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Research article

ENHANCEMENT OF WOUND CLOSURE IN DIABETIC MICE BY *ex vivo* EXPANDED CORD BLOOD CD34$^+$ CELLS

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Abstract: Diabetes can impair wound closure, which can give rise to major clinical problems. Most treatments for wound repair in diabetes remain ineffective. This study aimed to investigate the influence on wound closure of treatments using expanded human cord blood CD34$^+$ cells (CB-CD34$^+$ cells), freshly isolated CB-CD34$^+$ cells and a cytokine cocktail. The test subjects were mice with streptozotocin-induced diabetes. Wounds treated with fresh CB-CD34$^+$ cells showed more rapid repair than mice given the PBS control. Injection of expanded CB-CD34$^+$ cells improved wound closure significantly, whereas the injection of the cytokine cocktail alone did not improve wound repair. The results also demonstrated a significant decrease in epithelial gaps and advanced re-epithelialization over the wound bed area after treatment with either expanded CB-CD34$^+$ cells or freshly isolated cells compared with the control. In addition, treatments with both CB-CD34$^+$ cells and the cytokine cocktail were shown to promote recruitment of CD31$^+$-endothelial cells in the wounds. Both the CB-CD34$^+$ cell population and the cytokine treatments also enhanced the

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Abbreviations used: CB – cord blood; DAPI – 4',6-diamidino-2-phenylindole; Flt3-L – Flt-3 ligand; HSC – hematopoietic stem cell; IGF – insulin-like growth factor; IL – interleukin; MSC – mesenchymal stem cell; PB – peripheral blood; PDGF – platelet-derived growth factor; SCF – stem cell factor; STZ – streptozotocin; TGF-β – transforming growth factor-β; TPO – thrombopoietin; VEGF – vascular endothelial growth factor

recruitment of CD68-positive cells in the early stages (day 3) of treatment compared with PBS control, although the degree of this enhancement was found to decline in the later stages (day 9). These results demonstrated that expanded CB-$CD34^+$ cells or freshly isolated CB-$CD34^+$ cells could accelerate wound repair by increasing the recruitment of macrophages and capillaries and the re-epithelialization over the wound bed area. Our data suggest an effective role in wound closure for both *ex vivo* expanded CB-$CD34^+$ cells and freshly isolated cells, and these may serve as therapeutic options for wound treatment for diabetic patients. Wound closure acceleration by expanded CB-$CD34^+$ cells also breaks the insufficient quantity obstacle of stem cells per unit of cord blood and other stem cell sources, which indicates a broader potential for autologous transplantation.

Key words: $CD31^+$ cells, $CD34^+$ cells, $CD68^+$ cells, Cord blood, Diabetic mice, *Ex vivo* expansion, Hematopoietic stem cells, Macrophages, Stem cell therapy, Wound closure

INTRODUCTION

Diabetes is a chronic progressive disease with rising incidence worldwide over the last three decades [1, 2]. Diabetes patients often deal with non-closure of wounds and non-healing foot ulcerations, which contribute to the high mortality rate for this disease [3, 4]. Wound closure and healing comprise a series of orchestrated biological processes, mainly inflammation, proliferation, and maturation (tissue remodeling) [5]. Several biochemical pathways and cellular interactions are involved in these processes, as are substances such as cytokines and growth factors [5, 6]. The following all play important roles in wound healing: the epidermal growth factor family, the transforming growth factor-β (TGF-β) family, the fibroblast growth factor family, vascular endothelial growth factor (VEGF), granulocyte macrophage colony-stimulating factor, platelet-derived growth factor (PDGF), connective tissue growth factor, the interleukin (IL) family, the tumor necrosis factor-α (TNF-α) family, insulin-like growth factor (IGF), and nerve growth factor [7-12].

One of the major complications of diabetes mellitus is abnormal wound closure and healing, particularly in the case of foot ulcers. This can result in clinical problems, including lower-limb amputation, prolonged hospitalization and life-threatening sepsis [1]. Factors thought to play key roles in the delay of wound closure and healing in diabetes mellitus include: cellular dysfunction; alterations in growth factor; alterations in the signals that impair the migration and proliferation of keratinocytes and fibroblasts, the production of extracellular matrix, and angiogenesis; and the susceptibility of wound tissue to bacteria and infection [6, 13, 14]. In addition, the reduction in neuropeptide counts due to diabetic neuropathy affects wound closure and healing. Neuropeptides influence the stimulation of cell chemotaxis, proliferation and growth factor production [1].

Study of denervated wounds in rats demonstrated a significant reduction in wound closure and healing compared to control wounds, as manifested by delayed re-epithelialization and increased granulation due to failure of wound cell apoptosis [15]. Treatment of wounds requires an effective method with multiple steps of wound closure and healing to deal with all of these complications.

Hematopoietic stem cells (HSCs) are multipotent stem cells that possess the potential for self-renewal and differentiation into all mature blood cell types [16]. They have functions in homeostasis and the immune system. Moreover, some cytokines and growth factors produced by HSCs have mediatory roles in wound repair, e.g. IGF-1, TGF-β1, TNF-α, and IL-1 [12, 17, 18]. Thus, HSCs are of interest for cell-based therapy for the treatment of wounds.

Ex vivo expansion of human cord blood $CD34^+$ cells has been studied extensively for the treatment of blood diseases and autoimmune diseases. However, the mechanisms of action of $CD34^+$ cells in the cases of some diseases have not been fully explored.

Administration of human peripheral blood (PB) $CD34^+$ cells accelerated neovascularization and improved the healing of skin wounds in diabetic mice [19]. However, it has been shown that circulating $CD34^+$ cells from diabetic patients lack the potential to repair vasculature [20]. The side population of murine hematopoietic stem cells ($Sca\text{-}1^+c\text{-}kit^+Lin^{neg}/lowCD34^-$) was shown to harbor the potential to improve wound closure in diabetic mice [21]. Moreover, using a model of collagen covered with human fetal aorta-derived $CD133^+$ cells, improvement of ischemic ulcer healing and angiogenesis in diabetic mice was demonstrated [22]. However, there are difficulties in obtaining approval for clinical applications of this method [22]. A combination of human cord blood (CB) $CD34^+$ cells and $CD34^+$-derived endothelial cells encapsulated in a fibrin gel was shown to improve wound closure and healing in mice with diabetes induced by streptozotocin (STZ). The treatment decreased the inflammatory reaction and increased the neovascularization of the wound [23]. Recently, CB-$CD34^+$ cells were used to treat foot ulcers in a diabetic rat model [24]. The wound size and rate of epidermal healing were improved thanks to the acceleration of revascularization of the wounds.

We hypothesized whether local engraftment of expanded CB-$CD34^+$ cells or freshly isolated CB-$CD34^+$ cells directly to area surrounding the wound would accelerate its closure and healing. We used a diabetic mouse model for the investigation. Our results suggest the effectiveness of *ex vivo* expanded human CB-$CD34^+$ cells and freshly isolated human CB-$CD34^+$ cells in wound closure and healing in immunosuppressed diabetic mice.

MATERIALS AND METHODS

Human CB-CD34$^+$ cell collection and isolation

Human umbilical cord blood samples were obtained from patients at Por Pat Hospital with the informed consent of the patients and the approval of the Research Ethics Committee of Suranaree University of Technology, Thailand. Mononuclear cells were isolated from the cord blood by Ficoll-Hypaque centrifugation (density 1.077 g/ml, GE Healthcare). Then, CB-CD34$^+$ cells were purified using Dynal beads (Dynabeads M-450 CD34; Dynal) according to the manufacturer's instructions.

Ex vivo culture of CB-CD34$^+$ cells

Isolated CD34$^+$ cells were cultured at a density of 1 x 10^5 cell/ml in Iscove's modified Dulbecco's medium (IMDM) supplemented with 10% knockout serum replacement medium (Invitrogen) and a recombinant human cytokine cocktail containing Wnt1 (20 ng/ml), Flt3-ligand (Flt3-L; 100 ng/ml), stem cell factor (SCF; 100 ng/ml), interleukin-6 (IL-6; 100 ng/ml) and thrombopoietin (TPO; 10 ng/ml). All of the cytokines were from Peprotech Inc. Incubation was at 37ºC in 5% CO_2, 5% O_2 for 5 days. Expanded cells were washed in PBS twice and resuspended in PBS (1 x 10^5 cells in 100 μl per wound).

Diabetic mice model

The local committee on animal research approved all of the animal experiments and all of the procedures were performed in accordance with the *Guidelines for the Care and Use of the Animal Care Unit* of the Center for Scientific and Technological Equipment, Suranaree University of Technology. ICR male mice (average age: 8-9 weeks; body weight: 35-40 g) were intraperitoneally injected with streptozotocin (40 mg/kg in 0.1 M citrate buffer; Sigma) for five consecutive days. Blood samples were obtained from the tail of each mouse after 7 days and the glucose levels were measured using an Accu-Check Performa glucometer (Roche). The mice were considered diabetic when their non-fasting serum glucose level was higher than 300 mg/dl for two consecutive days [25]. Twenty-four hours before wounding, the mice were immunosuppressed with a subcutaneous injection of cyclosporine A (10 mg/kg). One day after wounding and every two days after that, the mice were injected with cyclosporine A (20 mg/kg) [26].

Wound closure assay

Immunosuppressed mice with STZ-induced diabetes were anesthetized with Zoletil (80 mg/kg; Virbac Laboratories). The hair on the dorso-lateral area was shaved and sterilized with 70% alcohol before two full-thickness excisional wounds were made with a standard skin biopsy punch (0.5 cm in diameter; Keyes).

There were three experimental groups. Group 1 mice were injected with freshly isolated CD34$^+$ cells (1 x 10^5 cells in 100 μl PBS per wound; n = 6). Group 2 mice were injected with 5-day expanded CD34$^+$ cells in a cytokine cocktail

(1×10^5 cells in 100 µl PBS per wound; n = 6). Group 3 mice were injected with the cytokine cocktail alone (20 ng Wnt1, 100 ng Flt3-L, 100 ng SCF, 100 ng IL-6 and 10 ng TPO in 100 µl PBS; n = 6). Each wound was subcutaneously injected in four different sites surrounding the wound area. As a control, mice with STZ-induced diabetes were injected with PBS alone (100 µl PBS per wound). The wounds were dressed with an occlusive polyurethane film (Tegaderm).

Wound area analysis

The open wound area was documented using a digital camera on days 0, 3, 5, 7, and 9, and then analyzed with Adobe Photoshop CS3 Extended software by calculating the pixel area from the traced wound margin and normalizing to the pixel count of the metric ruler scale. Then, the open wound area at each time point was expressed as the percentage of the original wound area (day 0).

Tissue preparation and epithelial gap determination

Three of the mice in each group were killed by cervical dislocation on day 5 and the other three on day 9. Wound tissue with a 0.3 cm margin of normal skin from both sides of dorso-lateral area was excised using a skin biopsy punch. A piece of each wound was excised and embedded with tissue freezing medium (Richard-Allan Scientific). Ten-micron thick freshly frozen tissue sections were cut perpendicularly to the wound surface with a microtome cryostat (HM 525, MICROM International GmbH). The sections were then subjected to further hematoxylin and eosin staining or to immunofluorescent analysis [27]. Hematoxylin and eosin staining was performed according to the manufacturer's instructions and the sections were mounted with mounting medium (Merck). The epithelial gaps were determined by measuring the distance between the newly encroaching epidermis with at least three cell layers on both sides (n = 6 per group) [28]. Image analysis software DP2-BSW Version 2.2 (Olympus Corporation) was used for the determination.

Histological analysis

To identify the capillary density and macrophage content within the wound area, immunofluorescent staining was performed on freshly frozen sections. Tissue sections (n = 6 per group) were fixed in cold acetone for 10 min and air-dried for 20 min. The sections were blocked for non-specific protein binding with 5% BSA (Sigma) in PBS for 10 min. Then the sections were incubated with the primary antibodies rat anti-mouse CD31 antibody or CD68 monoclonal antibody (1:100 in 1% BSA/PBS, Serotec) at 4ºC in a moist chamber overnight. The sections were washed three times with 0.1% Tween (Sigma) in PBS and the slides were incubated with the secondary antibody Dylight-488 conjugated goat anti-rat IgG (1:300 in 1% BSA/PBS, Serotec) in a moist dark chamber at room temperature for 1 h. After that, the slides were washed with 0.1% Tween in PBS three times prior to nucleus staining with DAPI (4',6-diamidino-2-phenylindole,1:100) in a moist dark chamber at room temperature for 5 min.

Negative slides were stained in parallel with primary rat IgG2a isotype control (1:300 in 1% BSA/PBS, Serotec) or without using the primary antibody to verify non-specific binding of the secondary antibody. Finally, the slides were mounted in fluorescence mounting medium (Vectashield) and the edges were sealed with nail polish.

Microscopic images of wound tissue sections were visualized using an Olympus BX51 fluorescent microscope (Olympus Corporation). Digital images were captured with an Olympus DP72 digital CCD camera (Olympus Corporation). The positive area of cells stained with either CD31 antibody or CD68 antibody was counted. To identify the localization of human fresh and expanded $CD34^+$ cells, immunofluorescent staining was performed on freshly frozen sections. PE-conjugated mouse anti-human CD34 (Pharmingen) was used for direct immunofluorescent staining and the sections were sealed with fluorescence mounting medium. Negative control slides were stained in parallel with the mouse IgG1 isotype control. The fluorescence was visualized using a fluorescent microscope.

Pathological analysis of tissue sections

Complete necropsies of the mice were performed after cervical dislocation and removal of the skin from the wound areas. All of the organs were fixed in 10% buffered formalin before the processing of paraffin-embedded sections. The sections were then stained with hematoxylin and eosin. A pathologist analyzed the slides.

Statistical analysis

Data are presented as the means ± SD. Differences between groups were analyzed using Student's *t*-test. p value < 0.05 was considered statistically significant. All of the statistical tests were done with SPSS software (version 17.0, SPSS Inc, USA).

RESULTS

Acceleration of wound closure by expanded and freshly isolated $CD34^+$ cells

Expanded $CD34^+$ cells were prepared by culturing freshly isolated $CD34^+$ cells with expansion media containing a 4FW cytokine cocktail as described in the Materials and Methods section. Our previous study showed that this expanded medium could increase the proliferation of $CD34^+$ cells about 6-fold by day 5 and 12-fold by day 7. These cells also exhibited the same hematopoietic stem cell characteristics as freshly isolated CB-$CD34^+$ cells with the cell surface markers: $CD34^+$, $CD38^-$, $CD71^{low}$, $CD33^{low}$, $CD3^-$ and $CD19^-$. Over 86% of the expanded cells still expressed $CD34^+$ after a 5-day culture. In addition, the expanded cells have the capacity to differentiate into all blood cell lineages, including erythroid, myeloid and lymphoid lineages. After expansion, the expanded cells were collected and injected into the wound area of immunosuppressed mice with STZ-induced diabetes.

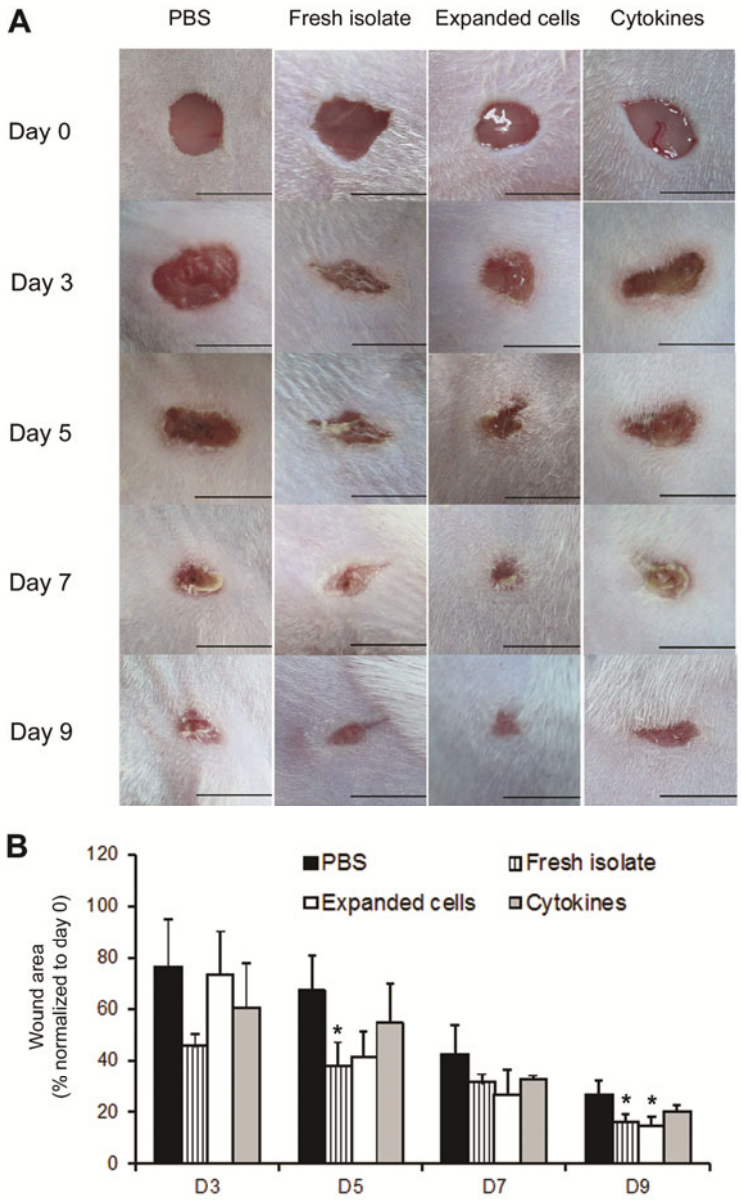

Fig. 1. Acceleration of wound closure in diabetic mice treated with freshly isolated and expanded CB-CD34$^+$ cells. A – Representative photographs of wound closure in mice treated with PBS, freshly isolated CB-CD34$^+$ cells, expanded CB-CD34$^+$ cells and cytokines alone on days 0, 3, 5 and 9 after wounding. Scale bar = 5 μm. B – Percentage of open wound area as normalized to that on day 0. Mice were treated with PBS, freshly isolated CB-CD34$^+$ cells, expanded CB-CD34$^+$ cells and cytokines around the full thickness of the dermal wound on day 0 (n = 6). The values are expressed as the means ± SD. *$p < 0.05$ compared with the results obtained with the PBS control on the same day.

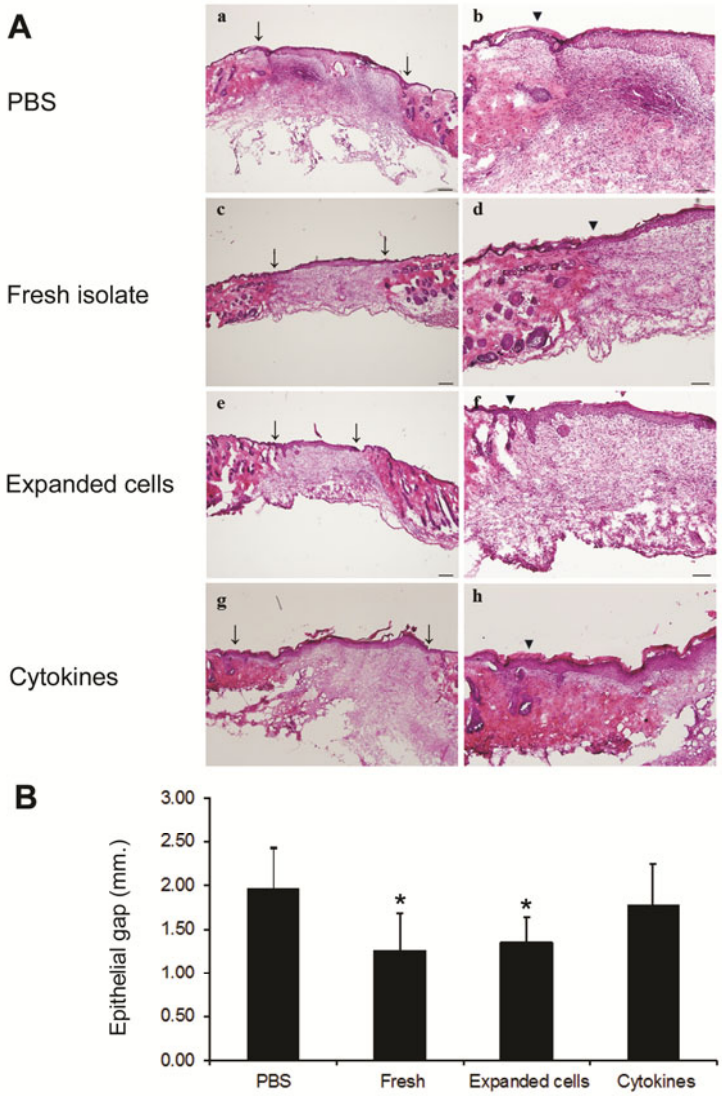

Fig. 2. Freshly isolated and expanded CB-CD34$^+$ cells improved wound closure and reduced the epithelial gap in diabetic mice. A – Histological analysis of day 9 wound sections from wounds treated with PBS (a), freshly isolated CB-CD34$^+$ cells (c), expanded CB-CD34$^+$ cells (e) and cytokines (g). The arrow indicates the length of the edge measurement of the epithelial gap. Scale bar = 200 μm. The corresponding higher power magnification images (b, d, f, h) show the right edge of the epithelial margin (arrow head) that connects to the original epidermis and is superior to the granulation tissue. Scale bar = 100 μm. B – Epithelial gap of the day 9 wounds treated with PBS, freshly isolated CB-CD34$^+$ cells, expanded CB-CD34$^+$ cells or cytokines alone. The values are expressed as the means ± SD (n = 6). *$p < 0.05$ compared with the results obtained with the PBS control.

All of the mice with diabetes had high blood glucose levels of 368 ± 50 mg/dl (normal blood glucose levels would be 144 ± 50 mg/dl). Each mouse had two wounds on both sides of its dorso-lateral area. PBS-treated wounds achieved 50% wound closure by day 7 (Fig. 1). Interestingly, when freshly isolated CB-$CD34^+$ cells were injected around the full-thickness dermal wounds, over 50% successful wound closures were obtained as early as day 3. Consequently, injection of freshly isolated CB-$CD34^+$ cells significantly accelerated wound closure by day 5 compared to the results for the PBS-treated group (fresh isolate: 38.3 ± 9.1%, PBS: 67.2 ± 13.6%; Fig. 1). This significant reduction in wound closure was also observed on day 9 after cell engraftment (freshly isolated cells: 16.4 ± 3.3%, PBS: 26.8 ± 5.3%). Similarly, wounds treated with expanded $CD34^+$ cells achieved 50% wound closure on day 5 (41.3 ± 10.3%) but this was not a statistically significant difference when compared to PBS-treated wounds (Fig. 1). However, the expanded $CD34^+$ cells enhanced wound closure significantly by day 9 (14.9 ± 3.6%) of the treatment compared to PBS-treated wounds. No significant acceleration of wound closure was observed after injection of the cytokine cocktail alone compared to the PBS-treated group throughout the study period.

Overall, treatment with either expanded CB-$CD34^+$ cells or freshly isolated CB-$CD34^+$ cells accelerated wound closure compared to the effects of treatment with cytokines alone or PBS. The group treated with expanded CB-$CD34^+$ cells showed a slightly higher rate of wound closure than that of the group treated with fresh CB-$CD34^+$ cells by day 9 of the treatment. At the end of the study period, all of the mice remained in a hyperglycemic condition (> 300 mg/dl) in all of the groups.

We next carried out histological examinations of the wound tissue sections to confirm the improvement in wound closure. The results revealed that there was advanced re-epithelialization over the wound bed area in the cell-treated wounds when compared to the results for the PBS-treated group (Fig. 2A). In addition, the epithelial gap range of wounds treated with both fresh CB-$CD34^+$ cells (1.2 ± 0.4 mm) and expanded CB-$CD34^+$ cells (1.3 ± 0.3 mm) demonstrated a significant reduction compared to that of PBS-treated wounds (2.0 ± 0.5 mm). The epithelial gap of cytokine-treated wounds (1.8 ± 0.5 mm) was not significantly different (Fig. 2B; n = 6) compared with that for the PBS-control group. These results confirmed the accelerative effect of expanded CB-$CD34^+$ cells or freshly isolated CB-$CD34^+$ cells on wound closure in diabetic mice.

Enhancement of macrophage recruitment and endothelial cell generation due to wound treatment with expanded and freshly isolated CB-$CD34^+$ cells

Macrophages and endothelial cells play crucial roles in the inflammation of injured tissue and neovascularization during wound closure, respectively. We assessed the content of both cell types using the immunofluorescent staining technique with CD68 (tissue macrophages) and CD31 (endothelial cells) antibodies. Immunofluorescent staining of tissue sections for CD68-positive macrophages revealed a significant increase by day 5 in the quantity of cells in all of the wounds treated with expanded CB-$CD34^+$ cells (104 ± 20 cells/HPF),

freshly isolated CB-CD34$^+$ cells (93 ± 8 cells/HPF) and the cytokine cocktail alone (96 ± 11 cells/ HPF) compared to the results for the PBS-treated wounds (81 ± 10 cells/HPF; Fig. 3). A significant decrease in number of macrophages was found on day 9 for all of the wounds treated with expanded CB-CD34$^+$ cells (65 ± 9 cells/HPF), freshly isolated CB-CD34$^+$ cells (61 ± 11 cells/HPF) and the cytokine cocktail (48 ± 12 cells/HPF) when compared to the PBS control group (92 ± 12 cells/HPF; Fig. 3). None of the three experimental groups had different numbers of macrophages to the others, but they did have a higher number than the PBS control on day 5. In addition, by day 9, all three experimental groups showed significantly higher levels of macrophages than the PBS control. The lowest level of macrophages was displayed by the mice treated with the cytokine cocktail alone. The level was only statistically lower than that of the mice treated with expanded CB-CD34$^+$ cells ($p < 0.05$).

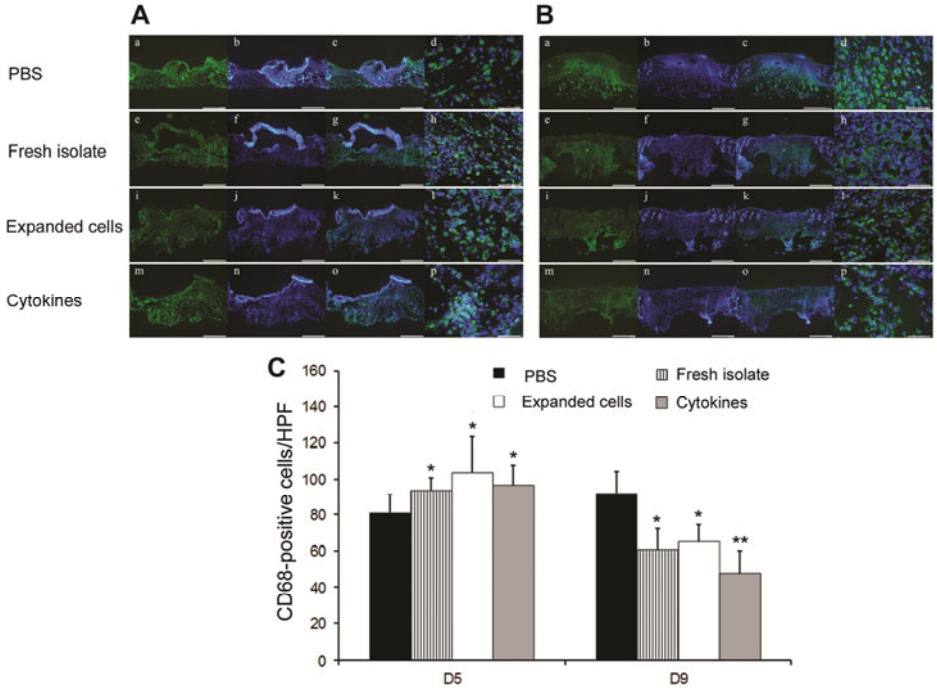

Fig. 3. Freshly isolated and expanded CB-CD34$^+$ cells improved macrophage migration into the wound sites as determined by immunofluorescent staining on days 5 (A) and 9 (B) for the macrophage marker CD68 (green) on wounds treated with PBS (a), freshly isolated CB-CD34$^+$ cells (e), expanded CB-CD34$^+$ cells (i) and cytokines alone (m). Counterstaining of the nuclei with DAPI (blue) on the same sections is shown in the second column (b, f, j, n). The overlay images (c, g, k, o) are shown in the third column. Scale bar = 400 μm. High magnification images of the CD68-positive cells are shown in the fourth column (d, h, l, p). Scale bar = 80 μm. C – Graph showing the CD68-positive cell number. The values are expressed as the means ± SD (n = 6). *$p < 0.05$ compared with the results for the PBS control. **$p < 0.05$ compared with the results obtained with expanded CB-CD34$^+$ cells the and PBS control.

Similarly, endothelial cell-specific staining for the CD31 antibody showed a significant increase in endothelial cell number in wounds treated with expanded CB-CD34$^+$ cells (41 ± 8 cells/HPF), freshly isolated CB-CD34$^+$ cells (37 ± 4 cells/HPF) and cytokines (27 ± 2 cells/HPF) compared to the results for PBS-treated wound tissues (14 ± 3 cells/HPF) by day 5 (Fig. 4). Interestingly, the endothelial cells were generated at the lateral transitional zone of the normal epidermis of the wounds treated with PBS, while the other experimental groups had the more advanced filtration of endothelial cells inside the wound tissue area and formed microvessels (Fig. 4). On day 9, the number of CD31-positive cells in wounds treated with expanded CB-CD34$^+$ cells (44 ± 8 cells/HPF), freshly isolated CB-CD34$^+$ cells (41 ± 5 cells/HPF) and cytokines (37 ± 5 cells/HPF) remained higher than those observed in PBS-treated wounds (27 ± 4 cells/HPF).

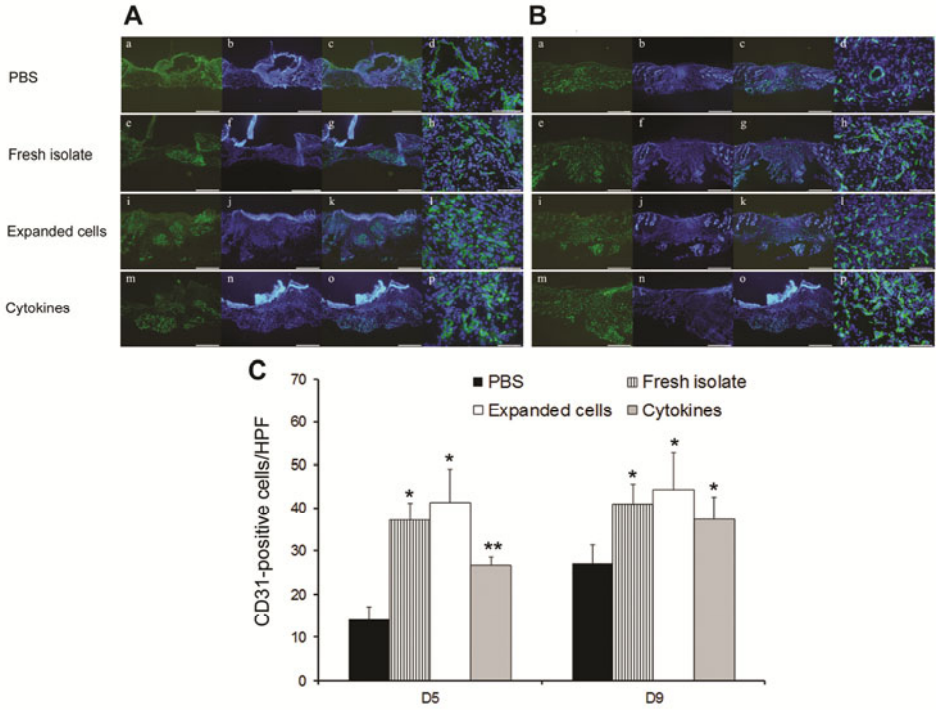

Fig. 4. Improvement of the endothelial cell content in the wound sites by treatment with freshly isolated or expanded CB-CD34$^+$ cells. Immunofluorescent staining of tissue sections on days 5 (A) and 9 (B) of treatment for the endothelial cell marker CD31 (green) in wound treated with PBS (a), freshly isolated CB-CD34$^+$ cells (e), expanded CB-CD34$^+$ cells (i) and cytokines alone (m). Counterstaining of nuclei with DAPI (blue) on the same sections are shown in the second column (b, f, j, n). The overlay images (c, g, k, o) are shown in the third column. Scale bar = 400 μm. High magnification images of CD31-positive cells are shown in the fourth column (d, h, l, p). Scale bar = 80 μm. C – Graph shows the CD31-positive cell number. Values are expressed as the means ± SD (n = 6). *$p < 0.05$ compared with the PBS control. **$p < 0.05$ compared with freshly isolated CB-CD34$^+$ cells, expanded CB-CD34$^+$ cells and the PBS control.

Moreover, all of the experimental groups showed a gradual increase in CD31-positive cells compared to the day 5 levels (Fig. 4). Overall, the groups treated with expanded and freshly isolated CB-CD34$^+$ cells displayed significantly higher quantities of the CD31-positive endothelial cells than the cytokine cocktail-treated and PBS-treated groups after 5 days of injections ($p < 0.05$) and the number observed in the mice treated with cytokines alone was higher than that for the PBS control ($p < 0.05$). On day 9 of dermal excision, none of the three experimental groups showed a difference in the number of endothelial cells between groups, but the amounts were significantly higher than those for the PBS control ($p < 0.05$).

Localization of injected human expanded CB-CD34$^+$ cells and freshly isolated CB-CD34$^+$ cells in diabetic mice

We further confined the localization of human CB-CD34$^+$ cells within the wounds and other organs using immunofluorescence analysis on days 5 and 9. The results demonstrated that the wound area and normal epidermis nearby contained small quantities of freshly isolated CB-CD34$^+$ cells and expanded CB-CD34$^+$ cells on days 5 and 9 (Supplemental Figs. 1 and 2 in supplementary material at http://dx.doi.org/10.2478/s11658-013-0089-9).

Pathology examinations

Paraffin sections were prepared from the mouse organs and then stained and analyzed for pathology changes. The assessed organs included the liver, spleen, pancreas, left and right kidneys, adrenal glands, mesentery, stomach/esophagus, small intestine, large intestine, left and right lungs, heart, bladder, left and right prostate glands, left and right brain hemispheres, left and right eyes, bone marrow, and femurs. The data revealed that all of the organs were normal and there were no pathological findings in the sections (Supplemental Table 1 in supplementary material at http://dx.doi.org/10.2478/s11658-013-0089-9). In addition, the paraffin-embedded sections did not show the localization of either freshly isolated human CB-CD34$^+$ cells or expanded human CB-CD34$^+$ cells in any of the tissues (assessed by immunofluorescent staining).

DISCUSSION

Delayed wound closure in diabetes is mainly caused by immune system defects that result in chronic wound problems. The mechanisms are considered to be mediated by changes in growth factor production, cellular responses to mediators, extracellular matrix deposition, angiogenesis and wound contraction [6]. In this study, we investigated the wound closure capacity of expanded CB-CD34$^+$ cells and freshly isolated CB-CD34$^+$ cells in diabetic mice. Wound assays were examined in immunosuppressed mice with streptozotocin-induced diabetes. STZ has toxic activity against pancreatic β-cells, in which it can induce a rapid and irreversible necrosis [25]. The effect can mimic the onset of human type 1 diabetes [25]. All of the experimental mice treated with expanded

CB-CD34$^+$ cells, freshly isolated CB-CD34$^+$ cells or the cytokine cocktail remained diabetic over the 9 days of the experiment (high blood glucose level, 551.4 ± 53.0 mg/dl), indicating that subcutaneous injection of the cells or cytokine cocktail near the wound area could not significantly decrease the blood glucose level in mice with STZ-induced diabetes within this short period of time. However, there have been reports of long-term clinical trials of type 1 diabetic treatment involving high-dose cyclophosphamide and anti-thymocyte globulin followed by intravenous autologous HSC transplantation. The data demonstrated that most patients were insulin-independent with the mean follow-up ranging from 7-30 months [29-31].

In this study, we performed experimental injections of cells or cytokines into the wounds via subcutaneous injection near the wound margin rather than injection into the wound directly in order to prevent the leakage of injected cells or fluid from the wound area. Theoretically, physiological wound healing starts from the wound margin [32]. Thus, our methodology of cell and cytokine injections could assist the starting process of wound closure. We also demonstrated for the first time that expanded CB-CD34$^+$ cells in a cytokine cocktail including Wnt1, Flt3-L, SCF, IL-6 and TPO had the effective capacity for wound closure. This effect has been demonstrated by the approximately 50% improvement in wound closure by day 5 after the engraftment of expanded CB-CD34$^+$ cells and the significantly enhanced wound closure by day 9 (about 80% wound closure) after the treatment compared to the results for the PBS-treated control mice. By contrast, wounds treated with the cytokine cocktail alone had no such significant effect. These findings also suggest that the injection of expanded CB-CD34$^+$ cells may serve as a novel therapeutic option for diabetic wound treatment. However, this therapeutic value does not apply to the injection of the cytokine cocktail alone.

The results also demonstrated that engraftment of freshly isolated CB-CD34$^+$ cells induced significant wound closure as early as day 5 and continued to improve wound closure until day 9. Freshly isolated and expanded CB-CD34$^+$ cell injections into the wounds similarly improved wound closure. The onset of wound closure in this study was earlier than in other studies, in which wound closure was observed on days 6 to 7 of treatments, including transplantations of cord blood mesenchymal stem cells (CB-MSCs), peripheral blood, and bone marrow hematopoietic stem cells [19, 21]. Pedroso *et al.* reported that 3D fibrin gel containing CB-CD34$^+$ did not accelerate wound closure while the gel containing a co-culture of CB-CD34$^+$ cells and CD34$^+$-derived endothelial cells could improve wound closure as early as 3 days post-injury [23]. However, the induction of wound closure was not statistically different compared to the control for the remaining time points [23]. There was a report demonstrating that transplantation of endothelial progenitor cells could enhance keratinocyte and fibroblast proliferation, inducing wound closure as early as day 3 post-transplantation [33]. These discrepancies between the rates of wound closure could be due to multiple factors, such as the quantity of the injected cells, the

different types of cell, the use of expanded or freshly isolated cells, the engraftment procedure, and the species of mouse.

Histological examination of the wound tissue sections also confirmed the improvement in wound closure mediated by engraftments of either expanded or freshly isolated CB-$CD34^+$ cells. The data indicated a lower epithelial gap between the two sites of the wound margins in both of the cell-treated groups compared to that found with the cytokine- and PBS-treated groups. In addition, a more finely regular arrangement of the complete epidermis lining the newly formed epidermal layer was found in wounds treated with fresh or expanded CB-$CD34^+$ cells on day 9. Neovascularization to the wound area was also exhibited in both cell transplantation groups.

Inflammatory and neovascularization processes are important in wound closure [34]. Further investigation involved examining the neovascularization and macrophage content in the wound tissue using the immunofluorescence method with endothelial CD31 and CD68 macrophage marker staining. We found that wounds engrafted with freshly isolated or expanded CB-$CD34^+$ cells or cytokine alone contained higher quantities of CD68-positive macrophages and CD31-positive endothelial cells than those of PBS-treated wounds after 5 days of treatment. By comparing the efficiency of freshly isolated and expanded CB-$CB34^+$ cell injections, we found that there was no statistically significant difference between the endothelial and macrophage contents within the wound areas between the two experimental groups. In addition, on day 5, the number of CD31-positive endothelial cells in the wounds treated with either freshly isolated or expanded CB-$CD34^+$ cells was significantly higher than that found with the cytokine-injected group, but the observation of CD68-positive macrophages did not show a difference. The presence of macrophages in the early stages of wound closure is a normal process that is not only involved in inflammation but also in wound closure processes. Macrophages generate some mediators, such as PDGF and VEGF, to promote granulation, and other growth factors for the fibroplasia and angiogenesis processes of wound closure [32]. These findings suggest that freshly isolated CB-$CD34^+$ cells, expanded CB-$CD34^+$ cells and cytokines alone could promote the macrophage and capillary recruitment and/or proliferation that contribute to re-epithelialization in the early process of wound closure. Interestingly, the macrophage levels had declined by day 9 in all of the experimental groups, but continued to be high in the PBS control group. Of all the experimental groups, the group treated with cytokines alone demonstrated the highest reduction in macrophage levels, and this was only statistically different from the reduction seen in the group receiving expanded CB-$CD34^+$ cells. The declining number of macrophages along with the increase in capillaries measured on day 9 suggested a decrease in inflammation processes, which may provide a more suitable environment for advanced wound closure processes to occur. These data correspond with those in a previous report that showed that injecting human PB-$CD34^+$ cells into the wounds of diabetic mice could accelerate revascularization and would healing [19]. Although our study

demonstrated that cytokine-treated wounds displayed similar macrophage and capillary contents to the wounds treated with freshly isolated and expanded CB-$CD34^+$ cells, the overall results for wound closure had not significantly improved at the end of the observations.

It has been proposed that stem cells can promote wound closure [19]. There are two possibilities whereby stem cells may accelerate the wound closure process. First, stem cells release growth factors, cytokines and mediators in a paracrine manner to stimulate the generation of neighboring tissues or cells [27]. Second, stem cells may either differentiate into regenerative cells in the wound site or stimulate tissue regeneration by collaborating with adjacent cells [35]. For the paracrine effect, one study showed that the conditioned medium of the hematopoietic progenitor cell line of DKmix cells could promote angiogenesis within wounds in murine models [27]. Moreover, conditioned media of human CB-derived endothelial progenitor cells have been found to promote keratinocyte, fibroblast and endothelial cell proliferations in a paracrine manner in the same action performed by the injection of endothelial progenitor cells into wounds on diabetic mice [33]. These data demonstrate that the incorporated stem cells release cytokines that act in a paracrine manner to enhance neovascularization, resulting in the remodeling of extracellular matrix and recruitment of circulating stem cells. In this study, the injection of cytokines alone to the wounds could enhance the recruitment of capillaries and macrophages. These data suggest that a cytokine cocktail containing Wnt1, SCF, Flt3-L, IL-6 and TPO exert chemotactic effects promoting macrophage and endothelial cell recruitment and might be the activating factors for endothelial cell proliferation toward neovascularization in the early stage of wound closure. However, the injection of cytokines alone may not be enough to induce a more advanced wound closure process in the maturation and remodeling phase, which may mediate in cases with keratinocyte defectiveness and collagen matrix contractions. In addition, the low amount of injected cytokines may also limit their efficiency.

Stem cell factor (SCF) is produced by keratinocytes in the epidermis and by endothelial cells, fibroblasts and mast cells in the dermis [36-38]. SCF is also found in the dermis during the early phase of normal wound closure but its level declines thereafter [39]. Additionally, SCF has been shown to function in anti-apoptosis of keratinocytes during wound closure [40]. It has been reported that human skin keratinocytes and fibroblasts express Flt3-L [41]. Studies on the infection of burns in a mouse model showed that treatment with Flt3-L could lead to an enhancement of the dendritic cell number and functions, which in turn could stimulate neutrophil recruitment and function in the immune response to the infection [42-44]. IL-6 is mainly secreted by epidermal keratinocytes, while macrophages, Langerhans cells and fibroblasts in the dermis are the other sources in the skin [45, 46]. Enhanced IL-6 expression in normal rat skin increased the epidermal proliferation and inflammation [47]. However, the conditioned medium of human ESC-derived endothelial precursor cells has been

shown to enhance normal wound closure in mice through the action of distinct secretory factors, such as epidermal growth factor, basic fibroblast growth factor, granulocyte-macrophage colony-stimulating factor, IL-6, IL-8, PDGF-AA and VEGF [48]. These data suggest that SCF, Flt3-L and IL-6 might mediate significantly in the early phase of the wound closure process. Many inflammatory cell types, such as mast cells and neutrophils, may be recruited during the inflammatory process and that could affect the outcome of wound closure processes. Thus, the improvement of wound closure might need more distinctly different cytokines to regulate and balance the healing process.

The canonical Wnt signaling pathway (mediated by β-catenin) is thought to play a role in dermal fibroblast proliferation and motility. It is elevated in fibroblasts during the proliferative phase of wound closure [49]. Moreover, the β-catenin level was found to enhance during the wound closure with a peak at 4 weeks following the injury and a return to the baseline level by 12 weeks [50]. The Wnt signal was found at an elevated level in the wound and was suggested to mediate in the maintenance and regeneration of hair follicles [51]. In addition, Wnt signaling has been shown to be the growth-inducer of stem cell compartments in skin with interfollicular progenitor cells expressing Wnt1, which can inhibit their growth and promote terminal differentiation [52]. Human adipose-derived stromal cells have been shown to produce cytokines in a paracrine manner. These induce the expression of Wnt1 in the de-epithelialized skin, mainly in the dermal fibroblasts [53]. These data suggest that Wnt signaling plays a role in skin regeneration and wound closure. Thus, the injection of a cytokine containing Wnt1 that mediates the canonical Wnt signaling may stimulate the regeneration of the skin to the fibroblast cells. However, other Wnt signals may be involved in wound repair in an orchestrating manner for the improvement of wound closure.

This study revealed the presence of human CB-$CD34^+$ cells inside the wound area and in the tissue surrounding the wound in the mouse groups treated with either freshly isolated or expanded CB-$CD34^+$ cells. The data suggest that these cells may exert their wound closure function partially through cooperation with other cells around the wound area or in a paracrine manner that assists in the recruitment of local stem cells, macrophages, endothelial cells, fibroblasts and other inflammatory cells to the wound site. Human $CD34^+$ cells and hematopoietic precursors secrete several cytokines and growth factors in an autocrine and/or paracrine manner, such as VEGF, HGF, FGF2, Flt3-L, IL-1, IL-16, TGFβ1, TGFβ2 and TPO [18, 54]. Some of those mediators are mediated in the wound healing process, such as IGF-1, TGF-β1, TNF-α, and IL-1 [17].

Analysis of tissue from all the major organs of the mice did not show distribution of human CB-$CD34^+$ cells or pathological changes beyond the wound area and surrounding skin. These results confirmed that engraftment of expanded or freshly isolated CB-$CD34^+$ cells is a safe procedure and effective treatment for local wound closure. Human CB-$CD34^+$ cells may also differentiate into endothelial cells during wound closure processes. Thus,

whether CB-CD34$^+$ cells could transdifferentiate into endothelial cells in the wound area should be explored further.

This study demonstrated that expanded CB-CD34$^+$ cells cultured in our new cytokine cocktail and freshly isolated CB-CD34$^+$ cells significantly accelerate the wound closure process in immunosuppressed mice with STZ-induced diabetes. The improvement is mediated by the recruitment of macrophages, increased neovascularization in the wound area, and advanced re-epithelialization over the wound bed area in cell-treated wounds. Transplanted cells may also mediate the wound closure process in cooperation with other cells surrounding the wound area or in an orchestrated manner with other cytokines both in paracrine or autocrine functions. Further study would be needed to clarify this. The implication is that there is a potential to improve wound closure treatment for diabetic patients by using fresh or expanded stem cells from non-invasive sources, such as cord blood. Wound closure acceleration by expanded CB-CD34$^+$ cells also breaks the insufficient quantity obstacle of stem cells per unit of cord blood and other stem cell sources, which indicates a broader potential for autologous transplantation.

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