Long-term clinical outcome after intramuscular transplantation of granulocyte colony stimulating factor-mobilized CD34 positive cells in patients with critical limb ischemia

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A B S T R A C T

Background: Our phase I/IIa clinical trial revealed that intramuscular transplantation of autologous, GCSF-mobilized CD34+ cells was safe, feasible and potentially effective at week 4 and 12 post cellular therapy in 17 patients with chronic critical limb ischemia (CLI) (5 patients with atherosclerotic peripheral arterial disease (PAD) and 12 with Buerger’s disease). However, long-term outcome of the cell therapy has yet to be reported.

Methods and results: Incidence of major clinical events and physiological parameters of limb ischemia were evaluated at week 52, 104, 156 and 208 post CD34+ cell therapy. No patients died by week 104, whereas 3 patients with PAD died by week 156 and 1 patient with Buerger’s disease died by week 208 due to cardiac complications. No patients underwent major amputation, whereas 1 patient with Buerger’s disease underwent unplanned minor amputation by week 104. CLI-free ratio was 88.2% at week 52 and 104, 156 and 208 post CD34+ cell therapy. No patients died by week 104, whereas 3 patients with PAD died by week 156 and 1 patient with Buerger’s disease died by week 208 due to cardiac complications. No patients underwent major amputation, whereas 1 patient with Buerger’s disease underwent unplanned minor amputation by week 104. CLI-free ratio was 88.2% at week 52 and 104, 92.3% at week 156 and 84.6% at week 208 in all patients. Significant improvement of toe brachial pressure index versus baseline was sustained up to week 208 and that of transcutaneous partial oxygen pressure was kept up to week 156. The Wong-Baker FACES pain rating scale, ulcer size and exercise tolerance significantly improved at week 52, the final evaluation time point, compared with baseline. Subgroup analysis revealed the similar outcome in patients with Buerger’s disease.

Conclusions: Favorable clinical outcomes as well as physiological evidences strongly indicate the long-term benefit of GCSF-mobilized CD34+ cell transplantation for retrieval from CLI, especially in patients with Buerger’s disease.
PAD patients represent ischemic rest pain and/or skin ulceration/gangrene, which condition is classified as critical limb ischemia (CLI) [1]. Prognosis of the CLI patients is quite poor in terms of both survival and limb salvage despite conventional therapeutic options such as medications, surgical treatments and endovascular interventions [2]. Especially, prognosis of CLI patients, in whom conventional revascularization is neither successful nor indicated, is extremely poor, and the development of novel strategy for blood flow recovery is urgently needed for such intractable patients.

Bone marrow (BM)-derived endothelial progenitor cells (EPCs), the small fraction (0.1–2%) of total mononuclear cells (MNCs), can promote physiological and pathophysiological neovascularization by their abundant potency of proliferation, migration, homing and differentiation into endothelial lineage [3]. Therapeutic potential of EPCs has been established by a number of preclinical studies for hindlimb, myocardial and cerebral ischemia [4–6]. Following these preclinical achievements, recent clinical studies have indicated potential effectiveness of transplantation of MNCs obtained from BM or peripheral blood (PB) for no-option patients with CLI [7,8]. Another report revealed that a small number of harvested CD34+ cells, an EPC-enriched fraction, was a negative prognostic factor associated with amputation and death following either BM- or PB-MNC transplantation in patients with CLI [9]. This finding suggests an important role of EPCs for therapeutic neovascularization and may provide a reasonable rationale of transplantation of EPCs isolated from crude MNCs in patients with CLI. Our group has reported the short-term outcome after intramuscular injection of granulocyte colony stimulating factor (GCSF)-mobilized CD34+ cells in no-option patients with CLI. The phase I/IIa clinical trial revealed safety, feasibility and potential effectiveness of CD34+ cell transplantation for 12 weeks following the cell therapy [10]. Burt et al. [11] also reported clinical outcome of intramuscular transplantation of GCSF-mobilized CD133+ cells, another EPC-enriched fraction, in nine patients with CLI (seven with atherosclerotic PAD, one with Buerger’s disease and one with thromboembolic disorder). One year post CD133+ cell therapy, seven of the nine patients were free from leg amputation, suggesting potential usefulness of the cell therapy for limb salvage. However, long-term outcome of these EPC therapies remains to be investigated.

In the present study, we evaluate the long-term safety and efficacy of intramuscular transplantation of GCSF-mobilized CD34+ cells in no-option patients with CLI for up to four years post cellular therapy.

2. Materials and methods

2.1. Patient enrollment and treatment procedures

Seventeen patients with CLI were enrolled in a phase I/IIa clinical trial of intramuscular transplantation of GCSF-mobilized CD34+ cells from November 2003 to January 2007 (clinical trial registration: NCT00221143). Details of the study protocol have been previously described [10]. In brief, the inclusion criteria were (a) atherosclerotic PAD or Buerger’s disease with ≥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 Rutherford category 4–6, (d) no indication for endovascular treatment or bypass surgery, and (e) aged 20–80 years. The exclusion criteria were mainly set to exclude patients at high risk for the adverse events of GCSF, apheresis and CD34+ cells.

Outline of the treatment procedures was as follows; All patients received subcutaneous continuous administration of GCSF (10 μg/kg per day for 5 days) to mobilize EPCs from BM. Leukapheresis (AS.TEC204; Fresenius HemoCare, Bad Homburg, Germany, http://www.fresenius.com) was performed to harvest PB-MNCs on day 5. The leukapheresis product was kept at room temperature overnight until the magnetic separation of CD34+ cells was started using CliniMACS Instrument, CD34 reagent, phosphate-buffered saline/EDTA buffer and tubing set (Miltenyi Biotec, Bergisch Gladbach, Germany, http://www.miltenyibiotec.com). Purified CD34+ cells dissolved in 10 ml saline were intramuscularly injected into 40 sites (30 sites in the calf, six sites in the sole, and four sites in the intertoe muscle) of the leg with more severe ischemia under spinal anesthesia. Dose of CD34+ cells were 10² cells/kg (Low) in six patients, 5 × 10³ cells/kg (Mid) in eight patients and 10⁴ cells/kg (Hi) in three patients.

2.2. Collection and evaluation of the data

According to the original study protocol, efficacy parameters such as Rutherford scale, the Wong-Baker’s FACES pain rating scale, ankle brachial pressure index (ABPI), toe brachial pressure index (TBPI), transcutaneous partial oxygen pressure (TcPO₂), ulcer size, total walking distance (TWD) and pain-free walking distance (PFWD) on treadmill were measured at baseline, week 4, 12 and 52 (year 1) post CD34+ cell therapy. In addition, after completing the original study period, Rutherford scale, ABPI, TBPI and TcPO₂ were also examined at week 104, 156 and 208 (year 2, 3 and 4, respectively) in survived patients. Because the efficacy score reflecting the improvement of the Wong-Baker’s FACES pain rating scale, TBPI and TWD [10] at week 4, 12 or 52 was similar in Low, Mid and Hi dose groups (data not shown), changes in each parameter were not compared between the three dose groups. Instead, these efficacy parameters in all patients were compared between baseline and each time point post CD34+ cell transplantation.

As for the safety evaluation, occurrence of the clinical events defined as 1) major amputation of the treated leg, 2) unplanned minor amputation of the treated leg, 3) death due to chronic CLI, 4) death due to cardiovascular diseases except chronic CLI, 5) death due to other causes and 6) retreatment of the treated leg by bypass surgery, endovascular intervention or any kind of cellular therapy as well as other serious adverse events were recorded until week 52 according to the original study protocol. Incidence of these clinical events was also followed until week 208 post CD34+ cell therapy. Overall survival ratio and the event-free survival ratio were evaluated up to week 208 post CD34+ cell therapy. According to the original study protocol, fundus oculi examination was performed at week 52 as well as baseline, week 4 and 12 in all patients to assess pathogenic retinal angiogenesis post CD34+ cell therapy. To evaluate the incidence of malignant tumor post cell therapy, fecal occult examination was performed at week 52 in all patients. Serum prostate specific antigen test was also examined in male patients and mammography and uterine cytology were undergone in female patients at week 52.

All efficacy parameters, overall and event-free survival ratios were evaluated in either all patients (n = 17) or patients with Buerger’s disease (n = 12). The efficacy and safety data at week 104, 156 and 208 were retrospectively collected from July 1 to August 31, 2010 by checking the medical records.

The study protocols for both initial clinical trial and the prospective data collection/analysis conformed to the Declaration of Helsinki and were approved by the Ethics Committee of Institute of Biomedical Research and Innovation on October 1, 2003 and June 24, 2010, respectively. Informed consent was obtained from each subject.

2.3. Statistical analysis

All data were shown as mean ± standard deviation (SD). Patient characteristics were compared between patients with...
atherosclerotic PAD and those with Buerger’s disease by Student’s unpaired t test and Fisher’s exact test. Serial changes of Rutherford scale were analyzed by Wilcoxon signed-rank test. The linear mixed model was applied in order to evaluate a longitudinal variation, and the differences between baseline and each time point after cell therapy were assessed by Dunnett–Hsu test. Overall and event-free survival ratios were analyzed by Kaplan–Meier method. The significance level was set at 0.05 for all statistical tests. Analyses were performed using SAS software, version 9.1.2 (SAS Institute, Cary, NC; http://www.sas.com).

3. Results

3.1. Baseline clinical characteristics

Baseline characteristics of the patients were shown in Table 1. There were notable differences between patients with atherosclerotic PAD and those with Buerger’s disease. Age and incidences of hypertension and diabetes mellitus were significantly higher in patients with atherosclerotic PAD compared with those with Buerger’s disease.

3.2. Efficacy evaluation

In all subjects, Rutherford scale was 4 in 7 patients and 5 in 10 at baseline. Following CD34+ cell therapy, Rutherford scale significantly improved by week 4 and the significant change sustained up to week 208 (Fig. 1A). The proportion of the patients with Rutherford scale 0–3 (CLI-free ratio) serially increased and peaked (92.3%, [95% CI 0.63–0.99]) at week 156 (Fig. 1B). These results suggest that autologous CD34+ cell transplantation may lead to significant retrieval from CLI in a month and the favorable outcome may sustain for 4 years post cellular therapy.

Subgroup analysis revealed the favorable results in patients with Buerger’s disease similarly as in all CLI patients; Rutherford scale significantly improved by week 8 and the improvement sustained until week 208 in patients with Buerger’s disease (Fig. 1C). CLI-free ratio serially increased and peaked (100%, [95% CI 0.71–1.00]) at week 156 (Fig. 1D).

To evaluate the severity of lower limb ischemia, we analyzed various physiological parameters including ABI, TBPI, and TcPO2. ABI, which was within normal range or mildly lowered in most patients at baseline, did not significantly change after the cell therapy in either all patients or patients with Buerger’s disease (Fig. 2A). TBPI significantly improved by week 12 in all patients and by week 52 in Buerger’s disease patients compared with baseline. These significant improvements sustained until week 208 (Fig. 2B). TcPO2 in both all patients and Buerger’s disease patients significantly improved by week 12 compared with baseline. These significant changes sustained until week 156 (Fig. 2C). The severity of rest pain was evaluated by the Wong-Baker’s FACES pain rating scale at baseline, week 4, 8, 12, 24 and 52. The Wong-Baker’s FACES pain rating scale in either all patients or Buerger’s disease patients significantly decreased at week 4 than baseline (Supplementary Fig. A). The significant improvement was continuously observed until week 52. Skin ulcers were present in 7 patients with Buerger’s disease and 3 patients with atherosclerotic PAD at baseline. The ulcer size in either all patients or Buerger’s disease patients significantly decreased at week 4 compared with baseline. The significant improvement continued until week 52 (Supplementary Fig. B). Ulcers completely healed in all patients with Buerger’s disease and 2 patients with atherosclerotic PAD by week 52. However, ulcers did not heal in 1 patient with atherosclerotic PAD until week 208. No recurrence ulcers developed up to week 208 in patients experiencing complete ulcer healing after CD34+ cell therapy. Regarding the exercise tolerance, TWD and PFWD were evaluated by treadmill test at baseline, week 4, 12 and 52. TWD and PFWD in either all patients or Buerger’s disease patients significantly increased by week 4 compared with baseline. These significant improvements continued until week 52 (Supplementary Fig. C, D).

These outcomes indicate that initial significant improvement of objective parameters of blood flow, ulcer size and exercise tolerance as well as subjective scale of rest pain in the treated legs may persist for years post CD34+ cell therapy. Subgroup analyses disclose similarly favorable outcomes in patients with Buerger’s disease.

3.3. Safety evaluation

Serious adverse events occurred in 5 patients from week 12 to week 208 after the cell therapy (Table 2). No patients died by week 104, whereas 3 patients with PAD died by week 156 and 1 patient with Buerger’s disease died by week 208. All causes of death were cardiac diseases unrelated to CD34+ cell therapy; heart failure in 3 patients and sudden death due to aortic valve stenosis in 1 patient. In a patient with Buerger’s disease, multiple ulcers except a toe gangrene healed by week 52, then minor amputation was performed at week 53 for the unhealed gangrene. Thus, no serious adverse events were causatively related to CD34+ cell therapy.

Kaplan–Meier analysis revealed that overall survival ratio was 75.0% at week 208 in all patients. The corresponding rate for patients with Buerger’s disease was 90.9% (Fig. 3A). Event-free survival rate at week 208 was 70.6% in all patients and 83.3% in patients with Buerger’s disease (Fig. 3B).

As previously reported [10], fundus oculi examination revealed no pathogenic angiogenesis in the retina at week 4 and 12 post CD34+ cell therapy in all patients. No development of retinopathy was also confirmed by fundus oculi test at week 52 in all patients. No malignant tumors were detected by fecal human hemoglobin test, urine cytology, chest and abdominal computed tomography in all patients, by serum prostate specific antigen test in male patients and by mammography and uterine cytology in female patients at week 52. No active retinopathy and malignant tumors were clinically identified in all patients until week 208.

4. Discussion

The present study is the first clinical investigation assessing the long-term efficacy and safety of GCSF-mobilized CD34+ cell
implantation in patients with CLI. The most striking outcome of this study is significant improvement of Rutherford scale and retrieval from CLI in most patients even 4 years post CD34\(^+\) cell therapy (CLI-free ratio was 92.3\% at week 156 and 84.6\% at week 208). Because mortality and major amputation ratio are extremely higher in CLI patients compared with non-CLI patients\(^2\), the reduction of the disease stage post CD34\(^+\) cell transplantation may lead to improvement of dismal prognosis of CLI. Previous investigators elucidating the usefulness of BM- or PB-MNCs in CLI patients for more than three years\(^{12-15}\) have never demonstrated the serial change in the disease severity following MNC therapy. The present study first disclosed the long-term benefit of cellular therapy for retrieval from CLI. As for the physiological parameters of lower limb ischemia, initial significant improvement of TBPI or TcPO\(_2\) sustained up to 4 or 3 years post CD34\(^+\) cell therapy, respectively. These findings suggest that CD34\(^+\) cell therapy may contribute to sustained improvement of the lower limb ischemia. The long-term results in the physiological assessments are clinically important because previous reports of MNC implantation lacked such physiological evaluation\(^{13,14}\) or failed to demonstrate the significant improvement of these parameters for years\(^ {12}\). The Wong-Baker’s FACES pain rating scale, ulcer size, TWD and PFWD, which were obtained from baseline to week 52, also showed significant improvement until the final evaluation point. These results suggest that blood flow recovery post CD34\(^+\) cell transplantation may result in rest pain reduction, ulcer healing and improvement of exercise tolerance, potentially contributing to the increase in CLI-free ratio.

Regarding the death and amputation following CD34\(^+\) cell therapy, overall mortality was 0\% until week 104, 17.6\% at week 156 and 23.5\% at week 208. No patients underwent major amputation, whereas 1 patient (5.9\%) with Buerger’s disease underwent unplanned minor amputation by week 104. Mortality at week 156 or 208 in this study seems to be comparable to that in the previous reports of BM-MNC\(^ {12}\) and GCSF-mobilized MNC therapies\(^ {14}\) and relatively lower than that in a study of non-mobilized MNC implantation\(^ {13}\). Major amputation ratio was extremely lower in the present study than the previous reports for MNC therapies. Because patients’ background such as proportion of atherosclerotic PAD or hemodialysis was not equivalent among these clinical trials, comparative study between CD34\(^+\) cell and MNC implantations will be needed to evaluate the superiority of the different therapeutic strategies.

A recent study\(^ {16}\) suggests 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 fundus oculi examinations revealed no active retinopathy after CD34\(^+\) cell transplantation in this study. No malignant tumor and arteriovenous shunt were also clinically identified during the follow-up period. These outcomes suggest that CD34\(^+\) cell therapy may not induce pathogenic angiogenesis-related adverse events.

**Fig. 1.** Serial changes in the proportion of Rutherford scale (0—6) and CLI-free ratio following CD34\(^+\) cell transplantation in all patients (n = 17 at week 0—104, n = 13 at 156—208) (A, B) and patients with Buerger’s disease (n = 12 at week 0—104, n = 11 at week 156—208) (C, D). *, p < 0.05 versus baseline; **, p < 0.01 versus baseline.
In the present study, overall survival ratio at week 208 was 75.0% in all patients and 90.9% in patients with Buerger’s disease. It is well known that Buerger’s disease patients have lower mortality and incidence of major amputation than atherosclerotic PAD patients, because Buerger’s disease is not generally associated with cardiovascular risk factors. In fact, patients with Buerger’s disease were younger and had less coronary risk factors such as diabetes and hypertension than those with atherosclerotic PAD in this study. Several investigators reported that the proliferative, migratory and vasculogenic potentials of EPCs are impaired in patients with diabetes mellitus, hypertension, smoking, and aging [17,18]. Moreover, it was reported that EPCs obtained from Buerger’s disease patients highly express endothelial lineage molecules compared with those from atherosclerotic PAD patients [19]. These findings suggest that differences in the number and function of EPCs and in the expression of EPC marker molecules might result in the superior efficacy of CD34+ cell therapy in patients with Buerger’s disease compared with atherosclerotic PAD. It would be ideal to evaluate the efficacy and safety of CD34+ cell transplantation in Buerger’s disease or atherosclerotic PAD, separately. However, subgroup analysis was performed only for patients with Buerger’s disease, because the number of atherosclerotic PAD was small (n = 5) in this study. The subgroup study revealed favorable outcomes of CD34+ cell therapy in patients with Buerger’s disease similarly in all subjects. These results indicate that CD34+ cell implantation may be safe and potentially effective for 3–4 years in CLI patients, especially in patients with Buerger’s disease. Long-term observation in a more number of patients with atherosclerotic PAD will be necessary to separately evaluate the clinical outcome post CD34+ cell therapy in the subpopulation of CLI.

As for the application of CD34+ cells for other ischemic diseases, Losordo et al. [20] recently reported that patients with refractory angina receiving intramyocardial injections of GCSF-mobilized CD34+ cells experienced significant improvement in angina frequency and exercise tolerance compared with those receiving

<table>
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<th>Diagnosis</th>
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<td>Death due to heart failure</td>
<td>909</td>
</tr>
<tr>
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<td>Death due to heart failure</td>
<td>1018</td>
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<tr>
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<td>Unexpected minor amputation</td>
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<td>Buerger’s disease</td>
<td>Death due to heart failure</td>
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<tr>
<td>Atherosclerotic PAD</td>
<td>Sudden death due to aortic valve stenosis</td>
<td>738</td>
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GCSF, granulocyte colony stimulating factor.

**Table 2**

Serious adverse events during 4-year follow-up after CD34+ cell therapy.

**Fig. 2.** Serial changes in ABPI (A), TBPI (B) and TcPO2 (C) during the 208-week follow-up period following CD34+ cell transplantation in all patients and patients with Buerger’s disease. *p < 0.05 versus baseline; **p < 0.01 versus baseline. ABPI, ankle brachial pressure index; TBPI, toe brachial pressure index; TcPO2, transcutaneous partial oxygen pressure. Data are shown as mean ± SD.

**Fig. 3.** Kaplan–Meier analysis. (A): Overall survival ratios after cell therapy in all patients and patients with Buerger’s disease. (B): Event-free survival ratios after cell therapy in all patients and patients with Buerger’s disease.
placebo. These results coincide with our present outcomes in CLI in terms of relief of ischemic muscle pain following CD34+ cell therapy. The favorable results in the phase II trial for angina may support therapeutic usefulness of this cell-based strategy for improvement of tissue ischemia in CLI. Recent preclinical studies indicate therapeutic potential of CD34+ cells not only for ischemic diseases but also other disorders such as unhealing fracture [21] and liver cirrhosis [22] through vasculogenesis and tissue regeneration. BM-derived CD34+ cells may be clinically applied for a variety of diseases because of their abundant regenerative property in the future.

The present study was not a randomized, controlled clinical trial. As described above, the number of subjects, especially patients with atherosclerotic PAD, was relatively small. In addition, clinical data at week 104, 156 and 208 were retrospectively collected and analyzed. Recently, results of several randomized and controlled clinical trials regarding BM- or GCSF-mobilized MNC implantation in CLI patients were reported [23–27]. In these studies, the MNC therapy group at least demonstrated trends towards improvement of amputation-free survival, ulcer healing or rest pain scale compared with placebo or standard of care group. Such promising results in the previous and the present studies warrant further randomized trials to compare the efficacy and safety of CD34+ cell transplantation versus crude MNC therapy in patients with CLI.

5. Conclusions

Transplantation of autologus and GCSF-mobilized CD34+ cells may lead to long-term improvement of lower limb ischemia, thereby contributing to rest pain reduction, ulcer healing and improvement of exercise tolerance. Considering the poor prognosis of CLI patients, especially those in whom conventional revascularization is not indicated, these favorable outcomes support that CD34+ cell therapy may be a promising therapeutic modality for such intractable patients.

Author disclosures statement

The authors have no conflicting financial interests.

Acknowledgments

This work was partly supported by Health and Labour Sciences Research Grants (H14-trans-001) from Japanese Ministry of Health, Labour and Welfare. The authors thank Ms. Noriko Kakuta for her secretarial assistance.

Appendix A. Supplementary data

Supplementary data related to this article can be found online at http://dx.doi.org/10.1016/j.atherosclerosis.2012.07.031.

References


