Tissue sheet engineered using human umbilical cord-derived mesenchymal stem cells improves diabetic wound healing

Jingbo Zhang  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Xiang Qu  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Junjun Li  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Akima Harada  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Ying Hua  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Noriko Yoshida  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Masako Ishida  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Yoshiki Sawa  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu

Li Liu  
li-liu@surg1.med.osaka-u.ac.jp  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu  
https://orcid.org/0000-0003-0808-5072

Shigeru Miyagawa  
Osaka University School of Medicine Graduate School of Medicine: Osaka Daigaku Daigakuin Igakukei Kenkyuka Igakubu
Research Article

Tissue sheet engineered using human umbilical cord-derived mesenchymal stem cells improves diabetic wound healing

Jingbo Zhang¹, Xiang Qu¹, Junjun Li¹, Akima Harada¹, Ying Hua¹, Noriko Yoshida¹, Masako Ishida¹, Yoshi Sawa¹*, Li Liu¹* and Shigeru Miyagawa¹*

¹ Department of Cardiovascular Surgery, Osaka University Graduate School of Medicine, 2-2 Yamada-oka, Osaka, 565-0871, Japan

Corresponding authors: Yoshi Sawa, Li Liu, and Shigeru Miyagawa

Email: miya-p@surg1.med.osaka-u.ac.jp; sawa-p@surg1.med.osaka-u.ac.jp; li-liu@surg1.med.osaka-u.ac.jp

Abstract

Background: Diabetic foot ulceration is a common chronic diabetic complication. Human umbilical cord-derived mesenchymal stem cells (hUC-MSCs) have been widely in regenerative medicine used owing to their multipotency and wide range of sources. In this study, we developed an hUC-MSC tissue sheet by combining hUC-MSCs and a poly (lactic-co-glycolic acid) (PLGA)-based scaffold.
Methods: hUC-MSC tissue sheets were formed and optimized by culturing hUC-MSCs on PLGA-based scaffolds. Cell apoptosis, collagen composition, and tissue sheet characterization were evaluated by immunostaining in vitro. Diabetic mice were subjected to a full-thickness skin biopsy experiment to generate diabetic wounds and treated with either the hUC-MSC tissue sheet, hUC-MSC injection, fiber only, or left untreated (control). Fourteen days after transplantation, wound area tissue was collected for histological and immunohistochemical analyses.

Results: The in vitro data showed that hUC-MSC tissue sheets formed thick and solid tissue sheets with abundant extracellular matrix (ECM). Additionally, hUC-MSC tissue sheet transplantation promoted diabetic wound healing in vivo, with improved re-epithelialization and collagen deposition. Remarkable blood vessel formation and maturation were also observed in the hUC-MSC tissue sheet group compared to the other groups. Furthermore, hUC-MSC transplantation alleviated inflammation in vivo.

Conclusion: Our findings suggest that hUC-MSCs cultured on PLGA scaffolds improve diabetic wound healing, collagen deposition, and angiogenesis. These data provide a novel and effective method for cell transplantation, and a new strategy for the treatment of diabetic skin wounds.

Keywords: Diabetic wound, Human umbilical cord-derived mesenchymal stem cells, PLGA scaffold, inflammation, collagen deposition, healing
Background

Diabetes mellitus is a serious and common chronic disease, posing a major threat to public health [1]. According to reports from the International Diabetes Federation (IDF), the global diabetes prevalence in individuals aged 20-79 years old in 2019 was 9.3% (438 million people) [2]. Diabetic foot ulceration (DFU) is a common chronic diabetic complication [3,4]. Patients with diabetes have a 25% lifetime risk of developing foot ulcers compared to those without diabetes[5]. It is estimated that one patient with diabetes undergoes a lower limb amputation every 30 s worldwide [5]. DFU is a non-healing wound that is caused by an imbalance of various mechanisms, such as hemostasis, inflammation, collagen deposition, and angiogenesis [6]. In particular, the reduction of angiogenic factors and decrease in blood supply are the major mechanisms of DFU [7]. Therefore, enhancing angiogenesis in the wounded area could be an effective strategy to accelerate wound healing.

In recent decades, growing evidence has demonstrated that mesenchymal stem cell (MSC)-based therapy can promote wound healing and inhibit scar formation [8–10]. Therefore, MSC transplantation is gaining increasing attention as a novel strategy for treating diabetic wounds [11]. However, several previous studies have indicated that the number and function of MSCs decrease with age or long-term disease. Because most diabetic patients are older adults, the majority of patients with diabetes lack sufficient and functional autologous stem cells for cell therapy of diabetic wounds [12–15]. Among the available sources of MSCs, the umbilical cord is economically
viable and productive. Human umbilical cord-derived MSCs (hUC-MSCs) have been reported to accelerate wound healing; however, there are few studies on their effects on diabetes mellitus [16]. Moreover, the mechanism by which hUC-MSCs accelerate diabetic wound healing remains unclear.

The injection of a single-cell suspension into the periphery of the wounded area is a useful method for the delivery of MSCs [17]. However, due to the mechanical stress of the syringe needle, the direct injection method destroys the integrity of the cell membrane, damages the connection between cells and the extracellular matrix (ECM), and leads to apoptosis and dysfunction of MSCs. Moreover, when the wound scale is large, peripheral injections cannot deliver the cells to the center of the wound. There are also some reports on the use of intravenous injections; however, this method could reduce the delivery efficiency of MSCs to the wounded area because some translated cells will be entrapped in the lungs, leading to their accumulation [18,19].

Poly (lactic-co-glycolic acid) (PLGA) is a biodegradable and biocompatible copolymer material that has been used in a host of Food and Drug Administration (FDA) approved therapeutic devices. In this study, we developed PLGA-based fibers as a scaffold to create hUC-MSC tissue sheets in vitro, which were transplanted in diabetic mice to assess their effects on diabetic wound healing. We optimized the conditions of scaffold preparation, cell culture, and tissue construction, and obtained 192 ± 14 μm thick tissue sheets. Before transplanting hUC-MSC tissue sheets on full-thickness excisional skin wounds of diabetic mice, the characteristics and properties
of the tissue sheets were tested and verified. hUC-MSC tissue sheets effectively promoted epithelial regeneration, collagen deposition, infiltration of micro-vessels, and vascular maturation. These findings may provide new clinically effective therapies for diabetic wounds.

Materials and methods

Construction of PLGA scaffold

For the construction of the scaffold, PLGA (75/25; Sigma-Aldrich, St. Louis, MO, USA) was mixed with hexafluoro-2-propanol (HFIP, Wako Pure Chemical Industries, Tokyo, Japan) in a centrifugal tube (1.2g:3 ml, w/v), and an automated electrospinning machine (NF-103, MECC, Fukuoka, Japan) was used to synthesize the fibers. The mixed solution was loaded into a 3-mL syringe to which a needle with a 0.6-mm inner diameter was attached to connect to the positive electrode of the high-voltage power supply (10 kV). A layer of aluminum foil was attached to the grounded drum. The drum was rotated at a speed of 1000 rpm and used to collect the PLGA scaffold. The distance between the needle tip and the drum was maintained at 15 cm. The spinning process lasted for 120 min. The fiber sheet was then transferred to a PDMS frame (1 cm ×1 cm) for subsequent cell seeding. After construction, the fibers were examined using a scanning electron microscope, as previously described [20].
Culture of hUC-MSCs

hUC-MSCs were provided by Cell Exosomes Therapeutics Co., Ltd. (Tokyo, Japan), and all experiments were approved by the Ethical Committee of Osaka University. Briefly, the cells were cultured in MSCs Xeno-Free culture medium (Takara Bio Inc., Shiga, Japan) at 37 °C in a 5% CO$_2$ incubator. The culture medium was changed every 2-3 d. Upon reaching 80-90% confluence, the cells were detached using TrypLE Select (Gibco, Waltham, MA, USA) for further expansion. All experiments used cells at passages 5-7.

Characterization of hUC-MSCs

Immunophenotyping was performed using flow cytometry (FACS II). Briefly, after seven passages, hUC-MSCs were harvested and dissociated into single cells, washed with phosphate-buffered saline (PBS), and stained with the following fluorescence-conjugated antibodies (Table 1): anti-hCD31-PE, anti-hCD34-PE, anti-hCD45-PE, anti-hCD73-PE, anti-hCD90 (Thy1)-PE, anti-hCD105-PE, anti-HLA-G-PE, anti-HLA-DR-PE, and anti-HLA-ABC-PE. Mouse IgG1 κ isotype was used to stain the cells as a control. The antibodies were purchased from BioLegend (San Diego, CA, USA). Data were analyzed using FlowJo software (BD, Franklin Lakes, NJ, USA).

The adipogenic, osteogenic, and chondrogenic differentiation potentials of hUC-MSCs were evaluated using a differentiation medium (PromoCell, Heidelberg, Germany) for 2, 2, and 3 weeks, respectively. Oil Red O, Alizarin Red S, and Alcian
Blue (Sigma-Aldrich) staining were performed to confirm the differentiation potential of hUC-MSCs.

**hUC-MSC tissue sheet formation**

hUC-MSCs were seeded onto a PLGA scaffold ($1-4 \times 10^6$ cells/cm$^2$). iMatrix-511 (Matrixome, Osaka, Japan) was added during cell seeding at a concentration of 10 μg/ml. The samples were then cultured in a 5% CO$_2$ humidified atmosphere at 37°C for 3-5 d before transplantation, and the medium was changed every 2 d.

**In vivo wound healing experiments in a db/db mouse model**

Animal experiments were performed according to the guidelines of Osaka University. All experiments were performed using male $db/db$ ($BKS.Cg-$+Leprdb/+Leprdb/Jcl) mice (10 weeks old, 46.2 ± 1.8 g weight; CLEA Japan, Inc, Shizuoka, Japan). The mice were observed for 10 d before skin wound creation.

Animals were anesthetized using isoflurane (1.5%; Mylan Inc, Canonsburg, PA, USA). After shaving the dorsal hair of the mice, two full-thickness excisional skin wounds (8 mm in diameter) were created on their backs. The mice were then randomly divided into four different groups (seven mice per group): (1) left untreated (control); (2) $1 \times 10^6$ hUC-MSCs cultured on PLGA scaffold-treated (hUC-MSC tissue sheet); (3) PLGA scaffold-treated (Fiber only); (4) $1 \times 10^6$ hUC-MSC suspension in 50 μL PBS subcutaneous administration (hUC-MSC injection). In the injection group, mice were subcutaneously injected with hUC-MSCs in PBS at five injection sites (10 μL per site).
After treatment, a transparent and semi-occlusive adhesive dressing (Tegaderm; 3M, Saint Paul, MO, USA) was applied to protect the wounds. The mice were housed individually. The wound area was photographed and measured on days 0, 3, 7, 10, and 14 after wounding. The pictures were then analyzed using image analysis software (Image J). Mice were sacrificed on day 14 after surgery, and wound skin tissues were harvested for histological analysis.

**Histological analysis**

hUC-MSC tissue sheets were fixed in 4% paraformaldehyde overnight at 4 °C to create frozen sections. TUNEL assay was performed using the Click-IT TUNEL kit (Alexa Fluor 647), following the manufacturer’s instructions (Invitrogen; Thermo Fisher Scientific, Waltham, MA, USA). Immunofluorescence staining was performed. The sections were incubated with primary antibodies (Table 1) overnight at 4 °C, washed with PBS, and then incubated with the respective secondary antibodies at 37 °C for 1 h. After counterstaining with 2-(4-amidinophenyl)-1H-indole-6-carboxamidine (DAPI) (Invitrogen) or Hoechst 33342 (Invitrogen), the sections were analyzed using a fluorescence microscope (Keyence BZ-X800 & NIKON A1). Skin samples from the wound site of each group of mice were fixed in formalin, transferred to ethanol, and embedded in paraffin. Serial sections were prepared at a 5-μm thickness from the middle of the wound. The sections were stained with
hematoxylin and eosin (H&E) and Masson’s trichrome. For immunofluorescence
staining, dewaxed paraffin sections were washed in PBS, and antigen retrieval was
performed in Target Retrieval Solution (pH= 6; DAKO Japan, Inc., Tokyo, Japan) at
121 °C for 10 min. Sections were stained as described above.

Statistical analysis

All quantitative data are presented as mean ± standard deviation (SD). Analysis of
variance (ANOVA) or t test was used to evaluate statistical significance, which was
defined by a p-value < 0.05 (significance was set at *p < 0.05, **p < 0.01, ***p <
0.001).

Results

Characterization of hUC-MSCs

hUC-MSCs were maintained in culture until the fifth passage and their
characteristics were confirmed based on the expression of MSC surface markers.
Flow cytometry analysis demonstrated that hUC-MSCs were remarkably positive for
CD73, CD90, CD105, and HLA-ABC, and remarkably negative for CD31, CD34,
CD45, HLA-G, and HLA-DR (Fig. 1a). The cells showed a typical spindle-like
morphology under a bright-field microscope (Fig. 1b). In addition, to examine
whether hUC-MSCs retained their adipogenic, osteogenic, and chondrogenic differentiation potential, in vitro direct differentiation assays were conducted after the fifth passage. The cells were positive for Oil Red O staining (adipogenic marker) (Fig. 1c), Alizarin S Red staining (osteogenic marker) (Fig. 1d), and Alcian Blue staining (chondrogenic markers) (Fig. 1e).

hUC-MSC tissue sheet construction and evaluation

The PLGA scaffold was used as a culture scaffold to guide tissue formation by hUC-MSCs (Fig. 2a). To explore the importance of fiber orientation for the formation of hUC-MSC tissue sheets, we compared two types of PLGA scaffolds, a vertical structure and a traditional parallel structure. Compared with the vertical type, the thickness of the tissue sheet in the parallel type was more uniform and flatter (Fig. S1). Therefore, we selected a parallel sheet to create a tissue sheet (Fig. 2b). The scanning electron microscope (SEM) image shows a parallel and evenly distributed construction (Fig. 2c), and the diameter of each fiber was 3622 ± 563 nm (Fig. 2d).

Furthermore, to determine the optimal cell concentration for creating hUC-MSC tissue sheets, we cultured three different concentrations of MSCs (1×10⁶ / 2×10⁶ / 4×10⁶ per cm²) on the PLGA scaffold and then evaluated the thickness of the tissue sheet (Fig. 2e, f). The results revealed that the thickness of the constructed tissue sheet increased with increasing seeded cell concentrations.
In addition, we performed a TUNEL immunofluorescence assay to detect the number of apoptotic hUC-MSCs in tissue sheets with different hUC-MSC concentrations. The percentage of TUNEL-positive MSCs increased to 26% in the tissue sheet group containing $4 \times 10^6$ cells. In contrast, the $1 \times 10^6$ and $2 \times 10^6$ cell groups exhibited a high survival level (3.4 ± 2.2% vs 5.7 ± 2.5% vs 26.1 ± 16.8 %), and no significant difference was observed between the groups (Fig. 2h, i). A higher proportion of collagen III aggregates in scarless fetal wound healing, whereas a higher proportion of collagen I is deposited in scarring adult wounds [21]. Thus, to evaluate collagen deposition at different cell concentrations, the tissue sheets were immunostained for collagen I and III. The ratio of collagen I to collagen III in the $4 \times 10^6$ cells group was higher than that in the $2 \times 10^6$ cells group; however, there was no significant difference between the $1 \times 10^6$ and the $2 \times 10^6$ cell groups (Fig. 2j, k). Therefore, we selected a cell concentration of $1 \times 10^6$/cm$^2$ to construct the tissue sheet and proceed with the transplantation experiments.

**hUC-MSC tissue sheet accelerates diabetic wound healing**

We used the $db/db$ diabetic mouse model to investigate the therapeutic potential of hUC-MSC tissue sheets in diabetic wound healing. Full-thickness skin wounds were created on the backs of the model mice, followed by treatment with an hUC-MSC tissue sheet, fiber only, or hUC-MSC injection on the wound site. We first compared the hUC-MSC tissue sheet ($1 \times 10^6$ and $2 \times 10^6$ cells) and control (left untreated) groups...
and observed that the tissue sheet-treated groups had remarkably accelerated wound healing. Interestingly, the average wound closure rate of the $1 \times 10^6$ cells group was faster than that of the $2 \times 10^6$ cell group, and a significant difference ($p < 0.05$) was observed on day 10 (Fig. S2a, b). Therefore, we used $1 \times 10^6$ cells for subsequent transplantation experiments. Notably, the hUC-MSC tissue sheet group had a remarkably better wound closure effect than the other groups on days 7, 10, and 14 after wounding. Moreover, the hUC-MSC injection group also had a better wound closure effect than the control group; however, only day 10 showed significant differences. Interestingly, the fiber-only group exhibited a slower healing rate than the control group, especially on day 7 (Fig. 3a). In addition, H&E staining revealed that compared to other groups, the hUC-MSC tissue sheet group exhibited a thicker epidermis and granulation tissue, completely covered the wound site (Fig. 3a, c), and had better wound re-epithelialization (Fig. 3b, d).

**hUC-MSC tissue sheet induces collagen synthesis in vivo**

Regeneration of the dermis requires reconstruction of the collagen structure, mediated by fibroblasts and myofibroblasts [21]. Thus, to further confirm the therapeutic effect of hUC-MSCs on diabetic wound healing, we investigated collagen deposition using Masson trichrome staining. As shown in Fig 4A and B, the wounded area in mice treated with the hUC-MSC tissue sheet or injections showed well-reorganized collagen fibers compared to the other groups; however, the hUC-MSC
tissue sheet group exhibited a more complex collagen fiber structure with thicker collagen bundles than the injection group. IF staining (Fig. 4c) of collagen I and III revealed that the wounded area treated with the hUC-MSC tissue sheet had a higher collagen III to collagen I ratio. This result was consistent with the data from the *in vitro* experiments (Fig. 2j, k). Therefore, we speculated that using hUC-MSC tissue sheets would likely cause the diabetic wounds to progress to scarless healing.

**hUC-MSC tissue sheet remarkably promotes new blood vessel formation and maturation in diabetic wounds**

To further study the mechanism of wound healing promotion by hUC-MSC tissue sheets, we evaluated whether the therapeutic effect of hUC-MSC tissue sheets on wound healing is mediated by promoting angiogenesis. CD31, a specific marker for capillary endothelial cells, is widely used to evaluate injury-associated angiogenesis. A significantly (*p* < 0.01) increased number of CD31-positive cells was observed in the wound center of the hUC-MSC tissue sheet and injection groups compared to that in the control and fiber-only groups (Fig. 5a and c). Furthermore, the number of CD31-positive cells in the hUC-MSC injection group was lower than that in the hUC-MSC tissue sheet group, suggesting that hUC-MSCs promote angiogenesis in the wound center, and hUC-MSCs cultured on the PLGA scaffold effectively enhanced this effect. Interestingly, there was no significant difference in the number of CD31-positive cells at the wound edge between the hUC-MSC tissue sheet and injection...
CD31-positive endothelial cells covered with α-SMA-positive mural cells are typically used to evaluate the matured vessels. Thus, we further analyzed α-SMA expression in these groups. The density of matured vessels in the wound center was significantly increased in both the hUC-MSC tissue sheet and injection groups compared to other groups, whereas the density of matured vessels in the wound edge was markedly increased only in the hUC-MSC tissue sheet group both at the wound center and edge (Fig. 5a, b, d and f). These results demonstrated that hUC-MSC tissue sheets could quickly and effectively promote the maturation of blood vessels in diabetic wound areas.

**hUC-MSC tissue sheet regulates the inflammatory response in diabetic wounds**

To evaluate the effects of transplanted hUC-MSC tissue sheets on the infiltration of macrophages on day 14 after treatment, wound tissues were immunostained for CD68, which is a tissue macrophage marker. As shown in Fig. 6a, b, large number of CD68-positive cells were observed at the wound sites of diabetic mice. The number of CD68-positive cells in the wound site of the hUC-MSC tissue sheet and injection groups was significantly lower than that in the control group. However, we could not determine the number of CD68-positive cells in the fiber-only group due to poor wound healing. Furthermore, no significant difference was observed between the two hUC-MSC groups. Collectively, these findings demonstrated that hUC-MSC tissue sheets or injections could decrease the number of macrophages in the local wound.
sites of diabetic mice, potentially contributing to the regulation of the inflammatory
response and enhancing diabetic wound healing.

Discussion

Wound healing is a complex biological process that involves numerous molecules. During this process, disruption of one or more stages can lead to delayed or non-healing of the wound. In particular, some chronic or serious diseases, such as burns and diabetes, are associated with remarkable impaired wound healing [22]. This impaired wound healing is mainly due to a reduction in growth factors, impaired angiogenesis, and reduced wound cell function [23]. Traditional treatment methods for skin injuries include adhesive wound dressings, hyperbaric oxygen therapy, negative pressure therapy, and autologous skin transplantation. However, these methods are limited [24]. Therefore, there is an urgent need to develop innovative and effective treatment strategies for patients with chronic and severe skin damage.

MSCs from different tissue coerces can promote the healing of diabetic wounds [25,26]. Among several different tissue-derived MSCs, UC-MSCs have a non-invasive advantage and are derived from neonatal tissues, and thus have a greater proliferative capacity, especially under hypoxic conditions [27]. Therefore, in our study, we selected hUC-MSCs for transplantation to accelerate diabetic wound healing.
The most commonly used method for cell transplantation is the injection of a single-cell suspension around the periphery of the wound area. However, owing to the lack of ECM and intercellular communication between cells, this method often leads to a low survival rate of cells after transplantation and a limited therapeutic effect. In previous studies, our research group has reported that in vitro construction of cells into tissues followed by transplantation greatly increased the cell survival rate in vivo and improved heart function and lower limb ischemia [20,28]. In this study, we used a fiber-based scaffold to construct an MSC tissue sheet with a 3D structure for transplantation. We hypothesized that tissue sheets could not only avoid disrupting the communication between cells but also maintain the integrity of the ECM, while also increasing the number of transplanted cells through the 3D structure, resulting in better therapeutic effects. Our results confirmed this hypothesis and showed that compared with traditional single-cell injection, transplantation of hUC-MSC tissue sheet can accelerate the healing speed of diabetic ulcer wounds, induce angiogenesis and maturation of the wound center and edge, improve collagen remodeling, and reduce scarring formation in the wounded area (Fig.4). Interestingly, we found that transplantation of fibers alone affected the healing rate of diabetic wounds but had no significant adverse effects on re-epithelialization, angiogenesis, or collagen remodeling (Fig.3-5). This is possibly because, without cells, the fiber could be directly in contact with and adheres to the affected part, preventing the contraction of the skin during the wound healing process. Reducing the fiber density may solve this
problem. Moreover, as diabetic wounds in humans do not shrink like those in mice, this problem may be negligible in the process of human diabetic wound healing. In addition, we evaluated the effect of the number of transplanted cells in the tissues and found that the tissue constructed with $4 \times 10^6$ cells had a higher rate of apoptosis than the $1 \times 10^6$ and $2 \times 10^6$ cell groups (Fig. 2). This is because the thickness of the tissue exceeds the diffusion limits of nutrients and oxygen. Although there was no significant difference in the apoptotic rate between tissues with $1 \times 10^6$ cells and $2 \times 10^6$ cells \textit{in vitro}, the group transplanted with $1 \times 10^6$ cells showed a more efficient therapeutic effect than the $2 \times 10^6$ cell group \textit{in vivo} (Fig. S2). This may be due to the harsh environment of the wounded area, especially in the early stage, which is accompanied by a large amount of tissue fluid exudation. The tissue fluid also contains a large number of inflammatory factors, which could affect the vascular construction between the graft and the host. If the tissue exceeds the appropriate thickness, the nutrient supply of the graft might be affected, thereby affecting the therapeutic effect in wound healing and potentially leading to a more severe inflammatory response. Therefore, it is important to carefully select the number of transplanted cells and the thickness of the tissues to induce the desired therapeutic effect.

The typical process of skin regeneration is divided into four overlapping phases: inflammation, angiogenesis, cell proliferation, and wound remodeling. During the cell proliferation phase, angiogenesis, collagen deposition, re-epithelialization, and wound
contraction occur simultaneously. Angiogenesis is crucial for wound healing and tissue repair [29]. Our data demonstrated that hUC-MSC tissue sheets promote angiogenesis in both the wound area and edge. Furthermore, the hUC-MSC tissue sheet also had an obvious effect on vascular maturation compared to single-cell injection (Fig. 5). The hUC-MSC tissue sheets not only accelerated wound healing but also effectively promoted re-epithelialization inside the wound area (Fig. 3). Therefore, our transplantation strategy not only allows cells to directly contact the ulcer wound but also allows the secreted factors of cells to directly act on the wound. In addition, this alternative strategy to single-cell transplantation could protect the integrity of the ECM between cells, thereby reducing the cell loss rate, improving cell survival after transplantation, and producing more factors that contribute to angiogenesis. Moreover, the remodeling phase of a wound is between 2 weeks to more than 1 year, and is closely related to the production and reorganization of the ECM; therefore, it has an important impact on the extent of scarring [30]. In our study, both the in vitro and in vivo results showed remarkable consistency. A large amount of ECM deposition was observed in the tissue sheet and transplantation site, and the proportion of collagen III was significantly higher than that of collagen I (Fig. 2 and 4). These results indicate that the transplanted tissue sheets effectively inhibited scarring.

Chronic tissue inflammation is a recognized feature of diabetes mellitus [31]. Therefore, improvement of the microenvironment of the wound area is a key factor in
wound healing and tissue remodeling [32]. Additionally, MSCs can play anti-
inflammatory and immunoregulatory roles [33]. Therefore, we investigated the
immunoregulatory function of hUC-MSC tissue sheets in the microenvironment of
diabetic wounds and found that hUC-MSC tissue sheets regulate the
microenvironment in the wound area by reducing the infiltration of macrophages.
However, there was no significant difference between the hUC-MSC tissue sheet and
injection groups (Fig. 6). This phenomenon may be because the two groups had the
same number of cells. Nevertheless, tissue repair and reconstruction require a certain
degree of inflammatory response to recruit more anti-inflammatory, pro-angiogenic,
and other factors to the wound area to promote wound healing.

Conclusion

In conclusion, our study demonstrated that hUC-MSC tissue sheets can effectively
accelerate the rate of wound healing, improve re-epithelialization of wound sites,
promote collagen deposition and remodeling, enhance angiogenesis and vessel
maturation, and regulate the immune microenvironment of the wounded area. Our
findings provide a new and effective method for cell transplantation and a new
strategy for the treatment of not only diabetic skin wounds but also burns and
traumatic skin injuries.

Supplementary information
Supplementary figures S1 and S2.

List of abbreviations

hUC-MSCs, human umbilical cord mesenchymal stem cells; PLGA, poly(lactide-co-glycolide; ECM, extracellular matrix; SEM, scanning electron microscope; H&E, hematoxylin and eosin.

Declarations

Ethics approval and consent to participate

All experiments using hUC-MSCs were approved by the Ethical Committee of Osaka University. All animal experiments were performed according to the guidelines of Osaka University.

Consent for publication

Not applicable.

Availability of data and materials

All data generated and/or analyzed in this study are included in this published article.

Competing interests
The authors declare that they have no competing nonfinancial interests to declare. Yoshiki Sawa was the chief advisor of the Cell Exosomes Therapeutics Co., Ltd.

**Funding**

This study was supported by Japan Society for the Promotion of Science (JSPS; 22H03157, 22K12801). This work was also supported by Cell Exosomes Therapeutics Co., Ltd.

**Authors' contributions**

L. L, Y. S, and S. M. conceived the project; J. Z., Y. S., L. L., and S. M. designed the experiments. J. L. synthesized the device. All authors contributed to the data analysis and interpretation. J.Z., L.L., Y.S. and S.M. wrote the manuscript.

**Acknowledgements**

Not applicable.

**References**


mesenchymal stem cells (MSC): A comparison of adult and neonatal tissue-derived MSC.


**Figure legends**

**Fig. 1 Characterization of human umbilical cord mesenchymal stem cells (hUC-MSCs).**

- Flow cytometry analysis using hUC-MSCs surface markers CD31, CD34, CD45, CD73, CD90, CD105, HLA-ABC, HLA-DR, and HLA-G.
- Bright-field microscopic
images show spindle-like hUC-MSCs. Scale bar = 100μm. c-e. Differentiation ability of hUC-MSCs. Adipocytes, osteoblasts, and chondrocytes were detected using Oil Red O, Alizarin Red, and Alcian Blue, respectively, Scale bar = 100μm, 100μm, and 200μm respectively.

Fig. 2 Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet construction and evaluation.

a. Schematic representation of tissue construction in vitro. b. Image of PLGA aligned fibers. Scale bar = 2mm. c. Scanning electron microscopy image of PLGA aligned fibers. Scale bar = 10μm. d. Quantitative analysis of the diameter of fibers in PLGA aligned fibers. e. Quantitative analysis of the thickness of hUC-MSC tissue sheets with different cell densities in (f) (n=10). f. Hematoxylin and eosin staining of hUC-MSC tissue sheets with different cell densities. Scale bar = 100μm. g. Immunostaining images of Vimentin (green) in hUC-MSC tissue sheet (1x10^6 cells). Scale bar = 100μm. h. TUNEL staining of hUC-MSC tissue sheet sections with different cell densities. Scale bar = 100μm. i. Quantitative analysis of cell death rate in different groups (n = 6). j. Immunostaining images of collagen I (green) and collagen III (red) in hUC-MSC tissue sheet section with different cell densities. Scale bar = 100μm. k. Quantitative analysis of the ratio of Col-I/Col-III in different groups (n=3). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance. ANOVA. *p < 0.05, **p < 0.01. PLGA, poly(lactide-co-glycolide).
Fig. 3 Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet accelerates diabetic wound healing.

a. Representative photographs of full-thickness excision wounds at 0, 7, 10, and 14 days after wounding. b. Hematoxylin and eosin staining of wound sections treated with hUC-MSC tissue sheet, fiber only, and hUC-MSCs injection 14 days after wounding. The double-headed black arrows indicate the edge of the scars. Scale bar = 2mm. c. Quantitative analysis of the rate of wound closure in each group (n=7). d. Quantitative analysis of the extent of re-epithelialization in (b) (n=5). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01

Fig. 4 Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet induces collagen synthesis in vivo.

a. Masson staining of wound sections in different groups on day 14 after wounding. Scale bar = 100μm. b. Quantitative analysis of collagen fiber deposition in different groups 14 days after wounding. c. Immunostaining analysis of collagen I (green) and III (red). Scale bar = 500μm. Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01

Fig. 5 Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet
remarkably promotes new blood vessel formation and maturation in diabetic wounds.

a. Representative images of tissue sections from the center of diabetic wound area 14 days after wounding (Scale bar = 500μm and 100μm). b. Representative images of tissue sections from the edge of the diabetic wound area on day 14 after wounding (Scale bar =500μm and 100μm). c. Quantitative analysis of vessel density of wound center in different groups 14 days after wounding (n=14). d. Quantitative analysis of matured vessel density of wound center in different groups 14 days after wounding (n=14). e. Quantitative analysis of vessel density of wound edge in different groups 14 days after wounding (n=14). f. Quantitative analysis of matured vessel density of wound edge in different groups 14 days after wounding (n=14). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01

Fig. 6 Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet regulates inflammation response in diabetic wounds.

a. Representative images of immunohistochemical sections in different groups on day 14 after wounding. Scale bar = 200μm. b. Quantitative analysis of immunohistochemical signals of CD68 in high power field images. (n = 5). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01
Fig. S1 Tissue sheet formation of human umbilical cord mesenchymal stem cell (hUC-MSC) using poly(lactide-co-glycolide) scaffold

Hematoxylin and eosin staining of hUC-MSC tissue sheets with different fiber structures (parallel and vertical). Scale bar = 100μm. The yellow arrowheads show a schematic of the two types of PLGA fiber structures.

Fig. S2 Tissue sheets with different cell numbers promote wound healing.

a. Representative photographs of full-thickness excision wounds on days 0, 7, 10, and 14 after wounding. b. Quantitative analysis of the rate of wound closure in each group. (n =7). Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01
Characterization of human umbilical cord mesenchymal stem cells (hUC-MSCs).

a. Flow cytometry analysis using hUC-MSCs surface markers CD31, CD34, CD45, CD73, CD90, CD105, HLA-ABC, HLA-DR, and HLA-G. b. Bright-field microscopic images show spindle-like hUC-MSCs. Scale bar = 100μm. c-e. Differentiation ability of hUC-MSCs. Adipocytes, osteoblasts, and chondrocytes were detected using Oil Red O, Alizarin Red, and Alcian Blue, respectively, Scale bar = 100μm, 100μm, and 200μm respectively.
Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet construction and evaluation.

a. Schematic representation of tissue construction in vitro. b. Image of PLGA aligned fibers. Scale bar = 2mm. c. Scanning electron microscopy image of PLGA aligned fibers. Scale bar = 10μm. d. Quantitative analysis of the diameter of fibers in PLGA aligned fibers. e. Quantitative analysis of the thickness of hUC-
MSC tissue sheets with different cell densities in (f) (n=10). f. Hematoxylin and eosin staining of hUC-MSC tissue sheets with different cell densities. Scale bar = 100μm. g. Immunostaining images of Vimentin (green) in hUC-MSC tissue sheet (1x10^6 cells). Scale bar = 100μm. h. TUNEL staining of hUC-MSC tissue sheet sections with different cell densities. Scale bar = 100μm. i. Quantitative analysis of cell death rate in different groups (n = 6). j. Immunostaining images of collagen I (green) and collagen III (red) in hUC-MSC tissue sheet section with different cell densities. Scale bar = 100μm. k. Quantitative analysis of the ratio of Col-I/Col-III in different groups (n=3). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance. ANOVA. *p < 0.05, **p < 0.01. PLGA, poly(lactide-co-glycolide).
Figure 3

Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet accelerates diabetic wound healing.

a. Representative photographs of full-thickness excision wounds at 0, 7, 10, and 14 days after wounding.
b. Hematoxylin and eosin staining of wound sections treated with hUC-MSC tissue sheet, fiber only, and hUC-MSCs injection 14 days after wounding. The double-headed black arrows indicate the edge of the scars. Scale bar = 2mm. c. Quantitative analysis of the rate of wound closure in each group (n=7). d. Quantitative analysis of the extent of re-epithelialization in (b) (n=5). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01

Figure 4

Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet induces collagen synthesis in vivo.

a. Masson staining of wound sections in different groups on day 14 after wounding. Scale bar = 100μm.
b. Quantitative analysis of collagen fiber deposition in different groups 14 days after wounding. c. Immunostaining analysis of collagen I (green) and III (red). Scale bar = 500μm. Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01

Figure 5

Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet remarkably promotes new blood vessel formation and maturation in diabetic wounds.

a. Representative images of tissue sections from the center of diabetic wound area 14 days after wounding (Scale bar = 500μm and 100μm). b. Representative images of tissue sections from the edge of the diabetic wound area on day 14 after wounding (Scale bar =500μm and 100μm). c. Quantitative analysis of vessel density of wound center in different groups 14 days after wounding (n=14). d. Quantitative analysis of matured vessel density of wound center in different groups 14 days after wounding (n=14). e. Quantitative analysis of vessel density of wound edge in different groups 14 days after wounding (n=14). f. Quantitative analysis of matured vessel density of wound edge in different groups 14 days after wounding (n=14). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01
Figure 6

Human umbilical cord mesenchymal stem cell (hUC-MSC) tissue sheet regulates inflammation response in diabetic wounds.

a. Representative images of immunohistochemical sections in different groups on day 14 after wounding. Scale bar = 200μm. b. Quantitative analysis of immunohistochemical signals of CD68 in high power field images. (n = 5). Results are presented as mean ± standard error of the mean. Significance was determined using analysis of variance ANOVA. *p < 0.05, **p < 0.01

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- AdditionalFigures.pdf
- LegendsforAdditionalfigures.pdf
- Table.pdf