at acute injured tissues and influenced by inflammatory stimuli for not neuronal but glial lineage induction. (2) NSCs

derived from different portion from specific lesion of subventricular zone or hippocampus in brain, and (3) ex vivo culture expanded NSCs were used in our study.

Our findings not only add to the multipotent repertoire established previously for NSCs but also provide in vitro and in vivo evidences for concurrent vasculogenesis and neurogenesis from common stem cells, which may contribute to reparative organogenesis. However, because not only neurospheres but also other spheres, ie, cardiospheres, are highly motile and prone to fuse, it is difficult to determine whether the spheres are clonal or oligoclonal in nature. Moreover, the multipotentiality of a single NSC cannot be assessed, and the possibility exists that cells in the spheres are unipotent, bipotent, or truly multipotent even though clonally isolated NSCs were examined in the experiments. In addition, based on recent evidences[35,36] in which close correlation between neurogenesis and vascular niche in the subventricular zone has been shown, we speculate that vascular niche is required to induce neurogenesis from adult NSCs. Other reports also demonstrate that endothelial cells play a critical role in the early stage of liver organogenesis or pancreatic differentiation, before blood vessel function.[6,7] The origin of vascular cells in forming a tissue or organ from stem cells, however, has not been identified completely. Angiogenesis and vasculogenesis are currently classified by the origin of preexisting mature endothelial cells and bone marrow–derived endothelial progenitor cells in adult organs.[21,37]

In this study, we have shown concurrent tissue regeneration from common stem cells and therapeutic potential of NSCs for neuronal tissue damage. Interestingly, although the favorable effect of NSCs on stroke is reported,[38] there was no therapeutic effect of NSC transplantation on myocardial infarction in our hands (data not shown), suggesting that NSCs have a therapeutic effect on neuronal tissue damage including mechanical injury and ischemia but not on nonneuronal tissue ischemia. The differential effects of NSCs on certain diseases might depend on different tissue regeneration process following injury. In addition, because not only in injured nerve but also ectopic NSC recruitment was observed when NSCs were infused systemically, local transplantation of NSCs might be more safe with better outcome as a therapeutic application. This vasculogenesis from somatic NSCs may be the third component of vascular formation in adult tissue regeneration specifically in neuronal tissue.

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Disclosures

None.

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Supplement Material

Cell cultures.

After exanguination via the cardiac apex to minimize contamination of brain tissue with peripheral blood, isolation of NSCs was carried out by previously established methods[1] using 4-6 week-old C57BL/6J or B6.129S7-*Gt(ROSA)26Sor*/J (Rosa 26, Jackson) mice. The obtained cells were suspended at a density of 1000-2500 cells/cm^2 or < 5 cells/ml. For clonal experiments, individual cells were transferred with a micropipette to the microwells of 96-well plates and allowed to form neurospheres. Each neurosphere was then dissociated and expanded. The NSC culture medium consisted of 20 ng/ml EGF (BectonDickinson), 20 ng/ml bFGF (Upstate Biotechnology), B27 supplement (Life Technologies), 2 mM glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin in DMEM/F12 (Life Technologies). Growth factors were added every other day and half the volume of the medium was changed every fourth day. EGM-2-MV BulletKit (Clonetics) was also used as vascular cell-orientated medium.

RT-PCR and quantitative real-time RT-PCR.

Total RNA was extracted from a single neurosphere or an attached colony derived from a single neurosphere with the RNAqueous kit (Ambion). After DNase I (Ambion) treatment, reverse transcription and PCR were performed using a Superscript Preamplification System Kit (Life Technologies) and Advantage cDNA Polymerase Mix (Clontech), respectively. The denaturing step was 95°C for 30 seconds; the annealing and extension was 64°C for 3minutes. After repeating 30 to 35 cycles, a prolonged extension step was carried out for 4 minutes. The amplified DNA was electrophoresed in 1.5 % agarose gel containing ethidium bromide and photographed under a UV transilluminator. For quantitative RT-PCR, the converted cDNA samples (2 μl) were amplified in triplicate by real-time PCR machine (ABI Prism 7700, Applied Biosystems) in a final volume of 20 μl using SYBR Green Master Mix reagent (Applied Biosystems) with gene-specific primers. Melting curve analysis was performed with Dissociation Curves software (Applied Biosystems) and the mean cycle threshold (Ct) values were used to calculate gene expressions with normalization to rat GAPDH (rGAPDH).

Northern-blot analysis.

Total RNA was extracted from neurospheres or lung tissue with the RNeasy kit (Qiagen). For Northern blots, 20 mg of total RNA was electrophoresed and transferred to a nylon membrane. Blots were hybridised with a 32P-labeled SM α-actin probe, washed and exposed to X-ray film.

Animal studies.

For animal studies, all experimental procedures were conducted in accordance with the Japanese Physiological Society Guidelines for the Care and Use of Laboratory Animals, and the study protocol was approved by the Ethical Committee in Institute of Biomedical Research and Innovation (IBRI) and RIKEN Center for Developmental Biology.

Nerve crush injury model.

The sciatic nerves of 8-10 week-old male C57BL/6J mice (Jackson) were subjected to unilateral nerve crush injury. Mice were anesthetized with 250mg/kg i.p. of 2, 2, 2-tribromoethanol (Avertin^{TR}, Sigma). The sciatic nerve was exposed after surgical incision of the overlying skin and muscles, and the nerve was crushed at mid-thigh level for 10 seconds, using a hemostat with tips covered by plastic tubing. The skin was closed with a surgical stapler. Mice were sacrificed at 2 weeks post-crush injury.

Neurophysiological measurements.

Sciatic nerve conduction velocity was measured using standard orthodromic surface recording techniques and a Teca TD-10 (Oxford Instruments) portable recording system in all mice at baseline (before treatment) and then at 2weeks and 4weeks after treatment as described previously.[2] Briefly, motor nerve conduction velocity (MCV) was calculated by dividing the distance between stimulating electrodes by the average latency difference between the peaks of the compound muscle action potentials (CMAPs) evoked from two sites (sciatic notch and ankle). Sensory nerve conduction velocity (SCV) was calculated by dividing the distance between stimulating and recording electrodes by the latency of the signal from the stimulation artifact to the onset of the peak signal. For each nerve, maximal velocities were determined bilaterally. All measured data from both sides are averaged.

Sublethal Forebrain Ischemia Model.

C57Black/Crj6 mice (Jackson), age 8-10 week-old male, were used to make the subletahl brain ischemic models as described previously.[3] Under anesthetization with 250mg/kg i.p. of 2, 2, 2-tribromoethanol (Avertin^{TR}, Sigma), bilateral common carotid arteries were isolated and clamped by hemostatic clips for 18 min. The skin was closed with a surgical stapler. Mice were sacrificed at 14 days post-ischemia.

Myocardial Infarction Model.

C57BL/6J mice (Jackson), age 8-10 weeks old, were anesthetized with 250mg/kg i.p. 2, 2, 2-tribromoethanol (Avertin^{TR}, Sigma) and underwent surgery for myocardial infarction as

described previously.[4] After induction of anesthesia, the mice are placed in a supine position and intubated with a 22G IV catheter (Johnson & Johnson). Respiration is controlled by mechanical ventilation using a rodent ventilator (Harvard Apparatus) (tidal volume 0.3ml, rate 10^5 strokes/min). Using a dissecting microscope (Olympus SZH 10), a left thoracotomy is performed in the fourth intercostal space. The medial aspect of the incision is extended cranially to form a flap that was retracted to expose the heart. A small opening is made in the pericardium and a 7-0 monofilament nylon suture on a curved taper needle is passed under the left anterior descending artery (LAD) just proximal to the first diagonal branch. Infarction was induced by LAD permanent ligation with the suture. The procedure is completed after closing the chest and eliminating thoracic air.

In Situ Fluorescent Staining.

Vascularity of tissues (sciatic nerve, heart, and liver) was examined by in situ fluorescent staining using an endothelial cell-specific marker FITC conjugated BS1-lectin (Vector Laboratories) as described previously.[4] After anesthesia, BS1-lectin (0.1mg per mouse) was injected to mice systemically via a left ventricle of the heart. Ten minutes later, the mice were sacrificed and tissues were harvested and fixed in 4% paraformaldehyde for 2 hours. After fixation, specimens were embedded in OCT compound for frozen cross-section or examined directly for whole mount sciatic nerve staining under computer-assisted fluorescent microscope. (Nikon)

Immunocytostaining and Immunohistochemistry.

Neurospheres were preserved in 2% paraformaldehyde in PBS for 10 min and then placed in HistoGel (Richard-Allan Scientific) before embedding in paraffin. In the nerve crush model, isolated sciatic nerves for whole mount staining were fixed by 2% paraformaldehyde for 10 min. For frozen sections, sciatic nerves and forebrains were placed in OCT compound (Sakura Finetechnical) and frozen by liquid nitrogen. Immunostaining of neurospheres was performed using antibodies to nestin (1:300, PharMingen), VEGF (1:100, PharMingen), HIF-1a (1:500, Sigma), SM α-actin (1:300, Sigma), calponin (1:300, DAKO), bacterial β-galactosidase (1:500, Cortex Biochem), CD31 (1:300, PharMingen), S-100 (1:400, PharMingen), GFAP (1:300, SantaCruz) and MAP-2 (1:300, SantaCruz).

Chemical staining using FITC-conjugated isolectin B4 (Vector Laboratories), or DiI-labeled acLDL (Biomedical Technologies) was performed as previously described.[4] After staining, samples were viewed with an inverted fluorescent microscope (Nikon).

Statistical Analysis.

All values were expressed as mean ± SEM. Statistical analyses were performed with commercially available software (GraphPad Prism™, MDF software, Inc.). Comparisons between multiple groups and two groups were tested for significance via analysis of variance (ANOVA) followed by post-hoc testing with the Tukey procedure and nonparametric Mann-Whitney test, respectively. A *P* value less than 0.05 was considered significant.

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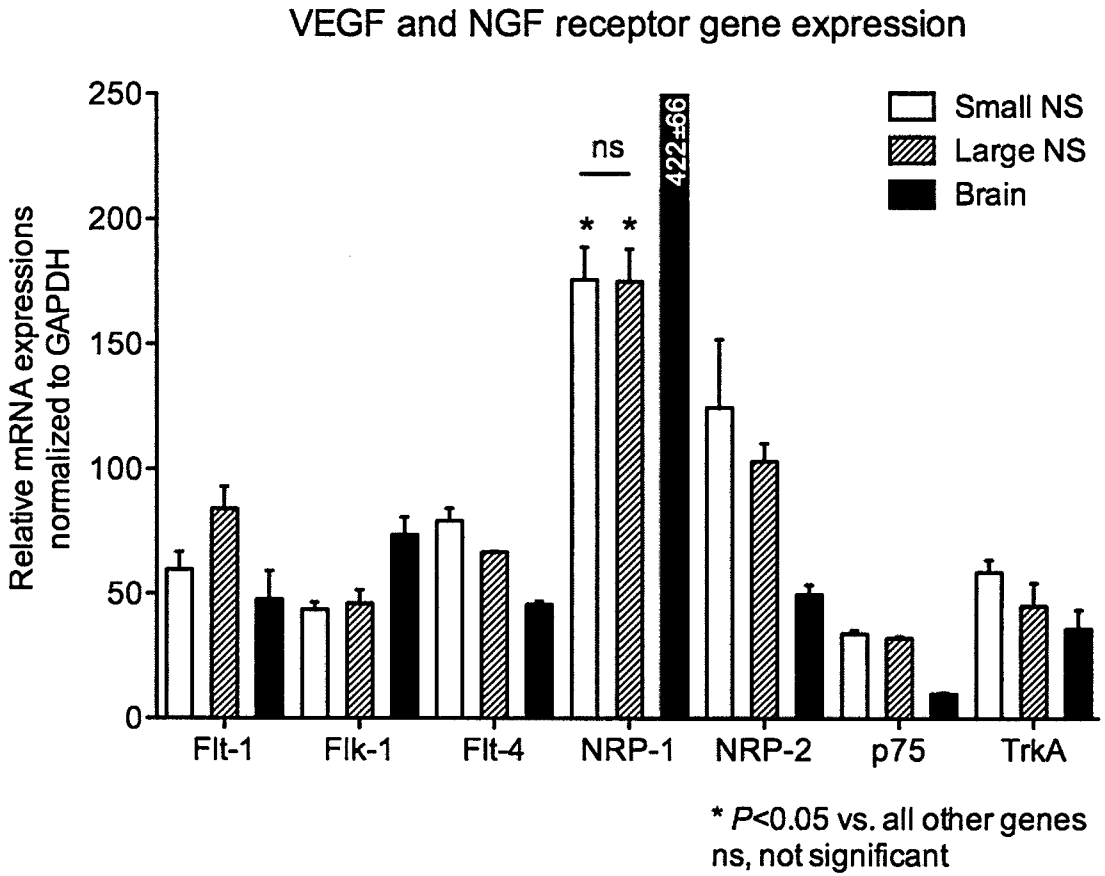
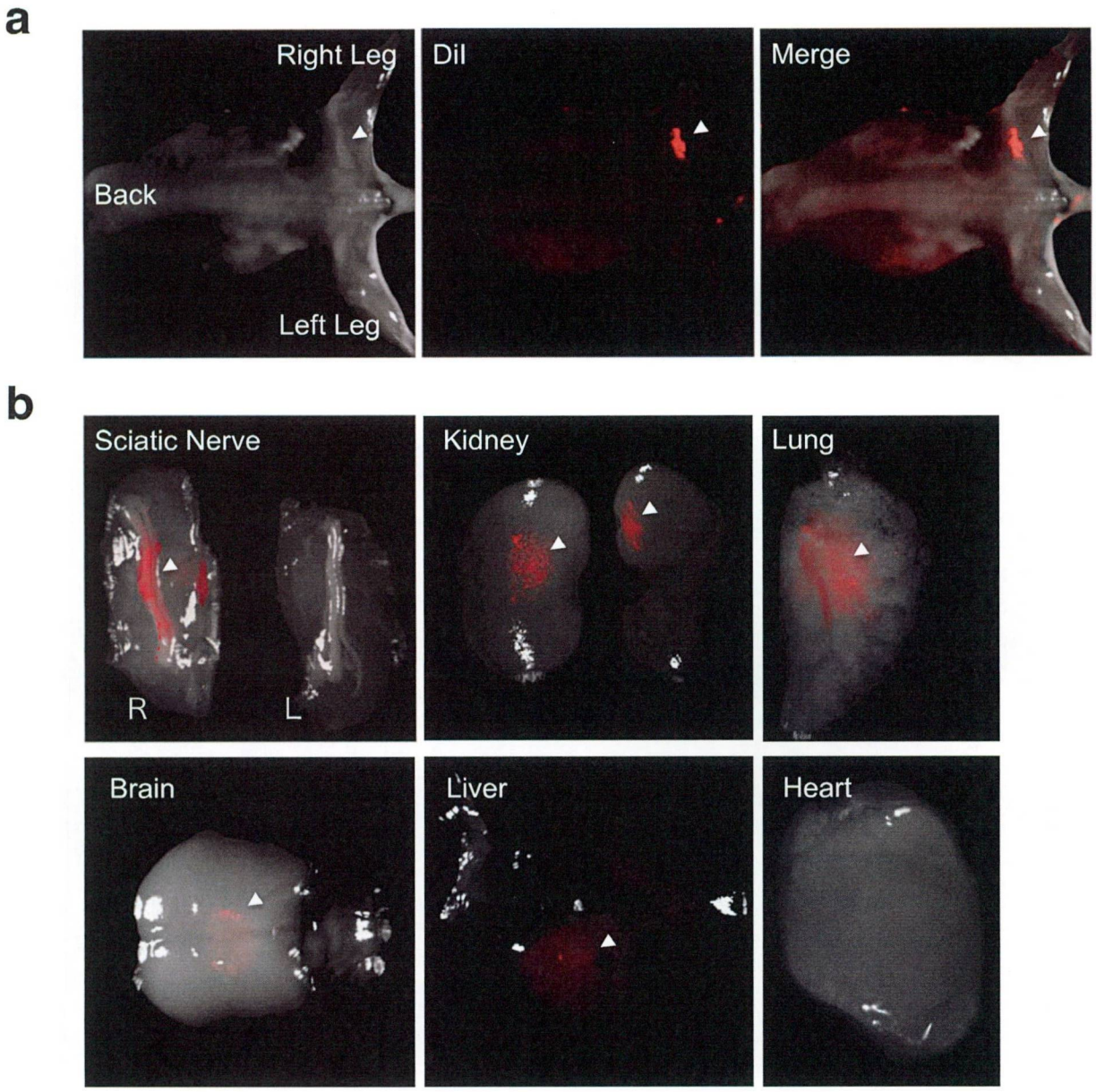
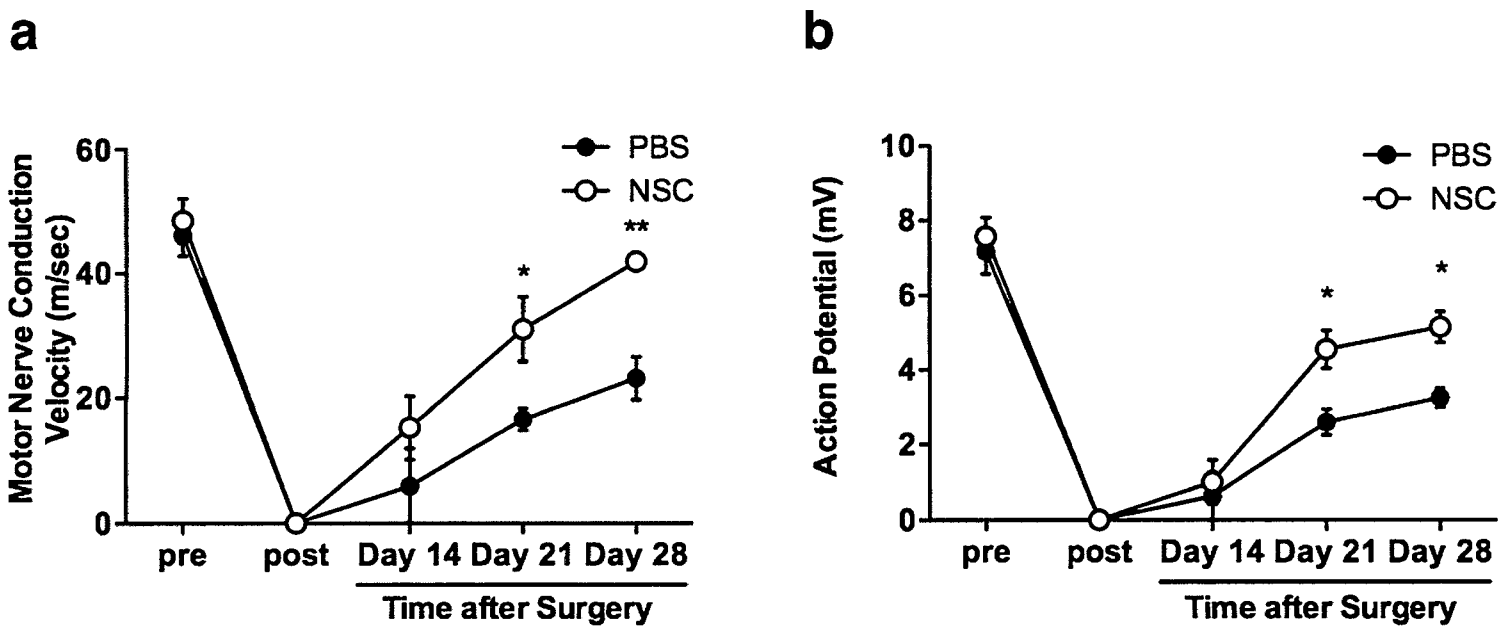


Online Figure I. VEGF and NGF receptor gene expressions in neurospheres. Small and large neurospheres were examined for the indicated VEGF and NGF co-receptor mRNA expressions by real-time RT-PCR. Brain tissue was used as a control. Each gene expression was expressed as a relative mRNA expression normalized to GAPDH, and over 50 is considered to be a significant expression as transcripts. NRP-1/-2: neuropilin-1/-2. The experiment was performed in triplicate, and RNA extracted from three samples was analyzed.



Online Figure II. Macroscopic red fluorescent detection following systemic DiI labeled NSC injection in whole mouse. a, DiI labeled NSCs were systemically injected to the mouse with right sciatic nerve crush injury. DiI positive NSC homing to major organs was assessed by fluorescent imaging system 2 weeks after cell injection. The whole back skin was removed to avoid capturing non-specific fluorescent signals, and NSC recruitment to tissue was recognized as red fluorescent signals on the back of the mouse. Arrow indicates the injured site with high red signal. Bar = 10 mm. b, Sciatic nerve with muscle, kidney, lung, brain, liver, and heart were harvested from the mouse and observed. Arrows indicate red signals that correspond to DiI labeled NSC recruited sites. Bar = 3 mm.



Online Figure III. Systemic NSC transplantation improved functional recovery in injured nerve. Either NSCs ($1x10^5$/mouse) or PBS (control) were injected to the mice with crushed nerve via a tail vein. To evaluate functional recovery in injured nerve, motor nerve conduction velocity (a) and action potential (b) were measured before (pre), immediately (post), 14, 21, 28 days after sciatic nerve crush injury. *, $P< 0.05$ and **, $P<0.01$ vs. PBS, n=4 in each group.

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TRANSLATIONAL AND CLINICAL RESEARCH

Intramuscular Transplantation of G-CSF-Mobilized CD34$^+$ Cells in Patients With Critical Limb Ischemia: A Phase I/IIa, Multicenter, Single-Blinded, Dose-Escalation Clinical Trial

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ABSTRACT

A number of preclinical studies have indicated the therapeutic potential of endothelial progenitor cells for vascular regeneration in ischemic diseases. A phase I/IIa clinical trial of transplantation of autologous CD34$^+$ cells, the endothelial and hematopoietic progenitor-enriched fraction, was performed in no-option patients with atherosclerotic peripheral artery disease or Buerger's disease with critical limb ischemia (CLI). CD34$^+$ cells were isolated from the G-CSF-mobilized apheresis product using a magnetic cell sorting system. CD34$^+$ cells (10^5/kg, $n = 6$; 5×10^5/kg, $n = 8$; or 10^6/kg, $n = 3$) were injected i.m. into the leg with more severe ischemia. The Efficacy Score, representing changes in the toe brachial pressure index (TBPI), Wong-Baker FACES pain rating scale, and total walking distance 12 weeks after cell transplantation, the primary endpoint, was positive, indicating improvement in limb ischemia in all patients, although no significant dose-response relationship was observed. During the 12-week observation after cell therapy, the Wong-Baker FACES pain rating scale, TBPI, transcutaneous partial oxygen pressure, total or pain-free walking distance, and ulcer size serially improved in all patients. No death or major amputation occurred, and severe adverse events were rare, although mild to moderate events relating to G-CSF and leukapheresis were frequent during the 12-week follow-up. In conclusion, the outcomes of this prospective clinical study indicate the safety and feasibility of CD34$^+$ cell therapy in patients with CLI. Favorable trends in efficacy parameters encourage a randomized and controlled trial in the future. STEM CELLS *2009;27:2857–2864*

Disclosure of potential conflicts of interest is found at the end of this article.

INTRODUCTION

Chronic ischemia in the lower extremities is mainly caused by arterial obstruction/stenosis in the leg. It is well known that atherosclerotic peripheral artery disease (PAD) is the most common cause of chronic limb ischemia, whereas Buerger's disease and collagen diseases, involving vasculitis in the small- and middle-sized arteries, also cause limb ischemia. The clinical consequences of chronic limb ischemia

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include intermittent claudication (IC), that is, pain with walking, and critical limb ischemia (CLI), as demonstrated by ischemic rest pain and/or loss of tissue integrity, including skin ulceration and gangrene. A large cohort study [1] indicated that 20%–50% of PAD patients are asymptomatic, 10%–35% of PAD patients have IC, 30%–40% of PAD patients have atypical leg pain, and only 1%–3% of PAD patients have CLI. The clinical course of PAD patients without CLI is generally stable; however, the prognosis for CLI patients is miserable. The 1-year mortality and major amputation rates are reportedly 25% and 30% in CLI patients, respectively [2]. Currently, surgical (bypass grafting or endoatherectomy) or endovascular (transluminal angioplasty, intravascular stents, or intra-arterial thrombolysis) therapy is believed to be the best option for limb salvage, in eligible patients. However, such conventional revascularization can be performed in only 50% of CLI patients, and sustained recovery from CLI is observed in only 25% of patients 1 year after the initial treatment. Among CLI patients without successful revascularization, 40% lose their legs and 20% die within 6 months [2]. Therefore, patients with CLI should be considered as different from PAD patients without CLI, and development of a novel therapeutic modality is urgently needed to improve the poor prognosis of CLI patients, especially those not eligible for conventional revascularization therapies.

Endothelial progenitor cells (EPCs) belong to an immature cell population that is capable of differentiating into mature endothelial cells [3]. In adults, EPCs mainly reside in bone marrow (BM) and are more proliferative and migrative than terminally differentiated endothelial cells [4]. EPCs can be clinically isolated as CD34$^+$ or AC133$^+$ mononuclear cells (MNCs) from adult BM or peripheral blood (PB) [3, 5]. Tissue ischemia or systemic administration of G-CSF, GM-CSF, vascular endothelial growth factor, or estrogen enhances mobilization of EPCs from BM into PB, and the mobilized EPCs specifically home to sites of nascent neovascularization, thereby contributing to vascular repair [6, 7]. The therapeutic concept of EPC transplantation for neovascularization has been established by a number of preclinical studies using animal models of hindlimb [8], myocardial [9] and cerebral [10] ischemia. In hindlimb ischemia, human EPCs are incorporated into ischemic tissue of immunodeficient animals following systemic administration, and participate in neovascularization with recipients' endothelial cells, resulting in enhancement of limb blood flow and a higher limb salvage rate [8]. These promising results have encouraged clinical application of EPC transplantation for improvement of the poor prognosis of CLI patients.

Here, we report a phase I/IIa clinical trial of i.m. transplantation of autologous and G-CSF-mobilized CD34$^+$ cells in patients with intractable CLI. G-CSF was used to efficiently mobilize BM EPCs to PB, and the mobilized CD34$^+$ cells were isolated as the EPC-enriched fraction. The study outcome indicates the safety and feasibility as well as favorable trend in efficacy of this novel cell therapy without a significant dose-response relationship in patients with CLI.

METHODS

Study Design and Criteria for Enrollment

This phase I/IIa clinical trial was designed as a single-blinded, dose-escalation study to evaluate the safety, feasibility, and efficacy of autologous and G-CSF-mobilized CD34$^+$ cells in no-option patients with CLI. The study protocol conformed to the Declaration of Helsinki and was approved by the ethics committees of the participating hospitals, the Institute of Biomedical Research and Innovation and Kobe City Medical Center General Hospital.

The inclusion criteria were (a) atherosclerotic PAD or Buerger's disease with $\geq$50% luminal stenosis in the leg arteries by digital subtraction angiography (DSA), (b) >6 months since the onset of lower limb ischemia, (c) CLI within category 4–6 on the Rutherford scale, (d) failure of or no indication for transluminal angioplasty/stenting and bypass surgery, (e) male or female aged 20–80 years, and (f) written informed consent. The exclusion criteria, which were mainly implemented to exclude patients at high risk for the adverse events of G-CSF, apheresis, and CD34$^+$ cells, and to precisely evaluate efficacy, are shown in supporting information Table 1. After evaluation of the eligibility of each candidate for this cell-based therapy by the case enrollment committee, appropriate case selection was confirmed at an independent case registration center set up in the Translational Research Informatics (TRI) Center. Cell therapy was performed only in patients in whom written informed consent was obtained after completion of case registration.

Because this was the first clinical trial of CD34$^+$ cell transplantation in patients with CLI, we designed the trial as a dose-escalation study for safety evaluation. Only when death and life-threatening adverse events (National Cancer Institute Common Terminology Criteria for Adverse Events [NCI CTCAE] grade $\geq$4) related to cell mobilization and harvest did not occur for 2 weeks after cell transplantation in the first patient was the second case registered. Also, only when no severe events (NCI CTCAE grade $\geq$4) were observed for 4 days following the cell therapy in the second to fourth patients was the next case registered. Case registration was to be canceled when any grade $\geq$4 event relating to cell mobilization and harvest was observed in one patient or grade $\geq$3 events occurred in three cases.

Treatment Procedures

All CLI patients enrolled in this study received s.c. administration of G-CSF to mobilize EPCs from BM. The regular dose of G-CSF was 10 μg/kg per day for 5 days, and leukapheresis (AS.TEC204; Fresenius HemoCare, Bad Homburg, Germany, http://www.fresenius.com) was performed to harvest PB MNCs on day 5. From the standpoint of safety in all patients, the dose of G-CSF was reduced to 5 μg/kg per day when the WBC in PB was $\geq$50,000/μl but <75,000/μl, according to the *Guideline for Mobilization and Harvest of Peripheral Blood Stem Cells from Healthy Donors for Allogenic Transplantation* by the Japanese Society of Hematopoietic Cell Transplantation and the Japanese Society of Transfusion Medicine. G-CSF was scheduled to be canceled when the WBC was $\geq$75,000/μl; however, the leukocyte count never exceeded 75,000/μl during the G-CSF administration period in any patient. The leukapheresis product was kept at a concentration of $\leq 2 \times 10^8$ cells/ml in autoplasma at room temperature overnight ($\leq$18 hours) until the magnetic separation of CD34$^+$ cells was started using a CliniMACS Instrument, CD34 reagent, phosphate-buffered saline/EDTA buffer, and tubing set (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). Purity of the isolated CD34$^+$ cells was examined by fluorescence-activated cell sorting (FACS) analysis using CD34-specific and CD45-specific monoclonal antibodies (Becton, Dickinson and Company, San Jose, CA, http://www.bd.com).

Cell transplantation was performed under spinal anesthesia. CD34$^+$ cells dissolved in 10 ml physiological saline were i.m. administered into 40 sites (0.25 ml/site) of the leg with more severe ischemia in each patient. The administration points consisted of 30 sites in the calf muscle, six sites in the sole muscle, and four sites in the intertoe muscle. The target dose of CD34$^+$ cells was determined in a dose-escalation manner as follows: 10^5 cells/kg (Lo group) in the initial five patients, 5×10^5 cells/kg (Mid group) in the next five patients, and 10^6 cells/kg (Hi group) in the last five patients. When the CD34$^+$ cell yield was less than

Table 1. Efficacy Score

Efficacy score category	
TWD score according to change in TWD from baseline (ΔTWD)	
Unable to walk → able to walk, or ΔTWD >50%	2
25% < ΔTWD ≤ 50%	1
Unable to walk → unable to walk, or −25% ≤ ΔTWD ≤ 25%	0
−50% ≤ ΔTWD < −25%	−1
ΔTWD < −50%	−2
TBPI score according to ΔTBPI from baseline	
0.15 < ΔTBPI	2
0.10 < ΔTBPI ≤ 0.15	1
−0.10 < ΔTBPI ≤ 0.10	0
−0.15 < ΔTBPI ≤ −0.10	−1
ΔTBPI ≤ −0.15	−2
Wong-Baker FACES Pain Rating Scale score according to change in score from baseline	
Improvement by ≥2 steps	2
Improvement by 1 step	1
No change	0
Worsening by 1 step	−1
Worsening by ≥2 steps	−2

The Efficacy Score = total walking distance (TWD) score + toe brachial pressure index (TBPI) score + Wong-Baker FACES Pain Rating Scale score. Range, −2 to +2 for each category.

the target dose, the next highest available dose was given to the patient.

Endpoints

Because no gold-standard endpoints have been established for such small-sized, early-phase clinical trials in patients with CLI, we originally prespecified the Efficacy Score as a surrogate endpoint so that we could simultaneously evaluate subjective and objective parameters in this study. The Efficacy Score was defined as the sum of three scores, each of which measured a difference in the parameters between baseline and 12 weeks after cell therapy: (a) total walking distance (TWD) on a standardized treadmill test, (b) the toe brachial pressure index (TBPI) in the lower limb receiving the i.m. injection of cells, and (c) the Wong-Baker FACES pain rating scale, for evaluation of ischemic pain in the treated leg. Because each score has a range of +2 to {minus]2 points (best response assigned +2 and worst outcome assigned {minus]2), the Efficacy Score sum of the three scores is in the range of +6 to −6 points (Table 1).

The primary analysis of this trial was a comparison of the Efficacy Score at week 12 after $CD34^+$ cell transplantation among the three groups based on cell dosage. The secondary endpoints for safety were adverse events, the severity of which was graded according to the NCI CTCAE (version 3.0). The secondary endpoints for efficacy were the following parameters in the treated leg: the Rutherford Score, Wong-Baker FACES pain rating scale score, skin ulcer size determined as the sum of the longest diameter of each skin lesion, TWD and pain-free walking distance (PFWD) on a standardized treadmill test, TBPI and ankle brachial pressure index (ABPI) (Form PWV/ABI; Omron Colin, San Antonio, TX, http://www.colinmedical.com), transcutaneous partial oxygen pressure ($TcPO_2$) (PO-850; Sumitomo Electric System Solutions, Tokyo, Japan, http://www.joki.seiss.co.jp) in room air and 10 minutes after inhalation of 5 l/minute of oxygen. To detect pathogenic angiogenesis in the retina, fundus oculi examination was performed before and after (week 4 and week 12) $CD34^+$ cell transplantation. DSA in the lower limbs was also performed before and 12 weeks after cell transplantation to evaluate the development of pathogenic angiogenesis.

Data Management and Statistical Analysis

Data were managed at an independent data center of the TRI Center following approval by the institutional ethics committee. Data were collected, stored, and managed using paper-based case report forms. The online Clinical Trial Data Management System (EPS Co, Ltd., http://www.eps.co.jp) was used as a database. Following data input, data cleaning and a logical check were performed to guarantee data quality.

This trial was designed to assess the dose-dependent efficacy of $CD34^+$ cell therapy. The Jonckheere-Terpstra (JT) trend test was used to compare the distributions of the Efficacy Score among increasing dose levels. The sample size was determined to have sufficient statistical power to reject the null hypothesis that there is no association between the Efficacy Score and the dose level. The alternative hypothesis was that the means of the distribution of the Efficacy Score in the Lo, Mid, and Hi groups were 1.0, 2.5, and 4.0, respectively. In this scenario, 15 patients (five in each dose group) provide a statistical power >95%. For another scenario, such that the means of the distribution of the Efficacy Score in the Lo, Mid, and Hi groups were 1.0, 2.0, and 3.0, respectively, the power can be assured to be 69%.

The JT test or Cochran-Artimatage test was applied to see the trend for each variable among increasing dose levels. Serial changes in continuous variables were evaluated by repeated measures analysis of variance. When a serial change was significant, differences between baseline and 4 or 12 weeks after cell therapy were assessed by Dunnet's test. Differences in $TcPO_2$ between the transplanted and contralateral leg were compared using a paired Wilcoxon test. All tests were two-sided, and p-values <.05 were considered to be statistically significant. Analyses were performed using SAS software, version 9.1 (SAS Institute, Cary, NC, http://www.sas.com).

Results

Patients

Seventeen patients with CLI were enrolled in this study from November 2003 to December 2006. Although the target number of patients was originally 15 ($n = 5$ for each dose cohort), one in six patients registered as a candidate for the Mid dose group in the end received the Lo dose of $CD34^+$ cells and three in six candidates for the Hi dose group received the Mid dose of $CD34^+$ cells because of a lower than expected cell yield. Therefore, the number of patients was six in the Lo dose group, eight in the Mid dose group, and three in the Hi dose group in this study. Patient enrollment was terminated in March 2007 because (a) more than the target number ($n = 15$) of patients was already enrolled and (b) the frequency of the lower cell yield, resulting in administration of the Mid dose, was as high as 50% in the Hi dose cohort (supporting information Fig. 1).

Baseline characteristics of the patients are summarized in Table 2. The incidences of PAD (versus Buerger's disease) and hypertension were significantly higher in the Lo dose group than in the Mid and Hi dose groups. Although patients in the Lo dose group had a tendency to be older and had a history of bypass surgery more frequently than those in the higher dose groups, these differences were not statistically significant. Sex, history of endovascular intervention, and the incidences of smoking, hyperlipidemia, diabetes mellitus, coronary artery disease, and cerebral artery disease were similar in all groups. Sixteen patients were current smokers. Although they had quit smoking at least 2 months prior to the cell therapy, the severity of the CLI did not improve before starting G-CSF administration in any patient. All patients received two or more kinds of antiplatelet drug throughout the study

Table 2. Baseline characteristics

	Lo dose (n = 6)	Mid dose (n = 8)	Hi dose (n = 3)	p-value
Age	58.3 ± 22.8	49.3 ± 19.8	33.3 ± 4.7	NS
Male/female	4/2	4/4	1/2	NS
PAD/BD	4/2	1/7	0/3	.019
Catheter intervention	0	1	0	NS
Bypass surgery	3	1	0	NS
Hypertension	4	1	0	.019
Diabetes mellitus	3	2	1	NS
Hyperlipidemia	1	1	0	NS
Smoking	6	7	3	NS
Coronary artery disease	1	1	0	NS
Cerebral artery disease	0	1	0	NS
Aspirin	5	4	1	NS
Prostanoid	3	4	3	NS
Serotonin $5HT_2$ antagonist	4	4	2	NS
Cilostazol	1	4	0	NS
Warfarin	2	0	0	NS
Statins	2	1	0	NS
ARB	2	0	0	NS

Abbreviations: ARB, angiotensin 1 receptor blockade; BD, Buerger's disease; NS, not significant; PAD, atherosclerotic peripheral arterial disease.

period. Concomitant drug therapy was not significantly different among the three groups.

Outcome of Mobilization, Harvest, and Isolation of CD34$^+$ Cells

In this study, the dose of G-CSF was adjusted according to the WBC each day. The dose was reduced to 5 μg/kg per day for 1 day in seven patients, for 2 days in four patients, and for 3 days in one patient. The apheresis product number was $(3.0 \pm 1.0) \times 10^{10}$ and the frequency of CD34$^+$ cells in the apheresis product was 1.4% ± 1.5% by FACS analysis. Although we hypothesized that we could obtain $>1 \times 10^6$ CD34$^+$ cells/kg from most patients, the CD34$^+$ cell yield was lower than the expected number in six patients (35.3%). Age and the incidence of PAD were significantly higher in those six patients than in the others, in whom $>1 \times 10^6$ CD34$^+$ cells/kg could be obtained. These data suggest that CD34$^+$ cell harvest/isolation may be less efficient in aged patients or in patients with PAD (versus Buerger's disease). FACS analysis revealed that the purity and viability of the CD34$^+$ fraction following magnetic sorting were 92.7% ± 16.4% and 87.3% ± 5.3%, respectively (supporting information Table 2 and Fig. 2).

Efficacy Evaluation

The Efficacy Score at week 12, the primary endpoint of this study, was ≥1 point in all patients, indicating improvement in chronic lower limb ischemia following transplantation of any dose of CD34$^+$ cells. The Efficacy Score at week 12 was not significantly different among the three dose groups (Fig. 1). Although changes in the TWD between baseline and week 12 increased in a dose-dependent manner (p = .029), changes in the Wong-Baker FACES pain rating scale score, TBPI, $TcPO_2$ before and after oxygen inhalation, PFWD, and ulcer size after 12 weeks were similar in all groups (data not shown).

Because no significant dose-response relationship was observed for most efficacy parameters, we compared each parameter at week 4 or week 12 with that at baseline in all patients. The Wong-Baker FACES pain rating scale score was significantly lower at week 4 or week 12 that at baseline ($p <$ .0001). The TBPI tended to be higher at week 4 (p = .066) and was significantly greater at week 12 ($p <$ .0001), compared with baseline. The ABPI, which was >1.0 even at baseline in eight of 17 patients, was not significantly different after cell therapy. The $TcPO_2$ before oxygen inhalation (in room air) was significantly greater at week 4 (p = .036) and even greater at week 12 ($p <$ .0001), compared with baseline. The $TcPO_2$ after oxygen inhalation also tended to be greater at week 4 (p = .089) and was significantly higher at week 12 ($p <$ .0001) than at baseline. Regarding the evaluation of exercise tolerance, the TWD using the treadmill test was significantly greater at both week 4 and week 12 than at baseline

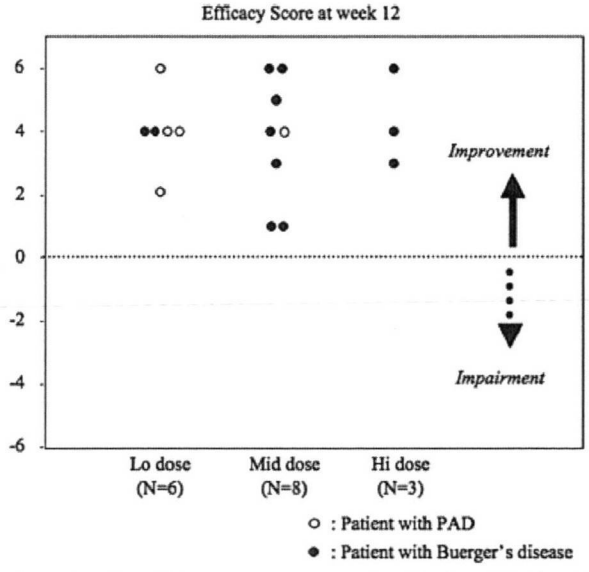

Figure 1. The Efficacy Score at week 12 following CD34$^+$ cell transplantation, which was the primary endpoint of this study, was positive, indicating improvement in limb ischemia in all patients. However, the Efficacy Score was not significantly different among the three dose groups. Abbreviations: PAD, peripheral artery disease.

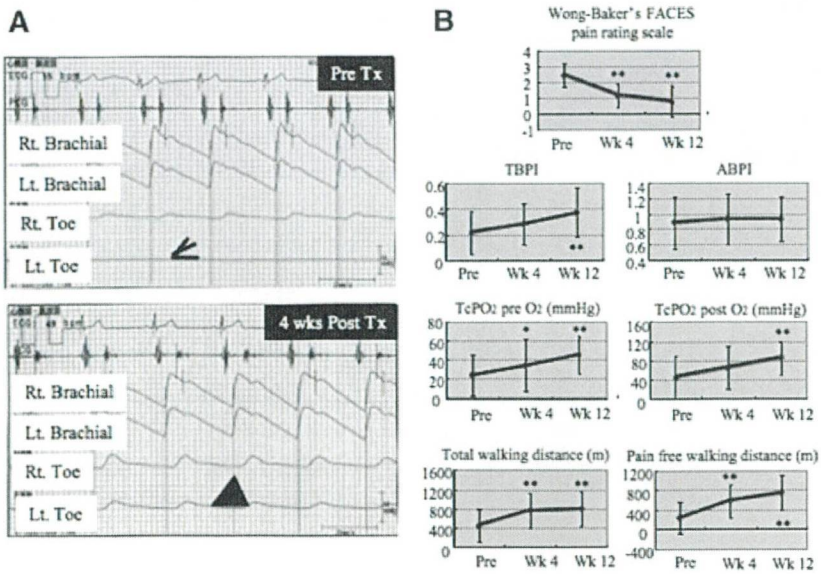

Figure 2. Improvement of efficacy parameters following $CD34^+$ cell therapy. **(A):** Representative recording of the pressure pulse wave in bilateral brachial and toe arteries (a male patient with Buerger's disease, aged 21 years). A nonpulsatile (flat) wave form (arrow) was observed in the left toe artery before cell therapy (Tx); however, recovery of the pulsatile pattern (arrowhead) was detected 4 weeks after Tx. **(B):** Serial changes in subjective and objective parameters of limb ischemia following $CD34^+$ cell transplantation in all patients ($n = 17$). $*p < .05$ versus baseline; $**p < .01$ versus baseline. Abbreviations: ABPI, ankle brachial pressure index; PAD, peripheral artery disease; TBPI, toe brachial pressure index; $TcPO_2$, transcutaneous partial oxygen pressure.

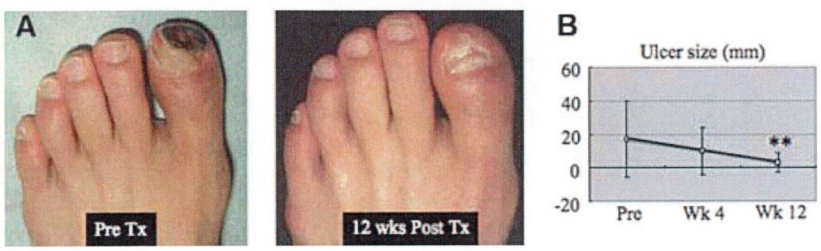

Figure 3. Healing of limb ulcer/gangrene after $CD34^+$ cell transplantation. **(A):** Representative pictures demonstrating healing of toe ulcer/gangrene following $CD34^+$ cell transplantation (a male patient with Buerger's disease, aged 36 years). **(B):** Serial changes in ulcer size in all patients. $**p <$ .01 versus baseline.

($p < .0001$). The PFWD was also greater at week 4 ($p = .003$) and at week 12 ($p < .0001$) (Fig. 2). Ulcer size tended to be smaller at week 4 ($p = .11$) and was significantly smaller at week 12 ($p = .001$) than at baseline (Fig. 3). These results suggest that both subjective and objective parameters of the severity of lower limb ischemia may significantly and serially improve after transplantation with $CD34^+$ cell therapy.

In this study, $CD34^+$ cells were transplanted only into the single limb with more severe ischemia. To assess the direct effect of the cell therapy on the transplanted limb, changes in $TcPO_2$ before and after oxygen inhalation between baseline and week 12 in the transplanted limb were compared with those in the nontransplanted (contralateral) leg in patients with bilateral limb ischemia ($n = 11$). The change in $TcPO_2$ before oxygen inhalation tended to be greater in the transplanted limb than in the nontransplanted limb ($p = .14$). The change in $TcPO_2$ after oxygen inhalation was significantly greater in the transplanted leg than in the contralateral leg ($p = .034$) (Fig. 4). These outcomes indicate that improvement in limb ischemia may be more prominent in the transplanted leg than in the contralateral one after cell therapy.

Because of the nature of a small-sized, phase I/IIa clinical trial, we did not evaluate the incidence of major adverse events such as death, major adverse cardiovascular event (MACE) and major amputation as hard endpoints in this study. However, no death by any cause or major/minor amputation occurred for 12 weeks after cell therapy in any patient.

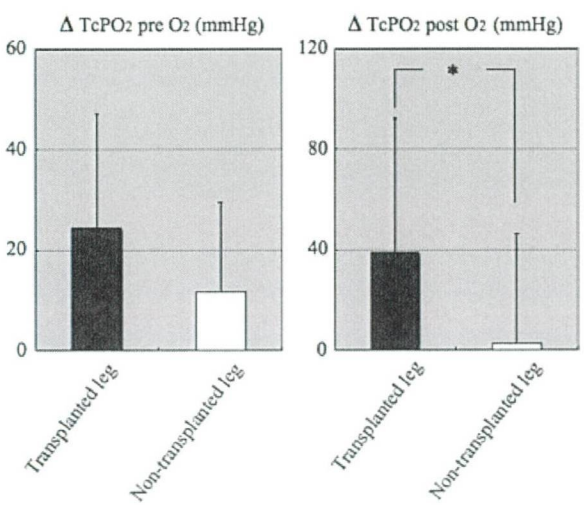

Figure 4. Change (Δ) in transcutaneous partial oxygen pressure ($TcPO_2$) before and after oxygen inhalation 12 weeks after $CD34^+$ cell transplantation in the transplanted and nontransplanted leg in patients with bilateral chronic limb ischemia ($n = 11$). $*p < .05$.

According to the inclusion criteria, all patients had Rutherford Score 4–6 CLI at baseline. However, seven of 17 patients (41.2%) at week 4 and 12 of 17 patients (70.6%) at

Table 3. Adverse events during the 12-week follow-up after CD34$^+$ cell transplantation

NCI CTCAE (version 3.0) grade	Adverse events
Grade 5 (death)	None
Grade 4 (life-threatening)	None
Grade 3 (severe)	Syncope ($n = 1$)
Grade 1–2 (mild to moderate)	Clinical symptoms
	Numbness[a] ($n = 9$)
	Fever[b] ($n = 7$)
	Bone pain[b] ($n = 7$)
	Headache[b] ($n = 5$)
	Lumbago[b] ($n = 3$)
	Tetany[a] ($n = 2$)
	Psoriasis vulgaris[b] ($n = 1$)
	Pulmonary congestion[a] ($n = 1$)
	Trichomonas vaginitis ($n = 1$)
	Idiopathic deafness ($n = 1$)
	Chronic cystitis ($n = 1$)
	Laboratory data abnormalities
	LDH elevation[b] ($n = 17$)
	ALP elevation[b] ($n = 15$)
	CRP elevation[b] ($n = 14$)
	ALT elevation[b] ($n = 9$)
	Uric acid elevation[b] ($n = 6$)
	Thrombocytopenia[a] ($n = 10$)

[a]Adverse events related to leukapheresis.
[b]Adverse events relating to G-CSF.
Abbreviations: ALP, alkaline phosphatase; ALT, alanine aminotransferase; CRP, c-reactive protein; LDH, lactate dehydrogenase; NCI CTCAE, National Cancer Institute Common Terminology Criteria for Adverse Events.

week 12 no longer had CLI. Considering the poor prognosis of CLI patients [2], especially those in whom conventional revascularization is not indicated, this outcome may suggest therapeutic potential for CD34$^+$ cell transplantation in such severe patients.

Safety Evaluation

Neither death (NCI CTCAE grade 5) nor life-threatening adverse events (grade 4) were observed during the 12-week follow-up after cell therapy. One severe adverse event (grade 3) was observed in a patient with cerebrovascular disease. That patient, who had a history of head tilt-induced syncope three times before study enrollment, experienced a similar syncope attack 11 days after cell transplantation. Because of the previous history and the nature of the syncope, the event was not considered to be related to the cell therapy.

As described above, severe adverse events were rare in this study. In contrast, mild to moderate adverse events (grade 1–2), especially G-CSF- or apheresis-related events, were frequent. All mild to moderate events were transient and disappeared without any permanent damage (Table 3).

The serum creatine phosphokinase (CPK) level was not elevated following G-CSF infusion in any patient. No anginal episode was observed during the study period in any patient. At week 4 and week 12, no electrocardiogram abnormality indicating de novo myocardial ischemia was identified. No adverse events stemming from spinal anesthesia were observed in any patient. There were no episodes of site infection following i.m. cell injections. Serial examinations of fundus oculi revealed no incidence of pathogenic angiogenesis in the retina following CD34$^+$ cell transplantation in any patient. DSA of the lower limbs also demonstrated no development of pathogenic angiogenesis, such as hemangioma or arteriovenous shunt formation, after cell therapy.

Discussion

To the best of our knowledge, the present study is the first clinical trial of transplantation of autologous and purified CD34$^+$ cells in patients with chronic ischemia in the lower extremities. As in previous studies of therapeutic angiogenesis by angiogenic growth factors [11, 12] or BM-derived MNCs [13, 14], no-option patients with CLI, who were at high risk for major amputation or death, were enrolled in this trial from an ethical point of view. We designed this small-sized, phase I/IIa clinical trial as an uncontrolled, but prospective, single-blinded, dose-escalation study to obtain useful information for a future phase IIb/III trial. In all patients, the primary endpoint, the Efficacy Score at week 12, was positive, indicating improvement in lower limb ischemia after cell therapy. In addition, both subjective and objective parameters of lower limb ischemia, such as the Wang-Baker FACES pain rating scale TBPI, $TcPO_2$, TWD, PFWD, and ulcer size, significantly and serially improved after transplantation of CD34$^+$ cells. Because this was not a randomized, controlled study, the possibility of a placebo effect after CD34$^+$ cell transplantation needs to be evaluated in a large-scale future trial. Another issue is that G-CSF might have a favorable effect on limb ischemia independently of CD34$^+$ cell therapy. Although G-CSF administration did not augment tissue perfusion in a preclinical model of chronic myocardial ischemia [15] and could not significantly improve left ventricular ejection fraction following acute myocardial infarction in recent double-blind, placebo-controlled, clinical trials [16, 17], the possibility that G-CSF could have an independent effect must be considered. In this study, improvement in $TcPO_2$ after oxygen inhalation was greater in the transplanted leg than in the contralateral leg in patients with bilateral limb ischemia. Because the contralateral leg could be identified as an internal control, this observation suggests that CD34$^+$ cells might have an independent effect on limb ischemia regardless of G-CSF administration. On the other hand, this result might be caused by more efficient recruitment of circulating EPCs into the more severely ischemic leg than into the contralateral limb. It would be indispensable to investigate the solo potential of CD34$^+$ cells in future randomized controlled trials.

The therapeutic potential of human CD34$^+$ cells for ischemic neovascularization and cardiac repair was dose dependent in an animal model of myocardial infarction [18]. In contrast, the effect of CD34$^+$ cells was not significantly different among different dose groups in a phase I/II, placebo-controlled, dose-ranging trial for patients with intractable chronic myocardial ischemia [19]. Because the clinical study for myocardial ischemia was not powered to detect a dose-response relationship, the dose-dependent effect of CD34$^+$ cells is still unclear in the clinical setting. Importantly, several investigators have reported that the proliferative, migratory, and vasculogenic functions of EPCs are impaired in patients with coronary risk factors such as diabetes, hypertension, smoking, and aging [20, 21]. Such interpatient differences in stem cell potential and variation in patient characteristics in each treatment group may be critical for evaluation of cell-based therapy. In the present study, patients with Buerger's disease were younger and had less coronary risk factors than those with PAD. The difference in such clinical backgrounds may have led to the result that the CD34$^+$ cell yield was significantly greater in patients with Buerger's disease than in those

with PAD. The effect of G-CSF for EPC mobilization is also an important factor. Previous clinical experience in the hematology field has revealed that $>3 \times 10^6$ $CD34^+$ cells/kg could be obtained from 80% of healthy donors following G-CSF administration (unpublished data from Kirin Pharmaceutical Inc.). Therefore, we hypothesized that $>10^6$ $CD34^+$ cells/kg would be harvested from most patients with CLI and that the patient number and characteristics would be equivalent in each group. However, the frequency of a $CD34^+$ cell yield $<10^6$ cells/kg was as high as 35%, especially in older patients with PAD. As a result, the incidences of PAD (versus Buerger's disease) and hypertension were higher in the Lo dose group, and only three patients with Buerger's disease actually received the Hi dose of $CD34^+$ cells. In the present study, such an unbalanced distribution in patient characteristics may have resulted in the lack of a significant dose-response relationship for $CD34^+$ cells for therapeutic neovascularization.

We quit the case enrollment when we noticed that only three of six patients in the Hi dose cohort could actually receive the target dose of $CD34^+$ cells. Aside from the lower than expected cell yield, confirmation of a high therapeutic response in the lower dose groups was another reason for study termination. Although we hypothesized that an Efficacy Score ≥ 4 might be observed in 0% of patients in the Lo dose group and in 10%–25% of patients in the Mid dose group at week 12, the incidence was as high as 83% in the Lo dose group and 63% in the Mid dose group. The favorable trend in the lower dose groups suggests the clinical efficacy of $CD34^+$ cells in CLI patients in whom at least 10^5 cells/kg (Lo dose group in this study) can be transplanted.

As for the safety evaluation, there were no severe adverse events for which a causal relationship to the cell therapy could not be denied. Although mild to moderate adverse events were frequent, these events were transient and expected. A recent clinical trial [22] suggested that pathogenic angiogenesis, such as arteriovenous shunt, might be an adverse event relating to BM MNC transplantation in patients with Buerger's disease. However, serial limb DSA and fundus oculi examinations demonstrated no pathogenic angiogenesis following $CD34^+$ cell transplantation in this study. No malignant tumor was also clinically identified during the study period. These outcomes suggest the safety and feasibility of this cell-based therapy in patients with CLI.

In conclusion, this prospective dose-escalation clinical trial revealed that transplantation of autologous and G-CSF-mobilized $CD34^+$ cells may be safe and feasible in no-option patients with CLI. Although both subjective and objective parameters of limb ischemia serially improved following the cell therapy, the efficacy of i.m. cell transplantation needs to be evaluated by comparison with an appropriate control group receiving G-CSF only or placebo. These findings encourage a randomized controlled phase IIb/III clinical trial in the future.

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Clinical trial registration: http://clinicaltrials.gov/ct/show/NCT00221143.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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