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Local Transplantation of Granulocyte Colony Stimulating Factor-Mobilized CD34$^+$ Cells for Patients With Femoral and Tibial Nonunion: Pilot Clinical Trial

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Key Words. CD34$^+$ cells • Clinical trial • G-CSF • Nonunion • Peripheral blood

ABSTRACT

Most bone fractures typically heal, although a significant proportion (5%–10%) of fractures fail to heal, resulting in delayed union or persistent nonunion. Some preclinical evidence shows the therapeutic potential of peripheral blood CD34$^+$ cells, a hematopoietic/endothelial progenitor cell-enriched population, for bone fracture healing; however, clinical outcome following transplantation of CD34$^+$ cells in patients with fracture has never been reported. We report a phase I/IIa clinical trial regarding transplantation of autologous, granulocyte colony stimulating factor-mobilized CD34$^+$ cells with atelocollagen scaffold for patients with femoral or tibial fracture nonunion (n = 7). The primary endpoint of this study is radiological fracture healing (union) by evaluating anteroposterior and lateral views at week 12 following cell therapy. For the safety evaluation, incidence, severity, and outcome of all adverse events were recorded. Radiological fracture healing at week 12 was achieved in five of seven cases (71.4%), which was greater than the threshold (18.1%) predefined by the historical outcome of the standard of care. The interval between cell transplantation and union, the secondary endpoint, was 12.6 ± 5.4 weeks (range, 8–24 weeks) for clinical healing and 16.1 ± 10.2 weeks (range, 8–36 weeks) for radiological healing. Neither deaths nor life-threatening adverse events were observed during the 1-year follow-up after the cell therapy. These results suggest feasibility, safety, and potential effectiveness of CD34$^+$ cell therapy in patients with nonunion. STEM CELLS TRANSLATIONAL MEDICINE *2014;3:1–7*

INTRODUCTION

Most bone fractures typically heal uneventfully, although a significant proportion (5%–10%) of fractures fails to heal, resulting in delayed union or persistent nonunion [1, 2]. Treatment of nonunion may require multiple operative procedures and prolonged hospitalization leading to years of disability until a union is obtained. Immune depression, hormonal milieu, malnutrition, mobility, high-energy fracture, extensive soft tissue damage, infection, irradiation, lack of contact between the bone ends, and the actual loss of bone substance are known to be risk factors for fracture-healing failure. In particular, severe skeletal injury by fracture under a compromised blood supply is a high risk for either delayed union or established nonunion [3–5]. An essential requirement for healing such intractable fracture is to restore the local blood flow, which has traditionally been accomplished through complex vascular procedures or soft tissue transfers with adequate blood supply [3–5].

Current clinical demands have been focused on cell-based therapies for bone formation as a category of regenerative medicine. Among those therapies, adult human peripheral blood (PB) CD34$^+$ cells have been reported to contain intensive endothelial progenitor cells (EPCs) as well as hematopoietic stem cells [6]. Tissue ischemia and cytokines such as granulocyte colony stimulating factor (G-CSF) mobilize EPCs from bone marrow (BM) into PB, and the mobilized EPCs specifically home to sites of nascent neovascularization and differentiate into mature endothelial cells (vasculogenesis) [7, 8]. Therapeutic potential of BM-derived CD34$^+$ cells for neovascularization in hind limb, myocardial, and cerebral ischemia has been demonstrated in both preclinical and clinical studies [9, 10].

Interestingly, recent reports indicate that BM-derived CD34$^+$ cells are capable of differentiating into osteogenic as well as hematopoietic and vasculogenic lineages [11–15]. We and other groups reported that fracture induces mobilization of EPCs from BM into PB and incorporation of the circulating EPCs into the fracture site [16–18]. Then, we performed a series of preclinical studies to demonstrate the therapeutic effect of CD34$^+$ cells for fracture healing. First, we demonstrated that systemic infusion of human circulating CD34$^+$ cells into immunodeficient rats with

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nonhealing fracture contributes to morphological and functional fracture healing by enhancing vasculogenesis and osteogenesis [19]. Next, we attempted local transplantation of CD34$^+$ cells with atelocollagen gel, a bioabsorbable scaffold, in the same animal model and demonstrated the similar effect at the lower dose compared with the systemic administration [20]. In addition, we reported the advantages of CD34$^+$ cell transplantation over mononuclear cells (MNCs) for fracture healing [21].

Based on this scientific background, we report a phase I/IIa clinical trial of transplantation of autologous, G-CSF-mobilized CD34$^+$ cells with atelocollagen scaffold in patients with femoral or tibial nonunion.

Materials and Methods

Study Design and Criteria for Subject Enrollment

This phase I/IIa clinical trial was designed as a nonrandomized, single-arm study to evaluate the safety, feasibility, and efficacy of autologous and G-CSF-mobilized CD34$^+$ cells in patients with femoral or tibial nonunion. 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 University Hospital. Finally, the institutions were allowed to start the clinical trial by the Japanese Ministry of Health, Labor, and Welfare.

The inclusion criteria were (a) tibial or femoral fracture; (b) noninfectious nonunion, defined as nonunited fracture for more than 9 months without evidence of progressive healing over the previous 3 months (according to the definition in the 1988 U.S. Food and Drug Administration guideline [22]); (c) male or female aged 20–70 years; and (d) 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 supplemental online 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.

Treatment Procedures

The scheme of the treatment procedure is shown in Figure 1. All nonunion patients enrolled in this study received subcutaneous administration of G-CSF to mobilize EPCs from BM. The basic dose of G-CSF was 5 μg/kg per day for 5 days. G-CSF was scheduled to be canceled when the white blood cell (WBC) count was $>$75,000 cells per microliter. Leukapheresis (AS.TEC204; Fresenius HemoCare, Bad Homburg, Germany, http://www.fresenius.com) was performed to harvest PB MNCs on day 5. From the safety standpoint, these procedures were performed 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. The leukapheresis product was kept at a concentration of $\leq 2.0 \times 10^8$ cells per milliliter 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/ethylenediaminetetraacetic acid 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). Immediately after the magnetic cell sorting, predefined dose (5.0×10^5 cells per kilogram) of CD34$^+$ cells were dissolved in 3 ml of atelocollagen gel (final concentration 1.5%; Koken, Tokyo, Japan, http://www.kokenmpc.co.jp/english), which was used as a bioabsorbable scaffold for retaining the cells at the transplanted site. In the previous clinical trial treating critical limb ischemia, efficacy and safety were confirmed with the transplantation of up to 1×10^6 cells per kilogram of CD34$^+$ cells and the use of 10 μg/kg G-CSF [9]. In addition, in the preclinical study using a rat-human cell xenotransplantation fracture model, transplantation of 5×10^5 cells per kilogram of CD34$^+$ cells exhibited higher efficacy than that of 5×10^4 cells with safety [20].

Cell transplantation and autologous bone grafting (ABG) was performed under general anesthesia. Following refreshing fibrous tissue at the nonunion site and the surrounding cortical bone and grafting autologous cancellous bone from iliac crest, CD34$^+$ cells dissolved in atelocollagen gel were locally administered into the fracture site using a syringe (Fig. 2). The original plate or intramedullary nail was replaced when the fracture fixation was unstable.

Endpoints

The primary endpoint of this study is radiological fracture healing (union) by evaluating anteroposterior and lateral views at week 12 following cell therapy. Radiological union was defined as bridging callus formation and absence of fracture line at the site of more than three out of four cortices, which was judged by an independent radiologist. Computed tomography was also used when radiographic imaging was inappropriate for detection of radiological healing. As a secondary endpoint, the interval between the treatment and radiological or clinical union was also assessed. Clinical union was defined as no tenderness and pain at the fracture site with weight bearing. Both radiological and clinical unions were assessed at weeks 8, 12, and 24 and at 1 year after treatment. When radiological healing was not achieved at the primary endpoint, additional assessment was performed until fracture union to assess real healing time.

For the safety evaluation, incidence, severity, and outcome of all adverse events were recorded. Screening for malignancy was performed at both baseline and 1 year after cell therapy.

Data Management and Statistical Analysis

Data were collected, stored, and managed using paper-based case report forms. Following data input, data cleaning and a logical check were performed to guarantee data quality

All data are shown as mean $\pm$ SD. Data were managed at an independent data center following approval by the institutional ethics committee.

The target number of cases was statistically determined based on the data of the historical control, for which cases were treated in the same institution by the same group of surgeons as the current trial, consisting of 11 patients with femoral or tibial nonunion (9 male and 2 female) aged 37.1 $\pm$ 14.9 years (range, 21–56 years). Demographic data and results are shown in Table 1. In the case series, 2 of 11 patients (18.1%) achieved union 12 weeks after the standard surgical treatment for nonunion. The healing ratio (18%) of the historical control was used as a threshold. In addition, the rate of fracture healing in this clinical trial was

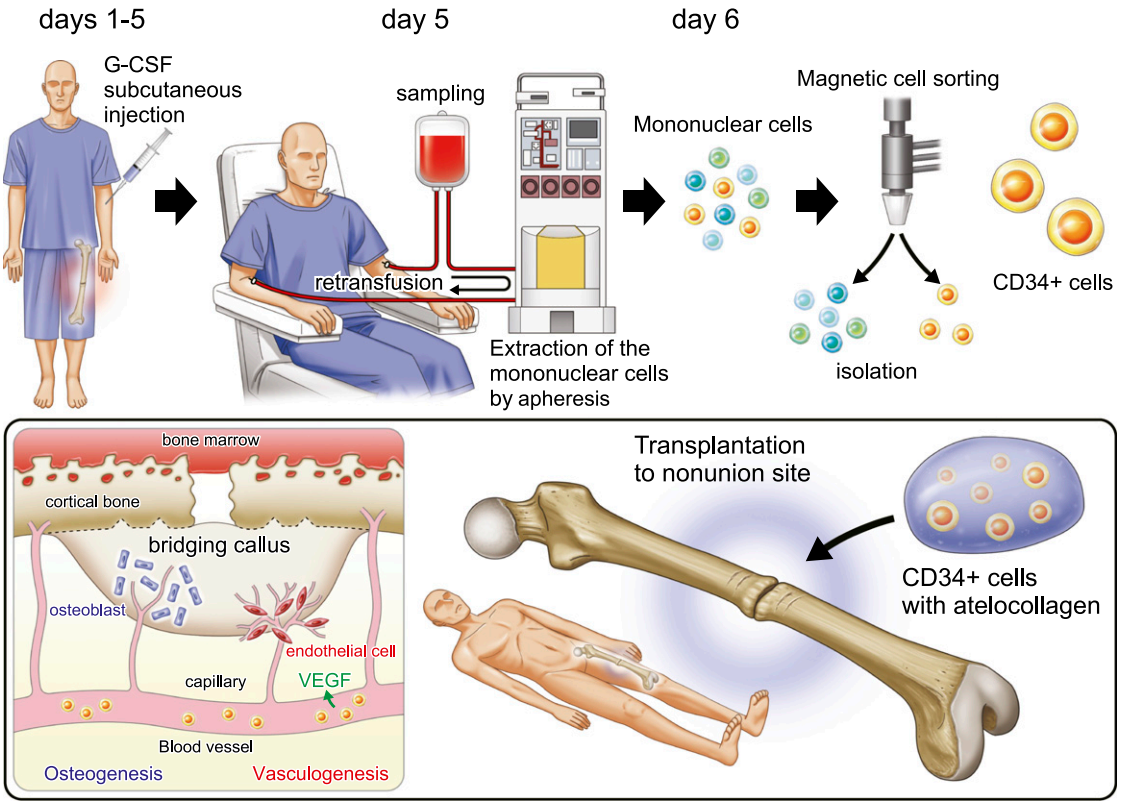

Figure 1. Schema of the treatment procedure. Abbreviations: G-CSF, granulocyte colony stimulating factor; VEGF, vascular endothelial growth factor.

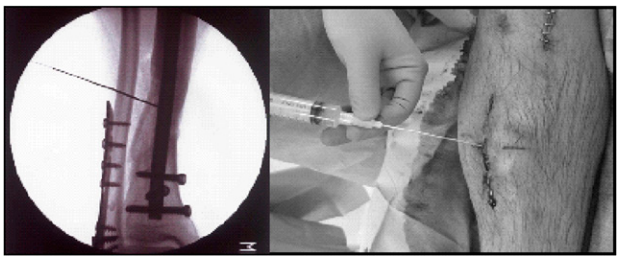

Figure 2. Procedure of $CD34^+$ cell transplantation. Following refreshing fibrous tissue at the nonunion site and the surrounding cortical bone and grafting autologous iliac bone, $CD34^+$ cells dissolved in 3 ml of atelocollagen gel were locally administered into the fracture site.

estimated to be 50% at 12 weeks after treatment on the basis of our preclinical study of human $CD34^+$ cell therapy using an immunodeficient rat model of nonunion. The Fleming single-stage procedure (one-sided α = 0.05, statistical power of 80%) revealed that 17 cases will be necessary to demonstrate the superior effectiveness of $CD34^+$ cell transplantation immediately after ABG over standard of care in this clinical trial.

Results

From March 2009 to March 2012, seven patients with nonunion were enrolled in this clinical trial. Although we extended the registration termination period from February 2011 to March 2012, we could not enroll the target number of patients (n = 17). We finally ended patient registration on March 31, 2012, and completed the last patient follow-up on August 10, 2012.

Baseline characteristics of each individual are shown in Table 2. Six patients were men and one was a woman, aged 33.9 $\pm$ 8.4 years (range, 20–45 years). The treatment site was the tibia in five patients (the tibial shaft was affected in four patients, and the tibial plateau was affected in one patient) and the femur in two patients (the femoral shaft was affected in one patient, and the femoral trochanteric area was affected in one patient). Three patients had closed injury and four had open injury; the severity of open injury was grade II in two patients, grade IIIA in one patient, and grade IIIB in one patient, according to the Gustilo-Anderson open fracture classification [23]. The initial surgical method before participating in this clinical trial was intramedullary nailing in three patients and plate open reduction and internal fixation in four patients. ABG had been applied unsuccessfully in two patients. This was the first time undergoing $CD34^+$ cell transplantation in all cases.

In all seven cases, $CD34^+$ cell transplantation was accompanied by ABG. Exchange nailing was performed in two patients, and revision plating was done in one patient. No revision of the existing fixation was performed in four patients.

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

G-CSF administration was not canceled in all patients because the WBC count never exceeded 75,000/μl during the administration period.

The apheresis product number was $2.6 \pm 0.7 \times 10^{10}$, and the frequency of $CD34^+$ cells in the apheresis product was 0.6 $\pm$ 0.4% by FACS analysis. FACS analysis revealed that the purity and viability of

Table 1. Baseline results of historical control

Case	Gender	Age (yr)	Fracture type	Anatomical site	Final operative interventions	Time to radiological union (weeks)
1	M	22	Grade I, oligotrophic	Tibia, shaft	ABG	12
2	M	23	Closed, hypertrophic	Tibia, shaft	IMN + ABG	12
3	M	45	Grade IIIB, oligotrophic	Tibia, shaft	IMN + ABG	40
4	M	57	Grade I, oligotrophic	Tibia, shaft	IMN + ABG	20
5	M	25	Closed, oligotrophic	Tibia, shaft	IMN + ABG	16
6	F	46	Grade IIIB, oligotrophic	Femur, shaft	ORIF + ABG	44
7	M	54	Closed, oligotrophic	Tibia, shaft	ORIF + ABG	44
8	M	36	Closed, oligotrophic	Femur, shaft	IMN + ABG	16
9	M	23	Grade I, atrophic	Tibia, shaft	ORIF + ABG	36
10	F	56	Closed, atrophic	Femur, shaft	IMN + ABG	40
11	M	21	Grade IIIC, oligotrophic	Femur, shaft	IMN + ABG	40

Abbreviations: ABG, autologous bone grafting; F, female; IMN, intramedullary nailing; M, male; ORIF, open reduction and internal fixation.

Table 2. Baseline characteristics of the individual patients

Case	Gender	Age (yr)	Fracture type	Anatomical site	Sequence of operative interventions
1	M	41	Closed, oligotrophic	Tibia, shaft	1. ORIF 2. Cell and ABG
2	F	38	Grade IIIA, oligotrophic	Femur, shaft	1. ORIF 2. Cell and ABG
3	M	31	Closed, oligotrophic	Femur, trochanteric	1. IMN 2. Exhange nailing, cell and ABG
4	M	34	Grade II, oligotrophic	Tibia, shaft	1. ORIF 2. Cell and ABG
5	M	28	Grade II, hypertrophic	Tibia, shaft	1. IMN 2. Dynamization 3. Exchange nailing 4. Dynamization 5. Exhange nailing, cell and ABG
6	M	20	Grade IIIB, defect	Tibia, shaft	1. IMN 2. Cell and ABG
7	M	45	Closed, defect	Tibia, plateau	1. ORIF 2. Revision plating, cell and ABG

Abbreviations: ABG, autologous bone grafting; F, female; IMN, intramedullary nailing; M, male; ORIF, open reduction and internal fixation.

the $CD34^+$ fraction following magnetic sorting were 78.0 $\pm$ 15.4% and 97.9 $\pm$ 1.3%, respectively (supplemental online Table 2).

Efficacy Evaluation

Radiological fracture healing at week 12, the primary endpoint of this study, was achieved in five of seven cases (71.4%) (Fig. 3). The two patients without fracture healing at week 12 had femoral fractures in which, ultimately, radiological healing was finally observed at weeks 19 and 36 (Fig. 3).

The interval between the cell transplantation and union, the secondary endpoint, was 12.6 $\pm$ 5.4 weeks (range, 8–24 weeks) for clinical healing and 16.1 $\pm$ 10.2 weeks (range, 8–36 weeks) for radiological healing (Table 3).

All patients were allowed to gait with partial weight bearing at week 6 and with full weight bearing at week 12 after the operation because clinical healing was achieved. At 12 weeks after the treatment, no patients complained of pain with full weight-bearing gait. All of the participants (six workers and one student) returned to their previous activities or occupations.

Safety Evaluation

Neither deaths nor life-threatening adverse events were observed during the 1-year follow-up after the cell therapy. Cervical dysplasia was found in a patient approximately 1 year after cell transplantation and was treated with a cervical conization. The operation was performed because a cervical smear test revealed suspicious carcinoma of the uterine cervix. After the operation, histological diagnosis of cervical dysplasia was confirmed. The causal relationship between this event and the cell therapy is unclear.

As described previously, severe adverse events were rare in this study. In contrast, mild to moderate adverse events, especially G-CSF- or apheresis-related events, were frequent. As for the unexpected, mild to moderate adverse events, a transient decline of WBC was only observed. All mild to moderate events were transient and disappeared without any permanent damage (Table 4).

Discussion

To the best of our knowledge, this study is the first clinical trial of transplantation of autologous, G-CSF-mobilized and purified $CD34^+$ cells in patients with tibial or femoral nonunion. In all seven patients, $CD34^+$ cell harvest, isolation, and transplantation were performed safely. The only serious adverse event in this study was cervical dysplasia 1 year after the treatment. No malignant tumor was clinically identified during the study period. Mild to moderate events relating to G-CSF and leukapheresis were frequent but transient. These outcomes indicate the feasibility and overall safety of $CD34^+$ cell therapy in patients with nonunion. In terms of effectiveness, the cell therapy combined with iliac ABG successfully achieved bone union in all seven cases and was confirmed

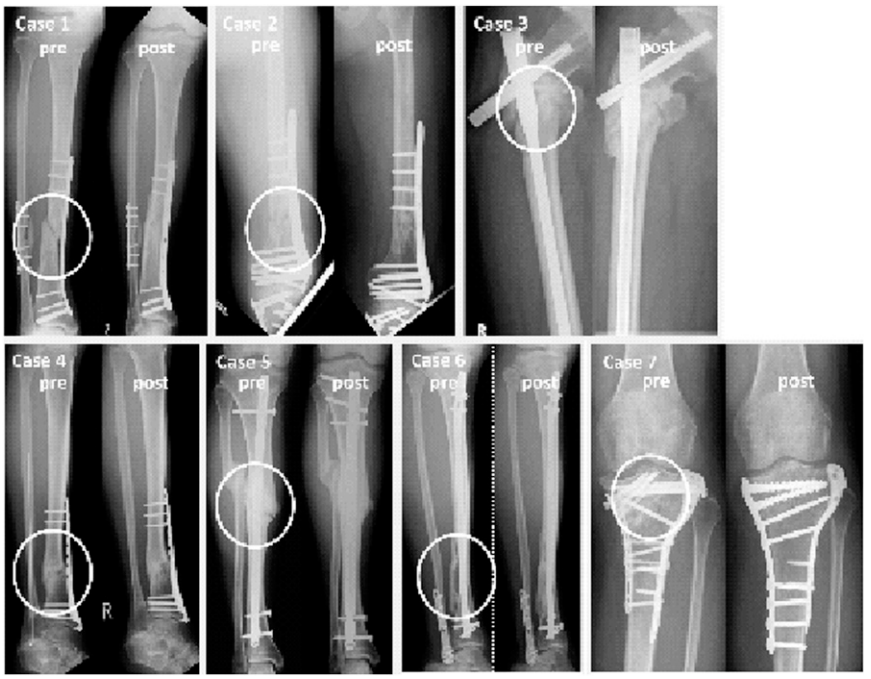

Figure 3. Preoperative and postoperative radiographs in all cases. All cases achieved union at final follow-up. Radiological fracture healing at week 12, the primary endpoint of this study, was achieved in five of seven cases. Two cases without fracture healing at week 12 (case 2 and case 3) had femoral fractures in which, ultimately, radiological healing was observed at weeks 19 and 36. Circles indicate the site of fracture.

Table 3. Radiological and clinical union of the individual patient

Case	Time to clinical union (weeks)	Time to radiological union (weeks)
1	12	12
2	12	19
3	24	38
4	12	12
5	12	12
6	8	8
7	8	12
Average	12.60 ± 5.38	16.10 ± 10.17

by clinical symptoms, radiograph, and computed tomography as early as 16.4 weeks on average after treatment. Radiological fracture healing at week 12, the primary endpoint, was confirmed in five of seven patients (71.4%). The radiological healing ratio was greater than both the predefined threshold (18%) based on the histological data and the estimated healing ratio (50%) based on the preclinical outcomes. Although this study was terminated before enrolling the target number of patients, these results suggest the potential effectiveness of $CD34^+$ cell therapy for nonunion; however, in the present study, ABG with $CD34^+$ cell therapy was applied for all seven cases. Consequently, we cannot simply evaluate the efficacy of the $CD34^+$ cell therapy apart from that of ABG alone or the combination of these therapies.

In recent years, cell therapy has been adopted in a clinical setting as an alternative and attractive strategy for bone fracture healing. Several research groups have demonstrated the usefulness of percutaneous total BM grafting for fracture healing [24–27]. Hernigou et al. reported that in 53 of 60 patients with noninfected nonunions of the tibia, bone union was achieved by percutaneous grafting of autologous total BM cells accompanied by external fixation or cast immobilization [26]. They concluded that percutaneous BM grafting is a "limited invasive technique" that is applicable under local anesthesia and functions as a simple, safe, inexpensive method in clinical cases of nonunion. Compared with transplantation of purified $CD34^+$ cells, crude BM cell therapy does not require the time and cost of magnetic cell sorting; however, our group reported that intramyocardial transplantation of human G-CSF-mobilized, total MNCs into rats with myocardial infarction represents a possible risk of severe hemorrhagic infarction through the excessive inflammation induced by abundant infiltration of hematopoietic cells [28]. Infusion of the crude BM cells might cause similarly unfavorable events in the case of fracture. In addition, we reported the advantages of $CD34^+$ cell transplantation over MNCs for fracture healing [21].

BM mesenchymal stem cells (MSCs) also have therapeutic potential for patients with fractures to reduce the time of healing and to treat nonunions. Quarto et al. were the first to report the clinical effectiveness and usefulness of BM MSCs associated with porous ceramic for large long-bone defects [29]. They treated three patients with the use of a traditional bone-graft approach and reported successful recovery 12–18 months after the treatment. Bajada et al. reported a case in which a 9-year tibial nonunion resistant to six previous surgical interventions healed 2 months after BM MSC therapy [30]. They used autologous BM MSCs expanded to 5.0×10^6 cells after 3 weeks of tissue culture, followed by a combination of calcium sulfate in pellet form along with MSCs [30]. Although these initial experiences suggest the clinical usefulness of BM MSC transplantation for nonunion, further investigations will be necessary to evaluate the safety and efficacy of the BM cell therapy.

As another novel strategy for the treatment of bone fracture nonunion, growth factors such as bone morphogenetic protein (BMP) 2 and 7 have also been gaining attention. The BMPs belong

Table 4. Summary of adverse events by severity

Adverse event (*n* = 7)		No. of patients with events	Severity (no. of events)			
			All	Mild	Moderate	Severe
SOC	PT		*n*	*n*	*n*	*n*
Blood and lymphatic system disorders	Splenomegaly	3	3	3	0	0
Investigations	Alanine aminotransferase increased	1	1	1	0	0
Investigations	Aspartate aminotransferase increased	1	1	1	0	0
Investigations	Blood bilirubin increased	2	2	2	0	0
Investigations	Blood calcium decreased	1	1	1	0	0
Investigations	Blood cholesterol increased	1	1	1	0	0
Investigations	Blood creatine phosphokinase increased	6	9	9	0	0
Investigations	Blood lactate dehydrogenase increased	6	6	6	0	0
Investigations	Blood potassium decreased	1	1	1	0	0
Investigations	Blood triglycerides increased	4	4	4	0	0
Investigations	Blood uric acid increased	4	5	5	0	0
Investigations	C-reactive protein increased	7	7	7	0	0
Investigations	γ-Glutamyltransferase increased	1	1	1	0	0
Investigations	Hematocrit decreased	7	7	7	0	0
Investigations	Hemoglobin decreased	7	7	7	0	0
Investigations	Platelet count decreased	7	7	7	0	0
Investigations	Protein total decreased	5	6	6	0	0
Investigations	Red blood cell count decreased	7	7	7	0	0
Investigations	White blood cell count decreased	1	1	1	0	0
Investigations	White blood cell count increased	1	1	1	0	0
Investigations	Platelet count increased	5	5	5	0	0
Investigations	Blood alkaline phosphatase increased	6	8	8	0	0
Investigations	Blood creatine phosphokinase decreased	1	1	1	0	0
Nervous system disorders	Hypoesthesia	1	1	1	0	0
Reproductive system and breast disorders	Cervical dysplasia	1	1	0	1	0
Vascular disorders	Deep vein thrombosis	1	1	1	0	0

Abbreviations: PT, preferred term; SOC, system organ class.

to the superfamily of transforming growth factor β and, like other cytokines, play a major role in controlling fracture healing. The osteoinductive effects of BMPs for fracture healing have been established in animal experiments, leading to the expectation of their clinical application in patients with delayed fracture healing [31, 32]. Regarding the use of BMP-7, there has been one prospective, randomized, and partially blinded clinical study investigating 122 patients (with 124 tibial nonunions) treated over a time span of 7 years in seven different trauma centers in the U.S. [31]. The insertion of an intramedullary rod accompanied by BMP-7 or ABG resulted in radiological healing in 62% (39 of 63) of the BMP-7 treated nonunions and 74% (45 of 61) of those receiving ABG at 9 months after the treatment. A review of the clinical application of BMP-7 recently published in the U.K. summarized that 74% of 384 nonunions treated with the use of BMP-7 required additional ABG [33]. These outcomes suggest that BMP-7 may not be superior to ABG for the treatment of nonunion.

Conclusion

The harvest, isolation, and transplantation of autologous, G-CSF-mobilized CD34$^+$ cells were first performed in patients with nonunion. All procedures were performed safely. Both clinical and radiological healing of the fracture was achieved in all subjects 16.4 weeks after the cell therapy and bone grafting. Promising outcomes in this phase I/IIa clinical trial encourage the application of the CD34$^+$ cells for nonunion or delayed union as a novel therapeutic modality. To elucidate the safety and efficacy of CD34$^+$ cell transplantation or combination of the cell therapy and ABG for bone fracture healing, randomized clinical trials with an appropriate control group receiving G-CSF only or placebo would be warranted. Further studies will be also needed to compare the therapeutic potential of CD34$^+$ cell therapy with that of other type of modalities such as BM MNCs, BM MSCs, and BMPs.

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Author Contributions

R.K. and T.M.: study design, surgery, manuscript drafting, final approval of manuscript; T.N. and S.Y.L.: surgery, final approval of manuscript; Y.K., T.F., and Y.M.: data collection, final approval of manuscript; S.K. and M.F.: data analysis, final approval of manuscript; T.A. and M.K.: study design, study conduct, data interpretation, revising manuscript content, final approval of manuscript; A.K.: study design, data interpretation, manuscript revision, final approval of manuscript.

Disclosure of Potential Conflicts of Interest

The authors indicate no potential conflicts of interest.

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