Original Article
Ulcer healing effect of autologous mixed sheets consisting of fibroblasts and peripheral blood mononuclear cells in rabbit ischemic hind limb

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Abstract: We developed mixed cell sheets consisting of fibroblasts and peripheral blood mononuclear cells that had high potency for secreting vascular endothelial growth factor. The purpose of this study was to confirm the therapeutic effects of mixed sheets in rabbits suffering from ulcers at the ischemic hind limbs. We used the ulcer model, which was constructed by implantation and sandwiching the skin between two magnets to be a representative of human refractory cutaneous ulcer. The ulcer healing rate of mixed cell sheets was higher than that of the control at an early stage of healing. The calf blood pressure and angiographic score, which were considered to reflect rough collateral blood flow, did not vary amongmixed cell sheets. However, through laser Doppler perfusion image, implantation of mixed cell sheets revealed a significant improvement in microvascular blood flow in the healed skin of the ischemic limb compared to trafermin, a recombinant human basic fibroblast growth factor, and the control. These results suggest that mixed cell may operate predominantly on the surface of the ischemic tissue by their angiogenic potency, thereby promoting healing of the ischemic ulcer. Mixed cell sheets could become a promising therapeutic material for refractory cutaneous ulcers.

Keywords: Cell sheet, fibroblast, peripheral blood mononuclear cells, skin ulcer, magnet, angiogenesis

Introduction

Chronic, non-healing wounds defined as barrier defects that do not heal within 3 months debilitate affected individuals. In these cases, there is a need for the development of a therapeutic challenge [1-3]. Almost all chronic wounds have a clear underlying cause, such as ischemic disorders containing arteriosclerosis obliterans (ASO), Buerger's disease, blue toe syndrome, diabetes mellitus, pressure, and congestive disorder containing venous insufficiency. With respect to ischemic disorder, many patients have undergone revascularization by surgery for blood flow improvement; however, some patients undergo limb amputation because of progressing infection and an insufficient response to pharmacotherapy if the skin ulcer does not heal [4]. To date, many curative materials have been developed for treating a skin ulcer, such as large varieties of dressing and collagen gels to maintain moistness of the ulcer surface to facilitate healing [5-7].

From the view point of angiogenesis to recover blood flow, various kinds of growth factors have been applied by protein administration or gene therapy to promote angiogenesis in the ischemic tissue [8-14]. In the field of angiogenic therapy, regenerative medicine has recently undergone rapid development [15]. Cell implantation methods using peripheral blood mononuclear cells (PBMNCs), endothelial progenitor cells, bone marrow mononuclear cells, bone marrow mesenchymal stem cells, and adipose-derived regenerative cells for the ischemic limb have been performed as clinical trials [16-22]. Although these therapies are effective for treating ulcers in a partial population of the patients, ulcers in many patients do not heal by
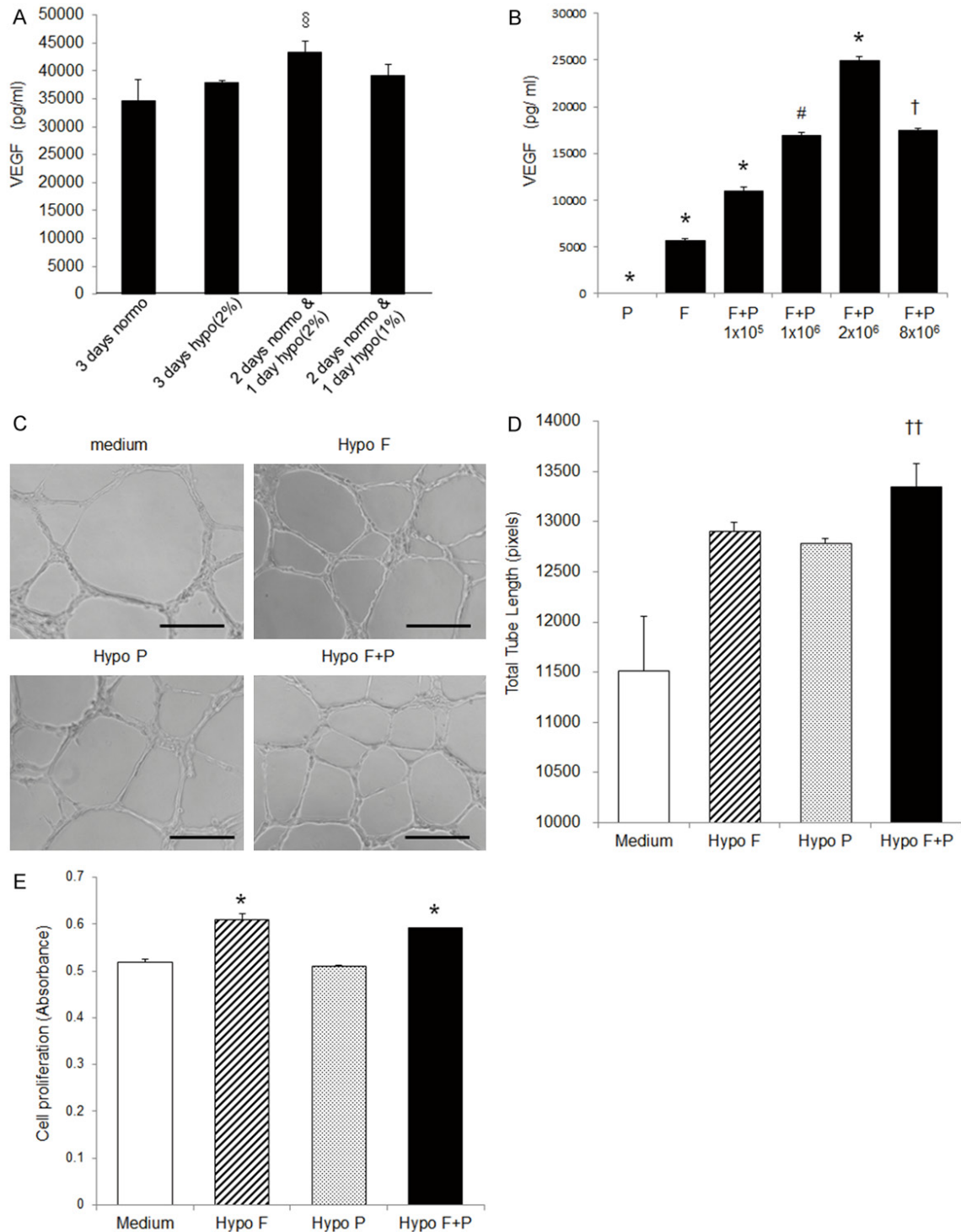
Figure 1. Synergetic effect of hypoxic preconditioning and cell mixing. A. Comparison of vascular endothelial growth factor (VEGF) secretion from fibroblasts incubated under four different conditions. Fibroblasts (5 × 10⁵ cell/well) were incubated for 3 d. The normoxic condition was 37°C, 5% CO_2, 20% O_2. The hypoxic condition was 33°C, 5% CO_2, 2% O_2, or 33°C, 5% CO_2, 1% O_2. §P < 0.05 vs. 3 d normo. B. Comparison of VEGF secretion from peripheral blood mononuclear cells (PBMNCs) and/or fibroblasts. Fibroblasts were co-cultured with different doses of PBMNCs (1 × 10⁵ cell/well, 1 × 10⁶ cell/well, 2 × 10⁶ cell/well, 8 × 10⁶ cell/well) for 3 d under a normoxic condition (37°C, 5% CO_2, 20% O_2). The VEGF concentration in the supernatant was measured by enzyme-linked immunosorbent assay (ELISA). *P < 0.01 vs. all of other groups; #P < 0.01 vs. all of other groups but F + P 8 × 10⁶; †P < 0.01 vs. all other groups but F + P 1 × 10⁶. C. Representative images of tube formation are shown. Scale bar shows 1,000 μm. D. Tube

formation assay was performed to evaluate the angiogenesis potency among the four groups. Total tube length per field is shown. ††P < 0.05 vs. medium. E. Fibroblast proliferative effects in four different conditioned media were evaluated by MTS assay. *P < 0.01 vs. medium and hypo P. P: PBMNC. F: Fibroblast. Hypo: 37°C, 5% CO_2, 20% O_2 for 2 d followed by 33°C, 5% CO_2, 2% O_2 for 1 d.
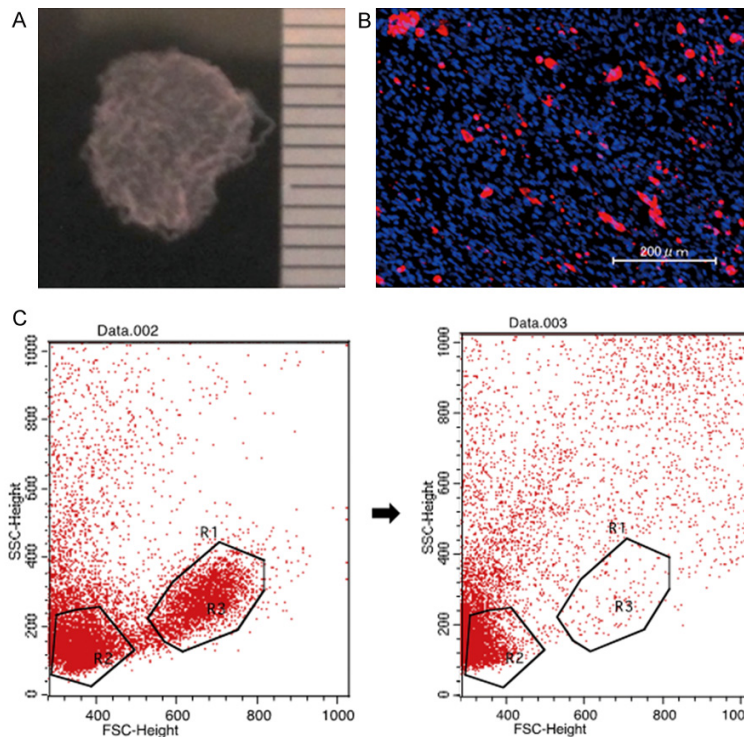
Figure 2. Mixed cell sheets consisting of fibroblasts and peripheral blood mononuclear cells (PBMNCs). A. The rabbit mixed cell sheet was detached from temperature-responsive culture dishes incubated at < 20°C. One small unit = 1 mm. B. Immunohistochemistry for PBMNCs (PKH26: red) and nuclear (4',6-diamidino-2-phenylindole (DAPI): blue) in rabbit mixed cell sheet revealed that PKH26-positive PBMNCs were present in the sheets. Scale bar = 200 mm. C. Fluorescence-activated cell sorting (FACS) analysis of PBMNCs before and after incubation for 24 h is shown. Left figure shows the distribution of freshly isolated PBMNCs and right figure shows that of floating cells after 24 h incubation of PBMNCs.

cell implantation, leading to infection and amputation.

Therefore, we considered the use of cell sheet technology to treat refractory cutaneous ulcers. We developed a mixed cell sheet consisting of fibroblasts and PBMNCs and verified the efficacy for ulcer treatment in mice [23]. The mixed cell sheet had high angiogenic potency, promoted wound closure, and led to a complete healing as the natural status. In the present study, we examined the therapeutic efficacy of mixed cell sheets in rabbits as the next step required before application of this mixed cell sheet to early-phase human trials.

Results

Angiogenic potency of mixed cell sheets

To assess the angiogenic effect of hypoxic-preconditioned mixed cell sheets, the concentration of vascular endothelial growth factor (VEGF) in the supernatant was measured using an enzyme-linked immunosorbent assay (ELISA). The concentration of VEGF secreted from fibroblasts was significantly higher in fibroblasts cultured under normoxic conditions (37°C, 20% O_2) for 2 d followed by hypoxic conditions (33°C, 2% O_2) for 1 d (**Figure 1A**) than that under other culture conditions. Even if fibroblasts were cultured under a hypoxic condition (33°C, 2% O_2) for 3 d or normoxic conditions for 2 d followed by hypoxic conditions (33°C, 1% O_2) for 1 d, there was no increase of VEGF secreted from fibroblasts compared to normoxic conditions for 3 d. In addition, 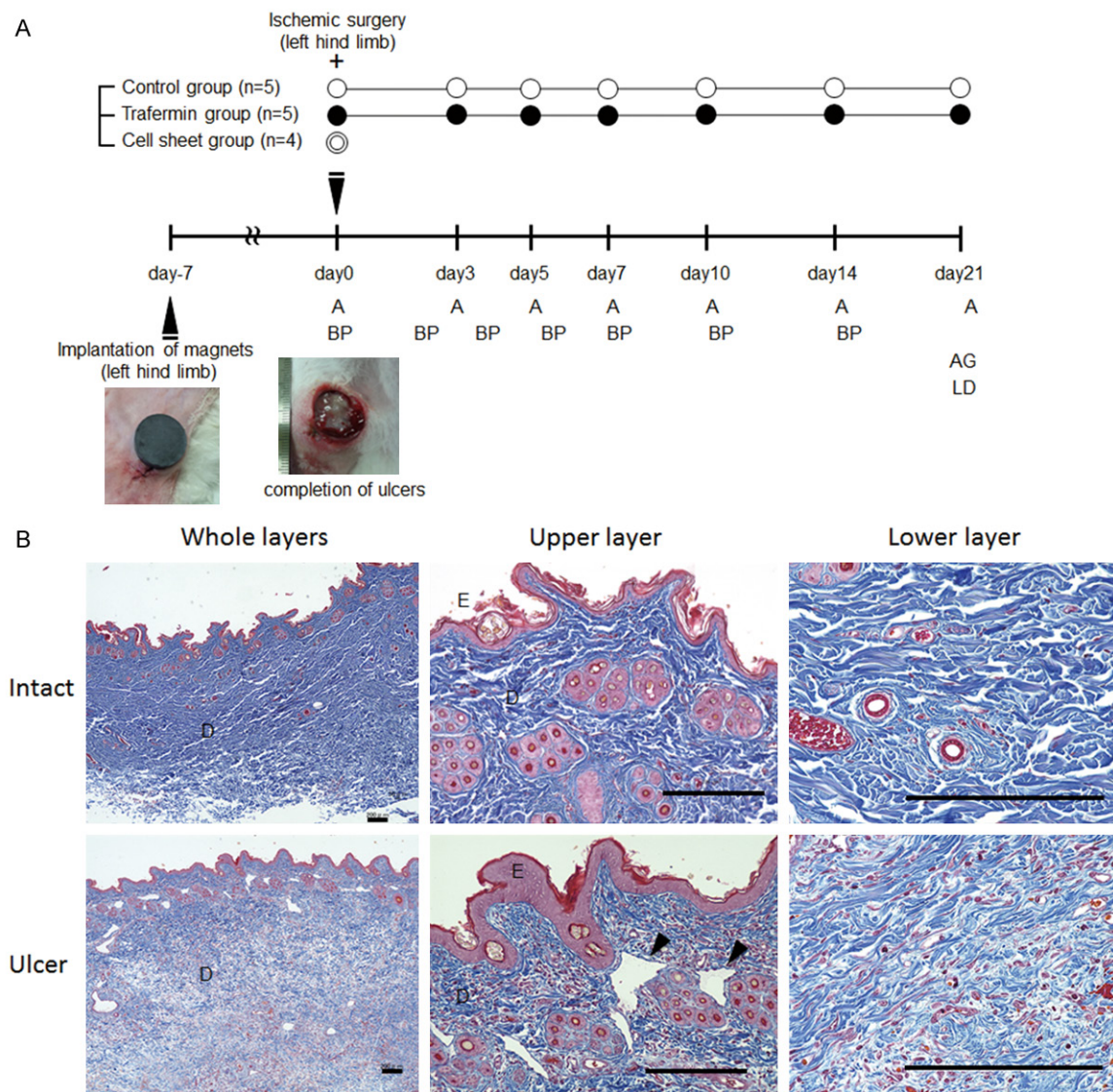although PBMNCs did not secrete VEGF in isolation, co-culture of PBMNCs and fibroblasts showed a dose-dependent increase in VEGF release (**Figure 1B**). However, too many PBMNCs, 8 × 10⁶ cells/well, reduced VEGF secretion. A tube formation assay showed that tube lengths formed by human umbilical vein endothelial cells (HUVECs) in conditioned medium from the co-culture of fibroblasts and PBMNCs were longer than those from conditioned media of fibroblasts or PBMNCs (**Figure 1C** and **1D**).

Cell proliferative potency of mixed cell sheets

To assess cell proliferation of fibroblasts by conditioned medium cultured under the opti-

Figure 3. Schedule of the present study and hind limb cutaneous ulcer model. A. Time schedule of the present study. Photographs show ulcers which were induced by implanting magnets under skin and sandwiching the skin between magnets for 7 d continuously. A: Area or ulcers. BP: Blood pressure. LD: Laser Doppler. AG: Angiography. ○: Application of saline. ●: Application of trafermin. ◎: Application of mixed cell sheet. B. Representative histological tissue sections showing the comparison of intact skin and skin around the ulcers by Masson's trichrome staining. In the upper layer, epidermis was thicker in the skin around the ulcer than intact skin and there were many dilated vessels (black arrowheads) in the skin around the ulcer. In the lower layer, infiltration of many inflammatory cells, edema, and hyperplasia of dermis in the skin around the ulcer were evident. D: Dermis, E: Epidermis. Scale bar = 200 mm.

mal culture condition for VEGF secretion, i.e., normoxic conditions (37°C, 20% O_2) for 2 d followed by hypoxic conditions (33°C, 2% O_2) for 1 d, a cell proliferation assay was conducted using conditioned media. Conditioned media cultured by fibroblasts and co-culture of fibroblasts and PBMNCs increased fibroblast proliferation to a degree equal compared to that of the control and PBMNC-conditioned media

(Figure 1E). PBMNCs-conditioned medium had no effect on fibroblast proliferation.

Constitution of mixed cell sheets

Mixed cell sheets were constructed using six well plates and peeled from the adhesive surface by temperature change. The rabbit sheets had a diameter of approximately 1 cm, and
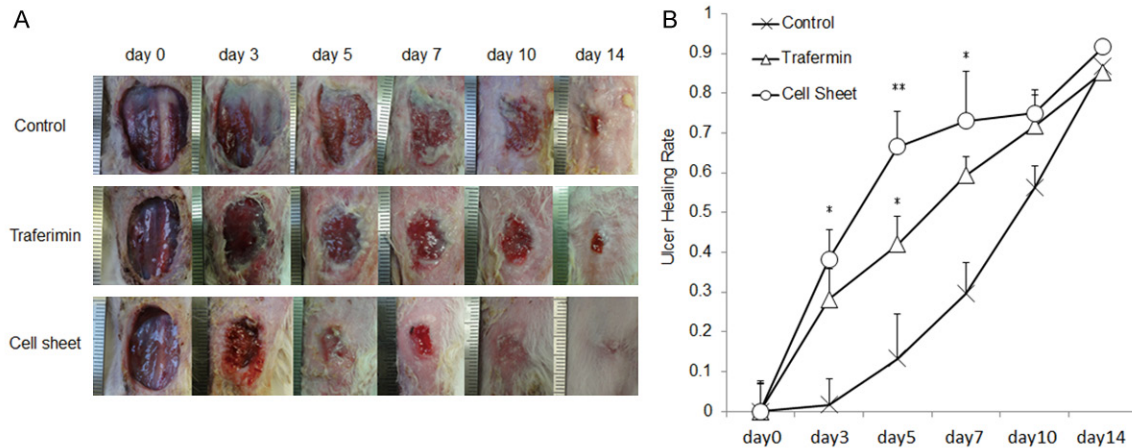
Figure 4. Therapeutic effect of autologous mixed cell sheets to ulcer healing. A. Representative images of the gross appearance of ulcers over time after treatment. B. Quantitative data of the proportion of the reduction relative to the initial ulcer area at different time points after treatment. Ulcer area was measured by Image J software. *$P < 0.05$ vs. control; **$P < 0.01$ vs. control (control; n = 5, trafermin; n = 5, cell sheet; n = 4).

formed a round shape (**Figure 2A**). To observe whether PBMNCs exist into cell sheets, mixed cell sheets were constructed from PBMNCs stained with PKH26. Immunohistochemistry of the mixed cell sheets revealed that there were many PKH26-positive PBMNCs in the sheets (**Figure 2B**). To assess the population of the PBMNCs, the distribution of PBMNCs was compared using fluorescence-activated cell sorting (FACS) analysis before and after incubation for 24 h. The cells in the area of monocytes before incubation almost disappeared after incubation (**Figure 2C**).

Therapeutic effect of mixed cell sheets in a rabbit hind limb ischemic and cutaneous ulcer model

To evaluate mixed cell sheets as a therapeutic material for treatment of cutaneous ischemic ulcers, autologous cell sheets originating from rabbits were transplanted onto the ulcers constructed from two magnets positioned in their left low extremity, in which ischemia was maintained by removing the left femoral arteries. This ulcer model was developed in our previous study in mice, and was also used in the present study. As a gross pathology, the edge of each ulcer in rabbits was impaired similar to that in the mouse model, with the ulcers induced by two magnets. The histology of each ulcer showed a thickened epidermis and dermis, many dilated vessels, infiltration of many inflammatory cells, and edema in the skin around the ulcer compared with intact skin (**Figure 3B**). The healing process, regarded as a reduction

in ulcer size, was then observed (**Figure 4A**). The wound healing rate in cell sheets was significantly higher than the control at day 3, 5, and 7. However, at day 10, the healing rates of trafermin and the control were the same as that of the cell sheet. The wound healing rate in trafermin was higher than that of the control only at day 5 (**Figure 4B**).

Effect of mixed cell sheets on the development of collateral flow

To roughly assess the development of collateral flow, calf blood pressure was measured chronologically by the ratio of systolic pressure of the ischemic (left) hind limb to that of the intact (right) hind limb. Calf blood pressure ratio was chronologically increased after ischemic surgery; however, there were no significant difference between cell sheet, trafermin, and control groups at the respective time points (**Figure 5A**). The collateral flow was then examined by angiography in the left medial thigh where the vessels were removed. The angiographic scores were at the same level between the three groups at day 21 (**Figure 5B**).

Effect of mixed cell sheets on the development of microcirculation in the skin after ulcer healing

To determine the reason for the cell sheet leading to a higher ulcer healing rate at the early stage of wound healing, blood flow in the skin where the ulcer had existed was examined. Perfusion rates were calculated by dividing aver-
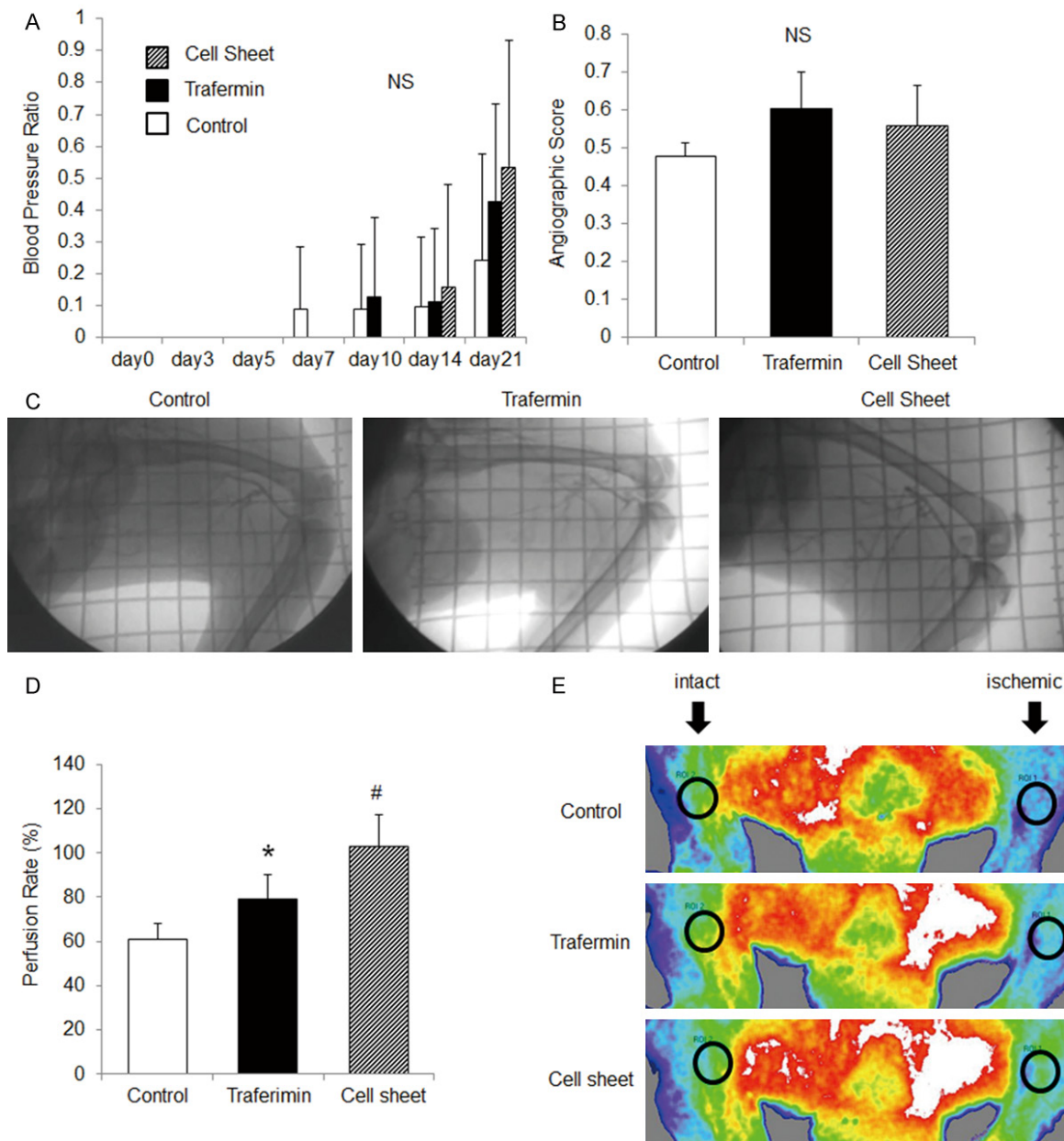
Figure 5. Angiogenic effect of autologous mixed cell sheets. A. Calf blood pressure ratio (ischemic/normal hind limb) was chronologically increased. (control; n = 5, trafermin; n = 4, cell sheet; n = 4). B. Angiographic score at day 21. (control; n = 3, trafermin; n = 4, cell sheet; n = 3). C. Representative angiography at day 21 in the left hind limb is shown. The 1 cm^2 grid plate was placed under the rabbit during angiography. Collateral vessels were recognized in all groups in the medial thigh. D. Perfusion rate was calculated by dividing average perfusion score in the ischemic hind limb (left) by that in the intact hind limb (right) at day 21. *$P < 0.01$ vs. control; #$P < 0.01$ vs. control and trafermin. (control; n = 5, trafermin; n = 5, cell sheet; n = 4). E. Representative laser Doppler perfusion images at day 21 in rabbit bilateral hind limbs are shown. High perfusion is indicated by red and low perfusion is indicated by blue. Right hind limbs are intact and left limbs are ischemic.

age perfusion score in the ischemic (left) hind limb by that in the intact (right) hind limb. Perfusion rates were highest in cell sheet, followed by in the trafermin group in which the perfusion rate was significantly higher at day 21 (**Figure 5D**).

Discussion

The present study demonstrated the preparation of rabbit mixed cell sheets consisting of fibroblasts and PBMNCs, and their curative effect for ulcer healing in rabbits. Ulcer healing

requires a well-orchestrated interplay of cell-matrix and cell-cell signaling. Fibroblasts play an important role in facilitating the interaction of extracellular matrix (ECM) with keratinocytes and endothelial cells for ulcer healing [24]. In addition, these three cells secrete vascularization-associated growth factors including VEGF, basic fibroblast growth factor (bFGF), and platelet-derived growth factor (PDGF) [25]. Fibroblasts can be isolated and cultured from various issues, and PBMNCs are easily harvested from humans clinically and can be transplanted to ischemic tissues as a key player for angiogenesis treatment [19-21]. Cell sheet technology has been a recent development. Instead, of detachment of cells by dispase or trypsin, a temperature-responsive culture dish was developed, making it possible to detach cells from dishes as cell sheets while maintaining the extracellular matrix [26]. By using this technology, fibroblasts as adherent cells formed cell sheets on our mixed cell sheets and were transplanted to ulcers as cell sheets with ECM. PBMNCs therefore contain cells with adhesive ability, which is due to a population of monocytes considered to contribute to angiogenesis [27]. Our data showing that monocytes adhered to dishes indicates that the population may be incorporated into the mixed cell sheets. In fact, in our previous study, CD11b-positive cells from PBMNCs were observed in the mixed cell sheets by immunohistochemistry in mice [23]. In addition, we have developed a novel and feasible protocol called "hypoxic preconditioning" to enhance the cellular functions of transplant cells through hemeoxygenase-1 and hexokinase-2 [28], and improved the retention of transplanted cells through C-X-C chemokine receptor type 4 (CXCR4) and integrin αM in mice [29, 30]. In our previous study using rabbits, hypoxic preconditioned PBMNCs enhanced cell adhesive ability and angiogenesis, and transplantation of the hypoxic preconditioned PBMNCs into the muscle improved the blood flow at the ischemic hind limb [31]. Therefore, our data suggest that hypoxic preconditioning is a powerful tool for improving therapeutic angiogenesis in severe ischemia patients. Therefore, this method was applied for our novel mixed cell sheets. In our previous study, mixed cell sheets secreted large amounts of VEGF rather than fibroblasts or PBMNCs, and hypoxic preconditioning for mixed cell sheets provided a larger increase in

VEGF release, which led to high wound healing potency [23]. The present study also demonstrated a positive relationship between the concentration of VEGF from fibroblasts and the number of suitable co-cultured PBMNCs, which might induce high angiogenic potency of mixed cell sheet in rabbits. Our result showed that hypoxic preconditioning was effective to increase VEGF secretion from fibroblasts.

To assess the effect of ulcer healing *in vivo*, we used the novel ulcer model which was considered relevant for human refractory cutaneous ulcer. This ulcer model in rabbits also showed dilated vessels and edema in the dermis, which were considered to reflect congestion. By using this model, we examined the improving potency of blood flow in the ischemic region and ulcer healing potency by transplantation of a mixed cell sheet. Although mixed cell sheets led to the significantly highest rate of ulcer wound healing compared to the control on days 3 and 7, healing speed in cell sheet-treated ulcers was suddenly decreased from day 7 onwards. This was considered to be because the mixed cell sheet might have been removed from the ulcer by peeling off with the dressing, because the mixed cell sheet was only applied once. If frequent application of the sheets is conducted, ulcers might heal within a shorter term than by single application, thereby allowing high healing potency against the ulcers to continue to a late stage of healing. Conversely, because trafermin was applied many times in the trafermin-treated group and since saline application was performed to prevent desiccation of the ulcer in the control group, the ulcer healing rates in these groups may have converged with that of the mixed cell sheet group from day 10 onwards. This result indicates the necessity to transplant the sheets many times for more efficient treatment.

In the analyses of calf blood pressure rate and angiography, there were no significant differences between mixed cell sheets, trafermin, and the control. However, the laser Doppler perfusion image showed an improvement of blood flow in mixed cell sheets. This indicates that a significant increase in microvascular *angiogenesis* occurred in the skin treated by mixed cell sheets. These results suggest that mixed cell sheets may predominantly operate on the surface of peripheral tissue of the ischemic region.

In the present study, our data clearly showed that the mixed cell sheet had a curative effect on ulcer healing by microvascular angiogenesis in the skin, and did not induce tumor development after transplantation (unpublished data). Our novel mixed cell sheet is expected to provide various benefits such as a natural cure of the wound, suppression of inflammation, and normalization of the impaired epidermis [23], thereby indicating that mixed cell sheets may provide a strong clinical material for treatment of refractory ulcers.

Materials and methods

Animals

Male New Zealand white rabbits (2.5-3.0 kg body weight, KBT Oriental, Saga, Japan) were used for the animal experiments. Animals were housed in a regulated environment (22°C ± 2°C), with a 12 h light/dark cycle (light cycle was from 8:00 am to 7:59 pm). All animal experiments were approved by the Institutional Animal Care and Use Committee of Yamaguchi University (#31-093). All surgeries were performed under general anesthesia and all efforts were made to minimize animal suffering.

Isolation of PBMNCs and fibroblasts

PBMNCs were isolated from rabbit peripheral blood using Lympholyte®-Rabbit (CedarLane Laboratories Ltd., Hornsby, Ontario, Canada) and cultured in Roswell Park Memorial Institute (RPMI)-1640 (Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 10% fetal bovine serum (FBS) (Thermo Fisher Scientific) and penicillin-streptomycin (Thermo Fisher Scientific). Fibroblasts were isolated from the ears of rabbits using collagenase (Wako, Osaka, Japan) and cultured in CTS™ AIM V® Medium (Thermo Fisher Scientific) supplemented with 10% FBS (Thermo Fisher Scientific).

Enzyme-linked immunosorbent assay (ELISA) for VEGF

To assess VEGF release from fibroblasts under different culture conditions, 3 mL of fibroblasts (5×10^5 cell/well) were seeded in a six well culture dish and incubated under four different conditions: 1) normoxic conditions (37°C, 20% O_2) for 3 d; 2) hypoxic conditions (33°C, 2% O_2) for 3 d; 3) normoxic conditions (37°C, 20% O_2) for 2 d followed hypoxic conditions (33°C, 2%

O_2) for 1 d, and; 4) normoxic conditions (37°C, 20% O_2) for 2 d followed by hypoxic conditions (33°C, 1% O_2) for 1 d. After incubation, the supernatant of each dish was collected, and the concentration of VEGF was measured using human the VEGF Quantikine immunoassay kit (R&D systems, Minneapolis, MN, USA).

To assess VEGF release from fibroblasts cultured with different doses of PBMNCs, 4 mL of fibroblasts (5×10^5 cell/well) and 4 mL of PBMNCs (1×10^5 cell/well, 1×10^6 cell/well, 2×10^6 cell/well, 8×10^6 cell/well) were co-cultured in a six well culture dish, and incubated under a normoxic condition (37°C, 20% O_2) for 3 d. For fibroblast cultures, 4 mL of fibroblasts (5×10^5 cell/well) and 4 mL of RPMI-1640 including supplements were added to the same well in a six well culture dish. For PBMNC cultures, 4 ml of PBMNCs (2×10^6 cell/well) and 4 mL of AIM V Medium including supplements were added to the same well in a six well culture dish. After incubation, ELISA was performed in the manner as described above.

Fibroblast proliferation assay

To evaluate the contribution to proliferation of fibroblasts by conditioned medium of fibroblasts and PBMNCs, a cell proliferation assay was performed. A volume of 150 μL of fibroblasts (2.5×10^3 cells/well) was plated onto a 96well plate and incubated with 50 μL conditioned medium for 48 h, following which 20 μL of Cell Titer 96 (Promega, USA) was added to each well, and absorbance was determined after 5 h.

The preparation of each conditioned medium is described below. For fresh medium, Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific) supplemented with 20% heat-inactivated FBS, MEM non-essential amino acids solution (Life Technologies), and penicillin-streptomycin and RPMI-1640 supplemented with 10% FBS and penicillin-streptomycin were mixed equally. For fibroblast conditioned medium, 1 mL of fibroblasts (1.25×10^5 cell/well) and 1 mL of RPMI-1640 including supplements were added to the same well in a 24 well culture dish. For PBMNC conditioned medium, 1 mL of PBMNCs (2×10^6 cell/well) and 1 mL of DMEM including supplements were added to the same well in a 24 well culture dish. For fibroblast and PBMNC conditioned medium, 1 mL of fibroblasts (1.25×10^5 cell/well) and 1 mL of PBMNCs (2×10^6 cell/well) were added

to the same well in a 24 well culture dish. Each supernatant was collected after incubation for 2 d under normoxic conditions (37°C in 20% O_2 and 5% CO_2) followed by 1 d under hypoxic conditions (33°C in 2% O_2 and 5% CO_2), respectively.

Tube formation assay

HUVECs were maintained in EGM™-2 medium (Lonza, Switzerland) supplemented with EGM™ BulletKit™ (Lonza). HUVECs were then trypsinized, resuspended in 10% FBS/AIM V Medium, and seeded onto Matrigel® (Corning)-coated 96 well plates at 2×10^4 cells/well (50 µL). Subsequently, 50 µL of conditioned medium from the fresh medium, fibroblast conditioned medium, PBMNC conditioned medium, or fibroblast and PBMNC conditioned medium were added into each of the wells. Images of tube formation were captured 12 h later and measured using the Angiogenesis Analyzer for ImageJ software (National Institutes of Health) [32, 33].

PKH-26 stained PBMNC contained in cell sheet

A volume of 1 mL PBMNCs (1×10^6 cell/well) labeled with a red fluorescent dye (PKH26; Sigma, St. Louis, MO, USA) and 1 mL of fibroblasts (2.5×10^5 cell/well) were co-cultured in a 12 well culture dish and incubated for 2 d under normoxic conditions (37°C in 20% O_2 and 5% CO_2) followed by 1 d under hypoxic conditions (33°C in 2% O_2 and 5% CO_2). After the dishes were washed with PBS, cells were stained by 4',6-diamidino-2-phenylindole (DAPI).

FACS

Freshly isolated PBMNCs were analyzed by FACS (FACScalibur, BD, San Jose, CA, USA). After PBMNCs were incubated in a 10 cm culture dish for 24 h, supernatant including floating cells was collected. FACS analysis was performed again using the same method and same gating as the analysis of fresh PBMNCs. During the analysis, only living cells were targeted.

Rabbit hind limb ischemic and cutaneous ulcer model

A 24 gauge intravenous catheter was inserted into the marginal ear vein for anesthetics and rabbits were anesthetized by an intravenous injection of ketamine (Daiichi Sankyo Propharma, 1 mg/kg) and xylazine (Bayer, 3 mg/kg). Repeated injections of ketamine and xylazine were administered as required to maintain a deep level of anesthesia. On day -7, excess hair was removed and depilated from the bilateral hind limbs [34]. The crucial incision was performed on the left low extremity, and a ferrite magnet (f20 mm, 5 mm thickness) was implanted under the skin through the incision, following which the incisional skin was sandwiched between the two magnets. On day 0, the magnets were removed, and an ulcer was created on the defected skin. Concurrently, through a longitudinal skin incision in the left thigh, the left femoral artery was ligated and all collateral branches were subsequently dissected free to induce ischemia.

Preparation and transplantation of cell sheets

For mixed cell sheets, 4 mL of fibroblasts (5×10^5 cell/well) and 4 mL of PBMNCs (2×10^6 cell/well) were added to the same well in UpCell® 6 multi-well plates (Cell Seed Inc., Tokyo, Japan) and incubated for 2 d under normoxic conditions (37°C in 20% O_2 and 5% CO_2) followed by 1 d under hypoxic conditions (33°C in 2% O_2 and 5% CO_2). Rabbits were divided into the following three groups: 1) mixed cell sheets (n = 4); 2) trafermin (n = 5), and; 3) control (n = 5). For the cell sheets group, a mixed cell sheet was placed onto the ulcer at day 0 only. For the trafermin group, trafermin was applied by spraying during every observation day using Fiblast Spray (Kaken Pharmaceutical Co., Ltd., Tokyo, Japan). For the control group, 100 µL of saline was applied at every observation day to prevent drying. All ulcers were covered with Sofratulle® (SANOFI, Paris, France), wrapped with OPSITE® FLEXIFIX GENTLE (Smith & Nephew Medical Limited, British), and fixed with bandage (Kawamoto, Osaka, Japan). The ulcer area was measured by Image J software at days 0, 3, 5, 7, 10, 14, and 21 under anesthesia.

Measurement of calf blood pressure ratio

Calf blood pressure was measured at days 0 (just after the operation), 3, 5, 7, 10, 14, and 21 in both the dorsal region of the foot with a sphygmomanometer (petMAP graphic II, CardioCommand Inc., Tampa, Florida, USA) under slight anesthesia (the inhalation of 4.0% sevoflurane). The systolic pressures in both limbs as

well as the ratio of systolic pressure of the ischemic (left) hind limb to that of the intact (right) hind limb were determined [35].

Angiographic analysis

Using angiography, we studied the development of collateral arteries on day 21. A 4-Fr angio-sheath was placed in the aortic archvia the left common carotid artery, and the contrast medium (10 mL, IOVERIN 300, TEVA, Japan) was injected as a bolus with hand injection under anesthesia. Using a 1 cm^2 grid overlay, quantitative angiographic analysis of the development of collateral vessel in the ischemic hind limb was performed, as described previously [35]. The ratio of grid intersections crossed by opacified arteries to the total number of grid intersections in the ischemic medial thigh was calculated to obtain the angiographic score.

Measurement of laser doppler perfusion image

Blood flow in the ischemic hind limb was measured using a laser Doppler perfusion imaging system (Omegazone laser Doppler blood-flow imaging system, Omegawave, Inc., Tokyo, Japan) at day 21 under slight anesthesia. Both intact (right) and ischemic (left) hind limbs were scanned, and mean perfusion scores were obtained from each limb. The perfusion ratesin ischemic hind limbs were evaluated by determining the percentage of blood flow expressed as the average perfusion score in the left hind limb normalized by that in the right.

Statistical analysis

Data analysis was performed using Stata/SE 12.1 software (StataCorp, College Station, Texas, USA). 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 (ANOVA) followed by a Bonferroni post-hoc test. $P < 0.05$ was considered statistically significant. Data are expressed as mean ± standard deviation (SD).

Disclosure of conflict of interest

None.

Authors' contribution

Y. T., K. U. and K. H. contributed conception and designation of this experiments. Y. T., K. U., M. S. and T. M. performed experiments. Y. T., K. U., T. H. and K. H. analyzed data. T. H., A. O., T. M. and N. M. contributed reagents/materials/analysis tools. Y. T., K. U. and K. H. wrote the manuscript. All authors discussed the results and approved the manuscript.

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