Keratinocyte-Like Cells Trans-Differentiated from Human Adipose-Derived Stem Cells, Facilitate Skin Wound Healing in Mice

Jonghun Kim1, Toshio Hasegawa1, Akino Wada1, Yuichiro Maeda1, Shigaku Ikeda1,2

1Department of Dermatology and Allergology, Juntendo University Graduate School of Medicine, 2Atopy (Allergy) Research Center, Juntendo University Graduate School of Medicine, Tokyo, Japan

Background: Mesenchymal stem cells (MSCs) have been reported to promote wound healing in both animal models and human studies. Among MSCs, adipose-derived stem cells (ADSCs) can be easily harvested in large quantities. Objective: We investigated whether skin wound healing in mice can be facilitated by keratinocyte-like cells differentiated from ADSCs (KC-ADSCs). Methods: For the wound contraction and epithelialization model, a 20 mm×20 mm full-thickness skin wound was made on the dorsum. For the wound epithelialization model, a 6 mm×6 mm full-thickness skin wound was made on the dorsum. A nitrile rubber stent with an inner diameter of 8 mm was sutured around the wounds to minimize wound contraction. Undifferentiated ADSCs (uADSCs) or KC-ADSCs was injected around the wound base in both models. To evaluate whether the injected ADSCs could enhance wound contraction in a skin wound, the contractile activity of ADSCs was assessed by an in vitro type I collagen gel contraction assay. Alpha-smooth muscle actin (α SMA) expressions in uADSCs and KC-ADSCs were also evaluated by flow cytometry and real-time polymerase chain reaction. Results: In a wound contraction and epithelialization model, KC-ADSCs further facilitated wound healing compared with uADSCs. In a wound epithelialization model, KC-ADSCs also further facilitated wound epithelialization compared with uADSCs. The contractile activity of KC-ADSCs was lower than that of uADSCs. The uADSCs expressed high levels of α SMA, which decreased after the differentiation into keratinocyte-like cells. Conclusion: Our results suggest that the wound healing effect of KC-ADSCs depends primarily on re-epithelialization rather than wound contraction. (Ann Dermatol 33(4) 324 ∼ 332, 2021)

Keywords: Keratinocytes, Mesenchymal stem cells, Wound healing

INTRODUCTION

Mesenchymal stem cells (MSCs) can be isolated from various tissues such as bone marrow, umbilical cord, placenta, fetal tissues, skin, and adipose tissues. Adipose-derived stem cells (ADSCs) are MSCs that can be easily harvested in large quantities and produce 500-times more colony-forming units than bone marrow-derived MSCs, which are the major source of MSCs for clinical use. Although the dermis can also be easily harvested, a previous study showed that dermal stem cells represent only 0.3% of the human dermal foreskin fibroblasts. Therefore, ADSCs have the potential to be a practical and promising tool for regenerative medicine. MSCs are immune-evasive cells because they do not express major histocompatibility complex (MHC) class II antigens and minimally express MHC class I antigens. This lack of immunogenicity enables the use of MSCs in clinical applications such as allogeneic cell transplantation.
MSCs secrete immunomodulatory, anti-inflammatory, pro-angiogenic, pro-mitogenic, and antibacterial factors such as transforming growth factor β1 (TGF-β1), hepatocyte growth factor, haemoxgenase-1, prostaglandin E2, interleukin (IL)-10, and HLA-G5-12. Of the different MSCs available, ADSCs show low immunogenicity and high immunosuppressive potential13-19.

MSCs have been reported to promote wound healing in both animal models and human studies20-27. The clinical utility of MSCs in wound healing is based on repairing and replacing cellular substrates, attenuating inflammation, increasing angiogenesis, and enhancing migration of reparative cells by cytokine/chemokine production. The differentiation potential of MSCs into keratinocytes is also required for re-epithelialization of wounds. It has previously been reported that keratinocyte progenitor cells reside in ADSCs, and ADSCs can further differentiate into keratinocyte-like cells28,29. ADSCs may be utilized in wound repairs, including those for deep wounds and burns. In this study, we investigated whether keratinocyte-like cells differentiated from ADSCs (KC-ADSCs) can facilitate skin wound healing in mice.

MATERIALS AND METHODS

Transdifferentiation of ADSCs into keratinocyte-like cells

ADSCs (Lonza Group AG, Basel, Switzerland) were co-cultured with normal human dermal fibroblasts (NHDFs) as previously reported28,30. Briefly, NHDFs were seeded in six-well plates (IWAKI, Shizuoka, Japan) and cultured in Dulbecco’s modified Eagle’s Medium (DMEM) (Gibco, Life Technologies, Carlsbad, CA, USA) containing 10% fetal bovine serum (Sigma-Aldrich, St. Louis, MO, USA) for 24 hours, while ADSCs were seeded on 0.4-μm Millipore filters hanging cell culture inserts (Merck Millipore, Darmstadt, Germany) coated with type IV collagen (Nitta Gelatin, Osaka, Japan) and placed onto the plates. All-trans retinoic acid (Sigma-Aldrich) was added at 1 μM to the upper chamber. After culturing for 3 days, 25 ng/ml of bone morphogenetic protein 4 (R&D Systems, Minneapolis, MN, USA) was added to the co-culture system and cultured on a dish coated with type IV collagen in KSFM for an additional 14 days.

Green fluorescent protein transfection into ADSCs

ADSCs were cultured at 60% to 80% confluence for transfection of green fluorescent protein (GFP). EmGFP Vector (Invitrogen, Carlsbad, CA, USA) was diluted with Lipofectamine RNAiMAX Reagent (Thermo Fisher Scientific) (1:1 ratio) before being added to the cells. Cells were incubated at 37°C for 48 hours and then the transfected cells were analyzed.

Mice

All animal experiments were performed according to the guidelines of the Ethical Committee of Juntendo University. All experimental mice were housed in cages with a 12-hour light-dark cycle at 22°C to 25°C with 55% to 60% humidity. Solid food and water were supplied ad libitum. Eight-week-old ICR-nu mice (Crlj:CD1-Foxn1nu) were purchased from Charles River Laboratories Japan (Yokohama, Japan). We used the ARRIVE checklists when writing our report31.

Wound healing model

For the wound contraction and epithelialization model, a 20 mm×20 mm full-thickness skin wound was made on the dorsum under systemic anesthesia with isoflurane (Phoenix Pharmaceuticals, Burlingame, CA, USA). Five hundred microliters of phosphate buffered saline (PBS) containing 1×10⁶ undifferentiated ADSCs (uADSCs) (n=6) or KC-ADSCs (n=6) was injected intradermally around the wound base at four sites. The wound was then covered by Tegaderm™ (3M, Saint Paul, MN, USA) to prevent licking and drying of the wound. In the control group (n=6), the same volume of PBS without cells was injected intradermally around the wound base at four sites.

Detection of ADSCs in the wound healing model

Wound specimens for histological analysis were harvested from the euthanized animals 2 days after cell injection. The specimens were frozen in optimal cutting temperature compound for cryosectioning. Three-micrometer thick sections were made by cutting from the central region of the wound. Frozen sections were embedded in 4% paraformaldehyde and nuclei were counterstained with 4,6-diamidino-2-phenylindole (DAPI). Sections were imaged and digitized in their entirety at 40-fold magnification (40×) with a BZ-700 microscope (Keyence, Osaka, Japan).

Wound epithelialization model

For the wound epithelialization model, a full-thickness skin wound was produced by 6-mm biopsy punches lateral to the midline of the back under systemic anesthesia with isoflurane (Phoenix Pharmaceuticals). A nitrite rubber stent with an inner diameter of 8 mm was sutured with 5-0 nylon (Ethicon, Somerville, NJ, USA) around the wounds to minimize wound contraction and to ensure healing by epithelialization. Two hundred and fifty microliters of PBS...
containing \(1 \times 10^6\) uADSCs (n=6) or KC-ADSCs (n=6) was injected intradermally around the wound base at four sites. The wound was then covered by Tegaderm™ (3M) to prevent licking and drying of the wound. In the control group (n=6), the same volume of PBS without cells was injected intradermally around the wound base at four sites.

**Detection of \(\alpha\)-smooth muscle actin-positive cells in wound epithelialization model by immunohistochemistry**

Alpha-smooth muscle actin (\(\alpha\) SMA), an actin isoform found in vascular smooth muscle cells, has been observed to be one of the most useful markers for myofibroblast differentiation\(^{32,33}\). \(\alpha\) SMA-positive cells in the wound healing model were detected by immunohistochemistry. Wound specimens were harvested from the euthanized animals 3, 5, and 7 days after cell injection for immunohistochemistry. The specimens were fixed with 4% paraformaldehyde/PBS and embedded in paraffin. The sample tissues were cut into 3- \(\mu\)m thick sections and stained with an anti-\(\alpha\) SMA rabbit monoclonal antibody (Abcam, Cambridge, MA, USA) (1:1,000). Each section was automatically stained using Benchmark (Ventana Medical Systems, Oro Valley, AZ, USA) and was imaged and digitized in its entirety at the 40 times magnification (40×) with a BZ-700 Analyzer (Keyence).

**Wound healing analyses**

The wounds of individual mice were photographed and the wound area was measured on days 1, 4, and 7 (wound healing model) and on days 0, 3, 5, and 7 (wound epithelialization model) after cell transplantation. Photographs were taken with a 20-megapixel digital camera (SX 720HS; Canon, Tokyo, Japan) from a distance of 3 cm, with the lens facing the wound vertically. The wound area was measured by tracing the wound margin and calculated using an image analysis program (Photoshop CC; Adobe Systems, San Jose, CA, USA) and Image J (National Institute of Health, Bethesda, MD, USA). The wound healing rate was calculated as follows: \(\{1-\text{[wound area/original wound area]}\} \times 100\) (%).

**Collagen gel contraction assay**

To evaluate whether the injected ADSCs could enhance wound contraction in a full-thickness skin wound, the contractile activity of ADSCs was assessed by an *in vitro* type I collagen gel contraction assay, which is used for evaluating contractile activity of fibroblasts\(^{34,36}\). Briefly, a collagen solution was prepared by mixing acid-soluble porcine type I collagen (3 mg/ml), a five-fold concentration of DMEM, and buffer solution (0.05 M NaOH, 2.2% NaHCO\(_3\), 200 mM HEPES) in the ratio of 7:2:1 (all purchased from Nitta Gelatin). A 2.0-ml mixture of cell suspension in serum-free adipose-derived stem cell basal medium (ADSC-BM) (Lonzar Group AG) and collagen solution was added to each well of a 12-well plate (IWAKI) and then gelled at 37°C for 30 to 40 minutes (final concentration \(1.0 \times 10^5\) cells/ml and 2.1 mg/ml collagen). A further 1 ml of serum-free ADSC-BM was then poured on to the gel to prevent the surface from dehydrating. After incubation of 1 hour, each gel was separated from the well, floated, and 10 ng/ml of recombinant human TGF-\(\beta\) 1 or vehicle control was applied. At 48 hours after the application of TGF-\(\beta\) 1 or vehicle control, the major and minor axes of each gel samples were measured, and the surface area was calculated. The contraction of the gel was expressed as a percentage of the surface area of the non-contracted state (defined as 100%). Both uADSCs and KC-ADSCs were assessed in this manner, and NHDF-embedded collagen gel was also assessed as a control. Twelve-well culture plates were used for each group (n=6).

**Detection of \(\alpha\)-smooth muscle actin in ADSCs by immunofluorescence and flow cytometry**

ADSCs and NHDFs were fixed with 4% paraformaldehyde, permeabilized with 0.1% Triton X-100, and immunostained for \(\alpha\) SMA using a rabbit monoclonal anti-\(\alpha\) SMA antibody (Abcam). The nuclei were counterstained with DAPI, and samples were mounted in Mounting Medium with DAPI H-1200 (Vector Laboratories, Burlingame, CA, USA) to detect fluorescence and visualized under a BZ-X700 microscope (Keyence). ADSCs and NHDFs were also incubated with anti-\(\alpha\) SMA rabbit monoclonal antibody (Abcam) (1:1,000), then incubated with goat anti-rabbit immunoglobulin G H&L FITC (Abcam), and analyzed by flow cytometry.

**Expression analysis of \(\alpha\)-smooth muscle actin mRNA in ADSCs by real-time polymerase chain reaction**

\(\alpha\) SMA mRNA expressions in uADSCs, KC-ADSCs, and NHDFs were evaluated by real-time polymerase chain reaction (PCR). Total RNA extracted from each cells was converted into cDNA using the ReverTra Ace qPCR RT kit (Toyobo, Osaka, Japan). TaqMan Master Mix (Applied Biosystems, Foster City, CA, USA) was used to amplify cDNA for 50 cycles on a StepOnePlus system (Applied Biosystems). \(\alpha\) SMA expression (using primer Hs00426835_g1; Applied Biosystems) was normalized to GAPDH levels, and the comparative cycle threshold (Ct) method using the formula \(2^{-\Delta \Delta Ct}\) was used to calculate the relative mRNA levels.
**Statistical analysis**

All experimental values were expressed as mean±standard deviation. Groups were compared using one-way ANOVA with Bonferroni post hoc analysis. Statistical significance was defined as a *p*-value < 0.05, **p*-value < 0.01.

**Ethical approval**

The study protocol was approved by the Ethics Committee of the Juntendo University Graduate School of Medicine (approval no. 2012020). All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. Data were analyzed in a blinded fashion, and procedures were carried out according to...
RESULTS

ADSCs facilitated wound healing in mice

Digital photographs of the wounds were obtained on days 1, 4, and 7 (Fig. 1A). The average wound healing rate of the mice injected with uADSCs was significantly higher than that of the control group on day 7 (Fig. 1B). The average wound healing rate of mice injected with KC-ADSCs was also significantly higher than that of the uADSCs-injected group on day 7 (Fig. 1B).

Before transplantation, it was confirmed that 80% to 90% of total ADSCs expressed GFP (Fig. 1C). After transplantation, GFP+ADSCs were sporadically identified in the dermis of the wound of both uADSCs-injected group and KC-ADSCs-injected group, while no GFP+ADSCs were detected in the wounds of controls (data not shown).

ADSCs facilitated wound epithelialization in mice

Digital photographs of the wounds were obtained on days 0, 3, 5, and 7 (Fig. 2A). As in the wound healing model, the average wound healing rate of the mice injected with uADSCs was significantly higher than that of the control group on day 7 (Fig. 2B). The average wound healing rate of mice injected with KC-ADSCs was also significantly higher than that of the uADSCs-injected group on day 7 (Fig. 2B). These results suggested that in this wound healing model, KC-ADSCs further promoted wound healing compared with uADSCs not only by epithelialization but also by granulation tissue contraction. Samples from the uADSCs-injected group showed an increase of αSMA-positive cells in the upper dermis compared with controls (Fig. 2C). There was no significant difference in the appearance of αSMA-positive cells between the uADSCs-injected group and KC-ADSCs-injected group.
KC-ADSCs have less contractile activity than uADSCs

The uADSCs-embedded collagen gels showed contraction rate of 79.6% ± 5.0% as compared with control NHDF-embedded collagen gels with contraction rate of 98.0% ± 1.7%. TGF-β1 further promoted contraction of uADSCs-embedded collagen gels to 61.6% ± 4.2%, while NHDF-embedded collagen gels with added TGF-β1 showed enhanced contraction to 96.2% ± 0% (Fig. 3A, B).

KC-ADSCs-embedded collagen gels showed contraction rate of 91.7% ± 4.7%, which was promoted to 74.6% ± 6.2% by the addition of TGF-β1 (Fig. 3A, B). The contractile activity of KC-ADSCs was lower than that of uADSCs. This result suggests that the effect of KC-ADSCs does not primarily depend on contractile activity but rather on promoting epithelialization.

**Fig. 3.** (A) The uADSC-embedded collagen gels showed enhanced contraction compared with NHDFs. However, KC-ADSCs-embedded collagen gels showed lower contraction than uADSCs-embedded collagen gels. TGF-β1 further promoted contraction of all collagen gels. (B) The uADSC-embedded collagen gels showed higher contraction rate of 79.6% ± 5.0%, while NHDF-embedded collagen gels showed contraction rate of 98.0% ± 1.7%. TGF-β1 further promoted contraction of uADSCs-embedded collagen gels of 61.6% ± 4.2%, while NHDF-embedded collagen gels showed less contraction rate of 96.2% ± 0% by TGF-β1. The KC-ADSC-embedded collagen gels showed contraction rate of 91.7% ± 4.7%, which was promoted to that of 74.6% ± 6.2% by TGF-β1. The contractile activity by KC-ADSCs was lower than that of uADSCs. (C) The uADSCs expressed higher levels of α-smooth muscle actin (αSMA) than NHDFs as determined by immunofluorescence microscopy. KC-ADSCs also expressed αSMA. (D) The uADSCs and KC-ADSCs expressed αSMA as determined by flow cytometry. (E) The expression level of αSMA mRNA in the uADSCs decreased after the differentiation into keratinocyte-like cells, although uADSCs expressed higher levels of αSMA mRNA than NHDF as determined by real-time polymerase chain reaction. NHDFs: normal human dermal fibroblasts, TGF: transforming growth factor, uADSCs: undifferentiated adipose-derived stem cells, KC-ADSCs: keratinocyte-like cells differentiated from ADSCs, αSMA: α-smooth muscle actin. Statistically significant (*p < 0.05, **p < 0.01).
The uADSCs expressed high levels of α-smooth muscle actin before differentiation into keratinocyte-like cells

The uADSCs expressed higher levels of α-SMA than NHDF as determined by immunofluorescence microscopy (Fig. 3C). The expression of α-SMA in ADSCs was also detected by flow cytometry (Fig. 3D). The levels of α-SMA mRNA in uADSCs as determined by real-time PCR were also higher than those in NHDFs, but they decreased after the differentiation into keratinocyte-like cells (Fig. 3E). These observations were consistent across three independent experiments performed using three different lots of ADSCs.

DISCUSSION

In general, the human skin consists of two layers: the epidermis and dermis. The epidermis acts as a barrier against envelopment factors such as pathogens and allergens, and regulates inside-out water loss or outside-in stimuli. On the other hand, the dermis is composed of connective tissue, nerves, vessels and appendages, and provides strength and flexibility to the skin. The epidermis is a rapidly regenerating tissue that is maintained by the continuous transformation of transient amplifying cells and epidermal stem cells located in the basal layer of the interfollicular epidermis, the bulge region of hair follicles, and sebaceous glands. Epidermal stem cells differentiate into keratinocytes that are directed to the upper layers of the epidermis and terminally form stratum corneum.

Wound healing is divided into three sequential phases: the inflammatory phase, the proliferative phase, and the remodeling phase. Fibroblasts and myofibroblasts play critical roles in both early and late phases, where they contribute to wound contraction, collagen deposition, and fibrosis. Because no epidermal stem cells remain in full-thickness skin defects, re-epithelialization from the inside of an ulcer hardly occurs. Therefore, the ulcer is supposed to be closed by cell migration of epidermal stem cells surrounding the ulcer, and by wound contraction. Conventional autologous split-thickness skin grafting is the gold standard for the treatment of a large skin defect. However, for patients with extensive skin defects, split-thickness skin grafting is limited due to donor skin availability. Various biological dressings, including autologous and allogeneic cultured skin substitutes, have been used for intractable ulcers. However, allogeneic cultured skin substitutes cannot be permanently adopted. Autologous cultured skin can serve as a permanent covering; however, it is cumbersome and time-consuming to create a cultured epidermis from autologous keratinocytes.

Stem cell therapy involving autologous and allogeneic ADSCs may be used for intractable skin ulcers as ADSCs promote wound healing and can differentiate into multiple cell lineages including keratinocytes. In this study, the effectiveness of KC-ADSCs in full-thickness wound healing was experimentally studied by allogeneic transplantation of ADSCs into a mouse skin ulcer model. The experimental groups included: no cells (control), uADSCs-injected group, and the KC-ADSCs-injected group. The wound healing in all groups was examined and compared clinically and histopathologically on days 1 to 7 after cell transplantation. Our results showed that uADSCs promoted wound contraction and healing compared with controls, and that KC-ADSCs further promoted wound reepithelization rather than wound contraction.

We also determined the effectiveness of ADSCs using an animal model in which wound contraction was suppressed by attaching a stent, enabling easy evaluation of re-epithelialization. In this model, uADSCs promoted wound healing, and KC-ADSCs further promoted wound healing. This may indicate that KC-ADSCs promote not only granulation tissue contraction but also re-epithelialization of the wound.

In wound healing model, the healing rate of uADSCs was lower than that of the control group on day 4. Similarly, the healing rate of uADSCs was lower than that of the control group on day 3 in wound epithelialization model. The transplanted ADSCs may not be effective before engraftment. In immunohistochemistry, there was no significant difference in hepatocyte growth factor, IL-10, matrix metalloproteinases-10, acellular dermal matrix, and VGEF staining between the ADSCs treated and control groups (data not shown). Although the collagen gel contraction assay revealed that the contractile activity of KC-ADSCs was lower than that of uADSCs, KC-ADSCs further promoted wound healing compared with uADSCs. Although uADSCs and KC-ADSCs showed a wound healing effect, and administration of ADSCs induced an increased number of α-SMA-positive cells, our results suggested that the wound healing effect of KC-ADSCs primarily depends on their re-epithelialization effect.

The use of ADSCs has several advantages, including their abundance in donors and their potential for easy isolation by minimally invasive methods such as liposuction. In addition, ADSCs can be used for the treatment of congenital or acquired large skin defects that cannot be treated with autologous split-thickness skin grafting, including epidermolysis bullosa and burns, due to not only their wound healing properties but also their potential for differentiation into keratinocytes. The method for the administration of MSCs is still debatable, and also establishment of an opti-
delivery procedure of ADSC by later-coming pre-clinical and clinical studies will be definitely required. The use of serum-free medium should be considered for clinical application to prevent infection.

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CONFLICTS OF INTEREST

The authors have nothing to disclose.

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DATA SHARING STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ORCID

Jonghun Kim, https://orcid.org/0000-0001-8462-5195
Toshio Hasegawa, https://orcid.org/0000-0001-9047-8427
Yuichiro Maeda, https://orcid.org/0000-0001-6960-3684
Shigaku Ikeda, https://orcid.org/0000-0002-7520-0707

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