Original Paper

Treatment of Cutaneous Ulcers with Multilayered Mixed Sheets of Autologous Fibroblasts and Peripheral Blood Mononuclear Cells

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Key Words

Cell sheets • Fibroblasts • Peripheral blood mononuclear cells • Skin ulcers • Angiogenesis

Abstract

Background/Aims: We have developed a mixed-cell sheet consisting of autologous fibroblasts and peripheral blood mononuclear cells with a high potency for angiogenesis and wound healing against refractory cutaneous ulcers in mouse and rabbit models. To increase the effectiveness of the mixed sheet, we developed a multilayered mixed sheet. **Methods:** We assessed the therapeutic effects of multilayered sheets on cutaneous ulcers in mice. Growth factors and chemokines were assessed by enzyme-linked immunosorbent assay. Angiogenesis and fibroblast migration were measured by using tube formation and migration assays. Wound healing rate of cutaneous ulcers was evaluated in mice with diabetes mellitus. **Results:** The concentration of secreted vascular endothelial growth factor, hepatocyte growth factor, transforming growth factor, C-X-C motif chemokine ligand (CXCL)-1, and CXCL-2 in multilayered sheets was much higher than that in single-layered mixed-cell sheets (single-layered sheets) and multilayered sheets of fibroblasts alone (fibroblast sheets). The supernatant in multilayered sheets enhanced angiogenic potency and fibroblast migration compared with single-layered and fibroblast sheets in an *in vitro* experiment. The wound healing rate in the multilayered sheet-treated group was higher compared with the no-treatment group (control) at the early stage of healing. Moreover, both vessel lumen area and microvessel density in tissues treated with multilayered sheets were significantly increased compared with tissues in the control group. **Conclusion:** Multilayered sheets promoted wound healing and microvascular angiogenesis in the skin by supplying growth factors and cytokines. Accordingly, our data suggest that multilayered sheets may be a promising therapeutic material for refractory cutaneous ulcers.

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Introduction

Refractory skin ulcers are caused by ischemic disorders including peripheral arterial disease (PAD), diabetes mellitus, and pressure and congestive disorders (e.g. venous insufficiency), and therapies are tailored according to the cause [1-4]. More than 200 million people are affected by PADs globally, including arteriosclerosis obliterans, Buerger's disease, and blue toe syndrome [5]. Although revascularisation via endovascular and bypass surgery methods is the best treatment for critical limb ischemia (CLI), complications can be encountered and some patients are not suitable candidates for revascularization [6]. Even if surgical treatment is successful, if a local circulatory disorder remains, the cutaneous ulcer might be refractory. Therapeutic angiogenesis is a novel strategy to treat patients with severe PAD with no other options by promoting the formation of collateral vessels and angiogenesis.

For patients who are not candidates for neovascularization, clinical trials of bone marrow cell transplantation in patients with CLI have revealed some degree of therapeutic effect for neovascularization [7]. To improve the survival rate of transplanted cells in ischemic tissue [8, 9], we previously reported a method to promote neovascularization termed 'hypoxic preconditioning' [10, 11]. To reduce invasiveness on collected transplanted cells, we used peripheral blood mononuclear cells (PBMNCs) instead of bone marrow cells and further examined the therapeutic effects of preconditioned PBMNCs on ischemic hindlimbs. Hypoxically pretreated PBMNCs improved microvessel density and limb blood flow in hindlimb ischemia models [12, 13].

Cell sheet technology has been developed to improve the engraftment of transplanted cells in grafted regions [14], and its therapeutic viability has been demonstrated in a variety of disease models [15-19]. A primary factor resulting in insufficient repair of refractory ulcers is the poor retention of grafted cells or artificial skins in wounded areas. Therefore, we considered the use of cell sheet technology to treat refractory cutaneous ulcers.

The normal healing process is regulated by a complex signaling network involving growth factors, cytokines, and chemokines and can be divided into three overlapping phases: (i) inflammatory, (ii) proliferative, and (iii) tissue remodeling phases [20]. In refractory cutaneous ulcers such as venous and diabetic leg ulcers, abnormal expression of growth factors induces chronic inflammation and delays the healing process [21, 22]. Vascular endothelial growth factor (VEGF), transforming growth factor (TGF- β 1), and platelet-derived growth factor BB (PDGF-BB) are necessary for the healing process from the inflammatory to the tissue repair phase (proliferative and tissue remodeling phases) [23, 24]. It has been reported that PBMNCs provide interleukins and growth factors such as TGF- β 1 and PDGF-BB that are required for the inflammatory phase of the normal healing process [22-25]. TGF- β 1 and PDGF-BB increase the expression levels of VEGF and collagen mRNAs in fibroblasts [26]. Accordingly, we developed a mixed-cell sheet consisting of autologous fibroblasts and PBMNCs that secrete necessary growth factors from the inflammatory phase to the tissue repair phase and confirmed its efficacy for ulcer treatment in mice and rabbits [26, 27]. The mixed-cell sheet had high angiogenic potency, promoted wound closure, and led to complete natural healing. Moreover, we sought to increase the effectiveness of the sheet, and so we developed a multilayered mixed sheet.

In the present study, we examined the therapeutic efficacy of multilayered mixed-cell sheets in mice. Multilayered mixed-cell sheets have the advantage of sustained growth factor secretion. In fact, the secretion of VEGF in mixed-cell sheets was significantly greater than in single-layered mixed sheets, suggesting that multilayered mixed-cell sheets might possess enhanced angiogenic activity. The therapeutic effect of multilayered sheet delivery in the treatment of cutaneous ulcers was similar to or better than that of single-layered sheets. Taken together, multilayered mixed-cell sheets may be a potentially useful novel tool to treat cutaneous ulcers.

Materials and Methods

Animals

Male C57BL/6 mice were purchased from Japan SLC, Inc. (Shizuoka, Japan). Male green fluorescent protein (GFP)-transgenic mice (C57BL6/Tg14) were provided by Masaru Okabe (Genome Research Centre, Osaka University, Osaka) [28]. All animal procedures were approved by the Institutional Animal Care and Use Committee of Yamaguchi University (#31-093). The study was conducted in accordance with relevant guidelines.

Isolation of PBMNCs and fibroblasts

PBMNCs were isolated from mouse peripheral blood using Lympholyte®-M (CedarLane Laboratories Ltd., Hornsby, Ontario, Canada) and cultured in CTSTM AIM V® medium (Thermo Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific). Fibroblasts were isolated from the tails of mice using collagenase (Wako, Osaka, Japan) and cultured in CTSTM AIM V® medium supplemented with 10% FBS.

Preparation of cell sheets

Single-layered mixed-cell sheets (1.25×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well; single-layered sheets) were cultured in UpCell® 24-well plates (Cell Seed, Inc., Tokyo, Japan). Multilayered sheets of fibroblasts alone (5.0×10^5 fibroblast cells/well and no PBMNCs; fibroblast sheets) and multilayered mixed-cell sheets (5.0×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well; multilayered sheets) were cultured in a standard 24-well culture dish. The three types of sheets were incubated under normoxic conditions (37°C, atmospheric O₂ concentration) for 2 days followed by hypoxic conditions (33°C, 2% O₂) for 1 day. For each culture, 2 mL CTSTM AIM V® medium and HFDM-1 (+) (Cell Science & Technology Institute, Sendai, Japan) supplemented with 5% FBS were used in the 24-well plates. After incubation, single-layered sheets were detached by placing them out of the incubator at room temperature. Fibroblast and multilayered sheets were detached using 10 PU/mL dispase.

Enzyme-linked immunosorbent assay (ELISA)

To assess VEGF secretion from sheets under different culture conditions, fibroblasts were seeded at different number of cells together with PBMNCs at 2.0×10^6 cells/well in standard 24-well plates. Cells were incubated under normoxic conditions (37°C, atmospheric O₂ concentration) for 2 days followed by hypoxic conditions (33°C, 2% O₂) for 1 day. Fibroblasts mixed with PBMNCs were seeded at 0, 1.25×10^5 , 2.5×10^5 , and 5.0×10^5 cells/well, because mixed-cell sheets with greater $\geq 1.0 \times 10^6$ fibroblast cells/well detached spontaneously during incubation. The supernatant of each well was collected, and the concentration of VEGF was measured using the mouse VEGF Quantikine immunoassay kit (R&D Systems, Minneapolis, MN). The supernatant in the 5.0×10^5 fibroblast cells/well without PBMNCs was collected as control. To assess VEGF, hepatocyte growth factor (HGF), TGF-β1, C-X-C motif chemokine ligand (CXCL)-1, CXCL-2, and interleukin-6 (IL-6) release from sheets cultured under different conditions, single-layered (1.25×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well), fibroblast (5.0×10^5 fibroblast cells/well and no PBMNCs), and multilayered (5.0×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well) sheets were cultured in 24-well plates and incubated under normoxic conditions for 2 days followed by hypoxic conditions for 1 day. For each culture, 2 mL CTSTM AIM V® medium and HFDM-1 (+) supplemented with 5% FBS were used in the 24-well plates. After incubation, ELISA was performed as described above. Mouse Quantikine immunoassay kits were used.

Tube formation assay

Human umbilical vascular endothelial cells (HUVECs) were maintained in EGMTM-2 medium (Lonza Ltd., Basel, Switzerland) supplemented with EGMTM BulletKit™ (Lonza), trypsinized, resuspended in 10% FBS/AIM V medium, and seeded onto Matrigel® (Corning Inc., Corning, NY)-coated 96-well plates at 2.0×10^4 cells/well (100 μL). Subsequently, 100 μL conditioned medium from the fresh medium, single-layered, fibroblast, and multilayered sheets were added into each well. Images of tube formation were captured after 12 h and measured using the Angiogenesis Analyzer for ImageJ software (National Institutes of Health, Bethesda, MD) [29, 30].

Migration assay

Fibroblasts were isolated from mouse tails as above and cultured in CTSTM AIM V® Medium containing 10% FBS. Fibroblasts were seeded into 24-well cell culture dishes at high confluence. Cells were scraped with 200 µL tips to create a scratch and then further cultured for 18 h in conditioned medium from single-layered, fibroblast, and multilayered sheets. Phase contrast images of the scratches were taken using a BZ-X710 All-in-One fluorescence microscope (Keyence Corp., Osaka, Japan) at 0 and 18 h after incubation. The wound area was measured using the BZ-X analyser software (Keyence).

TUNEL assay

Apoptotic cells were detected using the terminal deoxyribonucleotidyl transferase-mediated dUTP-digoxigenin nick end labelling (TUNEL) assay. Multi- and single-layered sheets were cultivated as above and transferred onto a piece of raw ham. Sheets were fixed with 10% formalin neutral buffer solution (Wako) and embedded in a paraffin block. Slides were deparaffinized, permeabilized with 0.1% Triton-X/protein blocking solution (Dako Japan, Inc., Kyoto, Japan), and then incubated with a reaction solution (In Situ Cell Death Detection Kit, Roche, Basel, Switzerland). In addition, cells were stained with 6-diamidino-2-phenylindole (DAPI) to visualize the nuclei. Immunofluorescence images were acquired using a BIOREVO microscopy system (BZ-9000 Generation II system; Keyence).

Cutaneous ulcer model

Mice were injected with streptozotocin (Sigma-Aldrich Corp., St. Louis, MO). Mice with blood glucose levels of more than 300 mg/dL were used for the animal experiments as diabetes mellitus (DM) mice and anaesthetized via the inhalation of 1.5% isoflurane during the surgical procedure. For mice with ulcers on the back, full-thickness skin defects 8 mm in diameter were created using a surgical technique. Sheets cultured under hypoxic conditions were transplanted onto the full-thickness skin defects. The area of the skin defect was measured using ImageJ software.

Transplantation of cell sheets

Mice were divided into the following four groups according to treatment: (1) single-layered sheets (n = 10), (2) fibroblast sheets (n = 10), (3) multilayered sheets (n = 9), and (4) no sheets (controls, n = 9). For the sheet-treated groups, the sheets were transported from plates to wounds using a tip (1000 µL) with the edge cut. All ulcers were covered with Urgotul® (Laboratoires Urgo, Chenove, France), wrapped with Derma-aid® (ALCARE Co. Ltd., Tokyo, Japan), and fixed with a bandage (ALCARE). The ulcer area was measured at days 0, 7, and 14, with the animal under anaesthesia, using Image J software.

Histological analysis

For hematoxylin and eosin (H&E) staining, after the skin defects had healed, cutaneous tissues were resected and fixed with formalin neutral buffer solution. For fluorescent immunostaining, cutaneous tissues were resected, fixed in 10% formalin neutral buffer solution overnight at room temperature, and embedded in a paraffin block. Slides were deparaffinized, treated with antigen retrieval solution (Dako), and incubated with blocking buffer (Dako). The following antibodies were used: anti-GFP pAb (598, Medical & Biological Laboratories, Nagoya, Aichi, Japan), anti-CD31 antibody (ab28364, Abcam, Cambridge, UK), and anti-rabbit IgG H&L (DyLight® 550; ab96884; Abcam).

Statistical analysis

Data were analyzed using Stata/SE 12.1 software (StataCorp, College Station, TX). Statistical significance between two groups was determined by two-tailed unpaired t-tests. Statistical significance among multiple groups was analyzed using one-way analysis of variance followed by a Bonferroni post hoc test. $P < 0.05$ was considered statistically significant.

Results

Preparation of multilayered mixed-cell sheets

We previously reported that mixed-cell sheets consist of autologous fibroblasts (1.25×10^5 cells) and PBMNCs (2.0×10^6 cells) on UpCell® 24-well plates [26]. To determine the maximum possible number of fibroblasts to configure mixed-cell sheets on these plates, we examined the number of fibroblasts present when the number of PBMNCs remained at 2.0×10^6 cells/well. Mixed-cell sheets detached naturally in the incubator if more than 1.25×10^5

fibroblast cells/well were seeded. Therefore, we could not peel the cell sheet off intentionally. Thus, we made multilayered mixed sheets in the standard 24-well plates. Fibroblasts were seeded in the range of 1.25×10^5 to 1.0×10^6 cells/well together with PBMNCs at 2.0×10^6 cells/well. Cells were incubated under normoxic conditions for 2 days followed by hypoxic conditions for 1 day. Mixed-cell sheets detached naturally in the incubator if more than or equal to 1.0×10^6 fibroblast cells/well were seeded. However, the multilayered sheets could not be detached with fibroblasts seeded at 1.25×10^5 , 2.5×10^5 , and 5.0×10^5 cells/well.

The concentration of VEGF in the supernatant on multilayered mixed cell sheets with 5.0×10^5 fibroblast cells/well was highest comparison with other experimental groups (Fig. 1A). The multilayered sheets consisting of 2.0×10^6 PBMNCs/well and 5.0×10^5 fibroblast cells/well were incubated with 10 PU/mL dispase for 40 min and peeled from the bottom of the culture dish with tweezers. The sheets had a diameter of approximately 8 mm and formed a round shape (Fig. 1B, left). Single-layered sheets were formed of 1.25×10^5 fibroblast cells and 2.0×10^6 PBMNCs using UpCell® 24-well plates (Fig. 1B, right). The multi- and single-layered sheets were transferred to a raw ham. Macroscopic images and H&E-stained samples of multi- and single-layered sheets are shown in Figs. 1B–D. To observe whether PBMNCs existed in multilayered sheets, these sheets were constructed from PBMNCs of GFP-positive mice. Immunohistochemistry revealed GFP-positive PBMNCs in the cell sheets (Fig. 1E).

Enhanced secretion of growth factors and cytokines in multilayered sheets

To assess the paracrine effect of multilayered sheets, the concentration of VEGF, HGF, TGF- β 1, CXCL-1, CXCL-2, and IL-6 in the supernatant was measured using an ELISA. The concentration of the growth factors and cytokines, except for IL-6, secreted from multilayered sheets was significantly higher than from single-layered and fibroblast sheets (only 5.0×10^5 fibroblast cells/well; Figs. 2A–F). The concentration of IL-6 from multilayered sheets was the same as from single-layered sheets.

Angiogenic potency of multilayered sheets

Tube formation assay was performed to investigate whether multilayered sheets enhanced angiogenic activity. HUVECs were

Fig. 1. Multilayered sheets consisting of fibroblasts and PBMNCs. (A) A greater number of fibroblasts in multilayered sheets resulted in increased VEGF secretion. Fibroblasts were seeded at different cell numbers together with PBMNCs at 2.0×10^6 cells/well in standard 24-well plates. Cells were incubated under normoxic conditions (37°C, atmospheric O₂ concentration) for 2 days followed by hypoxic conditions (33°C, 2% O₂) for 1 day. The number of fibroblasts mixed with PBMNCs were 0, 1.25×10^5 , 2.5×10^5 , and 5.0×10^5 cells/well. Concentration of VEGF in the supernatant were measured using ELISA. The supernatant in 5.0×10^5 fibroblast cells/well alone without PBMNCs was also collected as a control. *P<0.01 vs. 0 PBMNCs/well and 5.0×10^5 fibroblast cells/well; †P<0.01 vs. 2.0×10^6 PBMNCs/well and 0 fibroblast cells/well; ‡P<0.01 vs. 2.0×10^6 PBMNCs/well and 1.25×10^5 fibroblast cells/well; §P<0.01 vs. 2.0×10^6 PBMNCs/well and 2.5×10^5 fibroblast cells/well; ¶P<0.01 vs. 2.0×10^6 PBMNCs/well and 5.0×10^5 fibroblast cells/well. Error bars indicate the standard error. (B) Multilayered sheets were detached from standard culture dishes incubated with 10 PU/mL dispase (left). Single-layered sheets were detached from temperature-responsive culture dishes incubated at <20°C (right). (C, D) H&E-stained images after multi- (left) and single- (right)-layered sheets on raw ham in low (C) and high (D) power fields. (E) PBMNCs in multilayered sheets. PBMNCs were isolated from C57BL/6 GFP-transgenic mice and the sheets were stained using anti-GFP antibody. DAPI stained the nucleus of cells constituting multilayered sheets.

Fig. 2. Synergistic effect of multilayered sheets consisting of PBMNCs and fibroblasts. (A–F) VEGF, HGF, TGF- β 1, CXCL-1, CXCL-2, and IL-6 concentration in the supernatant of single-layered (1.25×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well), fibroblast (5.0×10^5 fibroblast cells/well and no PBMNCs), and multilayered (5.0×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well) sheets, which were cultured in 24-well plates and incubated under normoxic conditions (37°C, atmospheric O₂ concentration) for 2 days followed by hypoxic conditions (33°C, 2% O₂) for 1 day. Error bars indicate standard error. Single: single-layered sheets, Fibroblast: fibroblast sheets, Multi: multilayered sheets.

cultured with conditioned medium from single-layered, fibroblast, and multilayered sheets. Tube formation was significantly increased with conditioned medium from multilayered sheets after 12 h compared with fresh medium (Fig. 3A). This suggested that VEGF secreted in large amounts from multilayered sheets augmented angiogenic activity.

Fibroblast migration potency of multilayered sheets

Migration assay was performed to examine whether the multilayered sheets have potential fibroblast migration activity in a wound area. Primary mouse fibroblasts were seeded onto cell culture dishes and scratches were created in confluent cultures. The fibroblasts were then cultured with conditioned medium from sheets. Fibroblast migration was facilitated in the multilayered sheets after 18 h (Fig. 3B).

Cell viability of multilayered mixed sheets

TUNEL assay was performed to assess cell survival in the multilayered sheets transferred to a piece of raw ham (Fig. 3C). Although the number of fibroblasts was larger in multilayered than in single-layered sheets, the cell viability levels were the same.

Therapeutic effect of multilayered sheets in a mouse cutaneous ulcer model

To evaluate multilayered sheets as a therapeutic material for cutaneous ulcers, cell sheets derived from C57BL/6 mice were transplanted onto full-thickness skin defects on the backs of DM mice (Fig. 4A). At 7 days after initiation of therapy, the wound healing rate was significantly higher in the single- and multilayered groups than in control animals (Fig. 4B). At 14 days, although there was no significant difference in wound healing rate between the single-layered, fibroblast, and multilayered sheet groups, wound healing rates in the three groups were significantly higher than in the control group. Skin defects on the backs of DM mice were completely closed by 21 days after treatment in all four groups. However, histological analysis of the wounds revealed a clear difference in tissue regeneration between the three sheet groups and the control group. Although the anatomy of tissues from mice treated with the single- and multilayered sheets appeared normal, inflammatory-like cells were observed frequently in control tissues, indicating that abnormal tissue regeneration was induced in the control mice (Fig. 5A). CD31 staining revealed microvessel-like structures in tissues treated with multilayered sheets (Fig. 5B). Vessel lumen area in tissues treated with multilayered sheets was significantly larger than that in the control tissue. Moreover, microvessel density

Fig. 3. Supernatant from multilayered sheets enhanced angiogenic activity and fibroblast migration. (A) Multilayered sheets enhanced angiogenic activity. HUVECs were cultured in fresh medium or conditioned medium from single-layered, fibroblast, or multilayered sheets. The conditioned media from single-layered (1.25×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well), fibroblasts (5.0×10^5 fibroblast cells/well and no PBMNCs), and multilayered (5.0×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well) sheets were co-cultured in 24-well culture plates and incubated under normoxic conditions (37°C , atmospheric O_2 concentration) for 2 days followed by hypoxic conditions (33°C , 2% O_2) for 1 day. The number of junctions in the field was counted at $\times 10$ microscopic magnification; scale bar indicates 500 μm . (B) Cell proliferative activity of multilayered sheets was analyzed using migration assay. Confluent mouse fibroblasts were scraped to create a scratch approximately 1.0 mm in size and cells were cultivated further in conditioned medium for 18 h. Conditioned media of single-layered (1.25×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well), fibroblast (5.0×10^5 fibroblast cells/well and no PBMNCs), and multilayered (5.0×10^5 fibroblast cells/well and 2.0×10^6 PBMNCs/well) sheets were cultured in 24-well plates and incubated under normoxic conditions (37°C , atmospheric O_2 concentration) for 2 days followed by hypoxic conditions (33°C , 2% O_2) for 1 day. Wound closure area was measured and compared among the experimental groups. Cells were colored in green virtually to visualize the wound closure area; scale bar indicates 500 μm . (C) Apoptotic assay in multilayered and single-layered sheets. Arrowhead indicates TUNEL-positive cell. Error bars indicate standard error. Single: single-layered sheets, Fibroblast: fibroblast sheets, Multi: multilayered sheets.

in tissues treated with multilayered sheets was significantly higher than in the other tissues (Fig. 5C).

Discussion

We have described the development of mouse multilayered mixed-cell sheets consisting of autologous fibroblasts and PBMNCs, and their curative effect on ulcer healing in mice. Ulcer healing requires a well-orchestrated interplay of cell-matrix and cell-cell signalling. Fibroblasts have an important role in facilitating the interaction of extracellular matrix (ECM) with keratinocytes and endothelial cells for ulcer healing [31]. In addition,

Fig. 4. Therapeutic effect of multilayered sheets. (A) Wound healing after single-layered, fibroblast, and multilayered sheet transplantation onto full-thickness skin defects on the backs of DM mice at days 1, 7, and 14. Control shows DM mice that did not receive the experimental treatment. (B) Wound healing rate at different time points after treatment. Wound area was measured with Image J software. Error bars indicate standard error. Single: single-layered sheets, Fibroblast: fibroblast sheets, Multi: multilayered sheets.

tion, these three cell types secrete vascularization-associated growth factors, including VEGF, TGF- β 1, and HGF [32]. Fibroblasts can be isolated and cultured from various tissues, and PBMNCs are harvested easily from patients in the clinic and can be transplanted to ischemic tissues for angiogenesis treatment [33-35]. It is known that not only growth factors but also inflammatory cytokines and chemokines, such as IL-6 and CXCL, are essential for the normal wound-healing process [20, 36]. This multilayered mixed-cell sheet secretes these growth factors, inflammatory cytokines, and chemokines, which have the potential to augment the normal wound-healing process.

Cell sheet technology is a recent development. Instead of detaching cells with the conventional dispase or trypsin, a novel temperature-responsive culture dish enables detachment as a single continuous sheet of cells while maintaining the ECM [37]. Using this technology, fibroblasts, as adherent cells, formed layers on our mixed-cell sheets and these cell sheets with ECM were transplanted to ulcers. However, it is very difficult to form multilayered sheets with a temperature-responsive culture dish by using this method. Therefore, we attempted to make multilayered sheets on a typical culture dish and detach them using a small amount of dispase. With this method, the multilayered sheets incorporated a subset of PBMNCs as in our previous study [26], and the population of PBMNCs was considered to contribute to angiogenesis [38].

In addition, we developed a novel and feasible protocol called 'hypoxic preconditioning' to enhance the cellular functions of transplanted cells using hemeoxygenase-1 and hexokinase-2 [39], and improve the retention of transplanted cells through CXCR4 and integrin α M in mice [40, 41]. In our previous study in rabbits, hypoxic preconditioned PBMNCs enhanced cell adhesive ability and angiogenesis, and transplantation of the hypoxic preconditioned PBMNCs into muscle improved blood flow at the ischemic hindlimb [13]. Hypoxic preconditioning has also been reported to increase secretion of VEGF in single-layered sheets [26, 27]. Therefore, hypoxic preconditioning may be effective for promoting secretion of growth factors in multilayered sheets.

To assess the effect of ulcer healing *in vivo*, we used mice with streptozotocin-induced DM to slow wound healing. Three multilayered sheet-treated groups had a significantly higher rate of ulcer wound healing on day 14 compared with the control group. In particular, single-layered and multilayered sheet-treated groups also had significantly enhanced wound healing on day 7 compared with the control group. The facilitated wound healing in the two types of mixed sheet-treated groups reflected an increase in growth factors and cytokines. This finding might indicate that the three types of cell sheets secrete sufficient amounts of several growth factors, including VEGF, to promote wound healing. Conversely, there were no differences among the three sheet-treated groups on days 7 and 14. However,

Fig. 5. Angiogenesis in multilayered sheets. (A) H&E- and (B) CD31-stained images 21 days after single-layered, fibroblast, and multilayered sheet transplantation. CD31 is a marker of endothelial cells. (C) Comparison of vessel lumen area and microvessel density in tissues treated with multilayered sheets. Magnification $\times 40$. Error bars indicate the standard error. Single: single-layered sheets, Fibroblast: fibroblast sheets, Multi: multilayered sheets.

we observed a significant increase in mature microvascular angiogenesis in the skin treated with multilayered sheets as these sheets produce more growth factors, including VEGF, than single-layered sheets. There are no appropriate animal models that reflect human refractory cutaneous ulcers. Therefore, a skin defect model was used in this study. Hence, the efficacy of multilayered mixed sheets may not have been fully demonstrated. Based on these results, it is expected that multilayered sheets will provide effective treatment for refractory cutaneous ulcers in clinical trials.

In the present study, our data clearly showed that multilayered sheets had a curative effect on skin defect models for ulcer treatment. These sheets promoted wound healing and microvascular angiogenesis in the skin by supplying growth factors and cytokines. Our findings suggest that multilayered sheets may be a useful tool for treatment of refractory ulcers.

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Disclosure Statement

No conflict of interests exists.

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