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 ± 1.0) × 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 × 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 × 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

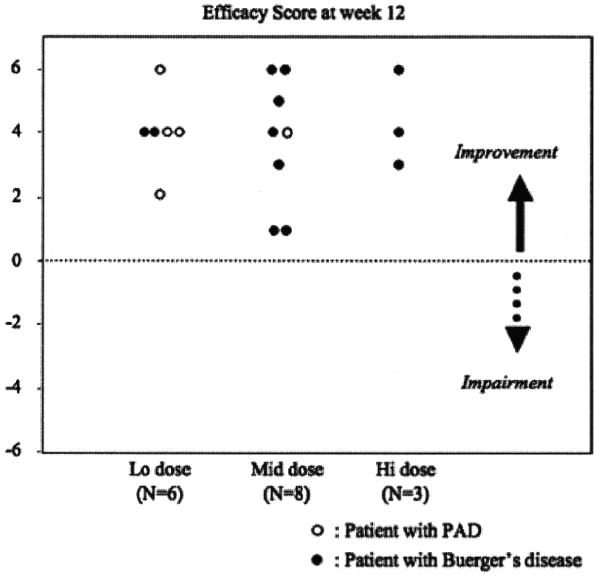

Figure 1. The Efficacy Score at week 12 following CD34$^+$ cell transplantation, which was the primary endpoint of this study, was positive, indicating improvement in limb ischemia in all patients. However, the Efficacy Score was not significantly different among the three dose groups. Abbreviations: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

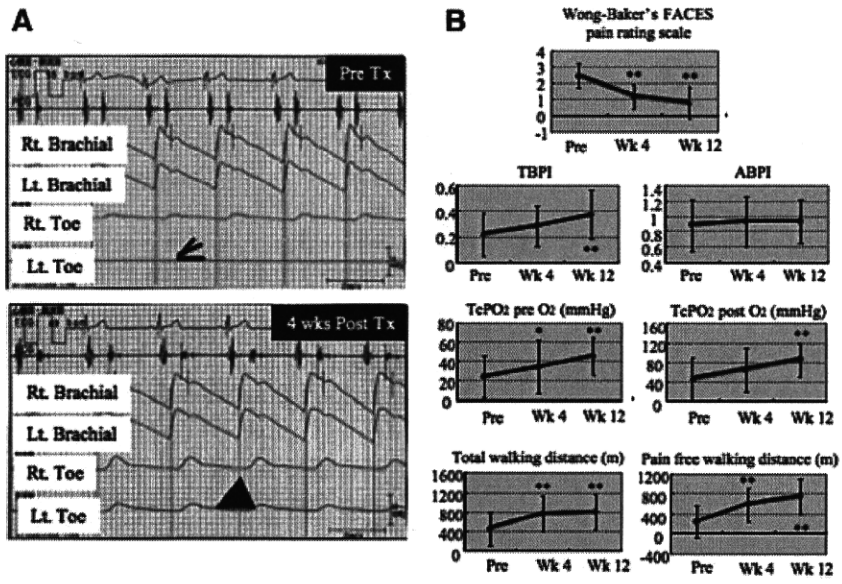

Figure 2. Improvement of efficacy parameters following $CD34^+$ cell therapy. **(A):** Representative recording of the pressure pulse wave in bilateral brachial and toe arteries (a male patient with Buerger's disease, aged 21 years). A nonpulsatile (flat) wave form (arrow) was observed in the left toe 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.

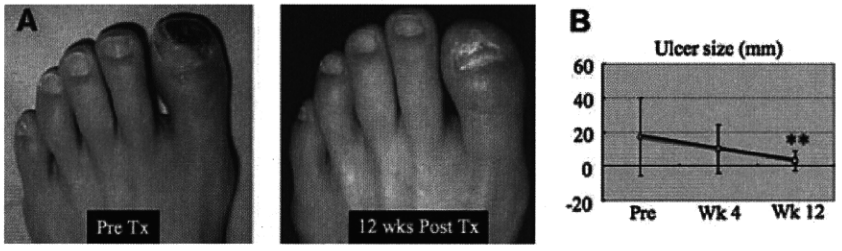

Figure 3. Healing of limb ulcer/gangrene after $CD34^+$ cell transplantation. **(A):** Representative pictures demonstrating healing of toe ulcer/gangrene following $CD34^+$ cell transplantation (a male patient with Buerger's disease, aged 36 years). **(B):** Serial changes in ulcer size in all patients.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

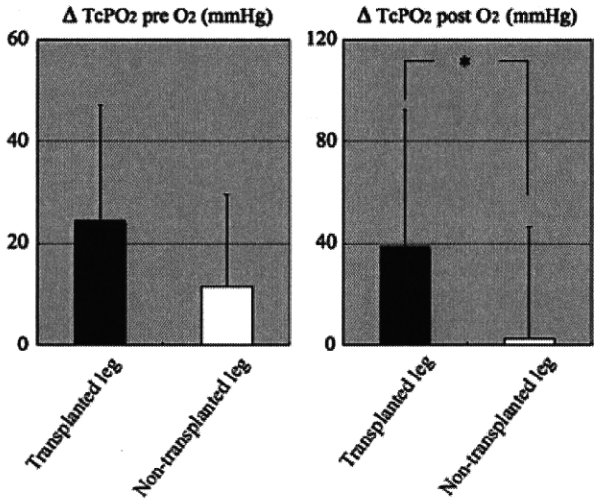

Figure 4. Change (Δ) in transcutaneous partial oxygen pressure ($TcPO_2$) before and after oxygen inhalation 12 weeks after $CD34^+$ cell transplantation in the transplanted and nontransplanted leg in patients with bilateral chronic limb ischemia ($n = 11$). $*p < .05$.

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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Therapeutic Potential of Unrestricted Somatic Stem Cells Isolated from Placental Cord Blood for Cardiac Repair Post Myocardial Infarction

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Therapeutic Potential of Unrestricted Somatic Stem Cells Isolated from Placental Cord Blood for Cardiac Repair Post Myocardial Infarction

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Objective—Unrestricted somatic stem cells (USSCs) were successfully identified from human cord blood. However, the efficacy of USSC transplantation for improving left ventricular (LV) function post myocardial infarction (MI) is still controversial.

Methods and Results—PBS, 1×10^6 human fibroblasts (Fbr), 1×10^5 USSCs (LD), or 1×10^6 USSCs (HD) were transplanted intramyocardially 20 minutes after ligating the LAD of nude rats. Echocardiography and a microtip conductance catheter at day 28 revealed a dose-dependent improvement of LV function after USSC transplantation. Necropsy examination revealed dose-dependent augmentation of capillary density and inhibition of LV fibrosis. Dual-label immunohistochemistry for cardiac troponin-I and human nuclear antigen (HNA) demonstrated that human cardiomyocytes (CMCs) were dose-dependently generated in ischemic myocardium 28 days after USSC transplantation. Similarly, dual-label immunostaining for smooth muscle actin and class I human leukocyte antigen or that for von Willebrand factor and HNA also revealed a dose-dependent vasculogenesis after USSC transplantation. RT-PCR indicated that expression of human-specific genes of CMCs, smooth muscle cells, and endothelial cell markers in infarcted myocardium were significantly augmented in USSC-treated animals compared with control groups.

Conclusions—USSC transplantation leads to functional improvement and recovery from MI and exhibits a significant and dose-dependent potential for concurrent cardiomyogenesis and vasculogenesis. (***Arterioscler Thromb Vasc Biol.* 2009; 29:1830-1835.**)

Key Words: USSC ■ cardiomyogenesis ■ vasculogenesis ■ cell therapy ■ myocardial infarction

Irreversible myocardial damage post myocardial infarction (MI) results in congestive heart failure (CHF), which is a growing worldwide clinical issue.[1] The long-standing axiom explaining the pathophysiology of the cardiac pump failure was the limited capacity of the damaged myocardium for self-repair and tissue regeneration.[2] Currently, no medication or therapeutic procedure applied clinically, except for cardiac transplantation, has significant efficacy for replacing the myocardial scar with functioning contractile tissue. Therefore, given the major morbidity and mortality associated with MI and CHF, new approaches have been sought to address the principal pathophysiological deficits responsible for these conditions, namely loss of blood vessels and cardiomyocytes (CMCs). Recently, the identification of stem cells capable of contributing to tissue regeneration has ignited significant interest in the possibility that cell therapy could have the potency of repairing damaged myocardium.

A multipotent stem cell population with high proliferative potential was isolated from human umbilical cord blood and termed unrestricted somatic stem cells (USSCs) by Kogler and colleagues.[3] USSCs have been suggested as a more immature cell type than bone marrow (BM) mesenchymal stem cells (MSCs), by the potential to differentiate into osteoblasts, chondrocytes, adipocytes, neurons, and CMCs. The cells exhibit an extended life span and longer telomeres when compared with the MSCs. In addition, these cells grow adherently and can be expanded up to 10^{15} cells without losing pluripotency in culture.[3,4] The application of USSCs did not induce macroscopic or microscopic tumors 6 months after transplantation into a fetal sheep model, suggesting long-term safety of the USSC therapy in normal heart tissue.[3] These data suggest that USSC transplantation could be a promising strategy for the regeneration of damaged mesenchymal tissue such as infarcted myocardium. Kim et al[5]

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performed intramyocardial injection of USSCs 4 weeks after MI in swine. Engrafted USSCs were immunohistochemically identified in the infarct region 4 weeks after cell transplantation, and regional and global LV function significantly improved in pigs receiving USSCs compared with those receiving media. However, the differentiation fate of the transplanted USSCs especially into CMCs, smooth muscle cells (SMCs), and endothelial cells (ECs), and the precise mechanism is still unclear. Moelker et al[6] evaluated the outcome of intracoronary delivery of USSCs 1 week after MI in swine. Intracoronary infusion of USSCs caused micro infarctions, resulting in increase in infarct size. Global and regional left ventricular (LV) function was similar in swine receiving USSCs and those receiving medium. Immunohistochemical examination revealed that CMC and EC markers were not expressed in USSCs surviving in the border zone myocardium. Although mechanisms underlying the discrepant results between Kim's and Moelker's studies are unclear, cell administration route or timing of cell delivery after MI may relate to the different outcomes. Another issue is that such large animal studies are not ideal for evaluating the autocrine and paracrine effects of engrafted USSCs because of the limiting availability of antibodies and primers, which specifically distinguish human proteins/genes from those of swine. Therefore, in this study, we performed a series of experiments using intramyocardial transplantation of USSCs into immunodeficient rats with acute MI to precisely elucidate the vasculogenic and cardiomyogenic potential of USSCs by physiological, histological, and molecular approaches.

Materials and Methods

Detailed procedures in histological, physiological, and molecular analyses are described in the supplemental materials (available online at http://atvb.ahajournals.org).

Experimental Animals

Female athymic nude rats (F344/N Jcl rnu/rnu, CLEA Japan Inc, Tokyo, Japan) aged 7 to 8 weeks and weighting ≈130 to 145 g were used in this study. The institutional animal care and use committees of RIKEN Center for Developmental Biology approved all animal procedures, including human cell transplantation.

Human Unrestricted Somatic Stem Cell Preparation

USSCs and human fibroblasts were isolated, cultured, and prepared as previously described.[3]

Induction of Myocardial Infarction and Cell Transplantation

Rats were anesthetized with ketamine and xylazine (60 mg/kg and 10 mg/kg, IP, respectively). MI was induced by ligating the left anterior descending coronary artery (LAD) as described previously.[7] Twenty minutes after the LAD ligation, the cells were then transplanted into the periinfarct zone by injection with a 27G needle in a series of 6×20 μL injections of 1×10^5 (LD) USSCs, 1×10^6 (HD) USSCs, or 1×10^6 human fibroblasts (Fbr) resuspended in 120 μL of PBS or the same volume of PBS without cells (n=16 in each group).

Physiological Assessment of LV Function

Transthoracic echocardiography was performed to evaluate LV function immediately before and 5 and 28 days after MI as described previously.[7] Immediately after the final echocardiography on day 28, the rats underwent cardiac catheterization for more invasive and precise assessment of global LV function as described previously.[7,8]

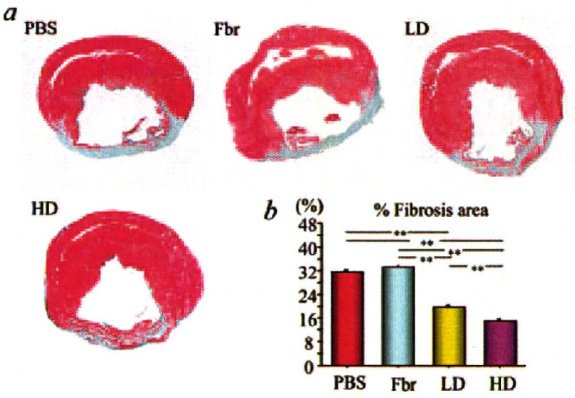

Figure 1. Histological evaluation of left ventricular (LV) remodeling after myocardial infarction (MI). a, Representative Masson-trichrome staining at day 28 in each group. Fbr indicates human fibroblasts; LD, 1×10^5 unrestricted somatic stem cells (USSCs); HD, 1×10^6 USSCs. b, Ratio of fibrosis area/ entire LV area (% fibrosis area) at day 28 in each group. $^{**}P<0.01$ (n=10 in each group).

Statistical Analysis

The results were statistically analyzed with the use of a software package (Statview 5.0, Abacus Concepts Inc). All values were expressed as mean±SE. Paired *t* tests were performed for comparison of data before and after treatment. The comparisons among 4 groups were made with 1-way ANOVAs. Post hoc analysis was performed by Scheffe test. Differences of $P<0.05$ were considered statistically significant.

Results

Morphometric Evaluation of Capillary Density and Infarct Size

LV remodeling as evaluated by % fibrosis area showed a dose-dependent inhibition in rats receiving USSCs ($P<0.01$ for HD versus LD, Fbr or PBS and LD versus Fbr or PBS). Percent fibrosis area was similar in Fbr and PBS groups (Figure 1).

Myocardial neovascularization assessed by capillary density on day 28 was enhanced in rats receiving USSC transplantation in a dose-dependent manner ($P<0.05$ for HD versus LD, $P<0.01$ for HD versus Fbr or PBS, and LD versus Fbr or PBS). Capillary density in Fbr group was similar as that in PBS group (supplemental Figure I).

Thus, transplantation of USSCs, not Fbr, significantly preserved LV structural integrity post MI. The histological efficacy of USSCs was dose-dependently observed.

Transplanted USSCs Dose-Dependently Preserve LV Function After MI

There were no significant differences in preoperative echocardiographic parameters, LVEDD, LVESD, FS, and RMWS among HD, LD, Fbr, and PBS groups (data not shown). Echocardiography on day 5 revealed that the functional parameters were also similar in all groups (data not shown). Left ventricular lateral wall motion on day 28 was better preserved in the USSC-treated groups compared with other groups (supplemental Figure IIa). Change in FS during 23

days (between day 5 and day 28 after cell transplantation) was significantly greater in the HD group than either Fbr or PBS group. Although a change in FS had a tendency to be greater in HD group than LD group, the difference was not statistically significant. The change in FS in Fbr group was also not significantly different from the PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and low versus Fbr or PBS). Similarly, the change in RWMS after transplantation was significantly lower (better preserved) in LD and HD groups compared with the Fbr or PBS group. The change in RWMS in the Fbr group was not significantly different from the PBS group ($P<0.05$ for HD versus LD and LD versus Fbr, $P<0.01$ for HD versus Fbr or PBS and LD versus PBS; supplemental Figure IIb).

Invasive hemodynamic study performed 4 weeks after transplantation revealed that heart rates were similar in each group (data not shown). The +dP/dt, absolute value of −dP/dt and EF were significantly greater in the HD group as compared to the LD, Fbr, or PBS groups. In addition, the LD group was significantly better than the Fbr or PBS group (+dP/dt: $P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; −dP/dt: $P<0.01$ for HD versus Fbr or PBS and $P<0.05$ for HD versus LD and LD versus Fbr or PBS; EF: $P<0.01$ for HD versus Fbr or PBS and $P<0.05$ for HD versus LD and LD versus Fbr or PBS). The LVEDP 4 weeks after MI was significantly lower in HD and LD groups compared to the Fbr and PBS groups ($P<0.01$ for HD versus Fbr or PBS and LD versus Fbr or PBS). However, LVEDP 4 weeks after MI in HD group was similar as that in LD group. The +dP/dt, −dP/dt, EF and LVEDP 4 weeks after transplantation in the Fbr group were not significantly different from those in PBS group (supplemental Figure IIc).

Based on these data, transplantation of USSCs, not Fbr, significantly preserved global and regional LV function post MI and the functional effect of USSC transplantation was generally dose-dependent, where the HD USSC group exhibited the greatest effect on cardiac functional improvements.

Transplanted USSCs Dose-Dependently Differentiate into CMCs

Double staining of GATA4 or Nkx2.5, an early cardiomyogenic marker, and HMA at day 5 revealed that GATA4 or Nkx2.5-positive immature cardiac stem/progenitor cells were negative for HMA in the PBS and Fbr groups. In contrast, in USSC-treated groups, double-positive cells for GATA4 or Nkx2.5 and HMA were observed as immature cardiac stem/progenitor cells derived from human USSCs (Figure 2a and 2b and supplemental Figure IIIa through IIIh). Double staining for cTn-I, a mature CMC marker, and HMA was performed to detect cardiomyogenic plasticity of transplanted USSCs at day 10. Human mitochondria-positive cells were identified in both Fbr and USSC groups, but human USSC-derived cardiomyogenic cells, which were double positive for HMA and cTn-I, were observed only in the USSC groups (Figure 2c). Differentiated human CMCs derived from the transplanted USSCs were mainly identified in the rat peri-infarct myocardium by double staining for cTn-I and HNA at day 28 (supplemental Figure IVa through IVe). The observation at day 28 was also confirmed by dual labeling for cTn-I

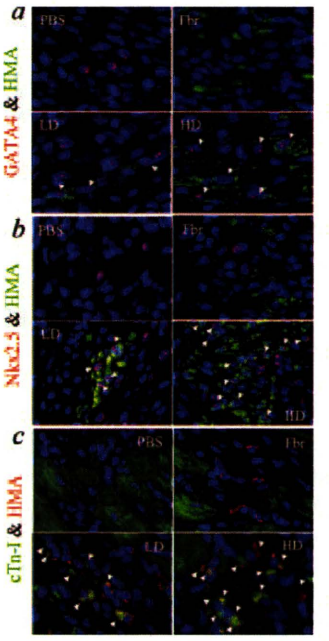

Figure 2. Representative immunofluorescent staining for immature cardiac markers and human mitochondria antigen (HMA) at days 5 and 10. a, Double immunofluorescent staining for GATA4 and HMA in each group at day 5. Few GATA4-positive immature CMCs (red) were identified, however no HMA-positive human cells (green) were detected in PBS group. HMA-positive and GATA4-negative cells or HMA-negative and GATA4-positive cells were observed in Fbr group. Few cells expressing both GATA4 and HMA were observed in LD group and relatively more double-positive cells were identified in HD group (×600). White arrows show nuclei of immature human CMCs. b, Double immunofluorescent staining for Nkx2.5 and HMA in each group at day 5. Few Nkx2.5-positive immature CMCs (red) were identified, however no HMA-positive human cells (green) were detected in PBS group. HMA-positive and Nkx2.5-negative cells or HMA-negative and Nkx2.5-positive cells were observed in Fbr group. Few cells expressing both Nkx2.5 and HMA were observed in LD group and relatively more double-positive cells were identified in HD group (×600). White arrows show nuclei of immature human CMCs. c, Representative double immunofluorescent staining for cardiac troponin-I (cTn-I; green) and HMA (red) in each group at day 10. Human CMCs expressing both cTn-I and HMA, were dose-dependently observed after USSC transplantation (×600). White arrows show human CMCs.

and HMA (supplemental Figure IIIi through IIIl). These findings suggest that USSCs have the potential to differentiate into mature CMCs after transplantation into infarcted myocardium. A dose-dependent distribution of human CMCs in rat myocardium was observed in samples stained with cTn-I and HNA (Figure 3a through 3d). In fact, the density of human CMCs in ischemic myocardium detected as double-positive cells for HNA and cTn-I were dose-dependently increased in ischemic myocardium at day 28 ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS). Total (both human and rat) CMC density was also dose-dependently augmented in ischemic myocardium at day 28 ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS), suggesting that the USSCs also support myocardial regeneration through a paracrine mechanism (Figure 3e). Frequency of the human CMCs to total (rat and human) CMCs was dose-dependently increased after USSC trans-

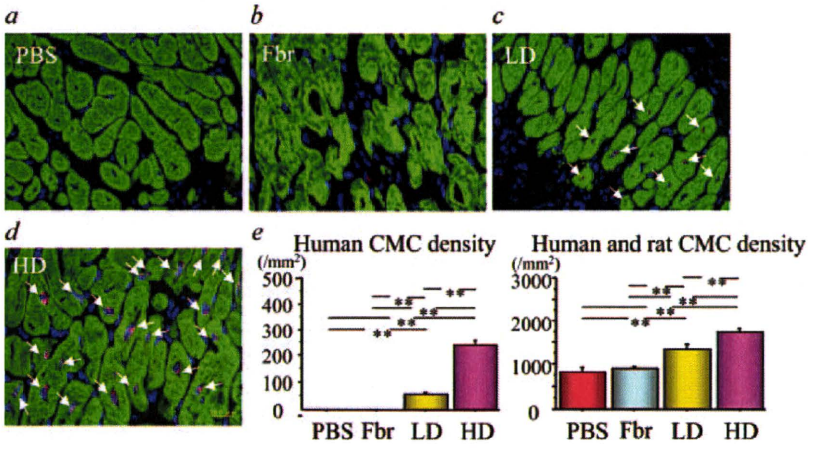

Figure 3. Histological evaluation of human CMC development in rat ischemic myocardium at day 28. a through d, Representative double immunofluorescent staining for cTn-I and HNA at day 28 in each group (×400). White arrows show nuclei of human CMCs. The double-positive cells for cTn-I and HNA derived from the transplanted USSCs were dose-dependently observed in ischemic myocardium (×400). e and f, Densities of human CMCs (the double-positive CMCs) and total (both human and rat) CMCs on day 28. *$P<0.05$; **$P<0.01$ (n=10 in all groups).

plantation ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; supplemental Figure Ve).

We explored whether USSC transplantation may contribute to cardiac repair post MI partially by stimulating proliferation of resident CMCs. Double staining for Ki67, a marker of proliferating cells, and cTn-I revealed dose-dependent distribution of Ki67-positive CMCs in the ischemic myocardium 7 days after USSC transplantation, but not PBS or Fbr administration (supplemental Figure VIa through VId). In fact, density of the proliferative CMCs in the ischemic myocardium at day 7 was significantly greater in HD group than LD, Fbr, and PBS groups and in LD group than Fbr and PBS groups ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; supplemental Figure VIe). These data indicate that transplanted USSCs may have the potential to stimulate proliferation of resident CMCs, thereby contribute to cardiac regeneration after MI.

The present results suggest that transplanted USSCs may have the potential to differentiate into mature CMCs and preserve the recipient's CMCs in the infarcted region. The data also demonstrate that a dose-dependent increase of the cardiac regenerative effect was observed between the two USSC transplant groups, whereas in the Fbr and PBS groups, no mature human CMCs were observed.

Transplanted USSCs Dose-Dependently Differentiate Into ECs

Differentiated human ECs derived from the transplanted USSCs were observed in the vasculatures within peri-infarct area by double staining for vWF and HNA (Figure 4a through 4d, supplemental Figure IVf through IVj). Density of the double-positive cells was greater in HD group than LD, Fbr, or PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS, $P<0.05$ for Fbr versus PBS). Density of total (both human and rat) ECs was also greater in HD group than LD, Fbr, or PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; Figure 4e).

Thus, locally transplanted USSCs were incorporated into sites of neovascularization, resulting in contribution to both vasculogenesis by USSCs and angiogenesis by rat ECs in ischemic myocardium.

Transplanted USSCs Dose-Dependently Differentiate Into SMCs

Human SMCs derived from the transplanted USSCs were mainly identified in vascular structures within peri-infarct area by double staining for SMA and HLA-ABC (Figure 5a through 5d, supplemental Figure IVk through IVo). Human SMCs were observed after USSC transplantation and similar to the CMC and EC analyses, a dose-dependent increase in

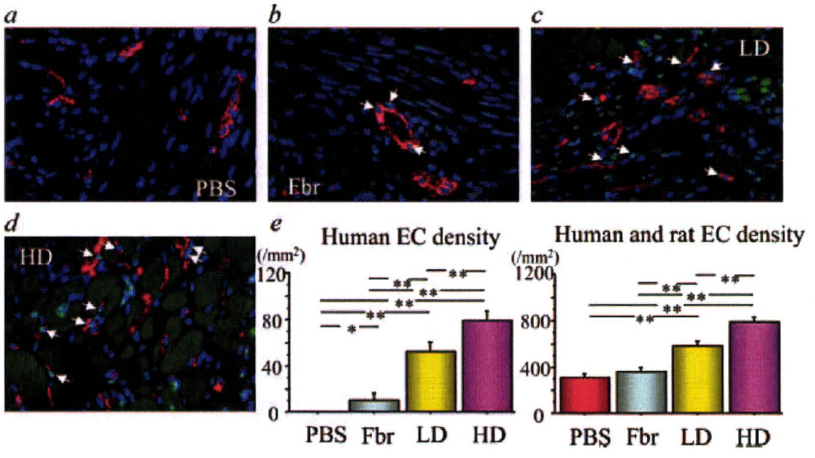

Figure 4. Histological evaluation of human endothelial cell (EC) development. a through d, Representative double immunofluorescent staining for von Willebrand factor (vWF; red) and human nuclear antigen (HNA; green) at day 28 in each group (×400). In PBS group (a), differentiated human ECs were not identified. In Fbr group (b), differentiated human ECs were rarely detected. In LD group (c), human ECs were more frequently demonstrated than Fbr and PBS groups. In HD group (d), human ECs were further more frequently identified than LD group. Arrows indicate human ECs. e, Densities of human ECs (the double-positive ECs) and total (both human and rat) ECs on day 28 in the ischemic myocardium. *$P<0.05$; **$P<0.01$ (n=10 in all groups).

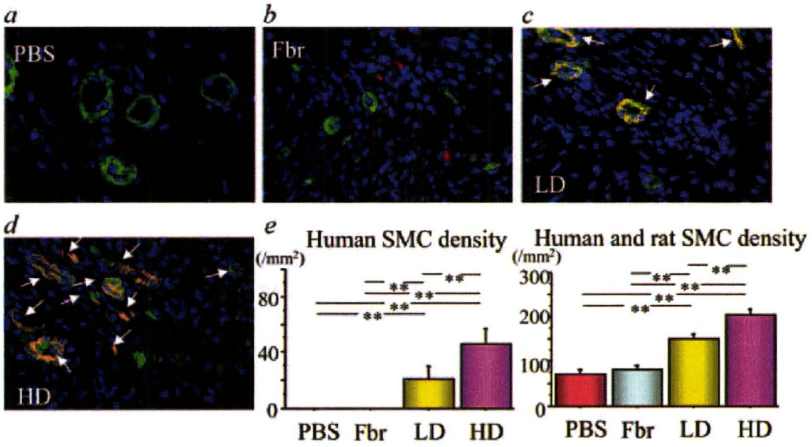

Figure 5. Histological evaluation of human smooth muscle cell (SMC) development. a through d, Representative double immunofluorescent staining for smooth muscle actin (SMA; green) and human leukocyte antigen (HLA)-ABC (red) at day 28 in each group (×400). Human SMCs were identified as double-positive cells (arrow). In PBS (a) and Fbr groups (b), differentiated human SMCs were not identified. In LD group (c), differentiated human SMCs were rarely identified. In HD group (d), human SMCs were more frequently identified than LD groups. e, Densities of human SMCs (the double-positive SMCs) and total (both human and rat) SMCs on day 28 in the ischemic myocardium. $^{**}P<0.01$ (n=10 in all groups).

SMCs was confirmed between the USSC transplant groups. In contrast, differentiated human SMCs were not identified in PBS and Fbr groups ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS). Total SMC density was also greater in HD group than LD, Fbr, or PBS group ($P<0.01$ for HD versus LD, Fbr, or PBS and LD versus Fbr or PBS; Figure 5e).

These findings suggest that transplanted USSCs exhibit a dose-dependent potency for differentiating into SMCs as well as preserving recipient SMCs in the infarcted myocardium.

Discussion

Cell transplantation is currently gaining a growing interest as a potent and novel means of improving prognosis of patients with cardiac failure. The basic assumption is that left ventricular dysfunction is largely attributable to the loss of a critical number of CMCs and that it can be partly reversed by implantation of new contractile cells into the postinfarction scars. The therapeutic strategy for cardiac failure with coronary artery disease should be focused on regenerating not only blood vessels but also cardiac muscle to improve the poor prognosis of the disease.

Many reports using various stem cells such as fat tissue–derived multipotent stem cells,[9] multipotent stem cells from BM or skeletal muscle,[10,11] and cardiac-resident progenitor cells,[12–15] which are capable of adopting the cardiomyogenic and vasculogenic fate, are also generating a great deal of interest. However, these novel cell therapies still have several problems for future clinical application. For example, techniques to efficiently and less invasively isolate, purify, and expand the numerically minor population of the potent stem cells will need to be optimized for clinical use, and experimental data from mammals larger than mice will surely be warranted. Moreover, other key questions such as (1) precise mechanism of tissue repair/regeneration and efficacy against LV dysfunction,[7,16–19] (2) optimization of cell dose, and (3) development of optimal delivery techniques also remains to be clarified.

Generally, umbilical cord blood is abundantly available, can be routinely harvested without any risk to the donors, and is seldom infected with agents, which give it a definite advantage for the development of cell therapeutics in regenerative medicine. In the case of autologous cell therapy, patients need to wait for the time of cell harvest, isolation, or expansion in a cell culture facility before undergoing transplantation. However, umbilical cord stem cells are routinely kept frozen after the whole procedure of the cell preparation is completed and therefore can be readily available for transplantation. The USSCs, which Kogler et al first identified from human cord blood in 2004, grow adherently, can be expanded up to 10^{15} cells without losing pluripotency in culture, and differentiates along mesodermal and endodermal lineages in animal models, suggesting significant potency of the USSC therapy in various clinical settings. In the present study, we have tried to confirm the multi-lineage developmental potency and the tissue plasticity of human USSCs after transplanting into immunodeficient (athymic nude) rats with acute MI. To detect the multi-lineage differentiation of the USSCs, we have performed not only immunohistochemistry but also RT-PCR for human-specific markers of CMCs, SMCs, and ECs. These sensitive assessments revealed dose-dependent augmentation of cardiomyogenesis and vasculogenesis of human USSCs in rat-infarcted myocardium. FISH analysis using human and rat genome probes indicated that cell fusion was not mainly involved in the process of the multi-lineage regeneration after transplantation of USSCs. The FISH analysis provided mechanistic information, indicating engraftment and differentiation versus cell fusion during cardiac regeneration by human USSCs. These results were consistent with the previous single cell PCR analysis in the case of sheep liver regeneration by human USSCs.[3] Immunohistochemical quantification of total (human and rat) CMCs, SMCs, or ECs in rat-infarcted myocardium also revealed dose-dependent preservation of the recipient cardiac cells probably because of a paracrine effect of the USSCs on recipient cell development. The multi-lineage potential was accompanied with dose-dependent enhancement of capillary density, inhibition of LV fibrosis, and preservation of LV function. These findings strongly suggest that USSCs may be useful for cardiomyogenic and vasculogenic regeneration in the infarcted myocardium by both autocrine and paracrine mechanisms. Considering future clinical application of the USSCs, a major limitation of the present study was a lack of assessing immune rejection, because the current study was performed using immunodeficient rats. Although a therapeutic effect of human USSCs using cyclosporine A immunosu-

pression was clearly demonstrated in a previous preclinical study using the swine model of chronic MI,[5] further investigation of long-term safety and efficacy of the cell therapy will be necessary, especially in the case of allogenic transplantation.

In conclusion, the multi-lineage differentiation potential of human USSCs for cardiomyogenic and vasculogenic regeneration of the infarcted myocardium was demonstrated by immunohistochemical and molecular assessments. The USSC therapy resulted in dose-dependent increase in capillary density, inhibition of LV remodeling, and improvement of LV function. Taken together with feasibility of the cell isolation and efficiency of the culture expansion, USSCs could be used as a highly valuable resource for cellular cardiomyoplasty in the future and could be the novel strategy to be translated from bench to bedside.

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Disclosures

Christina Willwerth, Stephan Wnendt, and William L. Fodor are employees of ViaCell Inc.

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Supplemental Materials

Supplementary Methods

Human Unrestricted Somatic Stem Cell Preparation

USSCs and human fibroblasts were isolated, cultured, and prepared as previously described [1]. The USSCs were characterized by cell surface markers using flow cytometry with antibodies against CD13, CD14, CD29, CD31, CD33, CD 34, CD44, CD45, CD49e, CD56, human leukocyte antigen class II (Beckmann Coulter) and CD133 (Miltenyi Biotech) according to the manufacturer's protocol. These cells were preserved at –80 °C until the day of cell transplantation.

Theses cells were washed with serum free Iscove's Modified Dulbecco's Medium (IMDM) twice by centrifugation, the supernatant was discarded, and the cells were resuspended in PBS. The number of surviving cells was enumerated using a hemacytometer and $1x10^6$ human fibroblasts, $1x10^5$ (Low dose, LD) or $1x10^6$ (High dose, HD) USSCs were prepared in 120 μl of PBS for transplantation.

(a) Physiological Assessment of LV Function Using Echocardiography and Micro-tip Conductance Catheter

Transthoracic echocardiography (SONOS 5500, Philips Medical Systems) was

performed to evaluate LV function immediately before and 5 and 28 days after MI as described previously [2]. Under general anesthesia with ketamine and xylazine, LV end-diastolic and end-systolic dimensions (LVEDD and LVESD, respectively) and fractional shortening (FS) were measured at the midpapillary muscle level. Regional wall motion score (RWMS) was evaluated per published criteria [3]. Immediately after the final echocardiography on day 28, the rats underwent cardiac catheterization for more invasive and precise assessment of global LV function as described previously [2 4]. A 2.0 Fr micromanometer-tipped conductance catheter (SPR 838, Millar Instruments Inc, Houston, Tx) was inserted via right carotid artery into the LV cavity. LV pressure and its derivative (LV dP/dt) were continuously monitored using a multiple recording system (Pressure-Volume Conductance System ARIA and Pressure-Volume Analysis Using P-V Analysis Software [Millar Instruments Inc, Houston, Tx] and Power Lab® DAQ System [ADInstrument, Australia]).

Heart rate (HR), LV end-diastolic pressure (LVEDP), LV ejection fraction (EF) and the maximum and minimum LV dP/dt (+dP/dt and -dP/dt, respectively) were continuously recorded for 20 minutes. All data were acquired under stable hemodynamic conditions.

All procedures and analyses were performed by an experienced researcher who was blinded to the treatments.

(b) Tissue harvesting

In each treatment group, ten rats were euthanized 28 days after transplantation with an over dose of ketamine and xylazine At necropsy, hearts were sliced in a broad-leaf fashion into 4 transverse sections from apex to base, embedded in OCT compound, snap frozen in liquid nitrogen, and stored at -80°C for Masson-trichrome staining, immunohistochemistry and Fluorescence In Situ Hybridization (FISH). Rat hearts in OCT blocks were sectioned, and 5 μm serial sections were collected on slides followed by fixation with 4.0% paraformaldehyde at 4°C for 5 minutes and stained immediately. Selective dissection of a portion of the myocardium surrounding the peri-infarct area was taken to isolate total RNA for reverse transcriptase-PCR (RT-PCR) experiments. In each group, three rats were euthanized at day 5 and additional three were also killed at day 10 for histological evaluation.

(c) Morphometric Evaluation of Capillary Density and Infarct Size

Histochemical staining with isolectin B4 (Vector Laboratories, Burlingame, CA) was performed, and capillary density was morphometrically evaluated by histological examination of 5 randomly selected fields of tissue sections recovered from segments of LV myocardium subserved by the occluded LAD. Capillaries were recognized as tubular structures positive for isolectin B4.

To elucidate the severity of myocardial fibrosis, Masson-trichrome staining was

performed on frozen sections from each tissue block, and the stained sections were used to measure the average ratio of fibrosis area to entire LV area (percent fibrosis area).

(d) Immunofluorescent staining

To detect transplanted human cells in rat ischemic myocardium, immunohistochemistry was performed with the following human-specific antibodies; human leukocyte antigen (HLA)-ABC (BD Pharmingen, San Diego, CA), human mitochondria antigen (HMA) (Chemicon International, Temecula, CA) and human nuclear antigen (HNA) (Chemicon International). Staining specificity of HLA-ABC, HMA and HNA against human cells and lack of cross reactivity to rat cells were confirmed by histochemical staining with the use of human and rat heart samples as described previously [25]. Double staining for GATA4 or Nkx2.5 (Santa Cruz, Santa Cruz, CA) and HMA was performed to detect human cells expressing early cardiac transcription factor in rat ischemic myocardium at day 5. Double staining for cardiac troponin-I (cTn-I) (Chemicon International) as mature cardiomyocyte marker and HMA or HNA was performed to detect cardiomyogenic plasticity of transplanted human cells at day 10 and 28. Dual-labeled immunohistochemistry with HLA-ABC and smooth muscle actin (SMA) was performed to detect double-positive cells as human SMCs in rat myocardium. Similarly, double immunohistochemistry with von Willebrand factor (vWF) (Chemicon International) and HNA was performed to detect double positive cells as human ECs in ischemic myocardium.

Double staining for Ki67 (Pharmingen, San Diego, CA) and cTn-I was performed to detect proliferative CMCs in the ischemic myocardium 5 days after USSC transplantation. The secondary antibodies for each immunostaining are as follows: Alexa Flour 594-conjugated goat anti-mouse IgG_1 (Molecular Probes, Carlsbad, CA) for HLA-ABC staining, Alexa Flour 488 and 594-conjugated goat anti-mouse IgG_1 (Molecular Probes) for HMA staining, Alexa Flour 594 -conjugated goat anti-rabbit IgG (H+L) (Molecular Probes) for GATA4 or Nkx2.5 staining, Alexa Flour 488-conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for cTn-I staining, Alexa Flour 488-conjugated goat anti-mouse IgG_{2a} (Molecular Probes) for SMA, Alexa Flour 488 and 594-conjugated goat anti-mouse IgG1 for HNA, Alexa Flour 594-conjugated goat anti-rabbit IgG for vWF (Molecular Probes) and Alexa Flour 596-conjugated goat anti-mouse IgG_1 (Invitrogen, Carlsbad, CA) for Ki67. DAPI solution was applied for 5 minutes for nuclear staining.

Number of total (both human and rat) CMCs was evaluated by counting cTn-I-positive cells in 5 randomly selected fields within rat ischemic myocardium at day 28. Similarly, the number of human CMCs in ischemic myocardium was examined by counting double-positive cells for HNA and cTn-I. Number of human SMCs detected as double-positive cells for HLA and SMA, and number of human ECs detected as double-positive cells for HNA and vWF 28 days after MI were also morphometrically quantified. Number of the proliferative CMCs in ischemic myocardium was examined by counting double-positive cells for Ki67 and cTn-I in 5 randomly selected fields within rat

ischemic myocardium at day 5.

***Fluorescence In Situ Hybridization* (FISH)**

To identify whether cardiac repair occurred through cell fusion or not, FISH was performed with biotin-conjugated DNA probe for human genome and digoxigenin-conjugated DNA probe for rat genome (Chromosome Science Lab, Sapporo, Japan) in MI tissue. Tissue sections were fixed immediately with Carnoy's fixation (MEOH and acetic acid) at room temperature for 10 minutes, dehydrated, and denatured according to the manufacture's protocol as described previously [2]. Sections were hybridized with the DNA probes for human and rat genome overnight at 37°C. After post-hybridization wash, Alexa 488-conjugated streptavidin and Cy-3-conjugated anti-digoxigenin was applied and slides were counterstained with DAPI, then fluorescent microscopically examined. The directly differentiated cells or the fused cells were identified as the cells positive for human genome only or the double-positive cells for both genomes, respectively. The directly differentiated or fused cells were quantified in 5 randomly selected fields in the ischemic myocardium at day 28.

(e) RT-PCR analysis of USSCs and ischemic heart tissue

Total RNA was obtained from USSCs before transplantation and from tissues of rat ischemic LV at day 28 using Tri-zol (Life Technologies) according to the manufacture's

instructions [2]. In brief, the first-strand cDNA was synthesized using the RNA LA PCR Kit Ver1.1 (Takara, Otsu, Japan), amplified by Taq DNA polymerase (Advantage–GC cDNA PCR Kit (Clontech, Mountain View, CA) and AmpliTaq Gold DNA polymerase (Applied Biosystems, Foster City, CA)). PCR was performed using a PCR thermalcycler (MJ Research PTC-225, Bio-Rad Laboratories, Waltham, MA). The human GAPDH (hGAPDH), total (human and rat) GAPDH, human CD34 (hCD34), human brain natriuretic peptide (hBNP), human cardiac troponin-I (hcTn-I), human myosin heavy chain-β (hMHC-β), human KDR (hKDR), human eNOS (heNOS) and human Nkx 2.5 (hNkx 2.5) were amplified by Taq DNA polymerase (AmpliTaq Gold DNA polymerase, Applied Biosystems) at the following conditions: 35 cycles of 30 seconds initial denaturation at 94°C, annealing at 56°C for 1 minute, and 30 seconds of extension at 72°C according to the manufacture's instructions. Human SMA (hSMA), human sm22α (hsm22α) and human CD31 (hCD31) were amplified by Taq DNA polymerase (Advantage–GC cDNA PCR Kit) under the following conditions: 37 cycles of 30 seconds initial denaturation at 94°C, annealing at 68°C for 3 minute, and 7 minutes of elongatation at 64°C according to the manufacture's instructions. Subsequently, PCR products were visualized in 1.5% ethidium bromide-stained agarose gels. Human heart RNA distributed from Clontech (premium RNA) was used as positive control.

Primers : To avoid interspecies cross-reactivity of the primer pairs between human and rat genes, we designed following human-specific primers using Oligo software