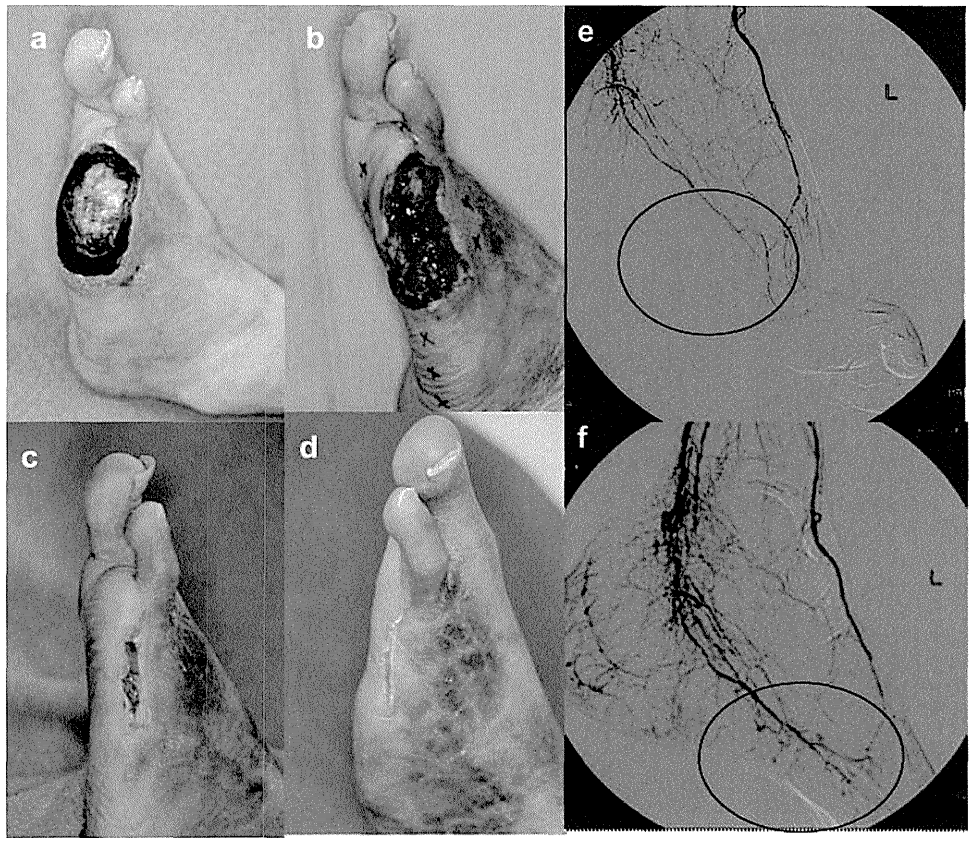

Figure 5. Case 5: A 63-year-old male with 20 years of diabetes and 4 years of CRF on hemodialysis. (a) Pretherapy: The ulcer located on the left third, fourth, and fifth toes to metatarsus did not heal for 26 weeks. SPP was 10 mmHg at this point. (b) The ulcer at time of debridement and CD34+ cell transplant. (c) At 12 weeks posttherapy with an SPP of 67 mmHg. (d) At 16 weeks posttherapy; time of complete wound closure. Currently, patient is ambulant for 2 years posttherapy without any recurrence and heterotopic ulcer with stable SPP of 66 ± 23 mmHg. (e) Angiography pretherapy; avascular area is marked with circle. (f) Angiography 12 weeks posttherapy; avascular area pretherapy has increased vascular perfusion after CD34+ cell therapy.

may contain cells not related to angiogenesis and wound healing, we believe that purified CD34+ cells are a more suitable source of stem cell therapy for angiogenesis and wound healing. Regarding this, although many investigators identify CD34+/CD133+/KDR+ cells as EPCs in basic research (5), isolating the rare cell population is clinically impractical due to the absence of a clinical-grade anti-KDR antibody. Therefore, we have chosen CD34+ cells as an enriched population of EPCs for cell isolation that was technically and clinically applicable.

Furthermore, animal studies have reported that purified CD34+ cells exhibit improved healing and vasculogenesis compared to nonpurified mononuclear cell transplantation (33). In a clinical randomized trial of direct intramyocardial injection of autologous mononuclear bone marrow cells during coronary artery bypass graft (CABG) to improve left ventricle function, it was reported that the "responder" group was transplanted with a cell population containing a significantly higher percentage and absolute number of CD34+ cells than nonresponders (11). Recently, Kawamoto et al. reported the safety and efficacy of G-CSF-mobilized CD34+ cell transplant to patients with critical limb ischemia (16). Therefore, we hypothesized that the autologous transplantation of purified CD34+ cells as enriched population of EPCs may be effective in the treatment of nonhealing chronic diabetic wounds with peripheral vascular disease. Since uncontrolled diabetes and high blood sugar level influence diabetic wound healing, our trial confirmed that the wounds were untreatable even after diabetic control and standard wound care. We designed this small size, phase I/IIa clinical trial as a prospective, uncontrolled, single-blinded study to obtain useful information for a future phase IIb/III trial. In this pilot study, we observed many clinical manifestations that significantly improved after autologous peripheral blood CD34+ cell transplant, such as wound closure, SPP, TcO_2, and lower limb pain. Measurements of ambulatory blood pressure (ABP) are reported to fail to reflect the severity of

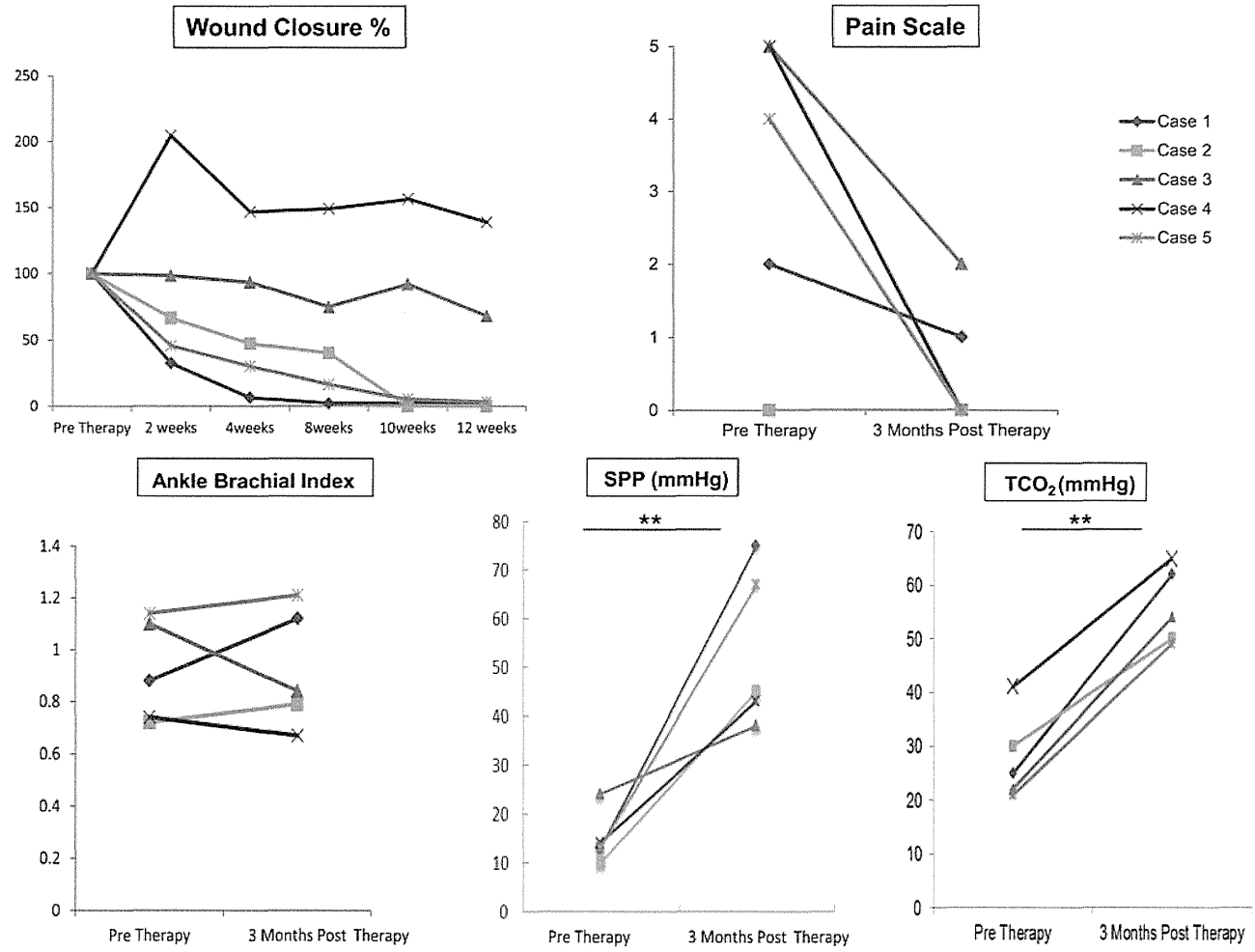

Figure 6. Improvement of efficacy parameters following $CD34^+$ therapy. Serial changes of subjective and objective parameters of wound healing and vascular perfusion of the treated foot. Percent wound closure was calculated by VH analyzer. Cases 1, 2, and 5 had near-complete wound closure after 12 weeks. Ankle brachial pressure index (ABI), SPP, and transcutaneous partial oxygen pressure (TcO_2) were performed to evaluate peripheral vascular perfusion. There was no significant change in resting ABI pretherapy and posttherapy (0.9 ± 0.2 vs. 0.9 ± 0.2), but SPP (14.5 ± 5.3 vs. 53.6 ± 16.3; $p < 0.01$) and TcO_2 (27.8 ± 8.2 vs. 56.0 ± 7.2; $p < 0.01$) proximal to the wound showed significant increase 12 weeks after the therapy in all patients. Pain level was evaluated using the Wong–Baker FACES Pain Rating Scale. All patients with pain pretransplantation showed a significant decrease in Pain Rating Scale (3.2 ± 2.1 vs. 0.6 ± 0.9; $p < 0.05$). SPP, skin perfusion pressure.

peripheral ischemia if the underlying vessels are calcified in patients who have diabetes or are receiving hemodialysis (25,35). In contrast to ABP, measurements of SPP and TcO_2 provide more accurate information even in noncompressive vessels, and false-positive results are rare (4,12). Since all patients in this trial were receiving hemodialysis due to chronic renal failure, skin perfusion pressure and TcO_2 measurements were used as a more reliable evaluator of peripheral vascular perfusion. Consequently, we did not observe significant changes in ABP in our cases, but a significant increase in SPP and TcO_2 was observed after receiving the therapy. Furthermore, patients of peripheral vascular disease with end-stage renal failure are reported to be less responsive to peripheral or bone marrow mononuclear cell therapy (24). Although all patients included in our trial were receiving hemodialysis, all patients had complete wound closure at an average of 18 weeks posttherapy and increased peripheral perfusion with no major amputations. This result suggests that purified $CD34^+$ cells may be the feasible cell therapy of these patients.

The role of G-CSF administration in the healing process in diabetic patients is not clear. There have been reports that G-CSF administration itself may accelerate wound healing (8). Some investigators reported that treatment by G-CSF improves symptoms but not signs of ischemic heart disease (36). However, in our cases, increase in SPP and TcO_2 was

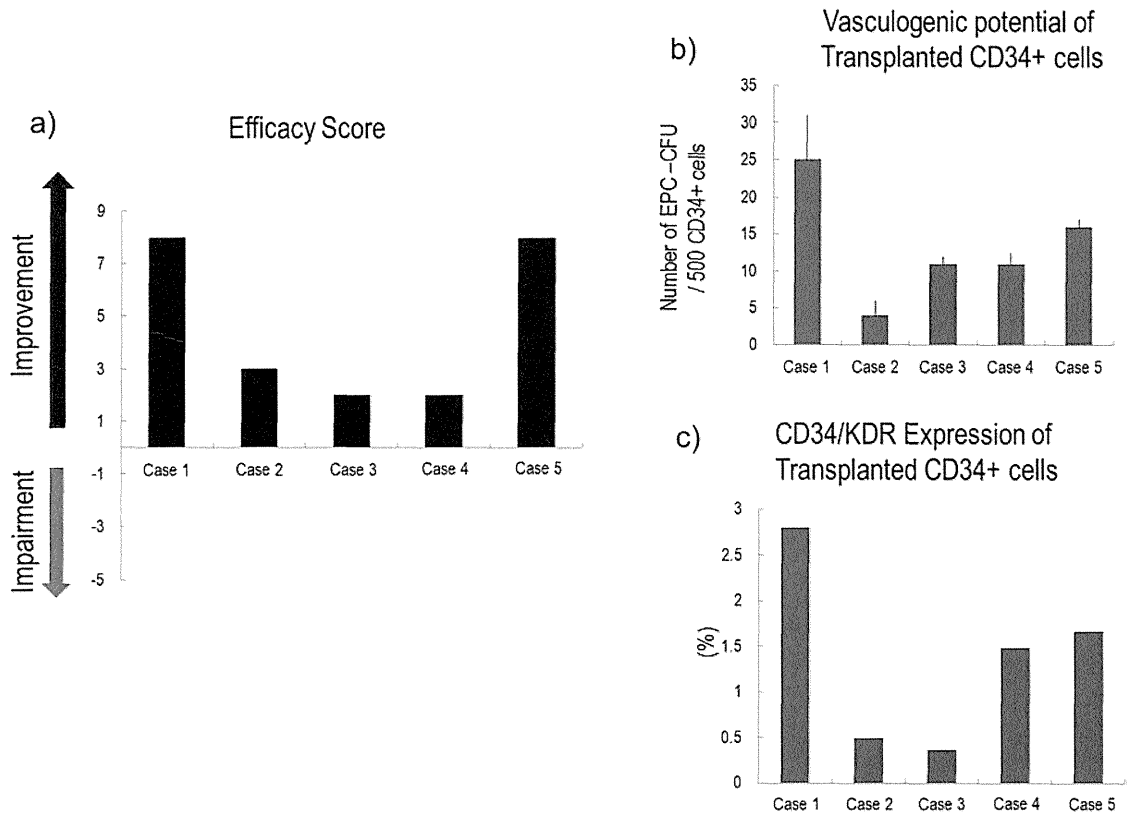

Figure 7. Efficacy score and vasculogeneic potential of transplanted CD34+ cells. (a) Efficacy score at 12 weeks following CD34+ transplantation. Efficacy score was positive for all cases indicating efficacy of the therapy. However, Cases 2, 3, and 4 showed less improvement compared to Cases 1 and 5. (b) Vasculogenic potential of transplanted CD34+ cells was evaluated by endothelial progenitor cell colony-forming assay (EPC-CFA). Number of total EPC colonies were counted per well of 500 CD34+ cells; $n=3$ per patient. The total colonies for all cases were as follows: Case 1: 25 ± 6, Case 2: 4 ± 2, Case 3: 11 ± 1, Case 4: 11 ± 1.5, Case 5: 16 ± 1. CFU, colony-forming units. (c) Flow cytometry of CD34+ and kinase insert domain receptor [KDR or vascular endothelial growth factor receptor 2 (VEGFR2)] double-positive cell percentage of transplanted EPCs is graphed. Similar to EPC-CFU, Case 1 demonstrated the highest CD34+/KDR percentage. Cases 1 and 5 with high efficacy score demonstrated a significantly higher number of total EPC-CFUs and CD34 and KDR double positivity, indicating that transplanting EPCs with higher vasculogenic function leads to better therapeutic outcome.

not seen in the nontreated side of the foot. In addition, the velocity measurement of dorsal artery by Doppler showed an increase only in the treated side of the foot (data not shown). The contralateral foot could be identified as an internal control; therefore, this observation suggests that CD34+ cells might have an improved neovascularization effect regardless of G-CSF administration.

One of the limitations of autologous EPC therapy for diabetic patients is impaired mobilization and function of diabetic EPCs (7). We and others have previously reported that both bone marrow and circulating diabetic EPCs have significantly lower vasculogenic potential compared to healthy EPCs (6,34). For that reason, the number of isolated peripheral blood CD34+ cells in our trial was lower compared to the number reported in a similar trial conducted by Kawamoto et al. with nondiabetic patients (16). In our trial, the vasculogenic potential of isolated and transplanted CD34+ cells was evaluated by EPC-CFU and a number of CD34/KDR double-positive cells. The patient with accelerated wound healing and positive prognosis without recurrence or heterotopic ulcers had a higher total number of EPC-CFU and CD34/KDR double-positive cells, indicating that the vasculogenic potential of transplanted cells is an important factor of effective autologous EPC therapy. These outcomes suggest the necessity to future investigate the relationship between patient background, EPC potential, and its efficacy.

The role of transplanted CD34+ cells in wound healing and postnatal neovascularization was not investigated in our study; however, we believe that accelerated wound healing and increased vascular perfusion were promoted with involvement of direct and indirect CD34+ cell contribution to neovascularization. In the context of EPC biology, CD34+ cells that contain enriched population

of EPCs can promote vasculogenesis by migration, proliferation, differentiation, and/or incorporation of bone marrow-derived EPCs into newly forming vasculature (1). This has been previously demonstrated by several studies using a well-established model using tunica interna endothelial cell kinase-dependent β-galactosidase reporter gene (Tie-2/LacZ) transgenic mice, allowing the detection of bone marrow EPCs in the targeted tissue (14,22). Transplantation of bone marrow-derived EPCs from Tie-2/LacZ mice accelerated wound healing and hind limb perfusion in wounded hind limb ischemia model by direct incorporation of EPCs into the neovessels in the granulation tissue using transgenic mice (3). We believe that not all EPCs incorporate into vasculature formation, but many of these cells greatly contribute to indirect contribution to vascular regeneration. EPCs activate the preexisting endothelial cells by producing various cytokines and other secreting proangiogenic factors in EPCs, such as VEGF, hepatocyte growth factor (HGF), insulin-like growth factor-1 (IGF-1), endothelial and induced nitric oxide synthase (eNOS/iNOS), angiopoietin 1 (Ang-1), and stem cell-derived factor-1 (SDF-1) (14,23).

As for the safety evaluation, there were no severe adverse events seen during and after cell therapy. Although mild adverse events were frequent, these were transient and expected. Exacerbation of diabetic retinopathy caused worry due to pathogenic angiogenesis such as arteriovenous shunt due to G-CSF administration and stem cell therapy. However, fundus oculi examinations demonstrated no pathogenic angiogenesis following $CD34^+$ cell transplant. There was no malignant tumor or angina pectoris and embolism identified during this trial. These results indicate that the autologous transplantation of mobilized peripheral blood $CD34^+$ cells is an effective and safe therapeutic approach for nonhealing chronic diabetic wounds.

In conclusion, we demonstrated that this prospective clinical trial of autologous peripheral blood $CD34^+$ cell transplant may be an alternative therapeutic option for nonhealing chronic diabetic wound for patients with peripheral vascular disease and chronic renal failure with hemodialysis. Future studies are needed to reveal its safety and efficacy in a larger number of patients and by comparison with an appropriate control group receiving G-CSF only or placebo.

CONCLUSIONS

Autologous peripheral blood $CD34^+$ cell transplant is a safe and effective therapy for nonhealing diabetic wound patients. Patients treated with $CD34^+$ cells with higher vasculogenic potential tend to have higher efficacy score and better prognosis. Furthermore, larger clinical studies with appropriate control groups are necessary to establish the safety and efficacy of this procedure.

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